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Population density and immune investment in different developmental stages of *Tenebrio molitor*

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Abstract

Immune responses are costly, so organisms should invest in immunity when environmental cues indicate a high risk of infection. The density-dependent prophylaxis (DDP) hypothesis proposes that because the risk of infection increases with population density, organisms should invest more in immunity under crowded conditions. The aims of this study were to investigate DDP in the mealworm beetle *Tenebrio molitor* and to assess whether density-dependent immune investment differs between developmental stages. *T. molitor* larvae weighing 50-130mg were assigned to either a low-, moderate-, or high-density treatment for 31 days. After which the phenoloxidase (PO) activity, encapsulation and capsule melanisation responses, and antimicrobial peptide (AMP) activity of any larvae, pupae and adults were measured. Development, adult phenotype, PO activity, AMP activity and capsule melanisation were unaffected by population density. Additionally, the encapsulation response declined with population density, coinciding with higher mortality in the high-density treatment than in the low- and moderate-density treatments. While there were no interactions between population density and developmental stage in determining immune investment, there were complex relationships between developmental stage and immune investment. PO activity of larvae was much higher than pupae or adults, whereas larval AMP activity was significantly higher than that of pupae, but not adults. Measures of encapsulation and capsule melanisation, however, were higher in pupae than in larvae. These results reflect the multifaceted nature of insect anti-pathogen defences and the intricacies of DDP in insects, and indicate a possible trade-off between the encapsulation response and competitive ability in *T. molitor*.

Introduction

Many organisms, in particular insects and especially insect pests of agricultural crops and stored products, experience significant fluctuations in population density during all or part of their life cycle (Nelson et al., 2013). Therefore, if the fitness costs or benefits conferred to an organism via the expression of a particular trait varies with the local conspecific population density, one would expect that trait to have evolved to be phenotypically plastic to population density, provided the organism has means to assess this (Via et al., 1995).

An organism that undergoes plastic phenotypic changes at high conspecific population densities displays density-dependent phase polyphenism. An organism's phenotype may be altered in many ways by their perception of conspecific population density, the most obvious being an overt colour change, as occurs in many species of Lepidoptera, Coleoptera and Orthoptera (Applebaum and Heifetz, 1999). For example, the nymphs of the desert locust, *Schistocerca gregaria* (Orthoptera: Acrididae), exist as a conspicuous yellow and black form under high conspecific population density and a cryptic green form under low population density (Wilson and Cotter, 2008). In addition to colour, other traits that show density-dependent expression include wing size, abundance of antennal sensilla, adult weight, adult longevity, developmental rate, and behaviour (Applebaum and Heifetz, 1999; Rosa et al., 2017; Barnes and Siva-Jothy, 2000; Simpson et al., 2001).

One trait that has been repeatedly shown to display density-dependence in insects is immune investment (Wilson and Cotter, 2008). The immune system of insects, while simpler than that of vertebrates due to their sole reliance on innate immune mechanisms, is complex and involves both constitutive and induced defences. One of the first lines of anti-pathogen defence are physical barriers that prevent pathogens from entering the haemocoel (the main body cavity). The primary physical barrier is the cuticle – a chitinous and hydrophobic material which can become thick and strong when sclerotised. The cuticle forms the insect exoskeleton and lines the foregut, hindgut and tracheal system. Even ingested pathogens must first cross both the cellular epithelium of the midgut and a non-cellular, chitinous peritrophic membrane before entering the haemocoel (Siva-Jothy et al., 2005). Pathogens that breach these physical barriers and enter the haemocoel, midgut or internal organs, elicit a variety of immune responses evolved to control and destroy infectious pathogens. Insects have a large repertoire for pathogen control and destruction, which can be broadly divided into eight non-mutually exclusive mechanisms: melanisation, encapsulation, phagocytosis, antimicrobial peptides (AMPs), nodulation, autophagy, apoptosis and RNA interference (RNAi; Hillyer, 2016)—the latter three being general responses to intracellular pathogens. Melanisation is an enzymatic process with roles in immunity, cuticle sclerotization, egg tanning, and wound healing (Nappi and Christensen, 2005; Cerenius et al., 2008). Pathogen death via melanisation occurs through the production of reactive species including superoxide and hydrogen peroxide (Gonzalez-Santoyo and Cordoba-Aguilar, 2012) and through starvation as the pathogen is isolated from the nutrient-rich haemolymph (Hillyer, 2016). Though many enzymes are involved in melanisation (Fig. 1), phenoloxidase (PO) is one of the most crucial and the one most commonly measured. Encapsulation involves the aggregation of haemocytes (the primary immune cells of invertebrates) around foreign material too large to be phagocytosed, which is often followed by the melanisation of the encapsulated structure (Hillyer, 2016). While some of the above immune mechanisms, including melanisation, show

constitutive and/or inducible activity, AMPs (secreted proteins usually between 2-20 kDa in mass with potent antibiotic activity; Schmid-Hempel, 2003) are produced only after pattern recognition receptors (PRRs) bind pathogen-associated molecular patterns (PAMPs; e.g. peptidoglycan or lipopolysaccharide). Often AMPs are specific to certain types of pathogens, for example, Coleopterans produce Defensin and Defensin-like peptides, which are generally effective against Gram-positive bacteria (though some are effective against Gram-negative bacteria; Hillyer, 2016). In addition to the aforementioned mechanisms, coagulation—the formation of an insoluble matrix within the haemolymph that principally stops bleeding and maintains haemocoelic turgor, likely also has roles in wounding healing and protection against infection (Barnes, 2000; Dushay, 2009). It should be noted, however, that the first line of anti-pathogen defence is arguably behavioural, rather than physical or immunological. For example, individuals may disperse away from dense aggregations and populations likely to have a high parasite load, and behavioural thermoregulation in insect hosts can elevate their body temperature to the detriment of specific parasites (Siva-Jothy et al., 2005).

The theory behind density-dependent immune investment, termed the density-dependent prophylaxis (DDP) hypothesis (Reeson et al., 1998), is relatively simple. Because it can usually be assumed that pathogens show density-dependent transmission (though some diseases may show density-independent, i.e. frequency-dependent, transmission; McCallum et al., 2001), the per capita risk of infection should increase with population density. Therefore, because investment in immunity is costly (Schmid-Hempel, 2003; Sheldon and Verhulst, 1996), natural selection should favour individuals using population density (or any other environmental condition associated with a high risk of infection) as a cue for infection risk and as a determinant of the need for immune investment. As such, immune investment should be phenotypically plastic to the local population density (i.e., the perceived risk of infection).

Evidence of DDP in insects has been forthcoming from a number of studies, mostly on Lepidopterans, with comparatively less on Orthopterans and Coleopterans and few on other insect taxa (Wilson and Cotter, 2008). For example, crowded *Spodoptera exempta* (Lepidoptera: Noctuidae) larvae become heavily melanised and show greatly increased resistance to a nuclear polyhedrosis virus when compared to isolated larvae, which is partially explained by heightened PO activity in melanic larvae (Reeson et al., 1998). The extremely phase-polyphenic desert locust, *S. gregaria*, shows increased resistance to the entomopathogenic fungus *Metarhizium anisopliae* and elevated antimicrobial activity when reared at high population density (Wilson et al., 2002), though it does not show any changes in PO activity, total haemocyte count, or encapsulation.

