The effect of bacterially-dense environments on the development and immune defences of the blowfly, *Lucilia sericata*

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Running title: Effect of bacteria on blowfly life cycle

Abstract

Competitive interactions between insects and microbes and the associated cost of development in bacterially-dense environments are investigated using the blowfly *Lucilia sericata* (Meigen) as a model. The effects of developing in a bacterially-dense environment are measured by assessing the fitness consequences of competition using the pathogen *Staphylococcus aureus.* Fitness is quantified in terms of larval survival, puparial development and adult emergence.

The influence of bacteria on larval immune defences is investigated using optical density to assess whether antibacterial potency of the larval excretion/secretion changes in response to the degree of contamination of the larval environment. The results demonstrate that bacterial presence has no detrimental effect on survival of *L. sericata* from egg to adult eclosion, or on puparial size. Additionally, the level of microbial contamination of larvae has no effect on the antibacterial potency of the larval excretion/secretion. These findings confirm that larval antibacterial activity is not induced by the presence of environmental bacteria but is produced constitutively.

Keywords: antimicrobial activity, constitutive response, insect immune defence, *Lucilia sericata*

Introduction

Carrion-feeding insects colonise and survive in bacterially-dense environments, such as that provided by a human or animal corpse. Blowflies (Calliphoridae) dominate the early stages of decomposition, assisting its progress as they feed (Putman, 1983). The demands of the fly larvae for nutritional resources are high (Ives, 1991) and there is intense intra- and inter-species competition within the Calliphoridae (Goodbrod & Goff, 1990; Prinkkila & Hanski, 1995; Smith & Wall, 1997; Dos Reis *et al.,* 1999). Such competition can have detrimental effects on larval survival, duration of the larval stage, puparial and adult size, adult longevity and fecundity.

Little research has been carried out on the competitive interactions between insect and microbe decomposers in the corpse environment. Previous research has concentrated on the costs associated with the internal immune responses of insects (Kraaijeveld & Godfray, 1997; Fellowes *et al.,* 1998; Kraaijeveld *et al.,* 2001a; Kraaijeveld *et al.,* 2001b; Armitage *et al.,* 2003; Sadd & Siva-Jothy, 2006; Haine *et al.,* 2008; Roth & Kurtz, 2008). However, competition with microbes in the external environment also incurs lifecycle costs for the insect. For example, the microbes in the corpse environment have a detrimental effect on the reproductive success and larval growth of the burying beetle, *Nicrophorus vespilloides* (Herbst) (Rozen *et al.,* 2008).

Several carrion-feeding insect species, both dipteran and coleopteran, show externalised antibacterial activity in their excretion/secretions (ES), which may be seen as the first line of defence against microbes in their environment (Scott, 1998; Suzuki, 2001; Hoback *et al.,* 2004; Cotter & Kilner, 2009; Barnes *et al.*, 2010).

Studies investigating the antibacterial activity of *Lucilia sericata* (Meigen) have been conducted in the context of maggot debridement therapy (where larvae are added to a wound to remove necrotic tissue, reduce the microbial load and promote healing) and show that ES produced by aseptically raised *L. sericata* larvae is active against several Gram-positive and Gram-negative bacteria (Simmons, 1935; Thomas *et al.,* 1999; Bexfield *et al.,* 2004; Kerridge *et al.*, 2005; Bexfield *et al*., 2008; Jaklic *et al*., 2008; van der Plas *et al.,* 2008). These results demonstrate that antibacterial activity is not induced by exposure to environmental bacteria. However they give no indication of bacterial effect on the potency of the ES. Additionally, the effect of such exposure to pathogenic bacteria, on the rate of survival to adulthood of these insects has not previously been investigated.

The present study examines the impact of a bacterially-dense environment on the survival and development of *L. sericata* larvae and the influence of this environment on the antibacterial potency of the ES produced by the larvae. The study has two aims; first to assess the costs of development in a bacterially-dense environment where cost is quantified in terms of successful larval development (reflected as puparial size) and survival throughout the lifecycle with and without the presence of bacteria. These parameters are chosen because they are important measures of fitness that correlate with success in terms of competition for reproductive opportunities amongst adults (Webber, 1955; Daniels *et al.,* 1991). The second aim is to assess the influence of bacteria on the effectiveness of the antibacterial activity of larval ES. This research extends that work conducted on internal interactions between the insect immune system and bacteria by exploring competition between insect and microbes in the external environment.

