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Abstract

Transmission of parasites and pathogens is generally positively density-dependent: as an insect population's density increases, the risk of an individual becoming attacked or infected also increases. In some insect species, individuals experiencing crowded conditions are more resistant to natural enemies than those experiencing low density conditions, and they are predicted to divert resources to increase resistance. This phenomenon is called density-dependent prophylaxis. Here, possible expression of prophylaxis in fifth-instar larvae of Beet Webworm, *Loxostege sticticalis*, to biocontrol agents was investigated under rearing densities of 1, 10, and 30 larvae per jar (650 mL). Larvae reared at the moderate density and those reared in isolation displayed the greatest and lowest resistance, respectively, to an entomopathogenic fungus and a parasitoid. Moreover, larvae from the moderate density treatment exhibited elevated phenoloxidase, total haemocyte count and antibacterial activity in the haemolymph, whereas phenoloxidase levels in the midgut were not affected. The results suggest that larval rearing density significantly affects the immune system, and they provide evidence for density-dependent prophylaxis of larval *L. sticticalis* against its biocontrol agents. These results have implications for understanding the population dynamics and biocontrol of beet webworm.

Keywords

antibacterial activity, density-dependent prophylaxis, larval density, *Loxostege sticticalis*, phase polyphenism, phenoloxidase

Disciplines

Agriculture | Entomology | Parasitology | Pathogenic Microbiology

Comments

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Density-dependent prophylaxis in crowded Beet Webworm, *Loxostege sticticalis* (Lepidoptera: Pyralidae) larvae to a parasitoid and a fungal pathogen

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Transmission of parasites and pathogens is generally positively density-dependent: as an insect population's density increases, the risk of an individual becoming attacked or infected also increases. In some insect species, individuals experiencing crowded conditions are more resistant to natural enemies than those experiencing low density conditions, and they are predicted to divert resources to increase resistance. This phenomenon is called density-dependent prophylaxis. Here, possible expression of prophylaxis in fifth-instar larvae of Beet Webworm, *Loxostege sticticalis*, to biocontrol agents was investigated under rearing densities of 1, 10, and 30 larvae per jar (650 mL). Larvae reared at the moderate density and those reared in isolation displayed the greatest and lowest resistance, respectively, to an entomopathogenic fungus and a parasitoid. Moreover, larvae from the moderate density treatment exhibited elevated phenoloxidase, total haemocyte count and antibacterial activity in the haemolymph, whereas phenoloxidase levels in the midgut were not affected. The results suggest that larval rearing density significantly affects the immune system, and they provide evidence for density-dependent prophylaxis of larval *L. sticticalis* against its biocontrol agents. These results have implications for understanding the population dynamics and biocontrol of beet webworm.

Keywords: antibacterial activity; density-dependent prophylaxis; larval density; *Loxostege sticticalis*; phase polyphenism; phenoloxidase

1. Introduction

Generally, the risk of an insect becoming infected by diseases (or attracted by parasites) increases with increasing population density, mainly because the per-capita risk tends to be positively density-dependent (Anderson and May 1981). Thus, it is found that insects which live in high-density populations have evolved adaptive prophylaxis mechanisms to cope with pathogens and parasites (Wilson and Reeson 1998). Individuals experiencing crowded conditions are predicted to be more resistant to natural enemies than those experiencing low-density conditions, through facultative physiological resource allocation to defense mechanisms. This phenomenon, termed “density-dependent prophylaxis” (Wilson and Reeson 1998), is predicted to be more common in outbreak pest species that exhibit phase polyphenism (i.e. the particular phenotype adopted by an insect, contingent on the population density it experiences during its early development), because they have the ability to detect and respond developmentally to crowded conditions. Density-dependent prophylaxis strongly affects host–parasite dynamics (White 1999). Understanding the density-dependent prophylactic responses of a species is essential for characterizing its population dynamics and the potential for its control using natural enemies.

An insect mounts an immune response when challenged with an infection (Hoffmann 2003). The insect immune system combines both cellular and humoral components (Cotter et al. 2004). Phenoloxidase (PO), total haemocyte count and antibacterial proteins are all important in defence against a range of pathogens and parasites (Wilson et al. 2002; Cotter et al. 2004). Reeson et al. (1998) found that PO level in the haemolymph of high-density phase *Spodoptera exempta* Walker was higher than in low-density phase individuals, which corresponded to increased resistance to nuclear polyhedrosis virus. High-density phase locusts had significantly higher haemolymph antibacterial activity than low-density phase locusts (Wilson et al. 2002). Thus, different components of the immune response can be responsible for observed levels of resistance in individuals from high-density populations (Cotter et al. 2004).

