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Density-dependent prophylaxis and condition-dependent immune function in Lepidopteran larvae: a multivariate approach

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Summary

1. The risk of parasitism and infectious disease is expected to increase with population density as a consequence of positive density-dependent transmission rates. Therefore, species that encounter large fluctuations in population density are predicted to exhibit plasticity in their immune system, such that investment in costly immune defences is adjusted to match the probability of exposure to parasites and pathogens (i.e. density-dependent prophylaxis).
2. Despite growing evidence that insects in high-density populations show the predicted increase in resistance to certain pathogens, few studies have examined the underlying alteration in immune function. As many of these species show increased cuticular melanism at high densities, the aim of this study was to use a multivariate approach to quantify relative variation in the allocation of resources to immunity associated with both rearing density (solitary vs. crowded) and cuticular colour (pale vs. dark) in a phase-polyphenic Lepidopteran species (*Spodoptera littoralis* Boisduval).
3. Relative to pale individuals, dark larvae (the high-density phenotype) exhibited higher haemolymph and cuticular phenoloxidase (PO) activity and a stronger melanotic encapsulation response to an artificial parasite inserted into the haemocoel. However, they also exhibited lower antibacterial (lysozyme-like) activity than pale larvae. Larval density *per se* had little effect on most of the immune parameters measured, though capsule melanization and antibacterial activity were significantly higher in solitary-reared than crowded larvae.
4. Correcting for variation in larval body condition, as estimated by weight and haemolymph protein levels, had little effect on these results, suggesting that variation in immune function across treatment groups cannot be explained by condition-dependence. These results are examined in relation to pathogen resistance, and the possibility of a trade-off within the immune system is discussed.

Key-words: condition-dependence, density-dependent prophylaxis, melanism, parasite resistance, trade-offs.

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Introduction

For many organisms, having the flexibility to cope with changes in the environment could influence their survival and reproductive success. Increasing population density can cause such environmental change as resources become scarce and the likelihood of infection by parasites and pathogens increases (Anderson & May 1981). Many insect species exhibit density-dependent phase-polyphenism, an adaptive response in which the phenotype adopted by an individual is contingent on the population density it experiences during its development (e.g. Long 1953; Pener 1991). In many locust, phasmid and Lepidopteran species, the 'phase' induced by crowding, often referred to as the 'gregaria' phase, is characterized by blackening or melanization of the cuticle, making these individuals darker and more conspicuous than 'solitaria' phase individuals.

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There have been many hypotheses to explain the adaptive value of melanism in the high-density phase, such as thermoregulatory benefits (Johnson *et al*. 1985; Goulson 1994; Gunn 1998) or a role in aposematic signalling (Iwao 1968; Wilson 2000). Thermoregulatory benefits have been identified for non-density-related melanism in the hoverfly *Episyrphus balteatus* (Marriott & Holloway 1998), but evidence in support of these hypotheses for density-dependent melanism is currently equivocal at best. The density-dependent prophylaxis (DDP) hypothesis provides an alternative, although not necessarily mutually exclusive, explanation for density-dependent melanism (Wilson & Reeson 1998). Maintaining an effective immune system is expected to be costly (Sheldon & Verhulst 1996). Therefore, individuals will benefit from increasing their allocation of resources to immune function when the risk of infection is high. The DDP hypothesis proposes that the increased levels of cuticular melanization observed in crowd-reared individuals is linked to disease resistance, such that crowded individuals invest more in immune function than those reared solitarily, in order to counter the increased risk of infection at high densities (Wilson & Reeson 1998).

However, immune function is expected to show condition-dependence, precisely because it is costly. Individuals in good condition, i.e. those with greater resources should be better able to mount an immune response than those in poor condition (Møller *et al*. 1998; Westneat & Birkhead 1998). In crowded conditions, resources are expected to be limited. If immune function is condition-dependent, increased investment in immunity under these circumstances should result in individuals having fewer resources to invest in other life-history traits.

Several studies have examined the effects of rearing density on pathogen resistance, particularly in Lepidopteran larvae. In these studies, resistance to entomopathogenic viruses tended to increase with rearing density both within (Kunimi & Yamada 1990; Goulson & Cory 1995) and across Lepidopteran species (Hochberg 1991). Although larvae of the cabbage moth, *Mamestra brassicae*, that were reared at exceptionally high densities, experienced an increase in susceptibility (Goulson & Cory 1995). The phenomenon has also been examined in the archetypal phase polyphenic species, the desert locust, *Schistocerca gregaria.* Solitaria locusts were significantly more susceptible to the entomopathogenic fungus *Metarhizium anisopliae* than gregaria locusts (Wilson *et al*. 2002).

In some studies, resistance to a specific pathogen has been compared between the colour phases while controlling for rearing density. Melanic *Mythimna separata* larvae were more resistant to an entomopathogenic fungus (Mitsui & Kunimi 1988) and a nucleopolyhedrovirus (NPV) (Kunimi & Yamada 1990); resistance to NPV was also higher in melanic *Spodoptera exempta* (Reeson *et al*. 1998). Cuticular melanism was found to be a better predictor of resistance to *Metarhizium anisopliae* than rearing density in the mealworm beetle *Tenebrio molitor*, as non-melanic beetles experienced significantly higher percentage mortality than melanic beetles – although melanic beetles were more common at high rearing densities (Barnes & Siva-Jothy 2000).

Despite the evidence that individuals reared at higher densities are relatively more resistant to disease, few studies have examined the underlying investment in immune function that is assumed to underpin this relationship. Moreover, those studies that have examined this investment have tended to concentrate on just one or two attributes of the immune system, rather than a comprehensive suite of immune traits.