Materials and Methods

Insect culturing

Colonies of *L. sericata* were maintained at the University of Lincoln under a lighting regime of LD 16 : 8h and at a temperature of 25 ± 3 °C. Larvae were reared on different diets according to the objectives of each experiment. Sterile larvae (experiment one and two) were fed an artificial diet combining 20% horse blood (Oxoid Ltd, Basingstoke, Hampshire, England) and 5% yeast agar (Oxoid Ltd, Basingstoke, Hampshire, England) (diet modified from the protocol of Daniels, *et al.,* 1991) which was sterilised by autoclaving before addition of larvae. Non-sterile larvae (experiment two) were fed a diet of *ad libitum* porcine liver.

Representative bacterial species

Staphylococcus aureus (ATCC 25923) was used as the bacterial species in these experiments. This bacterium is found in the nasal openings of 30% of the human population (Bexfield *et al.,* 2008) and has been isolated from human corpses during the first 24 h after death (Rose & Hockett, 1971; Niwayama, 1971). *Staphylococci* are also found in the oral cavity, upper respiratory tract and genital regions of the human body (Wilson, 2005), sites where blowfly eggs are likely to be deposited (Dix & Graham, 2000; Carvalho & Linhares, 2001; Grassberger & Frank, 2004; Perez *et al.,* 2005). Therefore, it is assumed that the larvae of an initial coloniser such as *L. sericata* will have contact with this pathogen as they emerge and consume a corpse.

Measuring survivability and larval growth

To assess the effect of bacteria on the survivability of *L. sericata*, larvae were fed on an artificial diet (comprising 20% horse blood and 5% yeast agar) containing *S. aureus*. Each Petri dish contained the same amount of artificial diet (20g) but varied

in the quantity of bacteria according to the treatment group (0%, 50% or 100% *S. aureus*).

Inocula for the artificial diet was prepared as follows: one colony of *S. aureus* was taken from a stock plate of nutrient agar (Oxoid Ltd, Basingstoke, Hampshire, England) and inoculated into 9 mL sterile water and diluted to 10^5 colony forming units (CFU) mL^{-1} . One loop (10 µL) of this suspension was added to each of nine artificial diets and spread over half of the agar (50% *S. aureus*). Two loops of this suspension were added to each of nine replicates and spread over the complete agar surface (100% *S. aureus*). Nine plates were left uninoculated to serve as the control (0% *S. aureus*). All plates were incubated at 37°C for 18 h to allow bacterial growth.

Eggs of *L. sericata* were collected and sterilized using 70% ethanol. A complete absence of bacteria was confirmed in the egg stage by spreading an aliquot of each egg batch onto agar plates and incubating for 24 to 48 h after which an absence of bacteria was confirmed. Hence, the only artificially introduced bacterial species in the larval environment was *S. aureus* and any effect on survivability could be attributed to the presence of this bacterial species alone.

Live first instar larvae that emerged from the sterile eggs were transferred to artificial diets (20 larvae per plate) and the dishes placed in a controlled environment with pots of water to provide local humidity. Larval development was monitored visually until the post-feeding stage. At this point, the lids were removed from the Petri dishes and the post-feeding larvae allowed to migrate into sawdust to pupate.

The proportions of surviving larvae in each treatment group (0% *S. aureus*, 50% *S. aureus* and 100% *S. aureus*) were counted at the 3rd instar, puparial and adult stage. Puparial cases of those larvae which successfully pupariated were recovered from

each treatment group and the length of each entire case measured (mm) using callipers.

Collection and preparation of larval excretion/secretion from bacterially-dense and bacterially-sterile environments

To compare the potency of antibacterial activity from larvae raised in sterile and non-sterile environments against *S. aureus,* ES was collected from 3rd instar larvae.

Larvae from previously sterilised eggs were raised aseptically on Petri dishes containing sterile artificial diets. Larvae from non-sterile environments were fed on porcine liver and no attempt was made to sterilise these larvae at any life stage.

The ES was collected by adding deionised water (dH_2O) to a weighed sample of larvae (1 g mL^{-1}) (Barnes *et al.*, 2010). Larvae were incubated at 30 °C for 60 min after which the ES was collected and micro-centrifuged at 7826 ***g*** for 5 min. The ES from non-sterile larvae was sterilized by filtration (0.20 µm). The ES was tested for sterility before use to ensure any bacterial growth recorded was solely due to *S. aureus*.

