

# Effect of diet and density on larval immune response and larval and adult host-preference in *Spodoptera littoralis*

Sophie Chattington



# **Effect of diet and density on larval immune response and larval and adult host-preference in *Spodoptera littoralis***

Inverkan av diet och densitet på immunförsvar och växtpreferens hos larver och adulter av *Spodoptera littoralis*

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## Abstract

Polyphagy presents difficulties to an insect, especially in terms of host choice. The adult insect must choose from a large number of possible host plant species, something which is considered to require extensive neural capacity to process. In order to decrease the amount of information to process, it has been suggested that polyphagous insects could rely on previous larval experience to make a choice.

In the polyphagous noctuid moth *Spodoptera littoralis* it has frequently been found that female oviposition choice is driven by 'larval memory', indicating that the adult chooses to oviposit upon the same host upon which it fed as a larva. However, is there a larval density effect on how the host plant is perceived, and does this effect adult behaviour?

In this study the insects were reared in three different treatments: low-density (optimal), high-density and starvation (both assumed suboptimal), on three different diets (cotton, cabbage, and maize). A development study was carried out whereby it was indeed found that low-density was optimal, in terms of fitness (pupal weight) and fecundity (egg load). High-density larvae were found have a higher investment in immune response, which coupled with the reduction in pupal weight and egg load, indicates that high larval density may cause a reallocation of energy and a negative effect on fitness and fecundity. Starvation was highly detrimental, having a high development time and death rate.

It was found that larval density did not have an effect on larval host choice, however, it was clear that the larvae were making an experience-based choice, irrespective of density. Adult oviposition choice, however, displayed a switch from choice in adults deriving from low-density treatments to the supposedly random laying seen in the adults originating from the high-density treatment. Furthermore, first choice in oviposition host showed a switch between low- and high-density reared insects, with high-density adults laying eggs on a non-experienced plant first.

I dedicate this work to my wonderful Granny - without your kindness and support I would never have been able to pursue my studies here in Sweden,

Thank you!



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## Introduction

Despite many different control measures, herbivorous insects continue to be a huge problem for crop farmers around the world, with estimates of up to 15% of crop yields being lost to insects yearly (van der Meijden, 2015). Most farmers rely upon chemical pesticides in order to control insect pest levels, but whilst these insecticides are not only expensive for the farmer (further emphasised by the requirement of frequent applications), they have potentially harmful effects upon the surrounding environment and ecosystem (van der Werf, 1996), and it is also possible that insects build up resistance against the control, forcing a continuous need for the development of new chemicals or methods (Sahek, 2010). Although it is probable that chemical pesticides will always remain an important part of controlling insect pest species there is an increasing focus on combining these chemical controls with biological controls in a strategy called Integrated Pest Management (IPM). Integrated Pest Management programmes have already been successfully implemented for a range of pest species on crops all around the globe (Schoonhoven et al., 1998), but it is an area of research that is becoming increasingly popular and important.

Whilst chemical pesticides control the insect populations by killing either the larval or adult stage, biological control methods seek rather to reduce the populations by introduction of a natural enemy (Chailleux et al. 2014), or by controlling the behaviour of the insects using natural chemicals, such as sex pheromones (Thomson et al., 2001; Witzgall, 2001). There are various ways of controlling insect species biologically including disrupting their mate-finding abilities (Nakanishi et al., 2013; Tabata et al., 2015; Thomson et al., 2001), and disrupting their host-finding abilities by semiochemicals (Wang et al., 2013). A further method is mixed planting approaches, whereby ‘trap crops’ and/or ‘inter crops’ draw the insects away from the commercial crop by the ‘push-pull strategy’ (Finch and Collier, 2011). The ‘push-pull strategy works by the fact that insects are repelled from the main crop by intercropping repellent plants, and attracted to the plants in the trap crop (Finch and Collier, 2011).

Integrated Pest Management could indeed be an effective approach in many agricultural systems across the globe. However, in order to begin even thinking about an IPM strategy, it is essential to improve our understanding of not only the life history of the pest insect in

question, but also the relationships between these insects and the plants, and the factors that could have an effect on these multi-trophic interactions (Schoonhoven et al, 2005).

### *Host-finding in herbivorous insects*

Most insects will detect their host plants by a combination of chemical and contact cues, initiated by a distance odour-mediated movement, and concluded in short-range odour-mediated or contact cues. In most environments, an insect can be confronted with a huge array of plants, many of them potential hosts, and must decide upon a plant with as much efficiency and accuracy as possible (Carrasco et al., 2015). Not all the plants in a given environment will make suitable hosts, either due to toxicity, low nutritional value, or high predation risk (Kaplan et al., 2014) therefore the insect cannot just simply choose the first plant that it comes across. Each plant is releasing its own unique blend of chemical volatiles, many of which are detectable by the insect's sensory system. These stimuli must be extracted from the surroundings, and converted into a relevant behavioural output (Schäpers et al., 2015). There are two main strategies to deal with host-finding, each having their advantages and disadvantages. Specialist (monophagous) species feed on a limited number of hosts, whereas generalists (polyphagous) species can feed on a wide range of plants (Cates, 1980).

Whilst generalists have the ability to thrive on a larger range of plants, they have many disadvantages when compared to specialist species. It is well known for any animal, including humans, that when there are a large number of options to choose from, decision-making becomes more difficult, and takes longer – a theory known as Hick's Law (Haynes, 2009; Hyman, 1953). Insects are not excluded from such a problem. Insects are bombarded with many different sensory cues, and it is advantageous to have the ability to distinguish between them and locate a suitable host plant efficiently and effectively. If an insect takes too long to find a host plant, it not only wastes energy, but also is increasing its risk of predation (Bernays, 2001). Speed can also be a necessity for example in gravid insects who have a restricted flight periods before needing to oviposit (Bernays, 2001). As mentioned above, different species of plants, as well as individuals within a species, have very different nutritional values, making it important that an insect not only chooses quickly, but also chooses an appropriate host (Bernays, 2001).

Examples of differences in host plant choice between closely related insect species that display differences in their feeding habits have been shown across different insect groups. For

example, when given the choice between their host plant *Urtica dioica* in different stages of health, the monophagous butterfly *Polygonia satyrus* would consistently choose to lay eggs on the healthy plants, whereas the more polyphagous species *Polygonia c-album*, was less likely to discriminate between the healthy and senescing plant. On a larval level, neither species are more suited to utilising the lower quality leaves, so it is clear that the monophagous species is more effective and discriminatory in their host plant choice (Janz and Nylin, 1997). This is also an example of where the ‘mother knows best’ hypothesis, or preference performance hypothesis (PPH), whereby the adult female will oviposit on a host-plant that is the most suitable for its larvae (Gripenberg et al., 2010; Janz, 2002), may be found to not be true (Gripenberg et al., 2010). There have been several other studies that reinforce this finding that polyphagous species often do not actively choose the most suitable host (Gómez Jiménez et al., 2014; Prager et al., 2014; Gamarale-Stille et al., 2013). Other studies, such as Bernays and Funks’ (1999) work with *Uroleucon ambrosiae*, have found that specialists are significantly faster at finding a host amongst non-hosts, when compared to generalists.

Predation is a substantial problem for herbivorous insects, with herbivores found to be outnumbered 2:1 in certain environments, and being attacked 100x more frequently during feeding than when not (Kaplan et al., 2014). It is therefore important to choose a host plant and to carry out the behaviour (feeding or ovipositing) as quickly as possible, in order to reduce the time spent with lower attentiveness to external environmental risks (Dukas, 1998). Furthermore, in terms of time efficiency, it is also advantageous to choose a nutritious host. Most insects rely upon high levels of protein to survive, and older leaves do not contain this (Davies, 2012). Therefore, feeding on such a host, the insect would have to eat a larger quantity (thus feeding, and therefore being more vulnerable to predators, for longer) in order to maintain essential nutrient levels (Bernays, 2001).

### *Advantages of polyphagy*

Despite the fact that it has many disadvantages when compared to monophagy, the fact that polyphagy exists, and not so rarely, suggests that there are certain fitness benefits to having a broad diet over a specialised one (Bernays, 2001). Advantages of polyphagy include the obvious wider range of hosts, increasing the likelihood of suitable host plants being available in the environment, but also could include immunity benefits. The European grapevine moth, *Lobesia botrana*, feeds on a mixed diet including a diverse range of locally rare species, despite the fact that grapevines (a suitable and highly abundant host plant) are readily

available in its native regions. Müller et al. (2015) found that when this species was fed on such a mixed diet, its haemocyte concentration and phenoloxidase activity were significantly higher than on those fed a diet of 'generalist' diet of grapevine. Haemocytes are integral to the immune response of an insect, responsible for the phagocytosis of viruses and pathogens (Müller et al., 2015).

### *Strategies to overcome problems of polyhagy*

These advantages explain the prevalence of polyphagy, but do not make up for the difficulties that being a generalist presents in host plant choice. As described above, efficiency of locating a host plant is of great importance in an ecological setting, and thus polyphagous insects must have developed certain mechanisms or strategies to either 1.) improve the efficiency of host finding, or 2.) minimise the problems associated with reduced efficiency. Indeed, it is thought that specialists and generalists employ two differing strategies for assessing sensory input and making a choice (Bernays, 2001). Specialists have simplified their decision-making processes through the evolution of increased sensitivity to a small number of stimuli that are relevant and important (Bernays, 2001). Being specialised in this manner needs fewer receptors dedicated to odour perception, and also requires a smaller memory capacity, allowing for more neural input to other tasks such as vigilance against possible predators or parasitoids (Bernays, 2001). With such an enhanced sensitivity to relevant odours, specialist species also benefit from faster decision-making (Bernays, 2001). Generalists, on the other hand, not having their sensory system focussed on particular odours, must cope with and decipher a larger quantity of information, for which they do not necessarily have the required neural capacity (Bernays, 2001).

Bernays (2001) suggests several possible solutions for this polyphagous' dilemma. Firstly, looking at a cellular level, when contact chemoreceptor cells respond independently to negative and positive inputs, an additive effect would cause a response suitable for the balance at that particular moment. This is commonly seen in phytophagous insects, with attractants enhancing, and deterrents reducing, input from positive neurons (Shields and Mitchell, 1995; White et al., 1990). It is thought that this mechanism is more common in polyphagous insects, and thus could be one way that a decision is reached more quickly (Blaney & Simmonds, 1990). Many insects prioritise certain behaviours at certain times, with modulation of neural circuitry resulting in individuals being less disturbed by other inputs while on a task (Bernays, 2001). There are thought to be many mechanisms allowing for such

narrowing of preferences, and one is thought to be the adjusted sensitivity of chemoreceptors to particular metabolites. In fact such metabolites, as well as other nutrients, are involved in actually activating feeding behaviour in many polyphagous species. For example, *Pieris rapae* neonates will feed on a non-host plant (cowpea) where they eclose, but later instars reject the plant. Later instars will however feed on cowpea when it has been treated with an aqueous extract of the host (cabbage) (Renwick & Lopez, 1999).

It is also considered, and proven, that learning also plays an important role in decision-making behaviour of polyphagous insects (Bernays, 2001). It has also been noted that behavioural plasticity is highly important for species that have unpredictable host availability. In these cases, the insects rely upon experience in order to hone their decision making by reducing their number of potential preferences. Associative learning, a mechanism whereby a certain experience coupled with a consistent reward results in learning, is seen in many insect groups. For example generalist flower-foraging bees will improve their foraging efficiency by choosing flowers that both have a particular shape (that consistently provides nectar), and that they have previously experience handling (Cnaani et al., 2006; Hammer and Menzel, 1995). It is thought that this form of adaptive learning is forced by memory constraints. Floral constancy allows for the bees to forage effectively despite lacking in the neural capacity to learn a wide range of flower forms.

Memory playing a function in polyphagous host-choice is not restricted to bees. Many recent studies have focussed on larval experience mediating adult oviposition choice in Lepidopteran species. Thöming et al. (2013) show that adults of the polyphagous moth *Spodoptera littoralis* have an innate preference hierarchy for host-plants, which would be one means of overcoming the challenges of decision making that generalist species experience. However it was also found that larval rearing could cause shifts within this hierarchy, indicating that this species has a certain level of plasticity when it comes to host choice. The plant upon which the larva is reared in most cases became the preferred plant of both the male and the female adults as a host plant for mating and oviposition. Other studies have also found that adult oviposition choice is mediated by larval experience (Moreau et al., 2008; Olsson et al., 2006; Anderson et al., 1995). Blackiston et al. (2008) subjected fifth instar *Manduca sexta* larvae to small electric shocks when in the presence of a specific odour, therefore establishing a conditioned aversion response. When the larvae were tested in a Y-tube choice, they actively avoided the odour associated with the shocks. Furthermore, adults

tested similarly in the Y-tube also displayed this aversion, indicating that the conditioned memory has been retained through pupation into adulthood. Some studies have found that this can be due to ‘chemical legacy’ – the exposure of newly emerged adults to the odours associated with their larval environment (Storeck et al., 2000). But Blackiston et al. (2008) removed the possibility that such a contact could occur, reinforcing the fact that there is indeed a transfer of memory through metamorphosis. They do however find that insects conditioned at an earlier instar carried the memory over only two larval moults, but did not show the aversion behaviour in the adult Y-tube assays.