While the aforementioned studies and many others clearly show adaptive prophylactic responses to population density, many others illustrate the intricacies of DDP in insects. For example, Silva et al. (2013) found changes in colour phenotype (increased frequency of cuticular melanisation) as well as increases in encapsulation and haemocyte density in crowded *Anticarsia gemmatalis* (Lepidoptera: Noctuidae) larvae. However, after comparing solitary larvae with larvae reared at two larvae/jar, and larvae reared at two, four and eight larvae/jar, they found that the increased frequency of melanic individuals, as well as the upregulation of encapsulation (but not haemocyte count) were better explained by the presence of conspecifics rather

than the density *per se*. Kong et al. (2018), however, appear to show the opposite, with *Mythimna separata* (Lepidoptera: Noctuidae) larvae showing higher PO activity in accordance with population density. Studies of DDP often fail to consider the issue of disentangling the presence of conspecifics from the population density, which could have major implications for the practical use of any results obtained (e.g., to biocontrol strategies), and further investigation into this area is required.

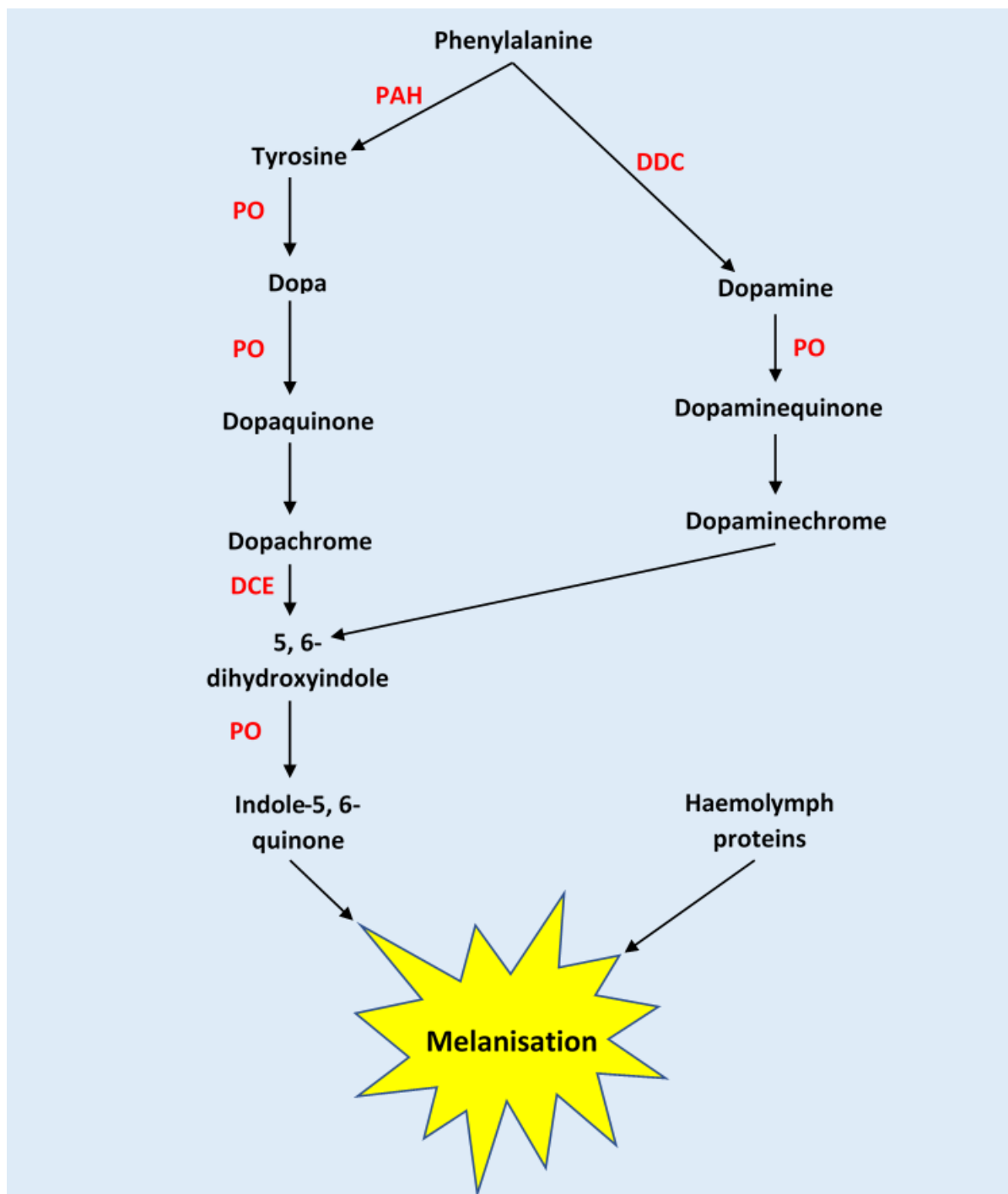


Figure 1: Simplified schematic of the melanisation pathway in insects, adapted from Hillyer (2016) and González-Santoyo & Córdoba-Aguilar (2011). Also included is the role of phenylalanine and the rate-limiting substrate of the melanisation pathway, tyrosine. PAH, phenylalanine hydroxylase; PO, phenoloxidase; DDC, dopa decarboxylase; DCE, dopachrome conversion enzyme.

The sensory information involved in initiating DDP has not been directly investigated, but the sensory basis of phase changes in insects displaying density-dependent phase polyphenism, particularly *S. gregaria*, has been investigated, and has been determined to be based mostly on tactile stimulation (Roessingh et al., 1998; Simpson et al., 2001). A tactile basis for the initiation of DDP could explain any changes in immune investment caused by the presence of conspecifics alone, as a single conspecific could potentially provide enough tactile stimulation to produce density-dependent physiological changes. Sensory information indicative of population density may be relayed to the immune system via the biogenic amine octopamine, as octopamine levels in *Mythimna separata* larvae increase with rearing density, and injection of larvae with the octopamine antagonist epinastine reduced PO activity and total haemocyte count (Kong et al., 2018). However, it should be noted that there is likely significant interspecific variation in both the required stimulus and the mechanism of relaying that stimulus to the immune system.

In addition to complexities in disentangling the presence of conspecifics from the population density, in several studies various immune parameters have been shown to decrease with population density. In many cases this is likely due to trade-offs with other immune responses, for example Silva et al. (2013) found that increased haemocyte count and encapsulation in crowded *A. gemmatalis* larvae was accompanied with decreased capsule melanisation. However, there often appears to be non-adaptive immune responses to population density, especially when the density is unnaturally high. For example, resistance to a nuclear polyhedrosis virus in larvae of the cabbage moth, *Mamestra brassicae* (Lepidoptera: Noctuidae) was higher in moderately crowded larvae (2-10 larvae/56ml plastic cup) than in solitary larvae (1 larvae/56ml plastic cup), but was lowest in very crowded larvae (20 larvae/56ml plastic cup), despite the fact that very crowded larvae showed the highest degree of cuticular melanisation (Goulson and Cory, 1995).