Density-dependent prophylaxis has been observed in a diversity of insect species, including *Spodoptera exempta* (Reeson et al. 1998), *Mythimna separata* Walker (Kunimi and Yamada 1990), *Spodoptera littoralis* Boisduval (Wilson et al. 2001), *Tenebrio molitor* L. (Barnes and Siva-Jothy 2000), *Locusta migratoria* L. (Wilson et al. 2002), and *Acromyrmex echinatior* Forel (Hughes et al. 2002). Most examples come from phase-polyphenic

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species. The Beet Webworm, *Loxostege sticticalis* L. (Lepidoptera: Pyralidae), is an important migratory pest (Luo et al. 1996; Luo 2004) that exhibits density-dependent phase polymorphism (Knorr et al. 2000). Larval colour forms, survival rates, detoxifying enzymatic activity and dopamine levels in haemolymph, diapause incidence, pupal weights, reproductive potential and flight potential in adults are affected by larval density (Alekseev et al. 1998; Knorr et al. 1993, 2000; Alekseev et al. 2008; Kong et al. 2010, 2011). Alekseev et al. (2008) reported that larval haemocyte encapsulation varied among different larval densities. Therefore, we hypothesized that *L. sticticalis* larvae reared at different densities may exhibit differential resistance to natural enemies.

Various insect natural enemies, including parasitoids (especially *Exorista civilis* Rondani, Diptera: Tachinidae), microsporidia, viruses, bacteria and fungi (especially *Beauveria bassiana* Balsamo, Hyphomycetales: Hypomycetes) attack beet webworm in the wild (Cui 1992; Li and Luo 2007). Parasitoids play an important role in the population dynamics of beet webworm (Mikhal'tsov and Khitsova 1980; Chen and Luo 2007), with > 67% parasitism levels reported for *E. civilis* (Li and Luo 2007). Therefore, any effects of larval density on a host's resistance to natural enemies could influence its population dynamics. The aim of this study was to investigate density-dependent prophylaxis in *L. sticticalis* larvae to parasites or pathogens under three rearing densities in the laboratory. We report herein the effects of larval density on: (i) the survival of larvae infected by *B. bassiana* or parasitized by *E. civilis*, and (ii) immunological variables, under laboratory conditions.

2. Materials and methods

2.1. Colony maintenance

A laboratory colony of *L. sticticalis* was initiated using diapausing larvae collected in Siziwangqi, Inner Mongolia, China, in 2007. The insects were maintained at room temperature and 16 h: 8 h light: dark regime until pupation and adult emergence. Adults emerging on the same day were collected and held together in a 2-L capacity plastic cage to allow mating and oviposition to occur. Adults were provided daily with 10% (wt/vol) glucose solution as supplemental food. Eggs were laid on nylon gauze lining the plastic cage. Larvae were maintained in 5-L capacity cages and fed daily with fresh leaves of normal host plant Lambsquarters, *Chenopodium album* L. When larvae stopped feeding, sterilized soil containing ~10% water was added to the cages to a depth of 10 cm to provide a substrate for cocoon formation, pupation, and adult emergence.

2.2. Test subjects

The *L. sticticalis* colony had been maintained for three generations when the experiments began. Newly hatched larvae were grouped and reared at three density treatments of 1, 10, or 30 larvae per 650 mL capacity jar (diameter

10 cm). The number of larvae for the two higher density treatments was maintained throughout the feeding period. Excess food was provided by adding fresh leaves every morning. All insects were maintained at a constant temperature of $22 \pm 1^\circ\text{C}$, 70% relative humidity, and a 16 h: 8 h light: dark regime. These conditions inhibit diapause and ensure maximum survival, development rate, and reproductive capacity (Luo and Li 1993a, 1993b).

2.3. Bioassays

2.3.1. Exposure to mortality agents

2.3.1.1. Entomopathogenic fungus. *Beauveria bassiana* was obtained from Shenwei Biological Company (Yancheng, Jiangsu, China). Fungal spores were suspended in sterile 0.1% Tween 80 solution (Polysorbate). The final concentration was adjusted to 1.6×10^7 conidia mL^{-1} for the bioassays. This spore dose consistently induced 40–60% mortality in preliminary experiments (H. Kong, unpublished data).