The insect immune system comprises a relatively simple, yet effective, combination of cellular and humoral components, which work together to fight infection. Phenoloxidase (PO), a key enzyme in the synthesis of the melanin pigment that darkens the cuticle of gregarious phase insects, has been implicated in resistance to a range of pathogens in the haemolymph, midgut and cuticle (Rowley, Brookman & Ratcliffe 1990; Ourth & Renis 1993; Hagen, Grunewald & Ham 1994; Hung & Boucias 1996; Washburn, Kirkpatrick & Volkman 1996; Wilson *et al*. 2001). The density of haemocytes in the haemolymph is indicative of the ability to encapsulate metazoan parasites (Eslin & Prévost 1996; Fellowes & Godfray 2000; Kraaijeveld, Limentani & Godfray 2001; Wilson *et al*. 2003), and the antibacterial proteins produced by the fat body are important in the defence against entomopathogenic bacteria (Gillespie, Kanost & Trenczek 1997), as well as providing resistance against other microorganisms, such as microfilariae (Ham, Yang & Nolan 1996; Lowenberger *et al*. 1996). Therefore, there are many different components of the immune response that could be responsible for the observed levels of resistance in high-density individuals.

Reeson *et al*. (1998) found that gregarious phase African armyworm, *Spodoptera exempta*, were more resistant to NPV and had higher levels of haemolymph PO than their solitary-phase counterparts. Moreover, PO levels in the cuticle of gregarious *S. exempta* were higher than in solitary larvae, which corresponded to increased resistance to both an entomopathogenic fungus and an ectoparasitoid (Wilson *et al*. 2001). However, the two studies on non-Lepidopteran species (mealworm beetles and desert locusts) found no significant difference in PO activity between rearing densities (Barnes & Siva-Jothy 2000; Wilson *et al*. 2002), although gregarious-phase locusts were found to have significantly higher haemolymph antibacterial activity than solitary-phase locusts (Wilson *et al*. 2002). Although there is some evidence from the above studies for increased investment in certain immune components at high densities, the DDP hypothesis assumes implicitly that all traits associated with resistance to diseases that are transmitted in a positively density-dependent manner are simultaneously up-regulated in response to the increased threat of infectious disease at high densities.

To test this hypothesis, and the condition-dependence of immune function, we used larvae of the Egyptian cotton leafworm, *Spodoptera littoralis* Boisduval, a Lepidopteran species that exhibits density-dependent phase polyphenism. At low densities, larvae tend to be pale brown–grey but when reared at high densities most larvae develop into the dark form, which has a highly melanized cuticle (Hodjat 1970). Although phenotypic expression is determined mainly by rearing density, it also has a significant genetic component (e.g. Tojo 1991; Goulson 1994), such that when larvae are reared solitarily, some individuals develop into the dark, melanized phenotype, whereas when larvae are reared in groups some individuals remain pale and non-melanic, similar to the typical solitary phenotype. This allows us to determine the relative importance of rearing density and colour in explaining adaptive variation in immune function.

Thus, the aims of the present study were twofold; first to gain a better understanding of the improved resistance of melanic, gregaria phase larvae by assessing the relative effects of rearing density and larval colour on a suite of immune-related traits. Secondly, we wished to determine the role of condition-dependence in the allocation of resources to the immune system. By taking a multivariate approach to both the experimental design and the data analysis, we hoped to determine whether all disease resistance traits are up-regulated simultaneously in response to an increased threat of infection (as assumed by the DDP hypothesis and the concept of 'immunocompetence'; Owens & Wilson 1999), or if the expression of some immune function traits is down-regulated at high densities, which would indicate possible trade-offs between different components of the insect immune system.

Materials and methods

INSECT REARING

The *Spodoptera littoralis* culture was established from eggs collected near Alexandria in Egypt in 1998 and high numbers were maintained at each generation to reduce inbreeding. First instar larvae of *S. littoralis* were placed in 25 mL pots, either singly (the 'solitary' treatment) or in groups of three (the 'crowded' treatment). Larvae were provided with artificial diet *ad libitum* (Cotter 2002) and reared at 25 °C under a 12-light : 12-dark light regime until the final instar. Within each rearing treatment, larvae were scored for colour (pale, medium or dark), and the medium larvae were discarded to give 60 non-melanic ('pale') larvae and 60 melanic ('dark') larvae per rearing density.

IMMUNE FUNCTION TRAITS

Haemolymph samples were collected from 240 *S. littoralis* larvae and the following immune function traits were assayed simultaneously in all larvae: total haemocyte count, antibacterial (lysozyme-like activity) activity, haemolymph PO activity, cuticular PO activity and midgut PO activity (for details, see below). In 50% of these larvae, a small piece of nylon was inserted into the haemocoel to mimic infection by a metazoan parasite and to stimulate a cellular encapsulation response, which was then quantified (see below). This method has been used in many previous studies as it has been shown that insects respond to inert implants and parasites in the same way (Gorman *et al*. 1996). The remaining larvae were not exposed to this immune insult and so it was possible to establish whether the artificial parasite resulted in any of the haemolymph immune parameters becoming up-regulated. In addition to measuring immune function traits, we also weighed the larvae and assayed the total protein of their haemolymph in order to determine whether the magnitudes of the immune traits were affected by the body condition of the insects, as might be expected if variation in immunocompetence merely reflects variation in larval body condition (Moller *et al*. 1998; Westneat & Birkhead 1998).