In summary...

### *Immune response*

As already mentioned, herbivorous insects are under pressure from a number of predators, parasitoids, and pathogens in their complex environment (Bernays, 2001). Whilst foraging and feeding many insects will have a reduced capacity to assess potential risk factors, and this coupled with a lack of an acquired immune system would seemingly indicate that they are at a disadvantage in any environment with parasitoids and pathogens present. Insects do however possess an innate immune system (González-Santayo and Córdoba-Aguilar, 2012; Rodríguez-Andrés et al., 2012). *Spodoptera littoralis* has been found to have the ability to phagocytize small organisms, and encapsulate larger organisms, that are recognized as being

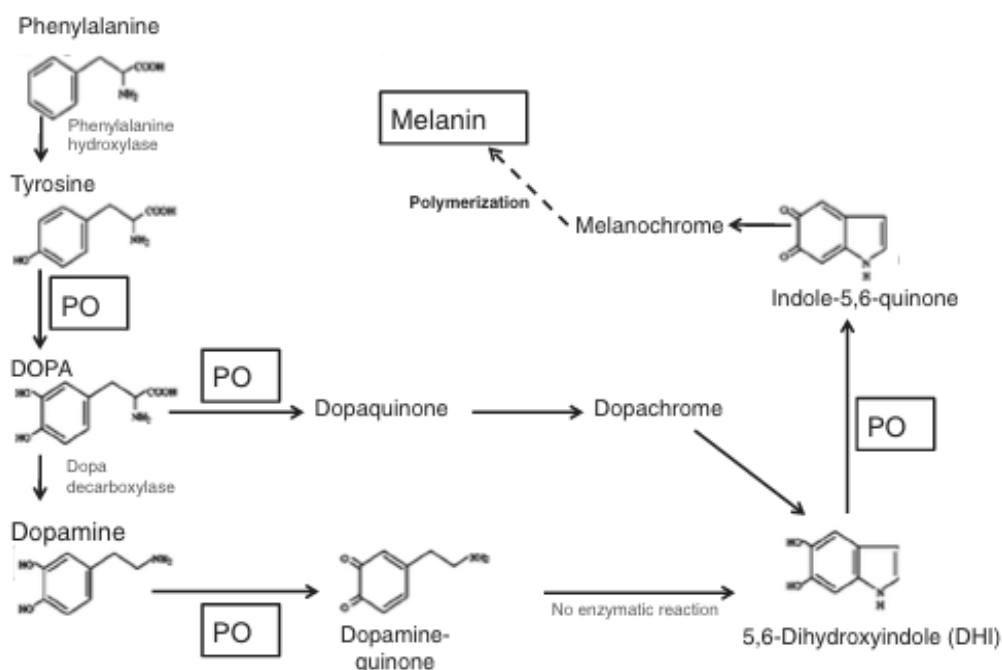


Figure 1. Melanin synthesis pathways, and the various ways that phenoloxidase (PO) is involved: in the conversion of Tyrosine to DOPA, DOPA (or alternatively dopamine) to dopaquinone, and 5,6-dihydroxyindole to indole-5,6-quinone. (González-Santayo and Córdoba-Aguilar, 2012).

foreign (Cotter & Wilson, 2002). Haemocytes, phagocytes of invertebrates, are present in the haemolymph of the insect, along with many proteins exhibiting antibacterial, antiviral, and antifungal properties. Phenoloxidase is one such important enzyme, which has been found to have a huge role in insect immunity, with a direct role in melanin synthesis, the key polymer involved in encapsulation of foreign entities such as parasitoid eggs.

Phenoloxidase (PO) is a key component in several steps of the melanisation pathway - for the hydroxylation of tyrosine to DOPA (the precursor of dopamine), the oxidation of DOPA (or dopamine in an alternative pathway) to dopaquinone, and then the conversion of 5,6-dihydroxyindole to indole-5,6-quinone (fig 1) (González-Santayo and Córdoba-Aguilar, 2012). Melanin is a chemical compound responsible for pigment formation, and is common across nature, for example in hair and skin colouration in mammals (Riley, 1997).

In invertebrates, melanin is not only important in pigmentation, but is also a defence against nematodes and parasitoids. Once a foreign object (e.g. parasitoid eggs) has been detected within the body of an insect, it becomes surrounded by haemocytes that are releasing chemoattractants that initiate the formation of a plasmatocyte wall around the target. Melanin production in the inner layers of this wall causes it to become thickened and hardened around the foreign body, preventing its growth and reproduction, and eventually killing the invading organism (fig 2) (González-Santayo and Córdoba-Aguilar, 2012).

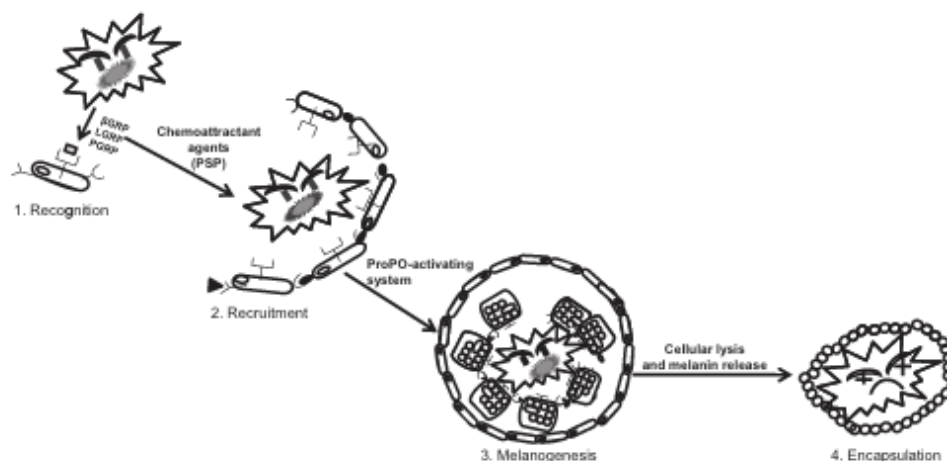


Figure 2. The four stages of melanisation: 1. specific molecules (such as lipopolysaccharides) recognise the presence of pathogens; 2. chemoattracted proteins recruit haemocytes to surround the pathogen; 3. melanogenesis occurs within the haemocytes; and 4. melanin is released and the pathogen is encapsulated. (González-Santayo and Córdoba-Aguilar, 2012).

Levels of PO have been found to vary between individuals of *S. littoralis*, even when treated and reared in the same way (Cotter & Wilson, 2002). This indicates that there is a genetic



heritability of immune function in this species. Cuticular melanisation has been found to be a quantifiable trait that can be correlated with PO activity, and thus a potential indicator for immune function, in the mealworm beetle *Tenebrio molitor* (Armitage and Siva-Jothy, 2005) as well as in *S. littoralis* (Cotter et al., 2008). Barnes and Siva-Jothy (2000) demonstrated that *T. molitor* larvae reared in high densities had higher resistance against pathogens, and that beetles with darker cuticles (i.e. higher levels of cuticular melanisation) were also more resistant. Cuticular melanisation is also seen to differ between different phases or in different circumstances in certain species. For example in the desert locust *Schistocerca gregaria*, the low-density nymphs are cryptic whilst the high-density nymphs have conspicuous yellow and black stripes, most probably to act as an aposematic warning, or for thermoregulatory reasons. *Spodoptera littoralis* has also been described by Cotter et al. (2008) as being a phase polyphenic species, also with different phenotypes dependent on larval density. It has also been suggested that this difference in cuticular melanism is related to thermoregulation or aposematism (Cotter et al., 2008) but Wilson and Reeson (1998) coined the idea of density dependent prophylaxis (DPP), whereby the insects in higher densities are at a higher risk of virus and disease, and thus invest more in their immune system (Cotter et al., 2004).

### *Research questions*

It has already been proven that insects carry memory from their larval experience through pupation that can have an effect upon their adult choice-making in terms of mate- and host-finding (Proffit et al., 2015). Blackiston et al. (2008) also demonstrated that conditioning to a stimulus could cause a repellent effect in both larval choice making and in the respective adult choice making. In *S. littoralis* it has been found that although adults have an innate host hierarchical preference, larval experience shifts the preference to the larval host plant in most cases. However no studies have looked at whether this choice preference can be affected by the quality of the experience. Will the larvae still choose the plant upon which it has been reared, if the experience has been suboptimal? And if so, will this behaviour be transferred to adulthood?

I subjected *S. littoralis* larvae to three differing treatments: solitary, high density, and starvation. This gave a range from no competition with adequate food (solitary), to a competition scenario with adequate food (high density), and no competition but inadequate food for proper development (starvation). Development data was collected in order to study the effects of each treatment on larval and pupal weight, and development time. Each

treatment was also repeated on three different species of agriculturally relevant plants - that have been found to be hosts for *S. littoralis*- cotton, cabbage, and maize, in order to test for any possible treatment-diet interactions upon choice behaviour.

Cotter et al. (2008) also found that *S. littoralis* have different levels of cuticular melanisation, and thus different allocations to immune response, when in solitary compared to in crowded phases. This was included in my study by recording cuticular melanisation and also artificial encapsulation levels, and to determine if there was perhaps also a diet effect upon melanisation levels.

### *Spodoptera littoralis*

I worked with the Egyptian Cotton Leafworm, *S. littoralis* Boisduval (Lepidoptera, Noctuidae), a noctuid moth that is an important agricultural pest across Africa and Mediterranean Europe. It has been spreading further north in Europe, and is considered a quarantine species in Europe and the United States (Ellis, 2004). Being highly polyphagous, with a staggering 88 host plant species across a range of families (Brown & Dewhurst, 1975), makes it a highly destructive species for a range of crops.

*S. littoralis* larvae are relatively immobile in comparison to the adult moths, so female oviposition site is important for the survival of the offspring (Renwick, 1989). *S. littoralis* do have an innate preference hierarchy, preferring clover to cowpea, cotton, and, cabbage respectively (in this order) (Thöming et al. 2013). However recent studies have found that this innate preference can be overridden by experience, for most plant species (Proffitt et al., 2015; Thöming et al. 2013), with cabbage being an exception, whereby preference is not increased following experience (Thöming et al. 2013).

I tested the larvae on three different host plants: *Gossypium hirsutum* L. (cotton), *Zea mays* L. (maize), and *Brassica oleracea* L. (cabbage). *Gossypium hirsutum* is one of the most widely cultivated species of cotton in the world, with 90% of global cotton production deriving from cultivars of this species (Wendel and Cron, 2003). *Zea mays* is one of the most important crops worldwide, for human consumption as well as animal fodder (Massey et al., 2014), with a wide range of forms depending on its final use. *Brassica oleracea* is another important crop species, with a high number of common, and popular, cultivars, including cabbage,

broccoli, and cauliflower (Rakow, 2004). Each of these species has a wide range of insect pests, one of which is *S. littoralis*.

It is hypothesised that high larval density could and starvation could induce repellence of the larval host plant, with the null hypothesis being that density and diet quality have no effect on how the host plant is perceived. It is also hypothesised that both density and diet could have an effect on immune response allocation, and larval development, with the null hypothesis being that there is no effect.

## Materials and Methods

### *Insects and plants*

Laboratory reared *S. littoralis* originating from a strain collected from the Alexandria region of Egypt were used in this project. Wild moths from Egypt are added into the laboratory culture at least once a year in order to maintain outcrossing within the population. The laboratory culture is reared on a semi-artificial diet (Hinks & Byers, 1976), but larvae used in the current study were reared on different plant species (cotton, cabbage, and maize – see below). Larvae were reared until pupation in a climate chamber set at  $25\pm 2^{\circ}\text{C}$ , with  $70\pm 2\%$  relative humidity (RH) and 16:8h light:dark (L:D) rhythm. Upon pupation, the pupae were sexed and males and females kept in separate climate chambers set at  $25\pm 2^{\circ}\text{C}$ ,  $70\pm 2\%$  RH and 16:8h L:D.

Cotton plants, *Gossypium hirsutum*, cabbage plants, *Brassica oleracea*, and maize plants, *Zea mays*, were cultivated from seed in a climate-controlled greenhouse at  $25\pm 2^{\circ}\text{C}$ ,  $70\pm 2\%$  RH, 12:12h L:D. The plants were grown in 1.5l pots in commercial compost (Specialblandning, Hasselfors Garden AB). Plants were used for feeding and experiments when they were ca. 6 weeks old, i.e. cotton and cabbage with approximately eight true leaves, and maize with approximately six true leaves.

All insects used in the larval- and oviposition preference assays were taken directly from the development assay. Those used for the immune assay were treated in the same manner as those in the development assay, but were not used after the encapsulation as this could potentially cause differences in behaviour and development.