In most studies of DDP in insects it is generally assumed that density-dependent immunological changes occur early in development. However, few studies have investigated the critical developmental periods involved, and none have directly investigated density-dependent changes in immune investment in the different developmental stages of holometabolous insects (i.e., none have investigated population density x developmental stage interactions in determining immune investment). Though in recent years there has been an interest in the interaction between population density and various other life history characteristics (e.g. sex; McNamara and Simmons, 2017), and environmental conditions (e.g. temperature; Silva and Elliot, 2016), in determining immune investment.

The mealworm beetle *Tenebrio molitor* (Coleoptera: Tenebrionidae) is an ideal model organism to study DDP. It is moderately phase polyphenic and displays extra-larval moults, reduced development time, increased adult weight, increased mortality and higher levels of cannibalism under high population density (Barnes and Siva-Jothy, 2000, Weaver and McFarlane, 1990). Additionally, *T. molitor* reared at high density display increased cuticular melanisation and resistance to the generalist insect pathogen *M. anisopliae* when compared to individuals reared in solitude, though it is unknown if they also display upregulated physiological or behavioural immune responses (Barnes and Siva-Jothy, 2000). Many Tenebrionids, including *T. molitor*, are also serious stored product pests, for example the red flour beetle

Tribolium castenum (Vigneron et al., 2019; Suiter et al., 2017). As such, studying DDP in *T. molitor* presents an opportunity both to study DDP in an organism likely to respond adaptively to population density (based on the predictions of the DDP hypothesis), and to consider the potential implications of any findings to biocontrol strategies. Also, because *T. molitor* is holometabolous, it is possible to investigate whether immune responses to population density vary between developmental stages. The aims of this study were to investigate the immune responses of *T. molitor* to conspecific population density, and to investigate whether density-dependent immune investment varies according to developmental stage (i.e. to determine whether there is an interaction between population density and developmental stage in determining immune investment). To achieve this, PO activity, AMP activity, the encapsulation response and the degree of capsule melanisation were investigated in populations of *T. molitor* maintained at low, moderate and high conspecific population density.

Materials and Methods

Experimental set-up

T. molitor larvae weighing 50-130mg were taken from a mixed colony maintained at the University of Plymouth and assigned randomly to one of three population density treatments, each consisting of 43 mealworms in differing amounts of bran: low-density (23.26g bran mealworm⁻¹), moderate-density (4.65g bran mealworm⁻¹), and high-density (2.33g bran mealworm⁻¹). All treatments were assigned 30g of apple at the start of the experiment as a source of water and were maintained in identical unlidded containers for 31 days, after which larvae and pupae were transferred randomly to one of two testing groups: encapsulation and capsule melanisation, or PO and AMPs. This was necessary as activation of the immune system during the encapsulation and capsule melanisation tests could have interfered with the results of the PO and AMP tests. Adults were observed for differences in body colour before being transferred solely to the PO and AMP testing group due to their incompatibility with the encapsulation and capsule melanisation test.

Encapsulation and capsule melanisation

Larvae and pupae were weighed and inserted with nylon filaments (length 3mm, diameter 0.3mm) between sternites five and six. After 24h, the filaments were carefully removed and photographed at two different angles using a Jiusion Digital Microscope, and the images were analysed in ImageJ. Each image was thresholded using an arbitrary grey value cut-off point derived from the mean grey value frequency distribution of unencapsulated filaments (122; Fig. 2) in order to separate the intensity of the darkening (representing the degree of capsule melanisation, though influenced by encapsulation and coagulation) from the area (representing the degree of encapsulation, though influenced by the coagulation response). After thresholding, the mean grey value and area of the thresholded region were measured. Grey values derived using this method are initially on a scale of 0-122, 0 being pure black and 122 being the grey value threshold. For ease of interpretation, mean grey values were transformed to a scale of 0-100, henceforth termed the capsule melanisation index, where 100 is pure black and 0 is the grey value cut-off point. As a means of standardisation, the area of the thresholded region was expressed as a percentage of the area of the entire filament (henceforth termed the encapsulation area).

Preparation of extracts for phenoloxidase and antimicrobial peptide tests

Individual *T. molitor* were frozen at -20°C, weighed, and homogenised in 100mM Hepes buffer (pH 6.9) at a ratio of 4:1 (v/w). Homogenates were centrifuged at 16 700 g_{av} for 5 minutes at 4°C, after which the supernatants were carefully removed and flushed gently with oxygen-free nitrogen for 30 minutes in order to prevent surface darkening of the extract via melanin formation while allowing for activation of phenoloxidase. After flushing with nitrogen, the extracts were frozen at -80°C.

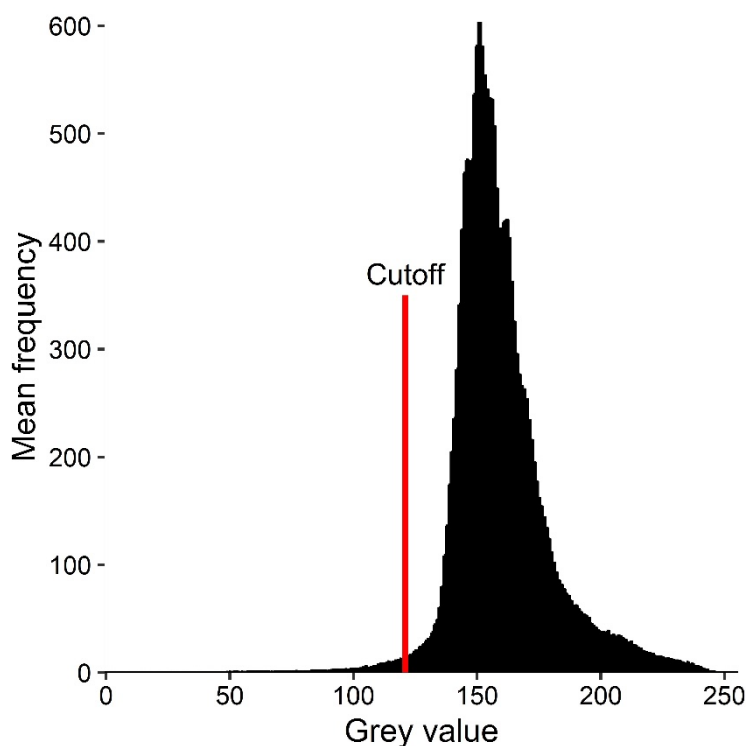


Figure 2: Mean frequency distribution of grey values of 16 control (unencapsulated) filaments, with the derived grey value cutoff point highlighted. Cutoff = mean - 2 × standard deviation = 122.