HAEMOLYMPH SAMPLING

At the onset of the final instar, all the larvae were weighed and a haemolymph sample taken from each individual by piercing the final proleg with a fine needle and allowing the haemolymph to pool onto parafilm. Each haemolymph sample was divided between three Eppendorfs. For the haemocyte counts, 10 μL of haemolymph were added to 5 μL of EDTA anticoagulant in phosphate buffered saline (PBS, pH 7·4; Sambrook, Fritsch & Maniatis 1989) and 5 μL of glycerol to protect the haemocytes during storage in the freezer. For the PO and protein assays, 8 μL of haemolymph were added to 400 μL of PBS, and the remaining haemolymph was left undiluted for the antibacterial assays. All the samples were then frozen at –20 °C until they were to be measured. After the haemolymph was sampled, larvae were kept singly for 24 h after which time a second haemolymph sample was taken. The haemolymph was divided up as before and the larvae frozen.

HAEMOCYTE COUNTS

Eight μL of the EDTA/glycerol/haemolymph mixture was pipetted onto each side of a haemocytometer with improved Neubauer ruling. Five non-adjacent squares were counted on each side of the haemocytometer and summed to give an estimate of the haemocyte density of each individual.

ANTIBACTERIAL ASSAYS

Lytic activity against the bacterium *Micrococcus lysodeikticus* was determined using a lytic zone assay. Agar plates containing 10 mL of 1% agar with 5 mg per ml freeze-dried *M. lysodeikticus* (Sigma) were prepared as

described in Kurtz *et al*. (2000). For each plate, approximately 20 holes with a diameter of 2 mm were punched in the agar and filled with 70% ethanol saturated with phenylthiourea (PTU), which inhibits melanization of the haemolymph. After the ethanol had evaporated, 1 μL of haemolymph was placed in each well, two replicates per sample. The plates were incubated at 33 °C for 24 h then photographed using a Polaroid DMC digital camera and the diameter of the clear zones calculated using Image Pro Plus software (Media Cybernetics). Standard curves were obtained using a serial dilution of hen egg white lysozyme. Concentration of 'hen egg white lysozyme equivalents' was then calculated.

HAEMOLYMPH PO AND PROTEIN ASSAYS

Haemolymph PO was measured using a modified version of the method described in Cotter & Wilson (2002). Samples were incubated for 20 min at 25 °C and the absorbance was measured on a Versamax tuneable microplate reader (Molecular Devices Corporation, Sunnyvale, CA, USA) at 492 nm. Previous results have shown the reaction to be in the linear phase during this time period (Cotter 2002). Protein levels were also measured in the samples as described previously (Cotter & Wilson 2002).

REPEATABILITY OF HAEMOLYMPH PARAMETERS

It is important to establish that the measures of the different immune parameters are repeatable if they are to be used as an indication of an individual's investment in immunity. The repeatability, *r* (Lessells & Boag 1987) was determined both within and across days. Two haemolymph samples were taken from each individual, 24 h apart. For the PO and protein assays, three measurements were taken for each sample and for the haemocyte counts and lytic assays; two measurements were taken for each sample. This allowed the within-sample repeatability to be calculated for each assay, giving an estimate of the accuracy of the measurement techniques. Taking measurements from the same individual on two different occasions allowed the calculation of the between-day repeatability, providing an estimate of fluctuations in the parameters over time. Separate repeatabilities were also calculated for 'challenged' larvae (i.e. those that had been implanted with a small piece of nylon) and 'non-challenged' larvae (i.e. those that did not receive an implant).

MIDGUT AND CUTICULAR PO ASSAYS

Midgut and cuticular PO activity was also measured using a modified version of the method described in Cotter & Wilson (2002). The midgut and cuticle were dissected and fixed. One half of each midgut and cuticle was placed in 250 and 500 μL of 20 mM *l*-DOPA, respectively. Absorbance was measured at 492 nm after 30 min using a Versamax tuneable microplate reader (Molecular Devices Corporation). PO activity was expressed as PO units per gram of cuticle/midgut.

ENCAPSULATION ASSAYS

Half of the larvae in each treatment group had a piece of nylon monofilament (Orvis fishing line, diameter 0·5 mm) approximately 3 mm long, inserted into the haemocoel after the first haemolymph sample had been taken. The nylon implants were dissected out after the larvae had been frozen, and were then mounted on slides and photographed using a Polaroid DMC digital camera. The level of melanization and the area of cell cover were separately quantified using Image Pro-Plus software (Media Cybernetics) as described in Cotter & Wilson (2002). Both these measures have been shown previously to correlate with a visual assessment of the level of encapsulation (Cotter & Wilson 2002).

Results

REPEATABILITY OF HAEMOLYMPH PARAMETERS

All the haemolymph parameters we measured (PO activity, protein levels, haemocyte density and antibacterial activity) showed high within-sample repeatability on both days ($r > 0{\cdot}82$). The between-day repeatabilities were all highly significant and ranged from 0·30 to 0·71 ($P < 0{\cdot}001$; Table 1), with the lowest estimates of *r* being obtained for haemocyte density. There was no significant difference between the estimates of *r* obtained for the 'challenged' and the 'non-challenged' groups for any of the measured parameters (Table 1), indicating that this treatment did not affect the repeatability of the assays.