### *Developmental study*

Upon eclosion, neonate larvae were randomly divided and transferred, using a fine paintbrush, into three boxes containing cotton, maize, or cabbage leaves. Upon reaching second instar, 2-4 days later dependent on diet, they were split into three treatments: ‘starve’, ‘low density’, or ‘high density’ in 100ml closed cups (fig. 3). Lids for the cups were punched with a 1.25cm-diameter hole, and a square of fine mesh (Sintab Produkt AB) placed underneath. Filter paper (Munktell Filter AB) was placed in the bottom of the cup and dampened with distilled water in order to prevent leaf-drying. Under the ‘starve’ treatment (fig. 3a) individual larvae were only fed a small quantity of food ( $\sim 1\text{cm}^2$  at early instars, increased to  $\sim 2\text{cm}^2$  at late instars) every 1-2 days, whereby they ran out of food between

feedings. The ‘low density’ treatment (fig. 3b) provided individual larvae with more than enough food. For the ‘high density’ treatment (fig. 3c) four larvae were placed in one pot, with more than enough food provided. Pilot assays were carried out previously in order to determine four as an optimal number for the high-density. Higher densities within a pot of this size resulted in such high mortality rates due to virus that these experiments would not be possible. Low- and high-density treatments were checked daily, with food added (or renewed in the case of drying out) in order that there was always food available.

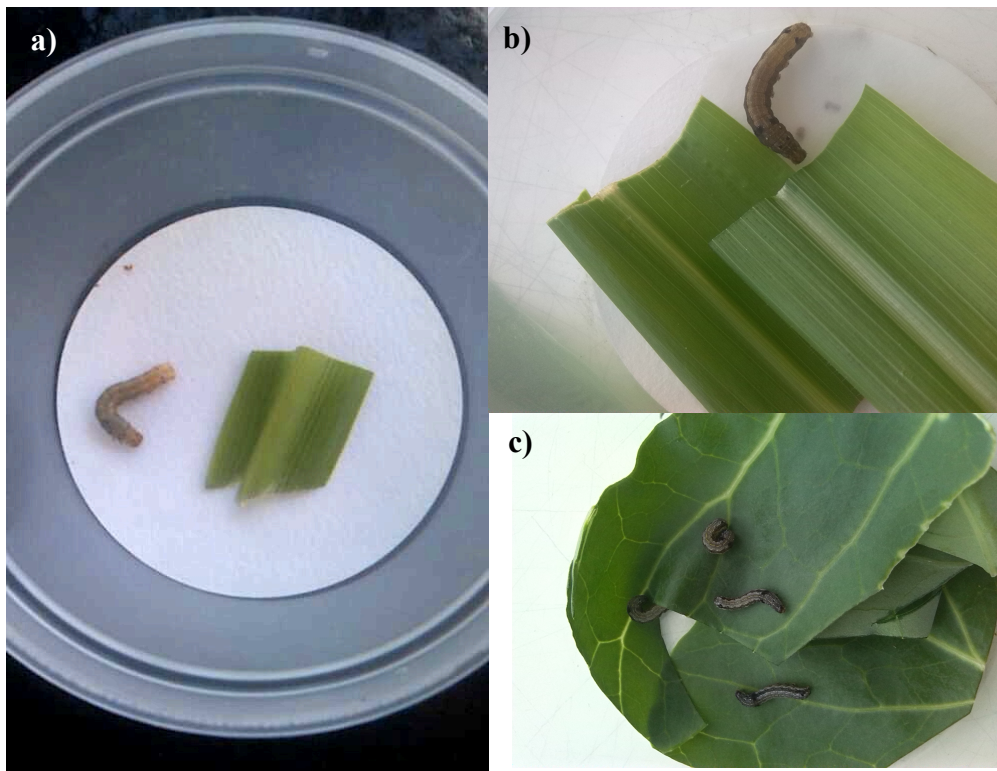


Figure 3. Examples of pots from the development assay: a) *Spodoptera littoralis* larva in the ‘starved’ maize treatment; b) *S. littoralis* larva in the ‘low density’ maize treatment; and c) *S. littoralis* larvae in the ‘high density’ cabbage treatment.

Larval weight (mg) was measured at days 10 and 14 after eclosion using scales (precise to  $\pm 0.1$ mg) (HR-200-EC, A&D Instruments Ltd.). The number of days until pupation, and pupal weight and sex, were also recorded.

### Larval preference

Host plant preference of IV<sup>th</sup>-V<sup>th</sup> instar larvae was tested in Y-tube olfactometer assays (fig. 4). Insects were starved for 2-3 hours in the experimentation room before testing. The experiment took place in a temperature and humidity controlled room (25±2°C, 70±2% RH). A choice between the plant upon which the individual was reared (‘experienced’) and an

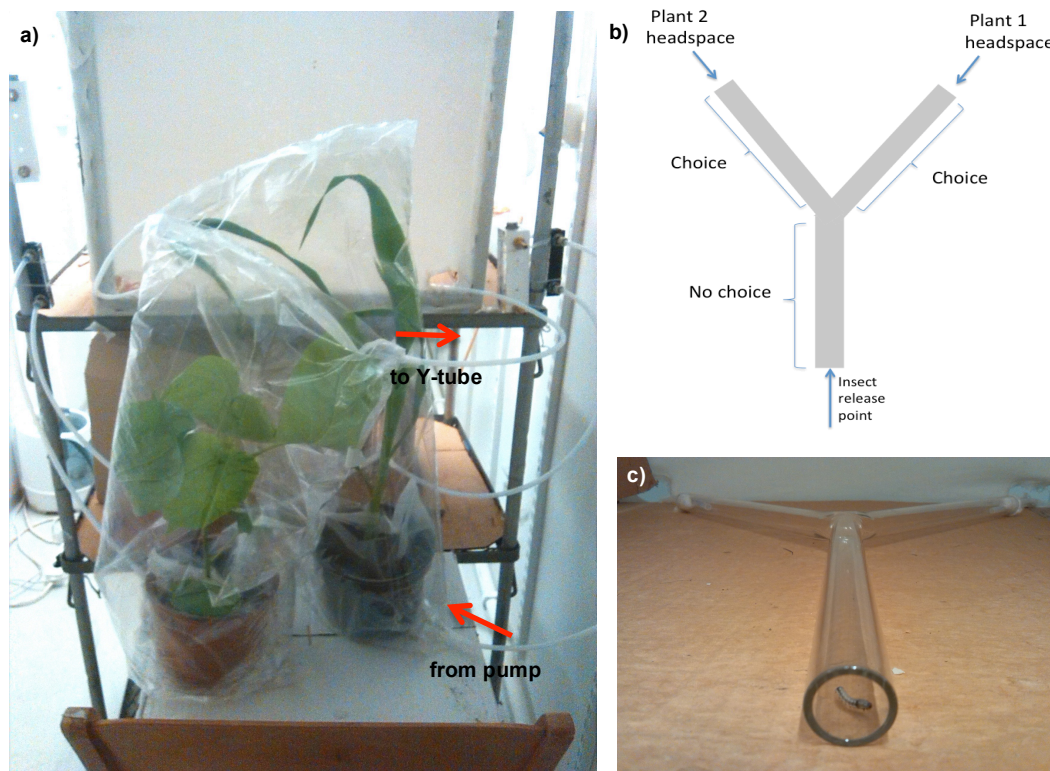


Figure 4. Larval choice assay. a) Set-up for the plant headspace. Cotton (left) and maize (right) plants in airtight odourless oven bags, connected by Teflon tubing to the pump (via flowmeter) and to the Y-tube (situated in the box behind); b) Pictorial diagram of the Y-tube. The insect is considered to have made a choice upon entering one of the branches within a period of 5 minutes; c) *Spodoptera littoralis* in the Y-tube.

‘unknown’ plant was given. The insects could not see or touch the plant, but had to rely upon the odour for making a choice. Undamaged plants with eight true leaves (or six in the case of maize) were used. The pots were sealed in 23x25cm food bags (Toppits, Melitta Group, Germany) in order that soil odour attraction was eliminated from the experiment. The plants were then individually sealed in airtight odourless 66x55cm cooking bags (Toppits, Melitta Group, Germany) into which a headspace of the plant can be collected and thus pushed into an arm of the glass Y-tube by means of Teflon tubing. The cooking bag was connected to the pump at one of the lower corners, and connected to the Y-tube from the opposite corner, in order to assure an even airstream across the plant (fig 4a).

The Y-tube olfactometer is comprised of a glass Y-shaped tube with each arm 20cm long and 1.5cm in diameter, connected as an airtight system using Teflon tubing to the odour source. A pump (Elite802) pushes air first through charcoal in order to eliminate contaminants, and then to the odour source via a flowmeter (ensuring a flow rate of 1.0). The Y-tube is in a box with a melanine top in order to ensure even light intensity. An insect is seen as having made a choice by entering one of the arms within a maximum period of five minutes. If the insect does not move into an arm, this is recorded as ‘no choice’ (fig 4b&c). The glass Y-tube was changed after every 20 insects, and a new one was also used for each plant combination in case of odour contamination. Y-tubes were cleaned with ethanol and burned in a 200°C oven in order to remove any contamination.

### ***Oviposition preference***

The oviposition assays were carried out in 32.5x32.5x32.5cm plastic cages (E6101 Bugdorm-

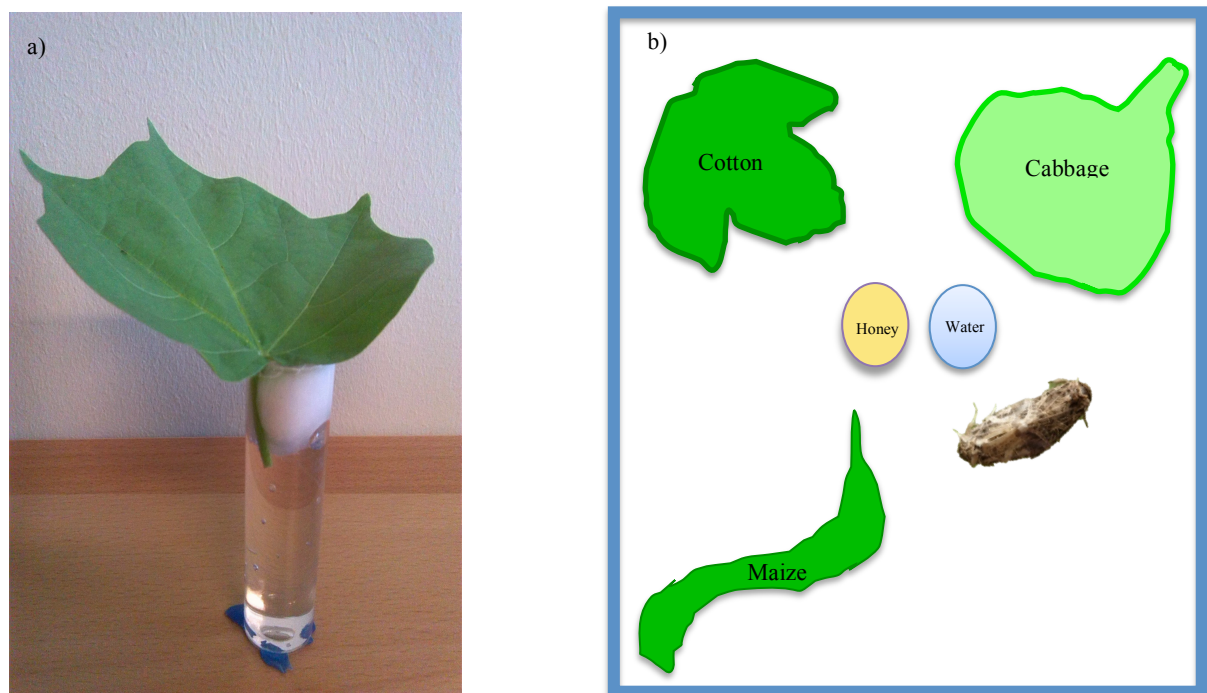


Figure 5. Oviposition assay. a) leaf set-up (*Gossypium hirsutum*); b) set up: detached leaves of cotton, cabbage, and maize placed in the corners of the bugdorm, touching the walls. Honey solution and water provided for the adults. Mating pair of *Spodoptera littoralis* placed directly into the cage and left for three days

43030, Taiwan) in a temperature-controlled greenhouse ( $25 \pm 2^\circ\text{C}$ ) with natural light. Single leaves were cut from plants with six true leaves and placed in drosophila vials of water plugged with cotton wool (fig 5a). The vials were secured to the floor of the cage using dental wax, with the leaves touching the cage side. Leaves were replaced every 24 hours. Water-



soaked, and honey-solution-soaked cotton was provided for the adult insects to feed and drink (fig 5b).

Two to four day-old females from the development study were mated with 2-day-old males from the laboratory artificial diet-fed rearing. The mating pair was put directly into the middle of the floor of the cage. The floor of the greenhouse was sprayed with water daily in order to maintain humidity at ca. 70RH.