Phenoloxidase activity

PO activity was assessed by following the production of dopachrome (Fig. 1) using a protocol developed by Moody and Billington (*pers. comm.*). 10 μ l of extract was added to 240 μ l of 100 mM sodium phosphate buffer (pH 6.9) containing 5.2 mM L-DOPA (prepared ten minutes prior to each assay). The absorbance of the reaction mixture was measured every 30s for 15 minutes at 367nm, 475nm and 600nm using a Spectramax 190 plate reader (Molecular Devices). The plate reader was set to shake the plates every 3s to ensure sufficient oxygenation of the wells. In an attempt to reduce interference from the intermediates of melanisation and from melanin itself, an adjusted A_{475} was calculated from the wavelength triplet:

$$A_{475} (adjusted) = A_{475} - (A_{600} + 0.54(A_{367} - A_{600}))$$

PO activity was then derived from the initial, linear part of the reaction using the extinction coefficient of dopachrome at this wavelength triplet ($3.29 \text{ mM}^{-1} \text{ cm}^{-1}$; Moody and Billington, *pers. comm.*).

Antimicrobial peptide activity

AMP activity was measured using a modified version of a standard protocol (Rustage, 2015). In order to reduce the risk of contamination, all extracts were transferred to sterile centrifuge tubes and centrifuged at 6000 g_{av} for 5 minutes. The

supernatants were then removed and used for the assay. *Arthrobacter globiformis* was grown in sterile Mueller-Hinton broth, and adjusted to give an $OD_{595} \approx 0.2$ (approximately 10^8 CFU ml^{-1}). 100 μ l amounts of broth culture were mixed with 10ml amounts of sterile Mueller-Hinton agar and poured into 9cm sterile petri dishes, which were swirled in order to create a thin layer of agar and ensure even dispersal of bacteria. After which seven evenly spaced 3mm wells were created. 3 μ l of extract was added to each well, bar the seventh which contained 3 μ l of tetracyclin ($125mg\ ml^{-1}$) as a positive control (Fig. 3). As a negative control, one plate contained six wells of sterile 100mM Hepes buffer (pH 6.9). Each plate was incubated at 25°C for 24h, after which the zone of clearance around each well was measured at the widest and narrowest points. As a means of standardisation, the AMP activity of each extract was expressed as a percentage of the zone of clearance produced by the corresponding positive control.

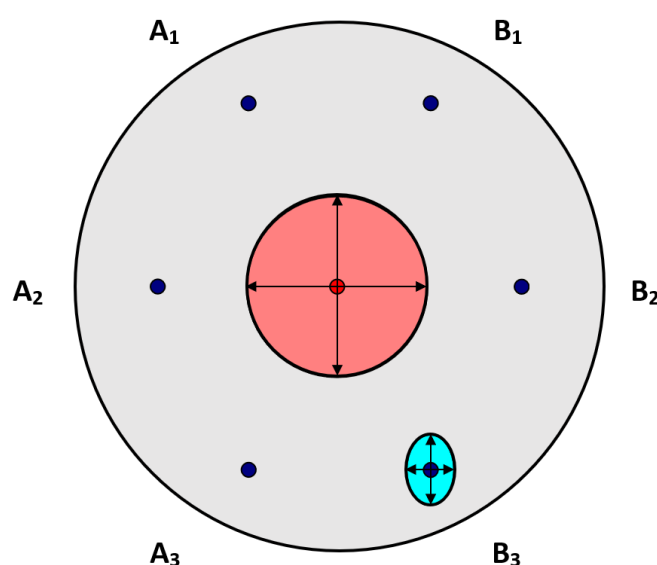


Figure 3: Layout of plates used in the antimicrobial peptides assay. All plates were 9cm in diameter and contained 10ml Mueller-Hinton agar mixed with 100 μ l *A. globiformis* broth culture (see section 2.5). Blue circles represent the positions of the samples (A, sample 1; B, sample 2; replicates in subscript), the central red circle represents the position of the positive control. Light blue and red circles represent the zones of clearance produced by the extracts and the positive control, respectively.

Statistical analysis

All analyses were conducted in R v.3.5.2. All frequency data were analysed using Pearson's Chi-squared tests. Unless otherwise stated, all remaining data were analysed using general linear models (GLMs), with population density and developmental stage included as an interaction term and body weight as a covariate. Model residuals were checked for normality using Anderson-Darling tests. Reported F and P values were obtained from the *car* package's *Anova()* function (specifying type II SS). Inter-treatment and inter-developmental stage differences were analysed using Tukey HSD post-hoc tests. Data reported in the text are means \pm standard error.

Ethics statement

No formal ethical approval was required for this study. Nevertheless, effort was made to ensure that all invertebrates used in this study were not subjected to unnecessary suffering and harm. Proceeding their use in this study, all larvae, pupae and adults were disposed of by freezing at -20°C.

Results

Development and mortality

Mortality was significantly higher in the high-density treatment than in the moderate- and low-density treatments ($\chi^2_{[2]}=6.99$, $P=0.031$; Table 1). Individuals in the low-, moderate- and high-density treatments did not appear to show altered development in response to their respective population densities, as evidenced by the findings that the relative proportions of larvae, pupae and adults ($\chi^2_{[4]}=1.89$, $P=0.755$; Fig. 4A), and adult weight ($F_{2, 23}=1.87$, $P=0.176$; one-way ANOVA; Fig. 4B) did not differ between treatments.

Table 1: Percentage mortality of *T. molitor* populations previously maintained for 31 days at low (23.26g bran/individual), moderate (4.66g bran/individual) and high (2.33g bran/individual) population density.