EFFECT OF 'CHALLENGE' ON IMMUNE PARAMETERS

First, we compared the levels of all immune parameters in the haemolymph (PO activity, antibacterial activity and haemocyte density) sampled from challenged and non-challenged larvae by testing the significance of the terms day, challenge, colour and rearing density and their interactions. Levels of all haemolymph immune parameters were lower on day 2 in the non-challenged larvae (Table 2). The only evidence for induction of immunity following the challenge to the immune system was for antibacterial activity, which increased significantly on day 2 in the challenged group (day × challenge interaction: $F_{1,454} = 13{\cdot}14$, $P < 0{\cdot}001$, Table 2). The other immune parameters decreased from day 1 to day 2, suggesting that these are not up-regulated over this time scale. All other interactions were not significant. In particular the change in immune parameters from day 1 to day 2 was independent of colour or rearing density. Therefore, the baseline levels (the measurements

Table 1. Effects of immune challenge on the repeatability of the haemolymph immune parameters. Between-day repeatabilities (± standard errors) were calculated for all measured haemolymph parameters (Lessells & Boag 1987). The repeatabilities represent a comparison of the mean values per sample obtained on day 1, with those obtained on day 2, for larvae that had received a nylon implant between each haemolymph sample (challenged) and for larvae that had not (non-challenged). The effects of the challenge to the immune system on the repeatability of each parameter were determined via *t*-tests. Repeatabilities were calculated on data after correcting for the variation between days in their mean values. NS, $P > 0{\cdot}05$; ***$P < 0{\cdot}001$

Repeatability	Haemocyte counts	Antibacterial activity	Haemolymph PO activity	Haemolymph protein
Between days: challenged	0·350 ± 0·080***	0·706 ± 0·046***	0·582 ± 0·060***	0·576 ± 0·061***
Between days: non-challenged	0·295 ± 0·084***	0·677 ± 0·050***	0·507 ± 0·068***	0·658 ± 0·052***
t-tests: challenged vs. non-challenged	$t_{238} = -0{\cdot}47$ NS	$t_{238} = -1{\cdot}46$ NS	$t_{238} = -0{\cdot}83$ NS	$t_{238} = -1{\cdot}02$ NS

Table 2. Effects of immune challenge on haemolymph immune parameters. The means (± standard errors) of each haemolymph immune parameter measured on each day for both the challenged and non-challenged treatment groups are given below. The significance of the terms day, challenge, colour and rearing density and their interactions were tested with ANOVAS. *F*-ratios and *P*-values of all significant interactions are reported below, all other interactions were not significant and so were removed from the model. NS, $P > 0{\cdot}05$; **$P < 0{\cdot}01$; ***$P < 0{\cdot}001$

		Haemocyte counts	Antibacterial activity	Haemolymph PO activity
Mean ± SE, non-challenged	Day 1	148·47 ± 4·84	1·45 ± 0·04	−0·69 ± 0·03
	Day 2	104·72 ± 3·99	1·30 ± 0·04	−0·85 ± 0·04
Mean ± SE, challenged	Day 1	142·42 ± 4·51	1·45 ± 0·03	−0·68 ± 0·03
	Day 2	85·91 ± 3·34	1·57 ± 0·04	−0·90 ± 0·04
F and *P*-values for each term in the model	Challenge	$F_{1,445} = 8{\cdot}69$**	$F_{1,454} = 11{\cdot}75$***	$F_{1,465} = 0{\cdot}32$ NS
	Day	$F_{1,445} = 141{\cdot}38$***	$F_{1,454} = 0{\cdot}27$ NS	$F_{1,465} = 30{\cdot}52$***
	Challenge: day	$F_{1,444} = 2{\cdot}30$ NS	$F_{1,454} = 13{\cdot}14$***	$F_{1,464} = 0{\cdot}75$ NS

Table 3. Correlations between immune parameters. Pearson's correlations between all the immune parameters using data from the first haemolymph sample only. To account for the problem of multiple testing, we used the method of Benjamini & Hochberg (1995) to control the false discovery rate, i.e. the expected proportion of false rejections within a class of rejected null hypotheses. Encapsulation traits, $n = 120$, $n = 240$ for all other traits. **$P < 0{\cdot}01$; ***$P < 0{\cdot}001$

	Haemolymph protein	Haemolymph PO	Midgut PO	Cuticular PO	Capsule melanization	Capsule size	Haemocyte density	Antibacterial activity
Larval weight	**0·39****	**−0·33****	0·05	**−0·49****	0·25	0·06	−0·05	−0·04
Haemolymph protein		−0·06	−0·03	**−0·26****	0·02	0·03	0·09	0·05
Haemolymph PO			0·01	**0·37****	0·18	−0·15	**−0·20****	−0·05
Midgut PO				−0·01	0·11	0·14	−0·04	0·06
Cuticular PO					0·01	−0·05	−0·12	−0·09
Capsule melanization						**0·25****	−0·11	0·19
Capsule size							0·04	−0·03
Haemocyte density								0·12

for day 1) of all the immune parameters were an adequate measure of an individual's investment in immune function and so were used in all subsequent analyses.

RELATIONSHIPS BETWEEN REARING DENSITY, LARVAL COLOUR AND 'CONDITION'

The first thing to be assessed was the effect of the two treatments (rearing density and larval colour) on 'condition', as measured by larval weight and haemolymph protein levels at the onset of the final instar. As these two traits were correlated positively (Table 3), the effects of rearing density and colour were analysed using multivariate analyses of variance (MANOVA). This allows multiple responses to be analysed as a single multivariate response, rather than a collection of univariate responses, so allowing the covariation between multiple measurements to be modelled explicitly. For statistically significant MANOVAS, univariate ANOVAS can then be performed for each response variable using sums of squares adjusted for the other dependent variables in the model.