Egg batches were collected every 24 hours for a period of 3 days. Collecting the eggs every day allowed for studying initial as well as overall preference. The eggs were scraped from the leaf using the non-sticky side of a 2"x3" Post-It note, and weighed using a scale (as above). Eggs were recorded to have been laid either on the leaf (including if they were laid on the cage directly behind the leaf), on the water- or honeypot, or just on the cage in no close vicinity to a leaf.

### ***Immune response***

The immune response of the larvae was assessed in two separate ways: cuticular melanisation, and an artificial encapsulation assay.

Artificial implants were made from nylon thread that was sliced using a razorblade into approximately 3mm-long pieces, with a knot tied 2/3 along. The knot allows for a piece of the nylon to remain on the outside of the body for easy collection of the implant (fig 6a). The implants were stored in 70% ethanol before being used.

The implants were inserted into 5<sup>th</sup> instar larvae, between the final pair of abdominal prolegs. A pin was used to make a small hole in the side of the larvae, in which to place the implant. The 2mm end of the implant was pushed using forceps into the hole as far as the knot, with close attention that it was inserted longitudinally, so as not to rupture the gut. The larva was then returned into its treatment cup. The implant was removed (pulled out using forceps) after 24h and placed into 70% ethanol. The level of encapsulation was analysed by photographing the implant (Nikon D200, attached to microscope (magnification x10) (SZ-PT Olympus) (fig. 5c) and ordering the images by darkness (by eye).





Figure 6. Immune function study: a) *Spodoptera littoralis* with an artificial insert placed between the two back prolegs, ready for removal. It is possible to see the black melanisation of the insert between the knot and the body of the insect; b) example of a melanised artificial insert (left) compared to an unused insert (right); c) Melanisation scale developed by Cotter et al. (2008), rating the insects on cuticular melanisation from ‘extra pale’ to ‘extra dark’ (XP = extra pale, P= pale, PI= pale intermediate, I= intermediate, DI= dark intermediate, D= dark, XD= extra dark) (Cotter et al., (2008))

The cuticular melanisation of 4<sup>th</sup>-5<sup>th</sup> instar larvae was scored according to a melanisation scale (Cotter et al., 2008) (fig 6c) ranging from extra pale (‘XP’) to extra dark (‘XD’). The artificial encapsulation assay follows an adaptation to the protocol by Cotter (2012).

### **Data analysis**

All statistical analyses were carried out using R statistical software (R version 3.1.2 “Pumpkin Helmet” run through RStudio version 0.98.1091). Tests were carried out comparing all three treatments, but also comparing only low- and high densities.

### **Development study**

The effect of each independent factor (diet, treatment, and diet-treatment interaction) on each of the development measures was tested using a two-way ANOVA, followed by a Tukey’s

HSD (Honestly Significant Difference) post hoc analysis test in order to identify where the significant (if applicable) differences lie.

#### *Larval preference*

Generalized linear models were carried out to test for significant differences between the choices made by larvae of the different treatments. The tests were also carried out to check for differences between ‘choice’ and ‘no choice’ behaviour.

#### *Adult oviposition preference*

Two comparisons were made of the oviposition data: total quantity of eggs laid, and first choice. These were tested for significance by using an ANOVA followed by Tukey’s HSD.

#### *Immune response assay*

The cuticular melanisation scores were converted into numerical scores (XP=1, P=2, PI=3, I=4, DI=5, D=6, XD=7) which were then analysed again by the non-parametric Kruskal-Wallis one-way analysis of variance.

The images of the artificial implants were not analysed per se, but differences can be seen by eye.

## Results

### Development study

#### Day 10 larval weight

Diet and density treatment both have a significant effect on larval weight at day 10 ( $F_{(2, 4.88)}$ ,  $p=0.0079$ , and  $F_{(2, 189.31)}$ ,  $p<2e-16$  respectively), with maize-fed larvae being significantly heavier than both cotton-fed ( $p=0.009$ ) and cabbage-fed ( $p=0.03$ ) larvae, and starved larvae being significantly lighter than low- ( $p<0.0001$ ) and high-density larvae ( $p<0.0001$ ) (fig 7). There is no significant difference between low- and high-density larvae ( $p=0.007$ ). There is also a significant interaction effect of diet and treatment ( $F_{(4, 4.45)}$ ,  $p=0.0021$ ).

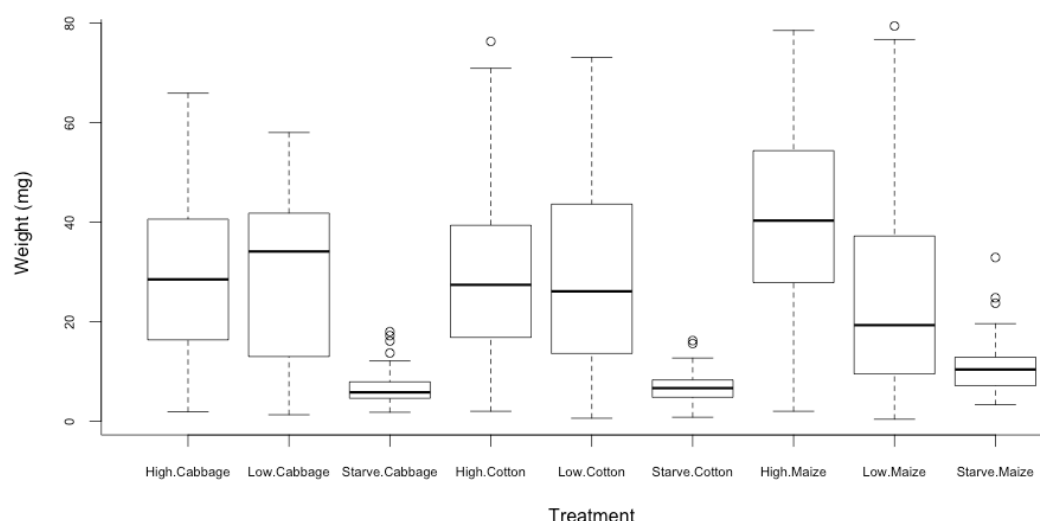


Figure 7. The weight of *Spodoptera littoralis* larvae at day 10 of their development from each diet and density treatment. (High cabbage:  $n=171$ , mean=33.74mg,  $SD=21.64$ ; low cabbage:  $n=50$ , mean=35.51mg,  $SD=20.72$ ; starve cabbage:  $n=33$ , mean=6.78mg,  $SD=3.49$ ; high cotton:  $n=115$ , mean=38.02mg,  $SD=35.71$ ; low cotton:  $n=69$ , mean=40.91mg,  $SD=36.39$ ; starve cotton:  $n=65$ , mean=6.89mg,  $SD=3.01$ ; high maize:  $n=87$ , mean=43.61mg,  $SD=23.13$ ; low maize:  $n=38$ , mean=29.78mg,  $SD=26.04$ ; starve maize:  $n=54$ , mean=10.83mg,  $SD=5.5$ )

#### Day 14 larval weight

Diet and density again both have a significant effect on the larval weight at day 14 ( $F_{(2, 47.94)}$ ,  $p<2e-16$ , and  $F_{(2, 434.51)}$ ,  $p<2e-16$ ) respectively. Interaction of these variables also has a significant effect upon the larval weight ( $F_{(4, 6.12)}$ ,  $p=7.79e-05$ ). Maize-fed larvae were found to be significantly lighter than both cotton- ( $p=0.002$ ) and cabbage-fed larvae ( $p<0.0001$ ), whilst cotton-fed larvae were significantly lighter than cabbage-fed larvae ( $p<0.0001$ ). Larvae undergoing the starvation treatment were significantly lighter than both low- ( $p<0.0001$ ) and high-density larvae ( $p<0.0001$ ), whilst the low- density larvae were also lighter than the high-density larvae ( $p=0.002$ ) (fig 8).

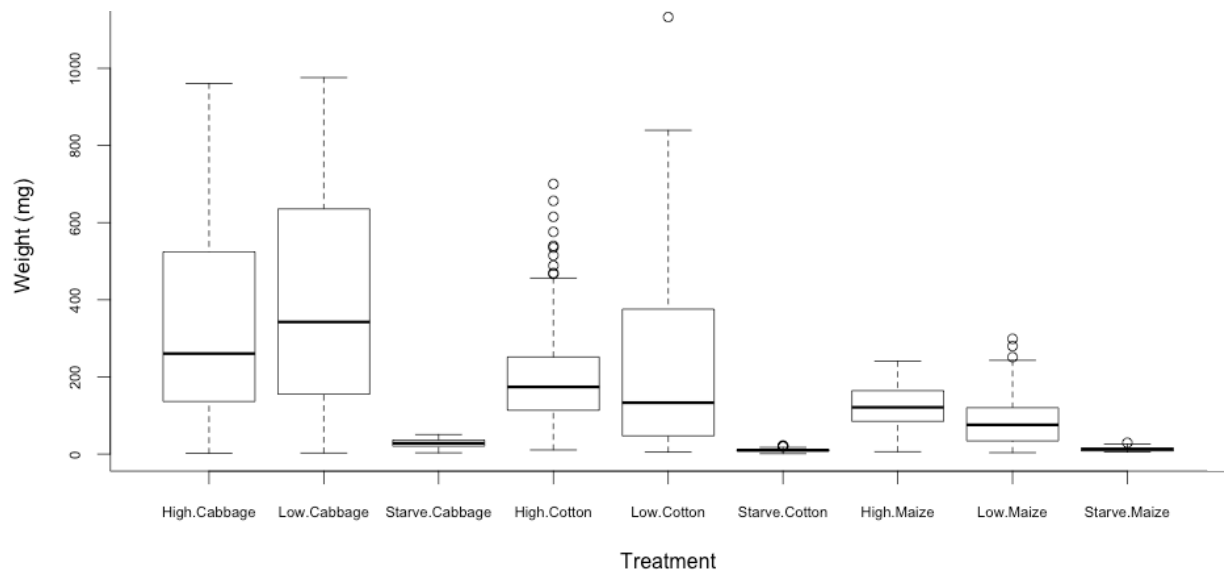


Figure 8. The weight of *Spodoptera littoralis* larvae at day 14 of their development from each diet and density treatment. (High cabbage:  $n=171$ , mean=346.2mg,  $SD=252.6$ ; low cabbage:  $n=50$ , mean=400.45mg,  $SD=284.65$ ; starve cabbage:  $n=33$ , mean=27.48mg,  $SD=11.66$ ; high cotton:  $n=115$ , mean=208.01mg,  $SD=146.76$ ; low cotton:  $n=69$ , mean=240.13mg,  $SD=250.87$ ; starve cotton:  $n=65$ , mean=10.16,  $SD=4.15$ ; high maize:  $n=87$ , mean=124.39mg,  $SD=58.36$ ; low maize:  $n=38$ , mean=94.48mg,  $SD=79.28$ ; starve maize:  $n=54$ , mean=12.48mg,  $SD=5.12$ )

### Starvation treatment

Cabbage was the only diet that produced enough pupae in the starvation treatment. Insects reared on cotton and maize ‘starvation’ treatment did not reach pupation due to high levels of failure before pupation, high levels of malformed pupae, or because the larvae were taking too long to develop for the timescale of this study. Therefore the data for pupal weight and development time including the starvation treatment was analysed only with the data from cabbage-reared insects. Otherwise, pupal weight and development time was analysed only by comparing low- and high-density treatments.

Treatment was found to have an overall statistical effect on both pupal weight ( $F_{(2, 193.2)}$ ,  $p<2e-16$ ) and overall development time ( $F_{(2, 675)}$ ,  $p<2e-16$ ) in insects reared on cabbage. Starved insects developed into significantly smaller pupae than those in low- ( $p<0.0001$ ) and high-density ( $p<0.0001$ ) treatments. Those in the low-density treatment were significantly larger than those in high-density ( $p<0.0001$ ) (fig 9).

No significant difference was seen in the development time of insects on the cabbage low- and high-density treatments ( $p=0.87$ ), but starved insects took significantly longer to develop than both low- ( $p<0.0001$ ) and high-density larvae (fig 7).