Population density	Mortality (%)
Low	7.0
Moderate	7.0
High	23.3

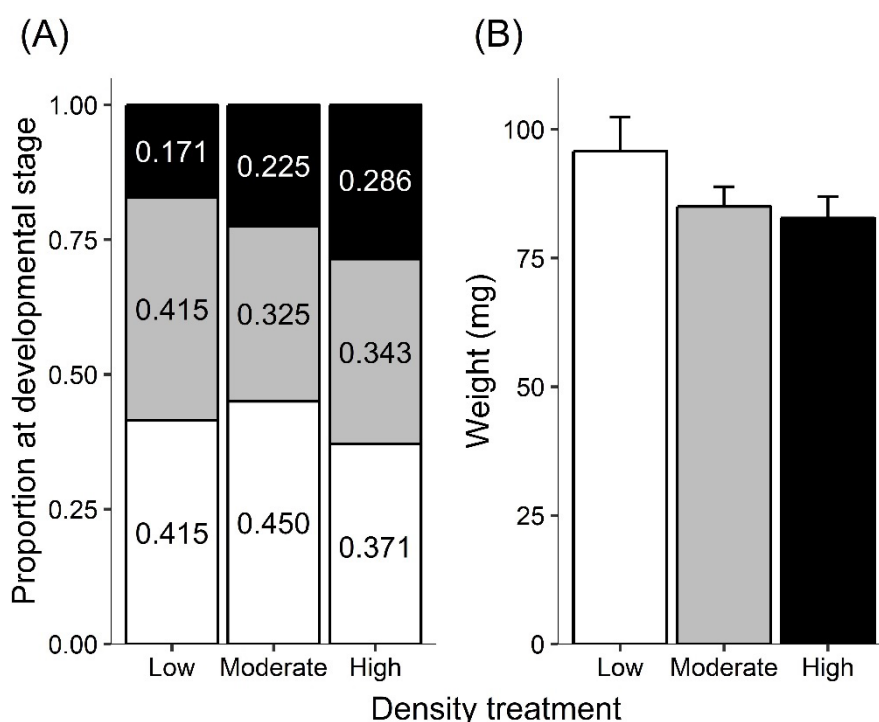


Figure 4: (A) Relative proportions of *T. molitor* larvae (white bars), pupae (grey bars) and adults (black bars) from populations of *T. molitor* previously kept for 31 days at low (23.26g bran/individual), moderate (4.65g bran/individual) and high (2.33g bran/individual) population density. Annotations represent exact proportions rounded to 3 d.p. (B) Weight of adults from populations of *T. molitor* previously kept for 31 days at low (white bar; 23.26g bran/individual), moderate (grey bar; 4.65g bran/individual) and high (black bar; 2.33g bran/individual) population density. Data are means \pm standard error.

Adult phenotype

Three adult phenotypes were clearly identified based on body colour: tan, brown and black. While tan beetles only appeared in the low-density treatment, the majority of adults in each treatment displayed the black phenotype (57.1%, 88.9% and 87.5% of adults in the low-, moderate- and high-density treatments, respectively), and the relative proportions of each phenotype did not significantly differ between treatments ($\chi^2_{[4]}=4.18$, $P=0.389$). The low proportion of tan and brown beetles did not permit proper analysis of the relationship between adult phenotype and PO and AMP activity.

Population density x developmental stage interactions

There were no interactions between population density and developmental stage in determining PO activity ($F_{4, 59}=0.69$, $P=0.605$), AMP activity ($F_{4, 60}=1.81$, $P=0.138$), the encapsulation area ($F_{2, 37}=2.55$, $P=0.091$), or the capsule melanisation index ($F_{2, 37}=1.22$, $P=0.306$). As such, the effects of population density and developmental stage on all immune parameters are henceforth interpreted separately.

Phenoloxidase activity

PO activity did not differ between individuals maintained at low, moderate and high population density ($F_{2, 59}=0.09$, $P=0.912$; Fig. 5A) but did differ between developmental stages ($F_{2, 59}=8.74$, $P<0.001$), being higher in larvae than in pupae and adults (Fig. 5B). Additionally, PO activity was not affected by body weight ($F_{1, 59}<0.01$, $P=0.921$).

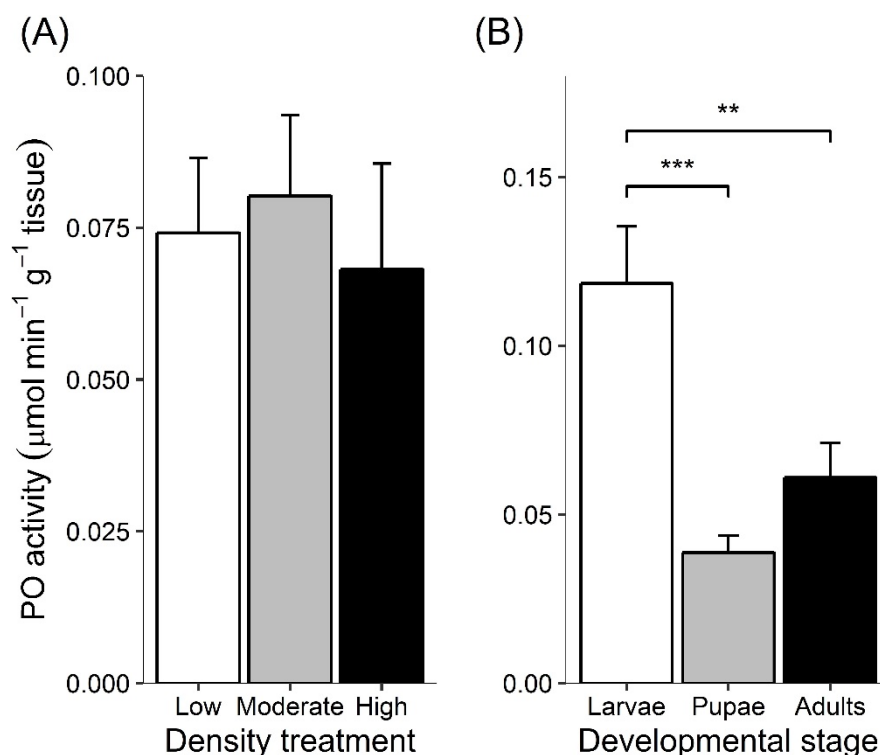


Figure 5: (A) Phenoloxidase (PO) activity ($\mu\text{mol dopachrome per minute per gram of insect tissue}$; see section 2.4) of *T. molitor* previously maintained for 31 days at low (white bar; 23.26g bran/individual), moderate (grey bar; 4.65g bran/individual) and high (black bar; 2.33g bran/individual) population density. (B) PO activity of *T. molitor* larvae (white bar), pupae (grey bar) and adults (black bar). Data are means \pm standard error. Annotations represent the results of a TukeyHSD post-hoc test. ***, $P<0.001$; **, $P<0.01$. No post-hoc test was performed with respect to population density as initial analysis found no significant differences between treatments ($F_{2, 59}=0.09$, $P=0.912$).

Antimicrobial peptide activity

AMP activity did not differ between individuals previously maintained at low, moderate and high population density ($F_{2, 60}=1.17$, $P=0.316$; Fig. 6A), and was not affected body weight ($F_{1, 60}=0.55$, $P=0.461$), but did differ between developmental stages ($F_{2, 60}=4.71$, $P=0.013$), with larvae showing higher activity than pupae and approximately equal activity to adults (Fig. 6B).