The effects of rearing density and larval colour on body condition were highly significant (rearing density:

$F_{2,231} = 68{\cdot}68$, $P < 0{\cdot}001$; larval colour: $F_{2,231} = 14{\cdot}53$, $P < 0{\cdot}001$). Univariate ANOVAs showed that solitary-reared larvae (s) were significantly heavier than crowd-reared larvae (c) (means ± SE for larval weight: $-0{\cdot}73 \pm 0{\cdot}028$ (s), $-1{\cdot}04 \pm 0{\cdot}028$ (c), $F_{1,232} = 125{\cdot}63$, $P < 0{\cdot}001$; means ± SE for haemolymph protein: $-1{\cdot}63 \pm 0{\cdot}048$ (s), $-1{\cdot}70 \pm 0{\cdot}048$ (c), $F_{1,232} = 1{\cdot}59$, $P > 0{\cdot}05$) and pale larvae (p) were significantly heavier and had significantly higher haemolymph protein levels than dark larvae (d) (means ± SE for larval weight: $-0{\cdot}84 \pm 0{\cdot}028$ (p), $-0{\cdot}93 \pm 0{\cdot}028$ (d), $F_{1,232} = 9{\cdot}91$, $P = 0{\cdot}002$; means ± SE for haemolymph protein: $-1{\cdot}54 \pm 0{\cdot}048$ (p), $-1{\cdot}79 \pm 0{\cdot}048$ (d), $F_{1,232} = 27{\cdot}91$, $P < 0{\cdot}001$). There was no interaction between colour and density.

This suggests that solitary-reared and pale larvae were in better 'condition' than crowd-reared and dark larvae despite all larvae being provided with food *ad libitum*. This may be either a consequence of non-adaptive differences in the levels of competition experienced by larvae in the different treatment groups or due to adaptive responses by the larvae to their perceived levels of crowding. In order to examine both scenarios, the influence of rearing density and colour on the immune parameters was analysed with and without the statistical elimination of 'condition' (see below).

EFFECTS OF REARING DENSITY AND LARVAL COLOUR ON IMMUNE PARAMETERS

As there were significant correlations between many of the immune parameters (Table 3), the effect of rearing density and larval colour on the different measurements of immune function were analysed using MANOVA. Their effects on cellular encapsulation (capsule melanization and capsule size) were analysed separately, as only half the larvae received a nylon implant and the reduction in sample size would have reduced the power to detect the effects of rearing density and larval colour on the other immune parameters.

There were significant effects of rearing density and larval colour on the immune parameters (encapsulation traits: density: $F_{2,106} = 14{\cdot}97$, $P < 0{\cdot}001$; colour: $F_{2,106} = 3{\cdot}26$, $P = 0{\cdot}043$; non-encapsulation traits: density: $F_{5,204} = 7{\cdot}84$, $P < 0{\cdot}001$; colour: $F_{5,204} = 7{\cdot}97$, $P < 0{\cdot}001$; density × colour: $F_{5,204} = 3{\cdot}56$, $P < 0{\cdot}01$; Table 4). Accounting for the effects of 'condition' had little effect on the immune traits (Table 4).

After correcting for variation in body condition in the univariate analyses, there was a significant effect of rearing density on haemolymph PO activity (Fig. 1a), antibacterial activity (Fig. 1e) and capsule melanization (Fig. 1k) with levels of these immune parameters being significantly higher in solitary-reared larvae. Haemolymph PO activity and capsule melanization were higher in dark larvae (Fig. 1b,l), while antibacterial activity was significantly higher in pale larvae (Fig. 1f). There was no significant effect of rearing density or larval colour on cuticular PO activity (Fig. 1c,d), haemocyte density (Fig. 1g,h) or capsule size (Fig. 1i,j). The only significant interaction between rearing density and colour was for midgut PO activity (Fig. 1m). This was due to midgut PO levels in the crowded-pale group being much lower than any of the other three treatment groups.

For most of the immune parameters, correcting for the relationship with condition did not change the results. However, while corrected haemolymph PO activity was significantly higher in solitary larvae, uncorrected haemolymph PO activity did not differ significantly between rearing densities (Fig. 2a). Conversely, while there was no significant effect of rearing density or larval colour on the corrected data, cuticular PO was significantly higher in crowded and dark larvae for the uncorrected data (Fig. 2c,d).

Discussion

The main aim of the present study was to quantify variation in the allocation of resources to immunity in relation to rearing density and larval colour, to gain a better understanding of the basis for the increased resistance to parasites and pathogens of melanic, gregaria phase larvae reported in a number of previous studies (Mitsui & Kunimi 1988; Kunimi & Yamada 1990; Hochberg 1991; Goulson & Cory 1995; Reeson *et al*. 1998; Barnes & Siva-Jothy 2000; Wilson *et al*. 2001). Our results suggest that while some immune function traits are elevated in crowded and melanic larvae, others are not (see below).

Table 4. Effects of rearing density and larval colour on immune function: multivariate analyses. Results of the MANOVAs on immune function. 'Encapsulation parameters' refers to capsule size and capsule melanization and 'Other immune parameters' refers to haemocyte counts, antibacterial activity, haemolymph PO, midgut PO and cuticular PO. The two encapsulation measurements were analysed separately as their sample size was only half that of the other immune parameters. *F*-values for weight and protein are based on Type I sums of squares, *F*-values for the immune parameters are based on Type III sums of squares. Values in italics show results with weight and protein first in the model, i.e. the effects of colour and rearing density over and above the effects of condition. NS $P > 0{\cdot}05$; *$P < 0{\cdot}05$; **$P < 0{\cdot}01$; ***$P < 0{\cdot}001$