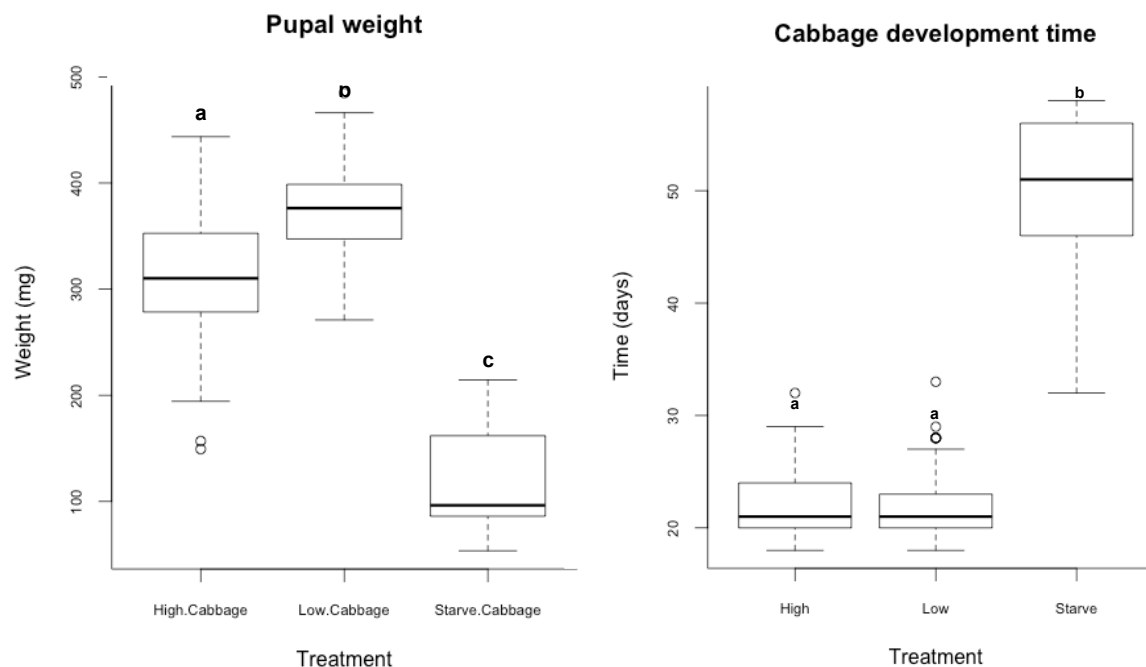


Figure 9. (left) Pupal weight (mg) for the different density treatments of cabbage-reared larvae. (right) Length of time (days) from neonate to pupation in cabbage-reared larvae. (*Weight*: High cabbage:  $n=138$ , mean=312.42mg,  $SD=55.37$ ; low cabbage:  $n=48$ , mean=376.99mg,  $SD=47.08$ ; starve cabbage:  $n=23$ , mean=117.72mg,  $SD=45.61$ ; *Development time*: High cabbage:  $n=138$ , mean=21.83 days,  $SD=2.62$ ; low cabbage:  $n=48$ , mean=22.14 days,  $SD=3.34$ ; starve cabbage:  $n=23$ , mean=49.86 days,  $SD=7.84$ )

## Immune response

### Melanisation

There is no significant effect of diet on larval cuticular melanisation (d.f.=2 ,  $H=291.6$ ,

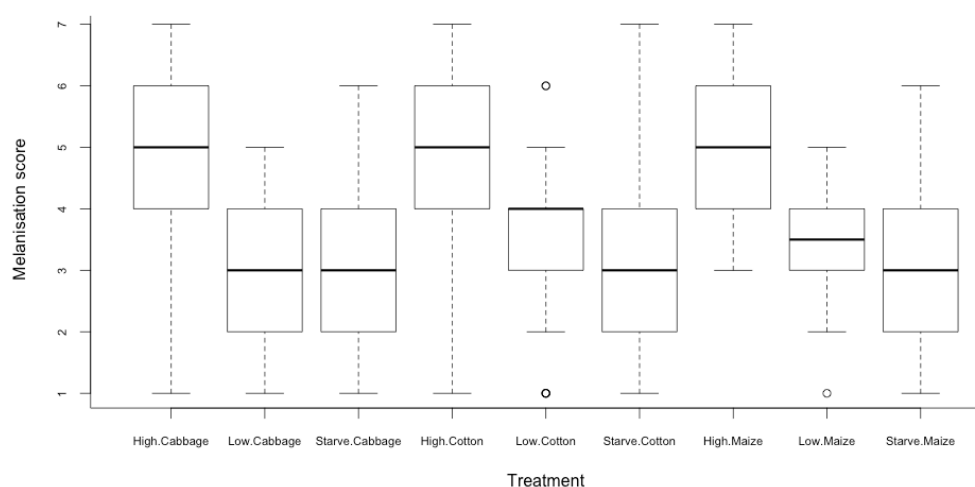


Figure 10. Mean scores of cuticular melanisation for each of the diet and density treatments. The scoring goes from XP-XD (numerically 1-6), whereby XP(1) is the palest, and XD (6) is the darkest. (*High cabbage*:  $n=190$ , median=5; *low cabbage*:  $n=59$ , median=3; *starve cabbage*:  $n=32$ , median=3; *high cotton*:  $n=127$ , median=5; *low cotton*:  $n=69$ , median=4; *starve cotton*:  $n=62$ , median=3; *high maize*:  $n=87$ , median=5; *low maize*:  $n=37$ , median=3.5; *starve maize*:  $n=71$ , median=3)

$p=0.916$ ) but there is a significant treatment effect on melanisation (d.f.=2 ,  $H=0.1749$ ,  $p=2.2e-16$ ) (fig 10).

When looking at figure 10 starved larvae are paler than both low- and high-density density larvae, and low-density larvae are paler than larvae reared in high-densities.

### *Artificial encapsulation*

Due to time constraints, for certain treatments or diets there are very few, or no, artificial capsules. However, from figures 11-13 it can be seen that the majority of starved cabbage and starved cotton show very little, or no, melanisation. In comparison the artificial capsules from the low-density larvae of both cotton and cabbage show higher levels of melanisation in most cases. Capsules from larvae reared in high-densities on cotton show perhaps marginally higher levels of melanisation (fig 12). However, maize shows very low levels of melanisation in both low- and high-densities (fig 13). In fact the low-density maize capsules could be comparable to the capsules from the starved treatment from cabbage and cotton, and the high-density maize comparable to the low-density from cotton and cabbage.

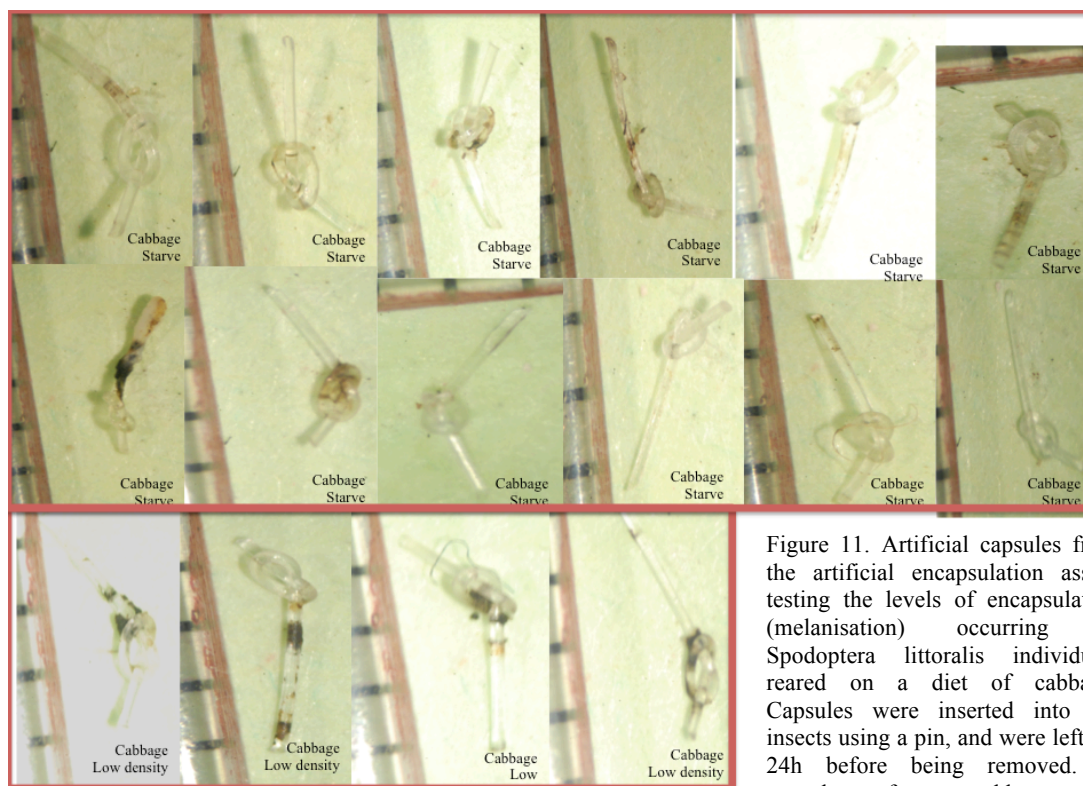


Figure 11. Artificial capsules from the artificial encapsulation assay, testing the levels of encapsulation (melanisation) occurring in *Spodoptera littoralis* individuals reared on a diet of cabbage. Capsules were inserted into the insects using a pin, and were left for 24h before being removed. a) capsules from cabbage-reared insects in the starved treatment; b) capsules from cabbage-reared insects in the low-density



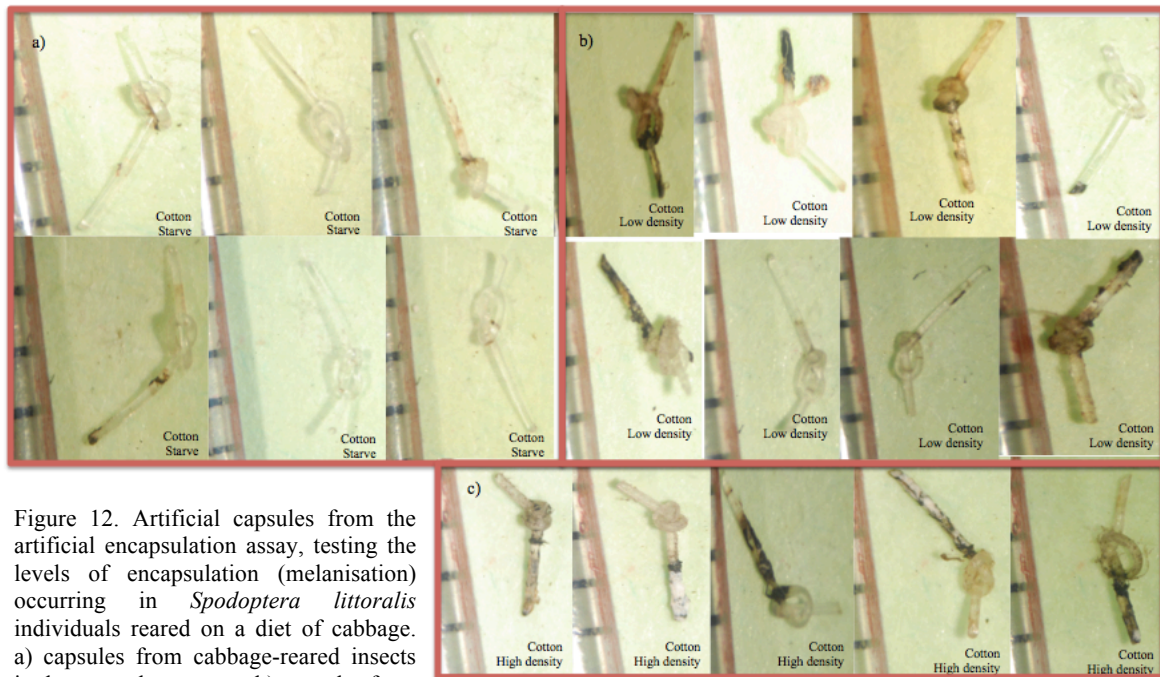


Figure 12. Artificial capsules from the artificial encapsulation assay, testing the levels of encapsulation (melanisation) occurring in *Spodoptera littoralis* individuals reared on a diet of cabbage. a) capsules from cabbage-reared insects in the starved treatment; b) capsules from cabbage-reared insects in the low-density;