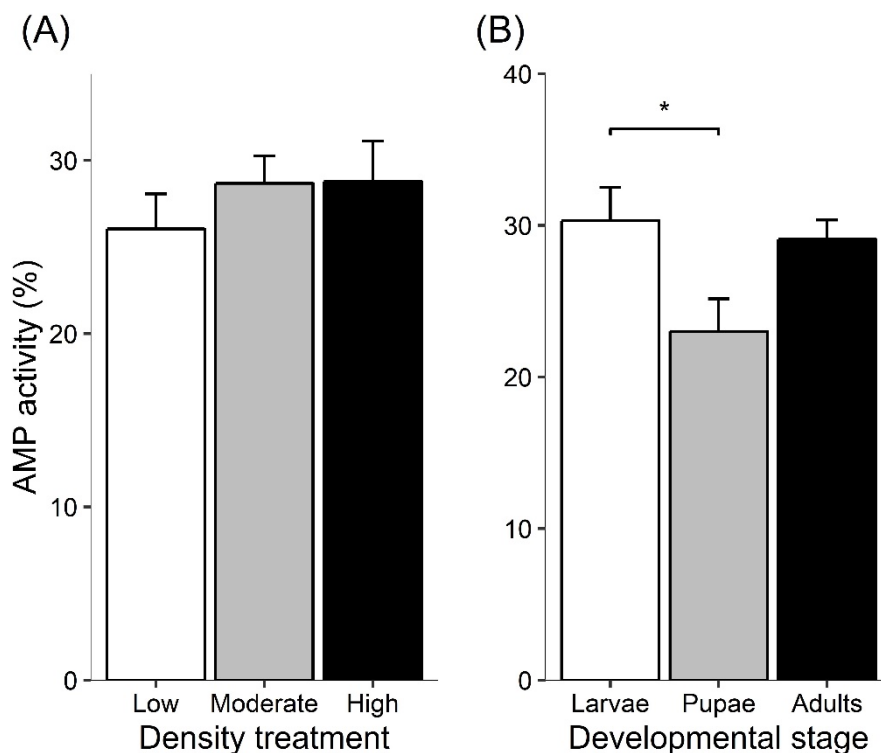


Figure 6: (A) Antimicrobial peptide (AMP) activity (percentage of clear zone produced by the respective tetracylin positive control; see section 2.5) in *T. molitor* previously maintained for 31 days at low (white bar; 23.26g bran/individual), moderate (grey bar; 4.65g bran/individual) and high (black bar; 2.33g bran/individual) population density. (B) AMP activity in *T. molitor* larvae (white bar), pupae (grey bar) and adults (black bar). Data are means \pm standard error. Annotations represent the results of a Tukey HSD post-hoc test; *, $P<0.05$. No post-hoc test was performed with respect to population density as initial analysis found no significant differences between treatments ($F_{2, 60}=1.17$, $P=0.316$).

Encapsulation and capsule melanisation

The degree of capsule melanisation did not differ between individuals previously maintained at low, moderate and high population density ($F_{2, 37}=0.95$, $P=0.395$; Fig. 7A), but was significantly higher in pupae than in larvae ($F_{1, 37}=12.13$, $P=0.001$; Fig. 7B). The encapsulation area, however, tended to be lower in individuals previously kept at high population density than in individuals previously kept at low population density ($F_{2, 37}=3.51$, $P=0.040$; Fig. 8A), and was significantly higher in pupae than in larvae ($F_{1, 37}=8.05$, $P=0.007$; Fig. 8B). Additionally, neither the encapsulation index ($F_{1, 37}=0.47$, $P=0.499$), nor the encapsulation area ($F_{1, 37}=1.89$, $P=0.178$) were significantly affected by body weight.

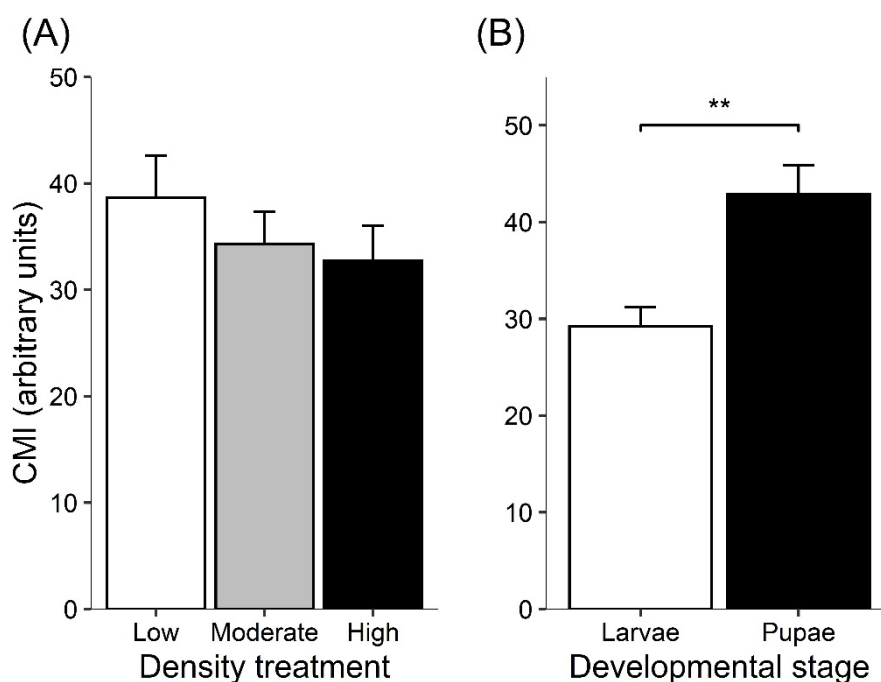


Figure 7: (A) Capsule melanisation index (CMI; arbitrary units; see section 2.2) of *T. molitor* previously maintained for 31 days at low (white bar; 23.26g bran/individual), moderate (grey bar; 4.65g bran/individual) and high (black bar; 2.33g bran/individual) population density. (B) Capsule melanisation index of *T. molitor* larvae (white bar) and pupae (black bar). Data are means \pm standard error. Annotations represent the results of GLM analysis; **, $P < 0.01$. No post-hoc test was performed with respect to population density as initial analysis found no significant differences between treatments ($F_{2, 37} = 0.95$, $P = 0.395$).

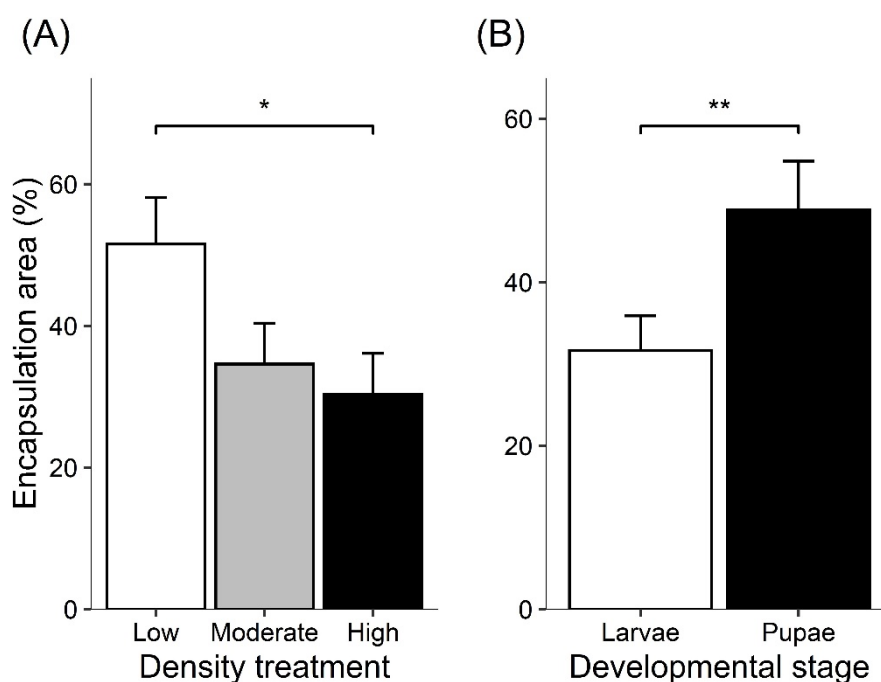


Figure 8: (A) Encapsulation area (% of total filament area; see section 2.2) of *T. molitor* previously kept for 31 days at low (white bar; 23.26g bran/individual), moderate (grey bar; 4.65g bran/individual) and high (black bar; 2.33g bran/individual) population density. (B) Encapsulation area of *T. molitor* larvae (white bar) and pupae (black bar). Data are means \pm standard error. Annotations represent the results of a Tukey HSD post-hoc test (A) and GLM analysis (B); **, $P < 0.01$; *, $P < 0.05$.