	F-values for each term in the model				
	Weight	Protein	Density	Colour	Density × colour
Encapsulation parameters	$F_{2,102} = 0{\cdot}08$ NS $F_{2,105} = \mathit{4{\cdot}04}$*	$F_{2,102} = 0{\cdot}16$ NS $F_{2,101} = \mathit{0{\cdot}74}$ NS	$F_{2,106} = 14{\cdot}97$*** $F_{2,105} = \mathit{10{\cdot}94}$***	$F_{2,106} = 3{\cdot}26$* $F_{2,105} = \mathit{3{\cdot}16}$*	$F_{2,101} = 0{\cdot}37$ NS $F_{2,101} = \mathit{1{\cdot}00}$ NS
Other immune parameters	$F_{5,204} = 15{\cdot}62$*** $F_{5,204} = \mathit{17{\cdot}87}$***	$F_{5,204} = 3{\cdot}95$** $F_{5,204} = \mathit{2{\cdot}07}$ NS	$F_{5,204} = 7{\cdot}84$*** $F_{5,204} = \mathit{8{\cdot}28}$***	$F_{5,204} = 7{\cdot}97$*** $F_{5,204} = \mathit{7{\cdot}17}$***	$F_{5,204} = 3{\cdot}56$** $F_{5,204} = \mathit{3{\cdot}56}$**