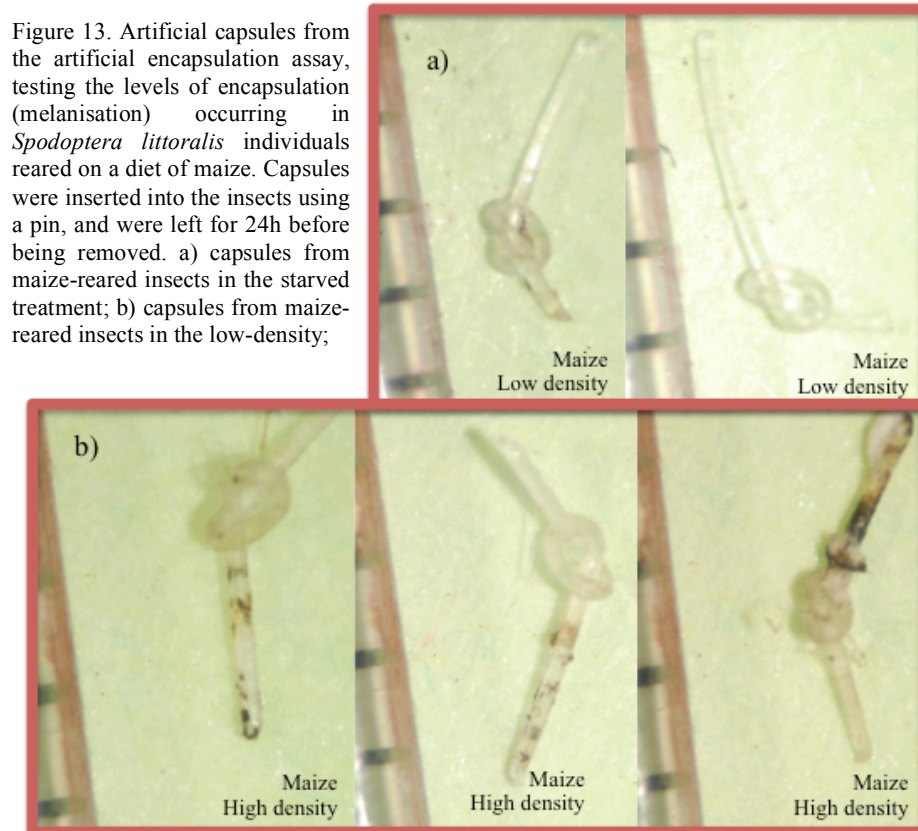


Figure 13. Artificial capsules from the artificial encapsulation assay, testing the levels of encapsulation (melanisation) occurring in *Spodoptera littoralis* individuals reared on a diet of maize. Capsules were inserted into the insects using a pin, and were left for 24h before being removed. a) capsules from maize-reared insects in the starved treatment; b) capsules from maize-reared insects in the low-density;

### *Larval preference*

Treatment was found to have a significant effect on whether or not an insect made a choice in the larval y-tube choice assays ( $F_{(2, 315.78)}$ ,  $p=6.031e-11$ ), whilst diet had no significant effect ( $F_{(2, 313.25)}$ ,  $p=0.282$ ). Both low- and high-density treatments saw a majority of individuals making a decision, with only a small number of individuals not doing so. Whilst there is little data for the starved insects (mainly due to the fact that they regularly did not choose, so the experiments were abandoned due to time constraints), it can be seen that they more often make no choice (fig. 14). For this reason, for all further y-tube analyses, the starved data was not included.

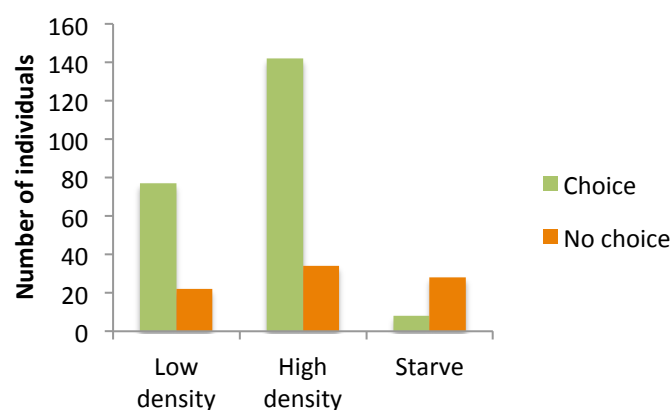


Figure 14. Number of individuals making a choice in the Y-tube behavioural assays, with a choice of the experienced host plant, and an unexperienced plant. (Low density:  $n=99$ ; high density:  $n=176$ ; starve:  $n=36$ )

In terms of time taken to make a decision (excluding all non-choice data), both diet ( $F_{(2, 23.342)}$ ,  $p=5.42e-10$ ) and treatment ( $F_{(1, 4.604)}$ ,  $p=0.0329$ ) were found to have a significant effect. No significant interaction effect of diet and treatment was found ( $F_{(2, 0.02)}$ ,  $p=0.9799$ ) (fig 15).

Tukey's post-hoc analysis shows that there is no significant difference in the time taken to choose between cotton and maize ( $p=0.575$ ), but that both maize- and cotton-fed larvae take significantly longer to choose than cabbage-fed larvae ( $p<0.0001$  and  $p<0.0001$  respectively). Low-density larvae took significantly longer (0.25 minutes) to choose than high density larvae ( $p=0.044$ ) (fig 15).



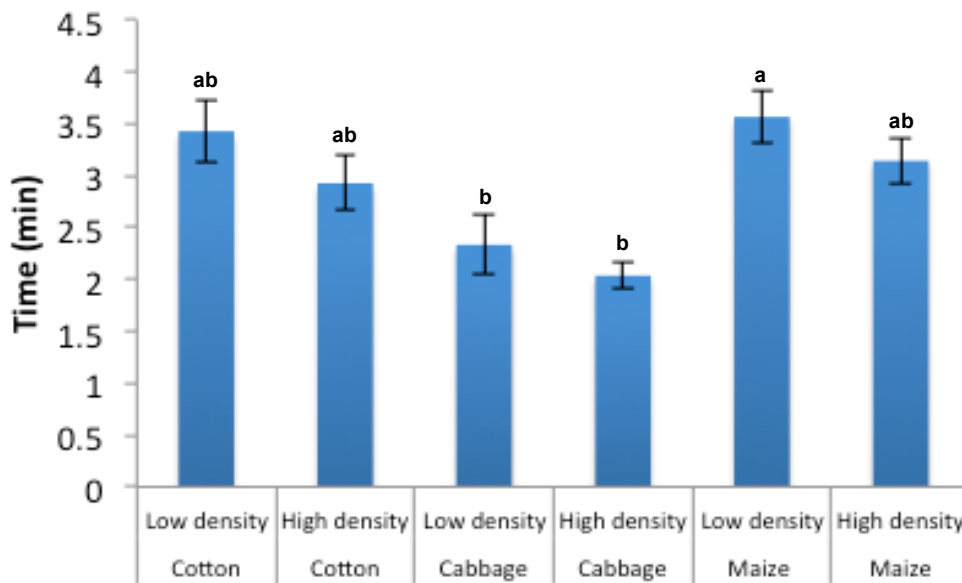


Figure 15. Time taken (minutes) to make a decision. Insects were given a maximum of five minutes before being removed. (High cabbage:  $n=125$ ; low cabbage:  $n=30$ ; high cotton:  $n=32$ ; low cotton:  $n=40$ ; high maize:  $n=42$ ; low maize:  $n=38$ )

#### *Y-tube experienced vs. non-experienced choice*

When pooled irrespective of choice comparison (cotton vs. cabbage, cotton vs. maize, and cabbage vs. maize), treatment ( $F_{(2, 308.64)}$ ,  $p=2.2e-16$ ), diet ( $F_{(2, 267.11)}$ ,  $p=9.593e-10$ ) has a significant effect on choice between experienced and non-experienced host plant. Treatment was not seen to have a significant effect ( $F_{(1, 266.33)}$ ,  $p=0.379$ ). There is however a significant interaction between diet and treatment ( $F_{(2, 258.74)}$ ,  $p=0.0335$ ). It appears from figure 14 that, in accordance with the statistics above, larvae will follow the odour of the diet that they have been reared on. It would appear that there is a slight increase of non-experienced host plant choice in the high-density larvae (except in the cotton-reared larvae), but this is not significant.

When analysing the data as separate y-tube choice assays (cotton vs. cabbage, cotton vs. maize, and cabbage vs. maize), diet nor treatment have a significant effect upon host choice in the cotton vs. cabbage choice ( $F_{(1, 93.69)}$ ,  $p=0.679$ ; and  $F_{(1, 93.861)}$ ,  $p=0.858$  respectively). In the cotton vs. maize choice test, diet was found to have a significant effect upon choice ( $F_{(1, 69.311)}$ ,  $p=0.0035$ ), whereas there was no significant difference caused by treatment ( $F_{(1, 77.832)}$ ,  $p=0.114$ ). For the cabbage vs. maize test, diet has a significant effect on choice ( $F_{(1, 112.66)}$ ,  $p=9.784e-06$ ), whilst treatment had no significant effect ( $F_{(1, 132.21)}$ ,  $p=0.461$ ).

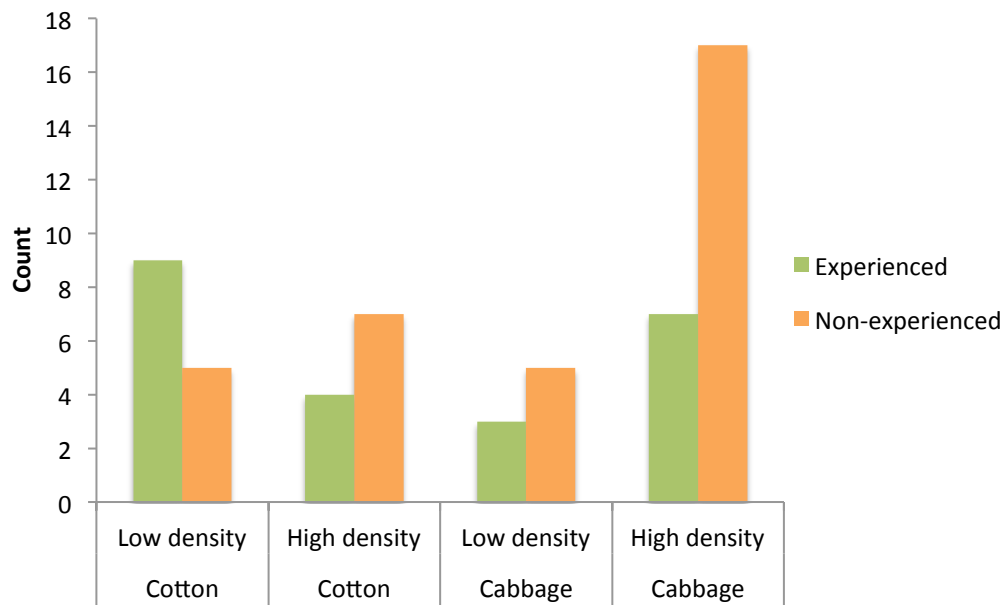


Figure 16. Decision making of *Spodoptera littoralis* larvae in the Y-tube, being given the choice between an experienced host-plant and an unexperienced plant. Larvae either made a choice, or ‘no choice’ if no decision was made within a five-minute time frame (High cabbage:  $n=125$ ; low cabbage:  $n=30$ ; high cotton:  $n=32$ ; low cotton:  $n=40$ ; high maize:  $n=42$ ; low maize:  $n=38$ )

Diet and density treatment were both found to have a significant effect ( $F_{(1, 54.82)}$ ,  $p=0.0167$ ;  $F_{(1, 69.311)}$ ,  $p=0.0027$ ;  $F_{(1,112.657)}$ ,  $p=9.784e-06$  respectively) upon whether the insect chose the plant that it had previously experienced or not (fig. 16).

### *Oviposition preference*

Only very few individuals from the ‘starved’ treatment made it to pupation, and fewer emerged as adults, and even fewer successfully mated. Therefore only low- and high-density treatments are taken into account in the analyses.

Comparing the oviposition first choice (excluding egg batches laid on the cage), there is no significant effect of diet or treatment ( $F_{(1, 74.367)}$ ,  $p=0.113$ ;  $F_{(1, 72.648)}$ ,  $p=0.1898$ ). However it can be seen from figure 17 that for cotton, there seems to be the possibility for a trend of moths that experienced low density larval rearing are more likely to choose the diet they previously experienced for their first egg-batch, whereas there is a shift in the moths from high-density larval rearing with more moths laying on the non-experienced host. In both cases for cabbage-reared insects, the moth laid more on the non-experienced plant (though no significance was found).