When most larvae of a treatment density had developed to the fifth instar, groups of ten larvae per density treatment were exposed to the fungus by dipping them collectively in the spore solution for 5 s followed by air-drying at room temperature. Each group of 10 larvae from a density treatment exposed to spores had a corresponding control group dipped in the same way but into sterile 0.1% Tween 80 solution only. After dipping, all exposed and control larvae were individually maintained in small glass jars under the same environmental conditions as above. The experiment was replicated 10 times, and thus 200 larvae in total were used for each density treatment (600 for the entire experiment). Survival rate was recorded daily from 4 d post-inoculation until all larvae either died or pupated. Infection of larvae that died was verified by checking for sporulation in cadavers placed in square Petri dishes containing 1% water agar.

2.3.1.2. Endoparasitoid. A laboratory colony of the endoparasitoid *E. civilis* was obtained from *L. sticticalis* larvae collected from the field in Siziwangqi of Inner Mongolia, China, in 2007. The parasitized larvae were maintained at room temperature and a 16 h: 8 h light: dark regime until pupation and adult emergence. Newly emerged adults (0–12 h post-emergence) were collected and held together in 2-L capacity plastic boxes for mating and oviposition. Adults were provided with 10% (wt/vol) honey solution as supplemental food. Eggs were laid on the body of *L. sticticalis* larvae. (Hatched larvae penetrate the host integument and complete their development within the host larva.)

When the parasitism experiment began, 10 fifth-instar larvae from each of the density treatments were exposed to one mated female fly in 1.5-L capacity plastic boxes. At this ratio of larvae per fly, most larvae are parasitized only once, that is, there is no superparasitism (H. Kong, unpublished data). Parasitism was confirmed by the presence of a white egg laid on the larval host's body. After

12 h, the parasitized larvae bearing one parasitoid egg were removed and maintained individually in a small glass jar with fresh leaves. Individual maintenance after parasitism was considered preferable to returning to initial rearing densities to ensure equivalent conditions for measuring the response among the density treatments, and to prevent larvae from dislodging one another's parasitoid eggs under crowded conditions. Corresponding groups of 10 control larvae were placed in the box but with no female fly. The experiment was replicated 10 times. Thus, 200 total larvae were used for each density treatment and control (600 for the entire experiment). Some parasitoid eggs hatched and developed into fly pupae, while others shrivelled and the host survived. The proportion of hosts surviving parasitism was used as our measure of host prophylaxis to endoparasitoids.

2.3.2. Immune assays

2.3.2.1. Haemolymph, cuticle and midgut sampling. On the second day of the fifth instar, larvae were cooled to torpor on ice and then sterilized by swabbing with 75% ethanol. An abdominal proleg was cut with fine scalpel, and haemolymph was collected by microcapillary pipette and transferred into 1.5-mL centrifuge tube on ice to prevent melanization (because the melanism response of haemolymph is constrained at low temperatures). Haemolymph samples collected from groups of 20 larvae per density were pooled as one replicate for the assays of PO and antibacterial activity and the counts of haemocytes. Each treatment was replicated 10 times. Following haemolymph extraction, the caterpillar's cuticle and midgut were isolated and washed thrice with phosphate solution. For each treatment, the cuticle and midgut from 10 larvae were respectively pooled as one replicate for the assays of cuticular and midgut PO activities. Cuticle and midgut pooled samples were separately homogenized using a Dounce® Active Motif homogenizer in an ice bath. After centrifugation (12,000g, 15 min, 4°C), the supernatant was collected and stored at -80°C for later use.

2.3.2.2. Haemolymph phenoloxidase activity. PO activity was analysed as described by Wilson et al. (2001). Briefly, 25 µl of haemolymph were added to 1475 µl of sodium phosphate solution in a plastic tube. Haemolymph PO activity was assayed spectrophotometrically by adding 100 µl of 10 mM L-Dopa to 100 µl of haemolymph phosphate solution, and the mixture was incubated for 3 min at 22°C. During this period, the reaction was maintained in the linear phase. Absorbance was read at 492 nm on a temperature-controlled INFINITE200 microplate reader (Tecan Corporation, Mannedorf, Switzerland) after 5 min of incubation. Protein content was measured in the samples following the method described by Bradford (1976). PO activity was expressed as PO units per milligram of protein, where 1 U was the amount of enzyme required to increase the absorbance by 0.001 min⁻¹.

2.3.2.3. Cuticular and midgut phenoloxidase activities. Cuticular and midgut PO activities were assayed as described above.