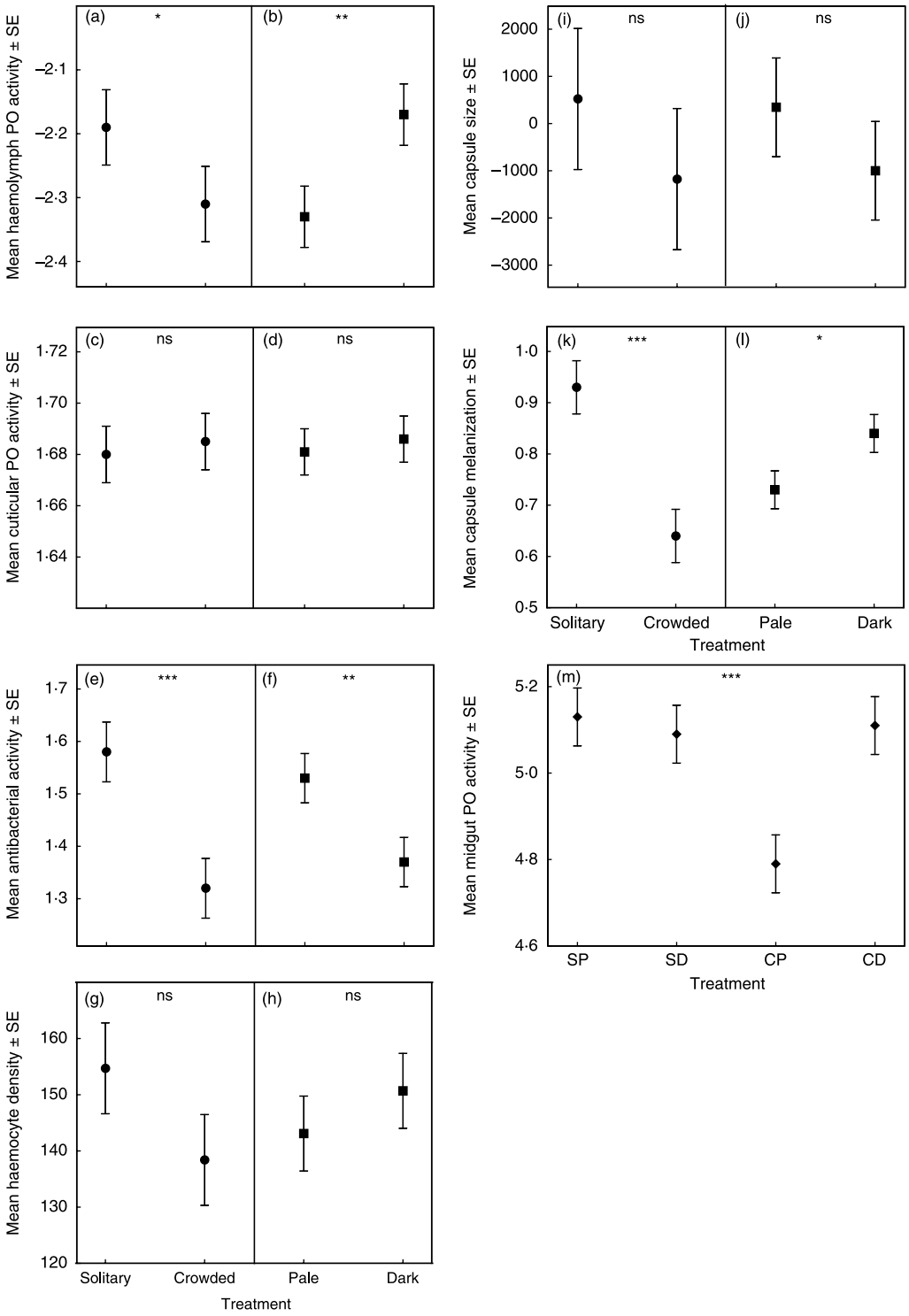

Fig. 1. Effects of rearing density and larval colour on the immune parameters, corrected for body condition. Variation in the immune parameters (after correcting for body condition) is plotted against rearing density (solitary or crowded) and larval colour (pale or dark). The bars represent the group means ± 1 SE, corrected for the other terms in the MANOVA model. The dependent variables were haemolymph PO, cuticular PO, antibacterial activity, haemocyte density, capsule size, capsule melanization and midgut PO; haemolymph protein and larval weight were included as explanatory variables along with rearing density and colour. The results of the univariate analyses for rearing density and larval colour were as follows: rearing density: (a) haemolymph PO: $F_{1,208} = 4{\cdot}09$, $P = 0{\cdot}044$; (c) cuticular PO: $F_{1,208} = 0{\cdot}08$, $P > 0{\cdot}05$; (e) antibacterial activity: $F_{1,208} = 20{\cdot}64$, $P < 0{\cdot}001$; (g) haemocyte density: $F_{1,208} = 3{\cdot}71$, $P = 0{\cdot}055$; (i) capsule size: $F_{1,106} = 1{\cdot}33$, $P > 0{\cdot}05$; (k) capsule melanization: $F_{1,106} = 22{\cdot}09$, $P < 0{\cdot}001$; larval colour: (b) haemolymph PO: $F_{1,208} = 10{\cdot}28$, $P = 0{\cdot}002$; (d) cuticular PO: $F_{1,208} = 0{\cdot}31$, $P > 0{\cdot}05$; (f) antibacterial activity: $F_{1,208} = 9{\cdot}86$, $P = 0{\cdot}002$; (h) haemocyte density: $F_{1,208} = 1{\cdot}33$, $P > 0{\cdot}05$; (j) capsule size: $F_{1,107} = 0{\cdot}79$, $P > 0{\cdot}05$; (l) capsule melanization: $F_{1,107} = 4{\cdot}34$, $P = 0{\cdot}040$. Midgut PO (m) was the only immune parameter for which there was a significant interaction term; rearing density: $F_{1,208} = 8{\cdot}37$, $P = 0{\cdot}004$, larval colour: $F_{1,208} = 9{\cdot}76$, $P = 0{\cdot}002$, interaction term: $F_{1,208} = 12{\cdot}59$, $P < 0{\cdot}001$. Thus, all four treatment group combinations are shown: solitary-pale (SP), solitary-dark (SD), crowded-pale (CP) and crowded-dark (CD). The F-values are based on type III sums of squares with condition terms first in the model. NS $P > 0{\cdot}05$; *$P < 0{\cdot}05$; **$P < 0{\cdot}01$; ***$P < 0{\cdot}001$.

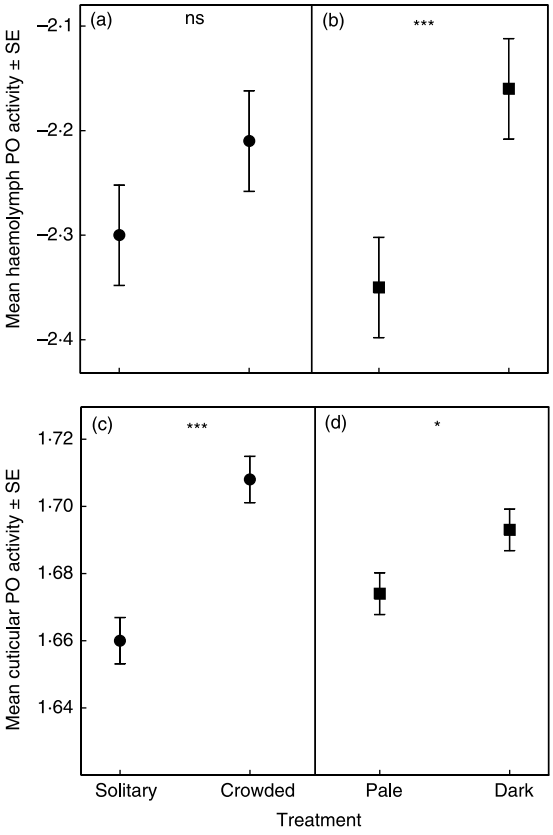

Fig. 2. Effects of rearing density and larval colour on the immune parameters, not corrected for condition. Variation in haemolymph and cuticular phenoloxidase activity is plotted against rearing density (solitary or crowded) and larval colour (pale or dark). The bars represent the group means ± 1 SE, corrected for the other terms in the MANOVA model. The dependent variables were haemolymph PO, midgut PO, cuticular PO, antibacterial activity and haemocyte density. Haemolymph protein and larval weight were included as explanatory variables along with rearing density and colour. The results of the univariate analyses for rearing density and larval colour were as follows: rearing density: (a) haemolymph PO: $F_{1,208} = 3{\cdot}64$, $P = 0{\cdot}057$; (c) cuticular PO: $F_{1,208} = 23{\cdot}80$, $P < 0{\cdot}001$; larval colour: (b) haemolymph PO: $F_{1,208} = 15{\cdot}00$, $P < 0{\cdot}001$; (d) cuticular PO: $F_{1,208} = 4{\cdot}95$, $P = 0{\cdot}027$. The F-values are based on type I sums of squares with condition terms last in the model. NS, $P > 0{\cdot}05$; *$P < 0{\cdot}05$; ***$P < 0{\cdot}001$.

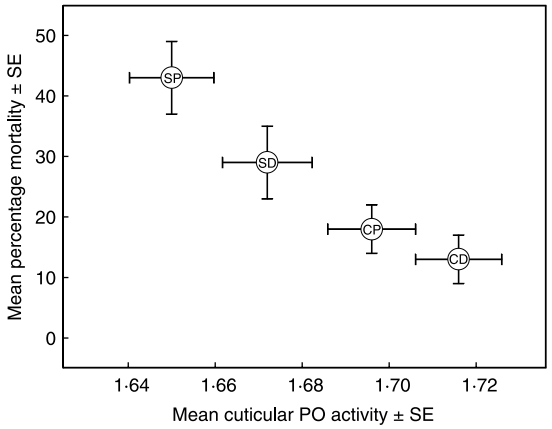

Fig. 3. Relationship between cuticular PO activity and resistance to fungal infection. The figure shows the relationship between susceptibility to fungal infection and levels of cuticular PO activity across treatment groups: solitary-pale (SP), solitary-dark (SD), crowded-pale (CP) and crowded-dark (CD). The proportion of larvae dying from fungal infection after exposure to *Beauvaria bassiana* was averaged over two fungal doses. The bars represent the group mean ± 1 SE. Fungus-induced mortality data are taken from Wilson *et al.* (2001).