Preparation of bacteria

One colony of *S. aureus* was removed from a stock plate of nutrient agar (Oxoid Ltd, Basingstoke, Hampshire, England) and inoculated into 20 mL sterile tryptone soya broth (TSB) (Oxoid Ltd, Basingstoke, Hampshire, England). The broth was incubated at 37 °C with aeration for 17 h. A sample of 0.1 mL of this overnight bacterial culture was transferred to 10 mL TSB and incubated at 37 °C with aeration for four h.

Assessment of the antibacterial potency of larval excretion/secretion from both environments

The ES was separated into 4 mL aliquots to investigate the antibacterial activity of ES from *L. sericata*. The purpose of this experiment was to model the antibacterial

activity of ES in the insects' natural environment. Therefore additional media was not employed in this assay as its effect on antibacterial activity of ES was unknown. In order to demonstrate the pattern of normal bacterial growth, 4 mL aliquots of TSB/ dH_2O were utilised as controls to replace the ES.

Twenty microlitres of *S. aureus* in TSB (10^5 CFU mL^{-1}) were added to all 4 mL aliquots. Optical density was used to measure bacterial growth over the experimental period (Thomas *et al.,* 1999; Bexfield *et al.,* 2004). An initial absorbance reading (time zero) was taken by diluting the sample in sterile dH_2O (1 : 10) in sterile universals and comparing it to a blank (with an identical content to the sample but without the bacteria) at 600 nm. Samples were incubated with aeration at 37 ºC for 24 h and readings were taken at 0, 1, 2, 4, 8, 18, 20 and 24 h after inoculation. The experiment was repeated three times and in each experiment samples were run in triplicate.

Results and Discussion

Experimental data fit the assumptions needed for parametric tests and statistical analyses were conducted using SPSS (version 14.0).

Effect of S. aureus on survivability and larval success

Under the environmental conditions provided in these experiments *S. aureus* is not detrimental to the growth, or survival, of *L. sericata* larvae to adulthood. The presence of bacteria had no significant effect on the number of survivors through the life stages between treatments (0% cover of *S. aureus*, 50% cover of *S. aureus* and 100% cover of *S. aureus*) indicating that *S. aureus* (10^5 CFU mL^{-1}) was not pathogenic to *L. sericata* (ANOVA: $F_{2,6}$=0.68, P = 0.54) (Fig. 1). Similarly, change in bacterial density did not affect the puparial size of *L. sericata* (ANOVA: $F_{2,89}$=1.09, P

= 0.34) (Table 1). These results indicate that *L. sericata* is able to tolerate the bacterial species frequenting its natural environment and that any nutrients resulting from the presence of bacteria are irrelevant in terms of enhancing larval growth.

These findings for *L. sericata* are in contrast to Rozen *et al.* (2008) who find evidence of a strong detrimental effect of microbial competition on the reproductive success and larval growth of the burying beetle, *N. vespilloides*. Burying beetles choose substrate with the lowest microbial load as a food source for their larvae (Rozen *et al.*, 2008). Such substrate choice has not been reported in *Lucilia* species and consequently, *L. sericata* larvae are likely to develop in suitable environments irrespective of the bacterial populations.

In the present study, larvae of *L. sericata* are able to tolerate the presence of pathogenic bacteria until they pupariate. Roth and Kurtz (2008) report that immune-challenged larvae of the red flour beetle, *Tribolium castaneum* (Herbst), pupate significantly earlier than controls. However, no difference in the speed of development is noted between the *L. sericata* larvae in sterile and non-sterile environments. In fact, during this period, the colonies of *S. aureus* are eradicated from the blood agar plates upon which the larvae are feeding. This observation suggests that larvae are not only able to tolerate the bacteria but can also destroy them.

Effect of S. aureus on antibacterial potency of larval ES

Research, mainly conducted within the context of maggot debridement therapy, proposes two possible mechanisms whereby *L. sericata* larvae reduce bacterial growth. Some studies show that bacteria, such as *E. coli* are consumed and eradicated in the acidic midgut of *L. sericata* (Robinson & Norwood, 1934; Greenberg, 1968; Mumcuoglu *et al*., 2001; Daeschlein *et al*., 2007), whereas others demonstrate that ES from *L. sericata* provides an effective control mechanism against *S. aureus* (Simmons, 1935; Thomas *et al.,* 1999; Bexfield *et al.,* 2004; Kerridge *et al.*, 2005; Jaklic *et al.,* 2008; van der Plas *et al.,* 2008). The ES recovered from 3rd instar *L. sericata* in the present study inhibited the growth of *S. aureus* for 24 h. These data demonstrate that *S. aureus* is susceptible to the antibacterial activity of ES, which plays an effective role in determining larval immunity against this bacterial species.