2.3.2.4. Total haemocyte count. The total haemocyte count was determined by adding 5 µl of haemolymph to the counting chambers of a haemocytometer. The haemocytes in the central square and four corners were counted under phase-contrast illumination and averaged to give an estimate of the number of haemocytes.

2.3.2.5. Antibacterial activity. Antibacterial activity against *Micrococcus lysodeikticus* Fleming (Actinomyceales: Micrococcaceae) was determined using a lytic zone assay. Test plates were prepared according to Wilson et al. (2002). Petri dishes were filled with 10 mL of agar suspension containing 1% agar, 5mg mL⁻¹ freeze-dried *M. lysodeikticus*, 0.1 mg mL⁻¹ streptomycin sulfate, and 67 mmol potassium phosphate buffer, pH 6.4. Holes (diameters 2 mm) were made by punching the agar layers and filling them with 1 µl of haemolymph. The plates were incubated at 33°C for 24 h and then photographed. The diameters of clear zones were measured using IMAGE PRO PLUS® Media Cybernetics. Standard curves were obtained by a serial dilution of hen egg white lysozyme, because there was a logarithmic correlation between the concentration of white lysozyme and the diameter of the inhibition zones, from which the concentration of the haemolymph sample was calculated.

2.4. Statistical analysis

Survival rates of larvae parasitized or infected by fungus at various densities were analysed by chi-squared analysis. Data for immune trait variables were assessed for their fit to a normal distribution using the Kolmogorov-Smirnov test. Based on the results, data for total haemocyte count and antibacterial activity were log₁₀-transformed before analysis. Differences among the three density treatments were tested using one-way ANOVA followed by the protected least significant difference (LSD) test. All statistical procedures were performed with SPSS 10.0.

3. Results

3.1. Survival after infection by entomopathogenic fungus

Survival rate of larvae infected by *B. bassiana* differed significantly among the three density treatments ($\chi^2 = 31.81$; $df = 2$; $P < 0.01$), and was the highest at 10 larvae per jar (71.43%) (Figure 1). This was greater than the larval survival rate at 30 larvae per jar (47.06%) and individually reared larvae (43.82%), but there was no significant difference between the latter two treatments. No mortality due to fungal infection was observed in the untreated control group.

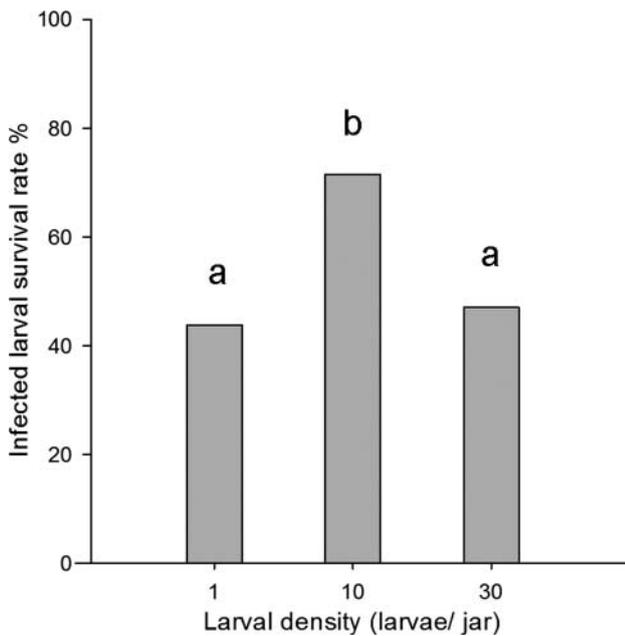


Figure 1. Effects of larval density on the survival of larval *Loxostege sticticalis* infected by entomopathogenic fungus *Beauveria bassiana*. Histograms with the same letter are not significantly different at the 0.01 level as determined by chi-squared test.

3.2. Survival after attack by endoparasitoids

Larval density significantly affected the survival rate of larvae parasitized by *E. civilis* ($\chi^2 = 15.77$; $df = 2$; $P < 0.01$) (Figure 2). The parasitized larvae reared at the density of 10 larvae per jar had the highest survival rate (79.17%) among the three density treatments. The survival rate of larvae reared at the density of 1 larva per jar