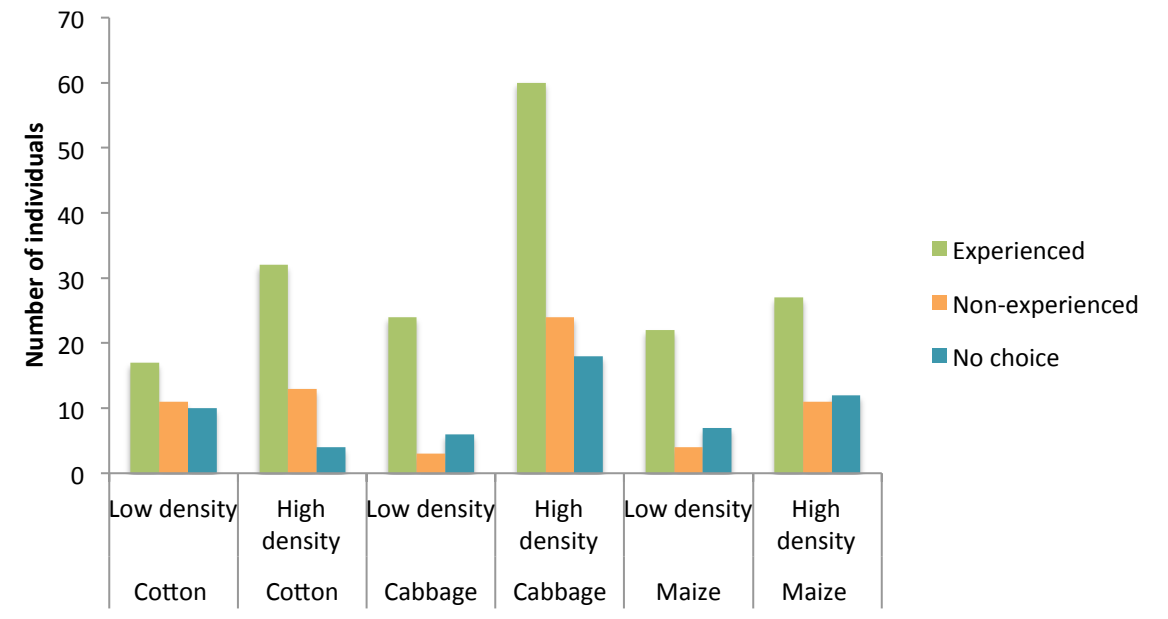


Figure 17. Number of adult *Spodoptera littoralis* females laying on each plant as their first choice. (High cabbage: n=24; low cabbage: n=8; high cotton: n=11; low cotton: n=14)

There is no significant difference between total egg load of females originating from the two different diets, cotton and cabbage, but there is a significant difference when considering whether the females were reared in low- or high- larval density. Insects that were reared in low larval densities lay a significantly higher total egg weight than those from the high-density larval treatment (fig 18)

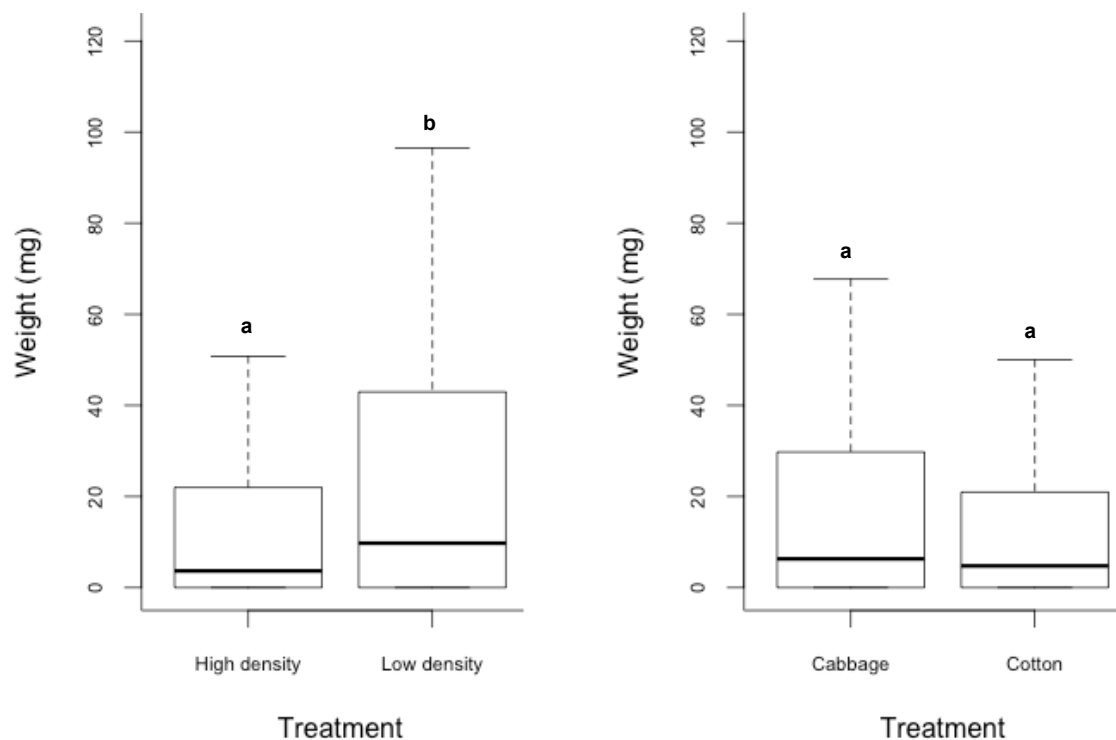


Figure 18. Weight (mg) of eggs laid per treatment and per diet. (High density: n=35, mean=13.8mg, SD=19.13; low density: n=22, mean=25.07mg, SD=32.76; cabbage: n=132, mean=18.99mg, SD=26.9; cotton: n=100, mean=16.17mg, SD=25.54)

### Average eggs laid per plant

The average weight (mg) of eggs laid on the different leaves is not significantly effected by diet ( $F_{(1, 0.821)}$ ,  $p=0.366$ ) but there seems to be an overall preference for plant species ( $F_{(3, 5.708)}$ ,  $p=0.0009$ ) (indicating that there is an overall innate-host plant hierarchy). Treatment also has a significant effect ( $F_{(1, 13.202)}$ ,  $p=0.0004$ ). There is also a significant interaction of plant and treatment ( $F_{(3, 4.244)}$ ,  $p=0.0061$ ). Low-density cabbage-reared insects laid a lower weight of eggs on maize than on either cotton ( $p=0.0047$ ) or cabbage ( $0.0047$ ), with a higher weight of eggs being laid on cotton than on cabbage ( $p= 0.032$ ). Low-density cabbage-reared larvae laid significantly heavier egg batches on cotton than low-density cotton-reared larvae do on cabbage, again indicating a hierarchy ( $p=0.0003$ ). Cabbage-reared low-density larvae laid significantly heavier total egg weight on cotton than cabbage-reared high-density larvae ( $p=0.0045$ ) (fig. 19).

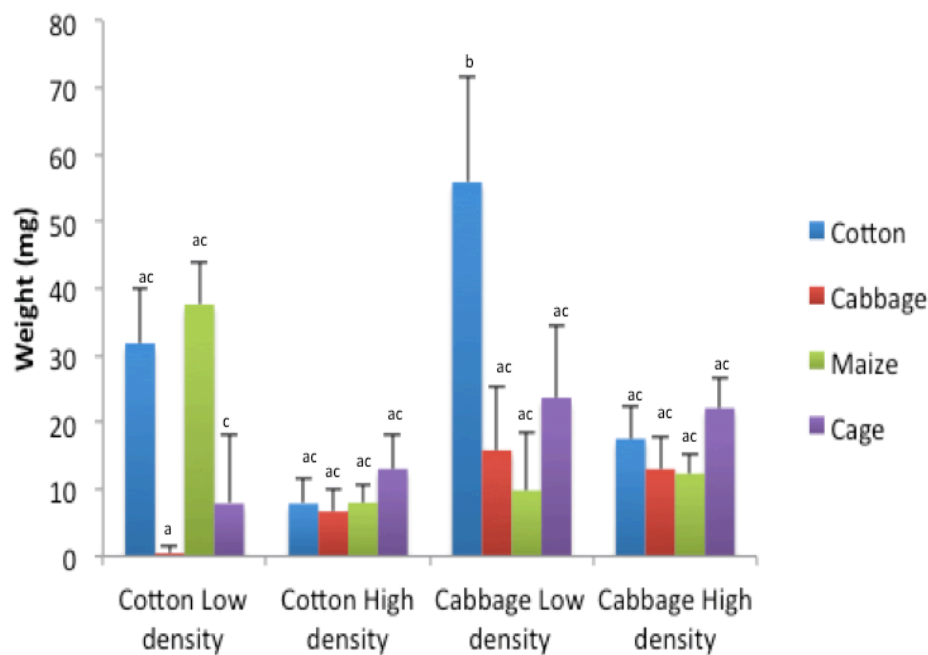


Figure 19. Average total egg weight laid by female *Spodoptera littoralis* that experienced different diets larval conditions (cabbage and cotton, and low- and high-density, respectively) on each plant (or cage) within the oviposition experiment. (Cotton low density:  $n=13$ ; cotton high density:  $n=12$ ; Cabbage low density:  $n=9$ ; Cabbage high density:  $n=24$ .)

## Discussion

It was long considered that insects follow the ‘mothers know best’, or preference performance hypothesis (PPH), whereby the adult female will oviposit on a host-plant that is most suitable for its larvae (Gripenberg et al., 2010; Janz, 2002). In most insects, the larval stage is comparatively much less mobile than the winged adult stage, such as is found in *S. littoralis*, and thus this seems like a reasonable adaptation. However, this has been recently disproved in several species, finding that the female may not actually oviposit on the optimal host for their larvae (Clark et al., 2011; Janz, 2002). Certain studies have actually found that the females have an innate preference hierarchy, where in some cases the female may actually prefer a host-plant that is not the most suitable for the development of its larvae (Thöming et al., 2013). An explanation for this could indeed be the fact that a species such as *S. littoralis*, being polyphagous, does not have the neural capacity to cope with the huge amount of sensory input from the complex environment surrounding them (Bernays, 2001). In this case, it is therefore reasonable to hypothesise that the insects have an innate preference for certain plants, thereby reducing the possible sensory input that must be deciphered. However, as well as having this innate preference, it has been found that adult females will lay on the same species of host-plant that it experienced during its larval phase (Anderson, 2013). However, as of yet there have been no studies looking at the effect of a perhaps negative experience during the larval stage on the adult oviposition choice.

This study attempted to demonstrate the effects of two different possible ‘negative’ experiences (starvation, and high-density), compared to what could be the optimal conditions for a larvae (low-density) (with less competition for food, and less likelihood of virus or pathogen transmission). Indeed, the results from the development part of this study clearly show that both the starvation and the high-density treatment do have an effect on the overall development of the insects, when compared to the ‘optimal’ low-density treatment. Despite the fact that both high- and low-density larvae were provided with an unlimited food supply, the high-density larvae weighed significantly more than the low-density larvae at day 14, for maize fed insects (fig. 8). This indicates that the insects in the high-density treatment are consuming a higher quantity food, which could be due to an effect of social interaction. This could be due to competition (i.e. eating more food more quickly due to the presence of other individuals, or a by-product of sociality, such as the need to allocate more resources to certain aspects of survival. Interestingly, despite the high-density larvae being larger at day 14 than those from the low-density, or not significantly different in the case for cabbage-fed larvae, there is no difference in development time of the two treatments (fig. 9), and in fact the pupal

weight of low-density larvae are significantly larger than the pupae of high-density larvae (fig. 9). Indeed, Kazimírová (1996) also found that *Mamestra brassicae* larvae reared in lower densities gave higher pupal weights. However, Kazimírová (1996) did not look at larval weight, and our findings for the pupal weight are the opposite to what would be expected when looking at the weights of larvae, where the high-density larvae weighed more than the low-density. Therefore there is some mechanism causing the weight gain for high-density larvae to be lost before their pupation. There could be several explanations for this, most probably something directly or indirectly linked to the social aspect of the high-density treatment. Energy could be used in physical social interaction, such as conspecific aggression (some insects will try to eat others (personal observation), which may cause defensive aggression), but also energy may need to be allocated in different ways in aggregated compared to in solitary conditions. Interestingly, when looking at the levels of melanisation the high-density larvae, in all diet treatments, display significantly higher melanisation levels than both low-density and starved larvae (fig. 11). When looking at the artificial-encapsulation assay, even without carrying out quantitative analysis, a trend can be seen whereby the inserts from high-density larvae were more melanised than those in low-density larvae, which were again more melanised than those in starved larvae (fig. 11, 12, 13). The results of both of these experiments are in accordance with the studies carried out by Cotter et al. (2004) whereby insects in higher density are more highly melanised, and also displaying higher rates of PO activity. As discussed before, this could be an indication of higher allocation of resources to immune response, due to a higher exposure to a range of viruses and pathogens. The fact that the high-density treated insects appear to allocate some of their energy to something other than development, and that they also show higher levels of melanisation indicates that there could be a very high possibility that the two are linked. It would be expected that it would be advantageous for any individual to allocate resources to immune response, but this study demonstrates the possibility that the implementation of such immune response does indeed require substantial energy input. This would indeed suggest why *S. littoralis* are found in the two phenotypic phases as described by Cotter et al. (2004).

The development study clearly shows that larvae that experience the ‘starve’ condition, with an inadequate food supply, are lighter at day 10 (fig 7) and markedly so at day 14 (fig 8). Very few insects fed on cotton and maize diets survived until pupation on the starve treatment, indicating that they require a higher quantity of these diets in order to survive and develop, in comparison to cabbage. This could be due to differing nutrient compositions

between the leaves of these species, and also that the leaves are of slightly different thickness, therefore even though the same size of leaf is fed for each diet treatment, it is highly possible that different levels of nutrition are being encountered. When comparing the development time of the cabbage-reared starved insects to cabbage-reared low- and high-density insects, the starved insects take, on average, 30 days longer to develop (over double the development time of larvae in the low- and high-density treatment) (fig 9). Furthermore, the pupal weight of the starved insects was substantially lower than high- and low- density pupae (fig 9). This is a clear indication that although the insect was successful in pupation, the lack of an adequate food supply throughout its development has had detrimental effects upon its health.