Discussion

Elevated mortality in group-reared insects, including *T. molitor*, has been demonstrated numerous times in previous studies (Kong et al. 2011, cited in Kong et al., 2013; Barnes and Siva-Jothy, 2000; Tschinkel and Willson, 1971). Where a clear immune response to high population density is found, this could be influenced by autoreactivity of the immune system (Schmid-Hempel, 2003, Pursall and Rolff, 2011). However, as this study found no evidence of immune upregulation *T. molitor* maintained at high-density, and because all treatments were provided with food in excess of what was required (and adult body weight did not differ between treatments, so the possibility of starvation can be dismissed), the elevated mortality in the high population density treatment is likely due to cannibalism. Cannibalism is common in densely aggregated *T. molitor* larvae, with larger larvae cannibalising relatively immobile prepupae and pupae, and, to a lesser extent, other larvae (Weaver and McFarlane, 1990; Tschinkel and Willson, 1971).

Contrary to other studies investigating the relationship between population density and immune investment in other insect species (see section 1; Wilson and Cotter, 2008), and *T. molitor* (Barnes and Siva-Jothy, 2000), this study found no direct evidence of DDP in *T. molitor*. One explanation could be that other mechanisms of disease resistance, separate from PO, AMPs, encapsulation and capsule melanisation, were upregulated in individuals kept at high and/or moderate population density. While this possibility cannot be excluded as this study did not measure, e.g., haemocyte densities, and ignored behavioural responses, it nevertheless seems unlikely, as this study also found no density-dependent changes in developmental rate or adult weight, contradicting the findings of previous studies (Tschinkel and Willson, 1971; Barnes and Siva-Jothy, 2000). Additionally, the relative proportions of each adult colour phenotype did not significantly differ between treatments, with the majority of adults across all population density treatments displaying the black phenotype, which has been shown to occur more frequently when larvae are reared to adulthood at high population density (Barnes and Siva-Jothy, 2000). In fact, Barnes and Siva-Jothy (2000) found that in populations of *T. molitor* reared to adulthood at high population density, only 15% displayed the black phenotype, almost four times lower than the proportion of black phenotypes observed in the low-density treatment used in this study. This is in spite of the fact that the absolute population density (in terms of the volume of substrate per larvae) was substantially higher in the high-density treatment of Barnes and Siva-Jothy (2000) than in the low-density treatment used in this study. Black beetles have thicker and more heavily melanised cuticles than tan beetles, which could potentially enhance their resistance to pathogens that enter via the cuticle (Silva et al., 2016; Barnes and Siva-Jothy, 2000). Considered together, all of this suggests that physiological changes associated with crowding may have been initiated across all population density treatments.

One possible explanation for this could be that individuals in all population density treatments initiated irreversible prophylactic mechanisms prior to the start of the experiment. This is a strong possibility as the individuals used in this experiment spent a large portion of their life cycle at relatively high density in the original mixed colony (personal observation; see section 2.1). However, density-dependent immunological responses can be altered when the population density is altered during development. For example, Kunimi and Yamada (1990) showed that *Mythimna separata* larvae reared in solitary conditions from the 2nd to 4th instar, and

in crowded conditions from the 4th to final instar (solitary-crowded), and vice versa (crowded-solitary) showed intermediate susceptibility to a nuclear polyhedrosis virus when compared to larvae reared completely in isolation (solitary-solitary; highest susceptibility) and larvae reared completely in crowded conditions (crowded-crowded; lowest susceptibility). While no similar experiment has been performed in *T. molitor*, it nevertheless indicates that it may be possible for *T. molitor* to alter its anti-pathogen defences later in development in response to a change in the local population density.

This study also showed that *T. molitor* maintained at high population density displayed a decreased encapsulation response when compared to individuals maintained at low population density. Downregulation of certain immune parameters under crowded conditions is common in insects displaying DDP (Silva et al., 2013; Silva and Elliot, 2016; Goulson and Cory, 1995), often as a result of trade-offs with other immune parameters, though the exact nature of the trade-offs (which responses are downregulated and which are upregulated) appears to exhibit significant interspecific variation (Wilson and Cotter, 2008). However, this study found no evidence of any trade-off within the immune system as no immune parameters were correspondingly upregulated.

Crowded conditions may result in the upregulation of physiological immune responses, but could also trigger behavioural responses, such as dispersion away from dense aggregations as a means to avoid a population likely to have a high parasite load (Schmid-Hempel, 2003; Elliot and Hart, 2010) and, especially in the case of *T. molitor* where cannibalism is common, a high level of unfavourable interactions between conspecifics (Tschinkel and Willson, 1971; Weaver and McFarlane, 1990). Dispersion would have been limited in the high-density treatment by the limited space. This observation, combined with evidence of elevated mortality, may indicate that the high-density conditions were stressful and as such resulted in an impairment of the encapsulation response, though it seems unlikely that stressed individuals would have an impaired encapsulation response and yet show no other indicator of stress such as reduced PO activity, AMP activity, capsule melanisation or adult weight. While this possibility cannot be dismissed, an alternative explanation could be that exposure to high population density resulted in the selective survival of individuals investing more in their competitive ability (i.e. their ability to tolerate the unfavourable conspecific interactions associated with high population density) and comparatively less in their encapsulation response. This is a plausible explanation as Kraaijeveld and Godfray (1997) demonstrated that *Drosophila melanogaster* artificially selected for an elevated encapsulation response also displayed reduced larval competitive ability when compared to control larvae, suggesting a trade-off between the two.

T. molitor has a natural tendency to form conspecific aggregations (Weaver and McFarlane, 1989). Therefore, even if individuals in the low- and moderate-density treatments dispersed away from dense aggregations, they would likely still aggregate in smaller groups. Within these aggregations, the degree of tactile stimulation between conspecifics may be quite high. As such, in this study, the degree of tactile stimulation, and consequently the perception of conspecific density, may have been similar across all population densities. If DDP is initiated via tactile stimulation in *T. molitor*, as occurs with other density-dependent responses, e.g. the delaying of pupation (Tschinkel and Willson, 1971), this could have resulted in the

upregulation of anti-pathogen defences across *all* population densities. Additionally, even if aggregation did not occur and *T. molitor* distributed uniformly throughout the bran, the degree of tactile stimulation in all treatments may have exceeded a threshold level required for the initiation of DDP. This could explain the lack of any difference in adult weight, developmental rate, PO activity, AMP activity and capsule melanisation between population density treatments, and why the majority of adults displayed the black, i.e., high-density (Barnes and Siva-Jothy, 2000), phenotype. In a similar vein, Silva et al. (2013) show that in *A. gemmatalis*, the presence of conspecifics is a better predictor of investment into encapsulation and haemocyte density than the conspecific density *per se*, which they suggest may be due to the presence of a single conspecific providing enough tactile stimulation to elicit immunological changes.