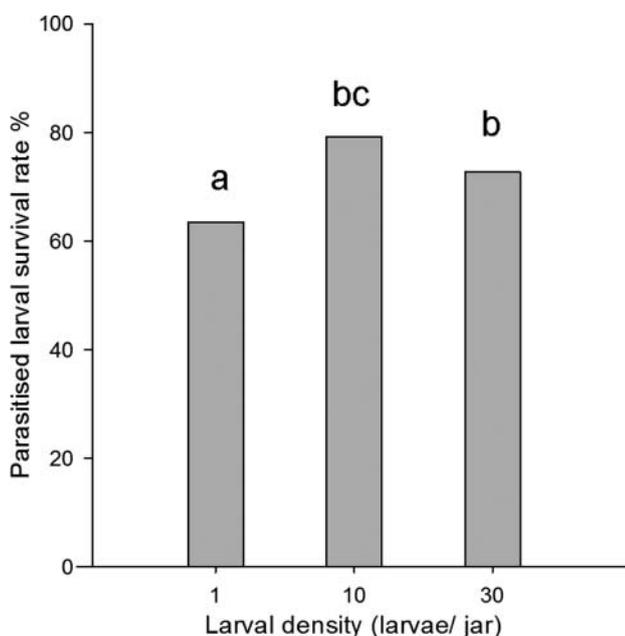


Figure 2. Effects of larval density on the survival of larval *Loxostege sticticalis* parasitized by parasitoid *Exorista civilis*. Histograms with the same letter are not significantly different at the 0.01 level as determined by chi-squared test.

Table 1. Phenoloxidase activities in the haemolymph, cuticle, and midgut of fifth-instar *Loxostege sticticalis* larvae reared under different densities.^a

Density (larvae/jar)	Phenoloxidase activity (U/mg)		
	Haemolymph	Cuticle	Midgut
1	0.058 ± 0.003 a	0.666 ± 0.020 a	0.423 ± 0.015 a
10	0.135 ± 0.010 b	0.889 ± 0.020 b	0.388 ± 0.012 b
30	0.065 ± 0.002 a	0.929 ± 0.010 b	0.387 ± 0.007 b

^aData are presented as mean ± standard error. Means in the same column followed by different letters are significantly different by protected LSD test ($P < 0.01$). Each treatment was repeated 10 times.

(63.47%) was significantly lower than that of larvae reared at a density of 10 (79.17%) or 30 larvae per jar (72.73%), but there was no significant difference between the latter two treatments. Unsurprisingly, no mortality due to parasitism was observed in the control group.

3.3 Immune assays

3.3.1. Phenoloxidase activity

Hemolymph PO activity differed significantly among the larval densities ($F = 51.49$; $df = 2, 27$; $P < 0.01$) (Table 1). The haemolymph PO activity of larvae reared at 10 larvae per jar was the highest, and it was significantly higher than those of larvae reared at the densities of 1 and 30 larvae per jar. Larval density also affected cuticular PO activity ($F = 68.74$; $df = 2, 27$; $P < 0.01$) (Table 1), with those of larvae reared at 10 or 30 larvae per jar being significantly greater than that of larvae reared individually. In contrast, PO activity in the midgut was not significantly affected by larval density ($F = 2.93$; $df = 2, 27$; $P > 0.01$) (Table 1).

3.3.2. Total haemocyte count

Larval density significantly affected the total haemocyte count in larvae ($F = 7.97$; $df = 2, 27$; $P < 0.01$) (Table 2). Larvae reared at 10 larvae per jar had a significantly higher total haemocyte count compared to those reared at the other two densities.

Table 2. Total haemocyte count and antibacterial activity of the haemolymph of fifth-instar *Loxostege sticticalis* larvae reared under different densities.^a

Density (larvae per jar)	Total haemocyte count (log mean no. per mL)	Antibacterial activity (log μ g of lysozyme equiv. per mL haemolymph)
1	6.31 ± 0.06 a	0.99 ± 0.06 a
10	6.64 ± 0.08 b	1.73 ± 0.05 b
30	6.41 ± 0.03 a	1.60 ± 0.07 b

^aData are presented as mean ± standard error. Means in the same column followed by different letters are significantly different by protected LSD test ($P < 0.01$). Each treatment was repeated 10 times.

3.3.3. Antibacterial activity

The antibacterial activity of larvae reared individually was significantly lower than those of larvae reared at the densities of 10 or 30 larvae per jar ($F = 41.69$; $df = 2, 27$; $P < 0.01$) (Table 2).

4. Discussion

Our results show that larval density has a significant effect upon resistance of *L. sticticalis* to its parasitoid and entomopathogenic fungus biocontrol agents, namely *E. civilis* and *B. bassiana*, respectively. Larvae reared at the medium density of 10 larvae per jar were the most resistant to the entomopathogenic fungus *B. bassiana* and the larval parasitoid *E. civilis*. Larvae reared at this density also had significantly higher levels of PO activity, total haemocyte count and antibacterial activity of haemolymph than those reared individually.