In terms of immune response in starved larvae, the melanisation score indicated that starved larvae showed low levels of melanisation (fig. 10), further backed up by the artificial encapsulation assay (fig. 10&11). This would be expected given the results from the development study, whereby it seems the starved larvae are not in good health (fig. 7,8,9). Indeed, Benrey and Denno (1997) found that *Pieris rapae* with an extended development time were more likely to be parasitized than those undergoing normal development time, due to the increased ‘window of vulnerability’ whereby the larvae is at the correct size for parasitizing. This would indicate that, despite the fact the larvae in the ‘starved’ treatment is solitary, it would be highly beneficial for insects with a prolonged development time, such as those in the starved treatment of this study, to have increased allocation to immune response, however it is clear that these insects do not have the health to do so

Diet had a significant effect on both larval size and on pupal weight with cabbage-fed larvae resulting in the highest larval- and pupal weight, and maize the smallest. This indeed indicates that maize is not an optimal host for *S. littoralis*, as also found by A. Roy (pers. comm.) probably due in part to its low water content. However, it is strange that the cotton-fed larvae produce smaller larvae and pupae than cabbage, which has been widely described as a suboptimal host for *S. littoralis*, and it also one host-plant for which preference is not increased with experience (Thöming, 2013). Cabbage is known to be a suboptimal host due to its high levels of toxic glucosinolates (Salama et al., 1971). However, there is further evidence showing that larvae and pupae that develop on cabbage weigh more than on other diets, including cotton (A. Roy, (pers. comm.); Shahout et al., 2011). Indeed Shahout et al. (2011) found cabbage to be the optimal host for *S. litura*, when it comes to pupal weight, from the diets being tested (cabbage, cowpea, and alligator weed). The nutritional value of a

host plant for a herbivorous insect is usually considered to be in the quantity of protein, however Barbehenn et al. (2013) suggests that it is not the quantity, but the quality (or a balance between the two) of the protein that is important. Perhaps it would be necessary to further study the nutritional values of each of the diets in our experiment in order to clearly understand any results and draw solid conclusions.

I therefore deduce, from the findings of the development assay, that the high-density and starved treatments could be compared to the optimal 'low-density' treatment. The starvation treatment can definitely be considered as a 'negative' experience due to the extreme differences in larval- and pupal weight, and development time. For the high-density treatment, it is difficult to ascertain whether it is an overall 'negative' experience, as they are nevertheless producing (seemingly) healthy adults.

#### *Larval choice*

When considering the diets together (excluding starvation, discussed later), there was no switch between low- and high-density larval choice (fig. 16). For both treatments, larvae were significantly more likely to choose the odour of the plant on which they were being reared, including cabbage. This is in accordance with studies on various polyphagous Lepidopteran species (Carlsson et al., 1999, Ting et al., 2002; Yamamoto, 1974) whereby larval host choice was seen to be induced by rearing conditions. When looking at each treatment in parallel with the rearing-diet, it is seen that in all cases, although 'experienced host' is more likely to be chosen, for high-density cabbage and maize reared insects, it appears that there is the possibility that there is a slightly higher chance that larvae will choose the non-experienced host when compared to low-density larvae (fig. 16). However, with having inconsistent replicate numbers between diet and treatments, this would need to be tested again. Nevertheless, the results indicate that, as expected, fourth instar larvae show higher preference for the host-plant upon which they have been reared, over an 'unknown' host, even for cabbage, irrespective of larval density.

#### *Adult oviposition choice*

The oviposition experiment however, although not significant, reveals the possibility for a switch in first-choice preference for the cotton-fed insects (fig 17). More adult females chose to lay their first egg-batch on a non-experienced plant option than on cotton in the high-



density treatment, compared to low-density-reared insects that laid more on cotton. Meanwhile, when it comes to cabbage-reared insects, both low- and high-density reared insects were more likely to choose the non-experienced leaf (fig. 17). This is in accordance with Thöming et al. (2013) as cabbage is lower in the preference hierarchy than both cotton and maize (even for cabbage-experienced insects). On the contrary, though, the cabbage reared larvae were still more likely to choose cabbage in the Y-tube than the unexperienced host (fig. 16), indicating that for larval choice previous experience overrides the innate preference hierarchy.

There is no significant difference between the number of females laying first-choice on experienced- and non-experienced host plant in the cabbage-reared low-density treatment, whilst in the high-density treatment the number choosing the non-experienced diet was significantly higher than choosing the experienced (fig. 17). This shows the possibility that there is a switch of preference based on larval rearing quality. However, more studies with a higher number of replicates need to be carried out in order to reveal whether this trend could indeed be something significant and worth considering. Also, the experiment was carried out with a choice of the experienced plant versus two non-experienced plants, which perhaps complicates the results, so it may be better to repeat this experiment with a two-way comparison, which would also be more easily comparable with the Y-tube data.

Adults from the low-density treatment laid on average significantly more eggs (in total, on all substrates) than those originating from the high-density treatment. This indicates that the insects being reared in low-densities perhaps have higher fecundity than those from crowded conditions (fig. 18). This is in accordance with Kaziřová (1996) who found that fecundity of *Mamestra brassicae* females was directly correlated with pupal weight, which in turn was related to larval density. In our study, there was no significant difference between the number of eggs laid by cotton- or cabbage-reared insects, indicating that although the cabbage larvae and pupae were larger than those from cotton, the overall fitness (if measured as egg-load) is not higher for cabbage over cotton. In terms of average eggs laid on each of the plants with regards to choice, cotton was significantly the most attractive choice for cabbage high-density adults, however this choice was seen to switch for the adults originating from a high-density background, whereby there was little difference between the species laid upon, with more eggs being laid on the cage than any individual plant (fig. 19). The results for high-density

adult egg laying are similar for those on the cotton treatment. This could be indicative of a shift in preference due to larval feeding experience. The high-density adults are more likely to spread their egg loads between different plants, whereas the low-density adults showed clear preference for one or two plant species (cotton for cabbage-reared insects, and cotton and maize for cotton-reared insects) (fig. 19). The fact that cotton is preferred for the cabbage-reared low-density insects is in accordance once again with the innate preference hierarchy described by Thöming et al. (2013). This indicates that perhaps the impacts upon fitness of a social, or crowded, experience during larval development, although not changing a preference in larval assays, makes for a shift from adult choice, to a spreading across all possible hosts. Indeed the first choice for oviposition is also switched from the experienced to the non-experienced, but with no large difference between the total quantities laid on all possible plants, it seems that the insect is choosing to lay its eggs across all possibilities, with no preference for experienced or non-experienced (or cage).

It has been hypothesized that oviposition (and mate-location) choice of adults could be influenced by where adult insects eclose, a phenomenon known as chemical legacy (Moreau et al., 2008; Bernays, 2001). The possibility for this to have an effect within this study was eliminated, as the pupae were removed from the box where they were being reared into a chemically neutral environment. However, the pupae were not cleaned, and there may have been some chemical from the pupation environment remaining on the surface of the pupae, but this is unlikely to have a significant effect on the adults choice (Bernays, 2001). This indicates that any choice being made (outwith their innate preferences) is likely to indeed be due to the transfer of information from the larval to the adult stage. It is considered that certain neurons in the insect brain could be retained over metamorphosis, which could explain such a memory transfer, but conclusive research into this area has not yet been carried out (Bernays, 2001).

For each of these experiments, it would be necessary for them to be repeated in order to ensure the results, especially the Y-tube and the oviposition. Due to the limited time period, insects were tested as they became ready for the experiments, which meant that there were much higher numbers tested for some treatments than others. Had it been apparent that the starved insects would take so long to develop, and there to be such a high mortality rate on maize, for example, more replications would have been set up for these tests earlier on in the experimental period. Also, for example, all four larvae from the ‘high-density’ pots were

used, which could be considered repeated measurements. This was done partly due to time-, plant-, and equipment-constraints, but it was also carried out in order to record each of their melanisation scores, as, as Cotter and Wilsom (2002) discuss, there is some genetic aspect to the melanised phenotype. In further experiments it would be advised to only use one insect from each pot, chosen randomly, and to have a higher replicate number in order to take into account the inherited aspect of phenotype. This being said, statistics were carried out to test for a 'pot-effect', and no significance was found, so for the purposes of this study, it can be ignored that individuals were derived from the same pot. Furthermore, as mentioned for the oviposition assay, it would be preferable to run these assays as dual-choice. The tri-choice was carried out due to time-constraints restricting the possibility to complete a high enough replicate repeat for all three combinations of plant choice (cotton vs. cabbage, cotton vs. maize, maize vs. cabbage). Of course, in future experiments, maize-reared adults would also be included, so all three comparisons would be valid. In terms of the starvation treatment, it would be interesting to be able to see the effect of such a low quality of diet on both the larval- and adult choice. It may be necessary that the insects be given a slightly higher quantity of food, for which the 'optimal' suboptimal quantity would need to be determined in order that a level of successful development and pupation was reached. Furthermore, in the oviposition experiments, the insects frequently oviposited on the artificial cage surface. It is possible that the cage seems like a suitable substrate for oviposition, so it could be advantageous to test different cage materials to find one that the adults do not find so favourable.

In terms of the artificial encapsulation assay, this study could be much developed. The melanisation score gave clear results, and it would be interesting to quantify the results using the artificial encapsulation technique. Software such as imageJ could be used to quantify the levels of darkness on the capsules after 24 hours (Cotter, 2012). It was also noticed that on one of the replicates that a much lower overall level of encapsulation took place, which may be due to the fact that the capsules were not as rough as the other sets. This is therefore something that may need to be standardised in future experiments. However, this study has found that it is possible to use the knotted-capsule technique, previously unused in larvae, which eliminates the need to dissect the capsule for extraction from the insect. Insects used for the encapsulation assay were not used for further study within this study, but it could be possible to run some experiments to ascertain whether this procedure has a behavioural effect on the larvae, and the adult. If not, then this could be used in direct conjunction with the other

experiments (development, Y-tube, and oviposition) in order to further study the links between immune allocation and development, and perhaps choice behaviour. A further experiment that could be carried out, of which pilot assays were begun, but time-constraints meant they had to be abandoned, was that of extracting the haemolymph and studying the composition and PO activity. This has been done by Cotter and Wilson (2002) who found that the PO activity is correlated with the melanisation score. However, it would be interesting to study this in relation to both diet and density, and the possible interactions between the two. In addition, it would be interesting to see the effect of the different plant species on specific immune function of *S. littoralis*. For example, it was found that the noctuid moth *Helicoverpa zea* is more susceptible to certain viruses when fed on one diet to another (Ali et al., 1998).

Unfortunately there was not enough data to analyse the effect of this starvation treatment on oviposition choice as very few insects successfully emerged, and if they did, they often did not successfully mate. This is a further indication that starvation had highly adverse consequences on their fitness. In terms of larval choice, we cannot say that they are more or less attracted to the diet that they have previously experienced compared to an ‘unknown’ host as they were more inclined to make no choice at all (fig. 14). This demonstrates that they are maybe in such a low level of health that they do not have the energy to follow an odour, or at least require more than the five-minute limit (although they made no indication that they would move). Together, this study cannot determine whether an inadequate food supply would alter insect preference in either larval- or adult stage, but it does however clearly indicate that if *S. littoralis* does not have sufficient food, that, even if it does manage to pupate, it does not necessarily mean that it will be a fit adult. It is however very unlikely that this would be likely to occur in natural conditions. *Spodoptera littoralis* is a highly polyphagous species, and therefore if there is an inadequate supply of its host plant, it will, if possible, migrate to another plant. Migration between plants, and even fields, has been seen in *Spodoptera* species in relation to their innate preference (Wilson and Gatehouse, 1992) so it is easily hypothesised that they would move to another plant if need be.

Although this study may not be directly applied, it is essential to understand the life histories of a target insect before realising the most effective method of control, and being able to implement IPM. This study builds upon previous work looking at immunity and memory transfer across metamorphosis in this noctuid moth.

## Conclusion

In conclusion, density is seen to have an interesting effect on the development of *S. littoralis*, with high-density larvae weighing the same as low-density *S. littoralis* larvae, but with the pupae of high-density insects being significantly smaller than those from low-density rearing. Melanisation (both cuticular and artificial encapsulation) show that *S. littoralis* invests more into immune response under the high density rearing conditions, as would be expected considering the heightened risk of viruses and pathogens. This could also explain the loss of energy to something other than development seen between the larval and pupal stages. Insects reared in low-density larval conditions have an overall higher fecundity than those from high-density conditions, as seen with the greater egg loads from low-density females.

Larval density does not affect larval choice, with the larvae more so choosing the diet of which it has prior experience. Mated *S. littoralis* females originating from low-density larval conditions show an overall preference for two of the host plants, whereas the individuals originating from high-density conditions tend to spread their egg load more evenly across all plants. Furthermore, for the insects reared on cotton, there was a switch between first choice for oviposition between low- and high-density, with insects originating from the low-density treatment ovipositing on the experienced plant, and those from the high-density ovipositing on a non-experienced plant. This could be an indication that the larval experience is being passed across metamorphosis and that the high-density treatment is being ‘remembered’ as a negative experience. More studies are required, however, to confirm this.

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