Though this study found no interactions between population density and developmental stage in determining immune investment, it nevertheless illustrated the complexity of stage-specific immune investment. Larvae showed much higher PO activity than pupae and adults, but had approximately equal AMP activity to adults, with pupae showing the lowest activity. PO is involved in several processes including cuticle sclerotization (Nappi and Christensen, 2005), which could contribute to the high level of PO activity in larvae, as larvae will likely have high PO activity immediately following larval moults in order to ensure timely cuticle sclerotization (Vigneron et al., 2019). Additionally, larvae showed lower encapsulation and capsule melanisation responses than pupae. Being sessile, pupae may invest relatively more in physiological immune responses than larvae due to their inability to utilise behavioural mechanisms, such as moving away from dense aggregations of conspecifics or populations likely to have a high pathogen load (Wilson-Rich et al., 2008). However, this does not seem to be the case in *T. molitor* as pupae had relatively low PO and AMP activity. This highlights a potential issue regarding the methods used in this study for measuring the encapsulation and capsule melanisation responses (see section 2.2), which are adapted from the standard method for assessing the encapsulation response used in most studies of insect immunity (Wilson-Rich et al., 2008; Silva et al., 2013; Silva and Elliot, 2016). Using this method, measures of encapsulation and capsule melanisation will inevitably be influenced by the coagulation response. Therefore, it is possible that relative to larvae, pupae mounted an elevated coagulation response to the breach of the cuticle (see section 2.2), rather than an elevated encapsulation and capsule melanisation response. An elevated coagulation response in pupae is a reasonable assumption as it could result from their heightened risk of cannibalism (Tschinkel and Willson, 1971), which in *T. molitor* entails the production of small holes in the integument from which haemolymph generally leaks out (Weaver and McFarlane, 1990). This suggests that, in some situations, more sophisticated methods for assessing the encapsulation response may be needed. Though as coagulation may have a role in anti-pathogen defence (Dushay, 2009), the distinction between the coagulation and encapsulation responses may not be of much importance anyway.

While the above factors likely play a part in shaping stage-specific immune investment, stage-specific immune investment exhibits a large degree of interspecific variation (Meylaers et al., 2007; Giglio and Giulianini, 2013; Wilson-Rich et al., 2008). For example, contrary to this study, Meylaers et al. (2007) found that when challenged with a solution of bacteria (*Escherichia coli* and *Micrococcus luteus*) and yeast (*Saccharomyces cerevisiae*), pupae of the wax moth, *Galleria mellonella*,

(Lepidoptera: Pyralidae) exhibit higher antimicrobial activity than larvae and adults. As such, stage-specific immune investment in *T. molitor* likely reflects a multitude of factors, including complex trade-offs between immune investment and developmental processes in larvae and pupae, trade-offs between immune investment and reproduction in adults, and, potentially, different selection pressures put upon each stage by their relative vulnerability to different types of pathogens (i.e. parasitic worms, fungi, bacteria, viruses, etc.; Wilson-Rich et al., 2008).

Conclusions

To summarise, this study found no direct evidence of DDP in *T. molitor* and instead indicated that high population density results in a reduced encapsulation response. Nevertheless, this study served to illustrate the intricacies of DDP and stage-specific immune investment, and the results of this study may find useful practical application. For example, it seems that in this study, *T. molitor* displayed phenotypic properties associated with high population density across all population density treatments. Whether due to larvae spending too much of their life cycle at high population density in the original colony, or the conspecific contact rate being similar across all treatments, this may suggest that DDP is of little practical importance in the biocontrol of *T. molitor*, as in a real-world situation where larvae may experience significant fluctuations in population density throughout their development, increasing conspecific density may be associated with a reduction in food supplies, etc., the criteria for the initiation of DDP may rarely be met. While often considered a pest, *T. molitor* is also used as a protein-rich food source (Vigneron et al., 2019), and therefore there is a need to maximise production by decreasing mortality, among other factors. In this context, the results from this study suggest that the population density of *T. molitor* should be carefully controlled in order to avoid the elevated mortality due to cannibalism and, potentially, parasitism (via a reduction in the encapsulation response), associated with high population density. Additionally, an understanding of stage-specific immune investment in *T. molitor*, as elucidated by this study, could be of great relevance to biocontrol strategies. For example, in order to prevent development to the reproductive stage and perhaps halt the continuation of a *T. molitor* infestation, the results of this study suggest that it may be best to use a bacterial pathogen as a biocontrol agent, as pupae displayed relatively low AMP activity and thus may be most susceptible to bacterial pathogens.

Future research

There are many future directions in the study of the relationship between population density and immunity. Firstly, there is a great need to expand the taxonomic range in future studies of DDP. Currently there is a strong taxonomic bias towards Lepidopterans, particularly those of the family Noctuidae (Wilson and Cotter, 2008). In addition to other insect taxa, consideration should be made of other groups of arthropods, many of which also experience large fluctuations in population density and may display density-dependent changes in physiology and immune investment. Several species of harvestmen (Order Opiliones), for example, form diurnal aggregations, and harvestmen of the suborder Eupnoi have shown record aggregations of up to 70000 individuals (Pinto-da-Rocha et al., 2007). Woodlice (Order Isopoda, suborder Oniscidea) are another group of arthropods that form aggregations of variable density (Broly et al., 2012), and may be good candidates for the future study of DDP. In addition to expanding the taxonomic range, the intricacies of the DDP hypothesis should be investigated further. The mechanistic basis of DDP remains unclear, and further investigation into this area is certainly required. In

addition, DDP-associated trade-offs should be investigated in a broader sense, one in which factors like reproductive output are considered, and one in which trade-offs are considered under specific circumstances (e.g. resource scarcity). Further investigation is also required into the influence of the presence of conspecifics, as opposed to the absolute density of conspecifics, on immune responses. Another surprisingly neglected line of inquiry that could be of great importance to biocontrol and the rearing of insects as food regards the critical developmental periods involved in sensing population density and initiating DDP. Finally, measures of immune responses in studies of DDP are currently more-or-less limited to cellular and humoral responses, with few studies considering physical responses (e.g. changes in cuticular properties) and fewer still considering behavioural and social responses (Elliot and Hart, 2010). This approach could entail ignoring swathes of an organism's anti-pathogen responses. In order to address this in future studies, it may be wise to consider the definition of immunocompetence put forth by Owens and Wilson (1999), wherein immunocompetence is defined as a measure of an organism's ability to minimise the *fitness costs* associated with an infection *via any means*, subsequent to controlling for prior exposure to relevant antigens. In this definition, immunocompetence comprises physiological immune responses, behavioural responses, social responses, and strategies that may reduce infection-associated fitness costs without increasing individual survival, e.g. increased reproductive effort to the detriment of physiological immunity.

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