The density-dependent prophylaxis hypothesis assumes that larvae experiencing crowded conditions become more resistant to parasites than those experiencing low-density conditions (Wilson and Reeson, 1998). In the fungal infection bioassay, the larval survival rate of *L. sticticalis* reared at the medium density of 10 larvae per jar was significantly higher than that of larvae reared individually. A similar result was obtained for survival of larvae attacked by parasitoids. These findings are consistent with previous reports on phase polyphenic species such as *Spodoptera exempta* (Wilson and Reeson 1998), *S. littoralis* (Wilson et al. 2001), and *S. gregaria* (Wilson et al. 2002), in which the prophylactic effect was significantly higher in larvae reared at higher density. Thus, the nature of the results suggests density-dependent prophylaxis is likely to be a universal phenomenon and an important characteristic in polyphenism species.

However, our results also showed that the prophylactic effects from being crowded as larvae are not always greater than that of solitary larvae. In both fungal infection and fly parasitism experiments, the level of larval prophylaxis dropped when the density increased to 30 larvae per jar. This result is consistent with the data obtained for *Mamestra brassicae* larvae, and their level of larval prophylaxis to nuclear polyhedrosis virus initially increased with rearing density, but decreased when the density was too high (Goulson and Cory 1995). All of these outcomes indicate that the effects of larval density on larval prophylaxis may depend on the density tolerance threshold of the given species. Moderate crowding triggers a shift to the expression of resistant behaviour, but physiological stress from overcrowding reduces the prophylactic ability to rescue the insect from infection. Our previous results indicated that the weight of pupae reared under crowded conditions larvae is significantly less than that of those from larvae reared under solitary conditions (Kong et al. 2011), suggesting there may not be sufficient energy to allocate for prophylaxis.

In this study, we found an association between the level of prophylaxis to the pathogen or parasitoid and the

strength of the immune response (measured by PO activity, total haemocyte count and antibacterial activity in the haemolymph) across the three rearing densities. Therefore, immune response may play an important role in the observed level of larval prophylaxis to parasites among the three different tissues of *L. sticticalis* tested under the different densities.

Under a high density of 30 larvae per jar, PO level, antibacterial activity and total haemocyte count in the haemolymph were not significantly different from the quantities recorded for just 1 larva per jar. This may reflect physiological stress of larvae from overcrowded conditions. Under high density of 30 larvae per jar, larval survival, pupal weight, adult fecundity and flight potential were all significantly reduced (Kong et al. 2010, 2011).

Cuticular PO activity increased with increasing larval density, and the activity of larvae reared at the different larval densities corresponded to the observed differences in larval melanization (H. Kong, unpublished data). This can be attributed to PO being a key enzyme in the synthesis of the melanin pigment, which makes the cuticle of crowded larvae turn black.

No significant difference in midgut PO activity among the three densities was detected in *L. sticticalis*. This indicates that PO activity in the midgut is not activated by larval crowding in *L. sticticalis*, and suggests the increase in PO activity in both the haemolymph and the cuticle may be a prophylactic defence response triggered by crowding.

The beet webworm is characterized by periodic heavy outbreaks alternating with more or less long periods of high population density (Frolov et al. 2008). Previous research has suggested that an increase in larval density triggers a positive feedback loop on population density within a certain range (Knor et al. 2000). In our study, larvae reared at the intermediate density of 10 larvae per jar exhibited the strongest prophylactic response, which also constitutes positive density-dependent feedback. This positive feedback mechanism may have evolved because overcrowded larvae have increased susceptibility to parasites. In addition, mathematical modelling has suggested that density-dependent prophylaxis could contribute to the unstable dynamics observed in insects, such as beet webworm, that show phase polyphenism (White and Wilson, 1999). Therefore, larval density may play an important role driving in population fluctuations through its effects on larval prophylaxis.

In conclusion, the *L. sticticalis* larvae used in this study exhibited density-dependent prophylaxis under an intermediate level of crowding. Larvae may use early crowding as a cue to mount a more vigorous immune response against a possible infection. However, the immune system of larvae from high-density populations might be compromised under conditions of increased physical stress. We agree with Wilson and Reeson (1998) that an adaptive investment in immune function in response to population densities may be a widespread phenomenon in some taxa, especially those that exhibit density-dependent phase polyphenism. Our results were obtained in a laboratory settings, but larval prophylaxis to

biocontrol agents with increasing population density will be further investigated by us using field populations.

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