CONDITION-DEPENDENT IMMUNE FUNCTION

We found additive effects of rearing density and larval colour on the suite of immune traits we measured. However, despite there being marked differences in body condition between the different treatment groups, statistically eliminating the effects of body condition in our analyses had little effect on the overall results. Dark and crowd-reared larvae had significantly higher levels of cuticular PO activity than pale and solitary-reared larvae. Moreover, there was a strong correspondence across treatment groups between the cuticular PO levels observed in the present study and the levels of resistance to a fungal pathogen observed by Wilson *et al.* (2001), in which survival after exposure to *Beauvaria bassiana* was highest in the crowded-dark group and lowest in the solitary-pale group (Fig. 3). This suggests that active PO in the cuticle may inhibit penetration of the cuticle by fungal hyphae (Gillespie *et al.* 2000). Alternatively, it may reflect the correlation between cuticular PO activity and haemolymph PO activity (Table 3).

After correcting for body condition, the differences in cuticular PO activity between the four treatment groups disappeared. However, it must be noted that the relationship between cuticular PO and body condition was negative (Table 3). In other words, larvae in good condition had reduced levels of PO activity in their cuticles. It is notable that haemolymph PO, the only other trait for which the results changed after accounting for differences in body condition, was also correlated negatively with body condition. Of the seven immune traits examined, only these two were related to body condition. Several studies examining the condition-dependence of immune function in vertebrates have found evidence for positive covariation between measures of immunity and body condition (Birkhead, Fletcher & Pellatt 1998; Gonzalez *et al.* 1999; Alonso-Alvarez & Tella 2001; Moller & Petrie 2002). We know of no studies using invertebrates that have examined condition-dependence of immunity in this manner. However, a study using *Bombus terrestris* found that inducing the antibacterial response in workers reduced their survival relative to controls, but only under conditions of starvation, suggesting that the costs of immune system activation are condition-dependent in this species (Moret & Schmid-Hempel 2000).

A positive relationship between condition and immune function is expected only if the variation in resource acquisition is greater that variation in resource allocation (van Noordwijk & de Jong 1986; Houle 1991). If

this is not the case then the trade-off between allocation of resources to condition or to immune function may become apparent. Therefore, it may be that crowded and dark larvae are in poorer condition precisely because they are diverting resources from growth to immune function. However, it would be interesting to see if these results held under conditions of food shortage, as may happen in natural conditions.

DENSITY-DEPENDENT PROPHYLAXIS

The density-dependent prophylaxis (DDP) hypothesis predicts an overall increase in immune function associated with the density of conspecifics. This study provides evidence that *S. littoralis* exhibits prophylactic investment in some disease resistance mechanisms but not others. We found that crowd-reared larvae had significantly higher cuticular PO activity than solitary-reared larvae. In addition, dark individuals (i.e. high-density phenotypes) had significantly higher PO activity in the haemolymph and cuticle and produced a stronger melanization response to an artificial parasite than pale larvae, which is broadly consistent with the DDP hypothesis and in agreement with results from previous studies on the closely related noctuid, *Spodoptera exempta* (Reeson *et al*. 1998; Wilson *et al*. 2001). However, contrary the DDP hypothesis, there was no significant difference between treatment groups in either their total haemocyte counts or the size of the cellular capsule surrounding an artificial parasite. More significantly, perhaps, both crowded and dark larvae exhibited significantly lower antibacterial activity. This suggests that larvae may be unable to invest in all types of immune response simultaneously and that there may be a phenotypic trade-off between antibacterial activity and PO activity, such that increased investment in one results in a decrease in the other.

Conclusions

This study provides evidence for the DDP hypothesis in that crowd-reared and dark larvae invest more in the phenoloxidase-related traits: haemolymph and cuticular PO and capsule melanization. However, it is unclear whether melanization of the cuticle is a by-product of this up-regulation of PO activity or if it has other adaptive benefits such as thermoregulation, which could also play a role in combating parasites (Blanford, Thomas & Langewald 1998; Wilson *et al*. 2002; Thomas & Blanford 2003). Contrary to the popular notion that an individual can invest in all aspects of immunity simultaneously (i.e. 'immunocompetence' (Folstad & Karter 1992; Sheldon & Verhulst 1996; Owens & Wilson 1999; Lochmiller & Deerenberg 2000)), these results suggest that there may be trade-offs *within* the immune system that may constrain this. It is widely accepted that innate immunity should be costly, although it has been argued that specific forms of immunity may be cost-free (Rigby, Hechinger & Stevens 2002). Many studies have demonstrated life-history costs associated with increased resistance but, with the exception of a few well-studied vertebrate models (Grencis 1997; Gehad *et al*. 1999; Ibanez *et al*. 1999; Gill *et al*. 2000; da Silva *et al*. 2001), most have overlooked the possible costs associated with reduced investment in other types of immune function. Trade-offs within the immune system would have important ramifications for evolutionary theories based around the concept of immunocompetence (Folstad & Karter 1992; Hamilton & Zuk 1982; Sheldon & Verhulst 1996). An analysis of the quantitative genetic architecture of the immune system is therefore required to clarify this situation.

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