Neither the sterility of the larvae or the larval environment influences the antibacterial potency of ES in the present study. A Repeated Measures ANOVA, conducted on the absorbance data over the 24-h experimental period, indicated that there was no significant difference between the effectiveness of ES from non-sterile larvae compared to ES from sterile larvae (ANOVA: $F_{1,16}$=0.12, P = 0.73). Additionally, there was no significant bacterial growth in either treatment over this period (ANOVA: $F_{7,16}$=0.41, P = 0.88). The change in bacterial growth in the two treatments over the 24-h experimental period is illustrated in Figure 2. These results show that the presence of bacteria does not appear to be required for the induction, or production, of the antibacterial activity of ES.

The results from this study are in accord with studies that report antibacterial activity in ES from aseptically-raised *L. sericata* larvae (Simmons, 1935; Thomas *et al.,* 1999; Bexfield *et al.,* 2004 and 2008; Jaklic *et al.,* 2008; van der Plas *et al.,* 2008). This result also suggests that there is no additional intensity of antibacterial factors in ES in response to the environmental presence of *S. aureus,* as there is in the haemolymph of *L. sericata* in response to a bacterial insult (Altincicek & Vilcinskas, 2009). The antibacterial activity of ES against *S. aureus* therefore appears to be present constitutively in these larvae. This is in contrast to the antibacterial activity in secretions from *N. vespilloides* which is only upregulated following discovery of a corpse (Cotter & Kilner, 2009). *Lucilia sericata* larvae have most likely evolved to have a high level of constitutive antibacterial capacity in response to developing in bacterially-dense environments.

It would seem that there are no detrimental effects in terms of survival associated with the production of externalised antibacterial activities against *S. aureus* in larval ES. This finding supports work by Armitage *et al.* (2008) who report no measurable costs for constitutive investment in immunity in *Tenebrio molitor* (Linnaeus). However, other bacterial species may differ in their effects on larval survivability or growth, showing less sensitivity to the ES. Roth and Kurtz (2008) find that *T. castaneum* produced fewer offspring when exposed to the Gram-negative *E. coli* but not to the Gram-positive *Bacillus thuringiensis*. Further work on the developmental success of *L. sericata* in the presence of other bacterial species is required to determine whether developing in an environment colonised by different pathogenic species has associated costs.

For carrion-feeding larvae, such as *L. sericata* that inhabit bacterially-dense environments natural selection ought to favour specific mechanisms of resistance to pathogens only when it is beneficial. Therefore, developing in a bacterially-dense environment should not warrant the costly use of the immune system and the constitutive production of antibacterial ES, where components also include enzymes utilized for digestion, is perhaps more efficient.

In summary, the present research provides evidence that *L. sericata* can develop normally in bacterially-laden environments. In addition, these experiments reveal that antibacterial activity against *S. aureus* is produced constitutively in larval ES and indicates that this form of immune defence is not costly to the growth or survival of the insect.

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Table 1: Mean puparial lengths of *Lucilia sericata* (Meigen) for control (0% *S. aureus*) and treatments (50% *S. aureus* and 100% *S. aureus*). Values for standard deviation and standard error are also presented.

Puparial sizes of *Lucilia sericata* (mm)	**0% *S. aureus***	**50% *S. aureus***	**100% *S. aureus***
Mean	7.39	7.20	7.21
Standard Deviation	0.47	0.53	0.67
Standard Error	0.09	0.10	0.12

Figure Legends

Figure 1. Mean (± SEM) number of surviving individuals of *Lucilia sericata* (Meigen) for control (0% *S. aureus,* open bar) and treatments (50% *S. aureus,* grey bar and 100% *S. aureus,* solid bar) at three developmental stages.

Figure 2. Mean (±SEM) growth curves for *Staphylococcus aureus* in excretion/secretion collected from aseptically reared larvae (sterile larvae) of *Lucilia sericata* (solid squares), or collected from non-sterile larvae and filter sterilized (non-sterile) larvae (solid triangles). Normal bacterial growth is represented by the control data (solid circles). The insert is a comparison of the growth of *S. aureus* in the two treatment groups (sterile larvae and non-sterile larvae) alone.