The immune response of larvae and pupae of the blowfly, *Calliphora vicina* (Diptera Calliphoridae), upon administered insult with *Escherichia coli*

by

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SUMMARY

Adult blowflies, *Calliphora vicina*, are exposed to bacteria-laden decay as adults and larvae. They are protected from infections in such habitats by a family of lytic proteins called cecropins. This study explores the relationship between developmental stage of the blowfly, and the strength of the immune response when insulted by the endogenous pathogen, *Escherichia coli*. Our data indicates that the protective function of cecropin B varies with developmental stage. Pupae that were not fully tanned exhibited the greatest response to an insult by *E. coli*. The exhibition of this high immune response is due in part to the high concentrations of cecropin B (and related proteins) manufactured by this instar.

INTRODUCTION

Blowflies (Diptera, Calliphoridae) are found in many terrestrial habitats, including rotting animal carcasses, excrement, and decaying plant material, where they are continually exposed to pathogenic microorganisms. In spite of this extreme habitat contamination, blowflies remain taxonomically diverse and numerically dominant in successional ecosystems. The impact of fluctuating and persistent microbe communities on flies results in directional selection for mechanisms to counter potentially lethal infections.

External structural barriers and a cascade of physiological responses contribute to insect immunity, which can be compared to responses during human infection (Dunn 1986). However, in contrast to what occurs in humans, insects lack a closed circulatory system, lymphocytes, and immunoglobins. Yet they are capable of sustained immunity and internal integrity. With an open hemocoel, once structural barriers are violated (Steiner *et al.* 1981), microbial infection can be diffuse, rapid and invasive. A suite of lytic peptides mobilize rapidly against a broad range of endogenous microorganismal associates, with limited specificity or memory (Boman 1995).

This point was appreciated in the 1800s when L. Cuénot established the role of phagocytosis in the defense system of insects. Only 20 years later, R.W. Glaser, A. Paillot, and S. Metalnikow, independently recognized the insect dependence on humoral immune reactions by observing the presence of potent antibacterial peptides in the hemolymph (Cociancich *et al.* 1994). We now understand that there is a large complex of antibacterial lytic peptides in insects (e.g., cecropins and defensins), some of which may be plesiomorphically shared with mammals (e.g., cecropins in pigs, Lee *et al.* 1989; defensins in humans, Boman 1995).

In the early 1980s, a family of lytic proteins was identified from the cecropia moth, *Hyalophora cecropia,* and designated as cecropins (Steiner *et al.* 1981). Cecropins work by disrupting the bacterial outer membrane leading to cytoplasm leakage and eventual lysis (Moore *et al.* 1996). Now it is understood that cecropins have a broad-spectrum of antibacterial action against both gram-positive and gram-negative bacteria and function as a principal defense mechanism in insects.

Medical and agricultural over-application of antibiotics has resulted in world-wide bacterial resistance, and cecropins represent a class of proteins which offer realistic functional alternatives (Moore *et al.* 1996). Some of the most innovative approaches include clinical application of cecropins for the treatment of burns, diabetic wounds, and eye infections (Boman 1995).

While previous studies have focused on the identification, structure, regulation, and genetic machinery of these distinctive proteins, there have been few studies concerning ontological and ecological aspects of cecropins *in situ.* Two issues arise when studying the immune system of insects. The first is the correlation between the stage of development and the response of the immune system. For many animals, there is a general decrease in the efficiency of the immune system with age (Miller 1996). In insects, the immune system exerts a maximal response to injury during times of larval ecdysis and pupal eclosion (Dunn 1986). These are periods of vulnerability as the structural cuticular barriers are least effective. The second is the correlation between the intensity of bacterial challenge and the resulting immune response. It has been postulated that insects exhibit a graded immune response, which implies that a small degree of challenge causes less response than that triggered by a larger challenge (Gillepsie *et al.*

1997). If the response is indeed graded, this would be conservative, as less resources would be expended on an infection of low intensity.

Calliphora vicina Robineau-Desvoidy (Diptera, Calliphoridae), a blowfly, is an ideal insect to use for this experiment because it commonly colonizes and oviposits on rotting carcasses where pathogens thrive. The purpose of this study is to observe the response of blowfly larvae and pupae when experimentally insulted with a pathogenic microorganism, *Escherichia coli*.

METHODS

Insects: The blowfly, *Calliphora vicina*, was the insect examined in this study. Approximately 100 larvae were obtained from Wards™ Biology. Some were used immediately, and the remaining stock was reared in a mesh cage to the adult stage. Larval stages were maintained in vermiculite substrate to reduce desiccation and to satisfy their natural burrowing instincts. Upon emergence of pupae, the adults were fed an artificial diet of granulated sucrose and an egg/sugar/water mixture. Beef liver was placed in the cage to encourage oviposition. This stock of blowflies was best maintained in minimal light.

Bacterial Medium for Inoculation: 10 ml of autoclaved LB-Broth and 100 µl of streptomycin-resistant *Escherichia coli* (K-12 strain S4362) from the bacteria stock solution were added to each of two 15 ml polypropylene tubes. The solutions were placed in an incubator-shaker at 37°C. One culture was removed when the optical density was 0.67, as measured using a Shimadzu UV-VIS spectrophotometer calibrated to 600 nm. The second was removed at 0.88 OD_{600}. 1 ml of each of these was centrifuged, the pellet re-suspended in 1 ml of PBS, and the solution was stored on crushed ice until inoculation of the insects.

Agarose Plate Preparation: For each trial of this experiment, an agarose plate was prepared as follows: a 0.7% agarose solution was heated and temperature equalized, 1,050 µl of filter-sterilized lysozyme (10 mg/ml) was added to an 85 mm petri dish, 21 µl of streptomycin solution (10 mg/ml) was added to the bottom of a 20 ml bijoux bottle,

and 3 µl of *E. coli* (0.67 or 0.88 OD_{600}) was gently placed on the side of the bottle to prevent mixing with the streptomycin. When the agarose was considerably cooled, 21 ml was added to the bottle, and the contents were mixed with gentle agitation. This solution was transferred to the petri dish, the mixture was swirled, and the plate was refrigerated at about 35° F for 24 hrs. After this time, small wells were made in the Agarose using a 1000 µl pipette tip. Care was taken to penetrate the agar without touching the bottom of the plate, and the resulting wells were approximately 2 mm in diameter.

Microinjection Apparatus: Organisms were injected using a Narishige hydraulic microinjection system. Injection needles were prepared using IM-CFS capillary tubes and the Narishige heating element. The 1 µl injection quantities were obtained by measuring 1 µl of the desired solution onto moisture resistant laboratory film (Parafilm™) and subsequently drawing this volume into the needle. Upon injection, pressure from the syringe filled with mineral oil provided sufficient force to move the liquid into the organism.

Injection Procedure: From the stock of larvae, 21 third-instar larvae were selected and placed in a separate dish. To slow the animal's movement, the larvae were placed on ice. 1 µl of *E. coli* (0.67 OD_{600}) was injected into each of 15 larvae. The injections were made between body segments to reduce leakage of hemolymph. For control purposes, three larvae were sham-punctured, but not microinjected, and three were handled but not punctured. Each larva was then placed in a separate Stendor dish containing fresh vermiculite. These dishes were placed in an enclosed polystyrene box, at room

temperature for 24 hrs. This protocol was replicated by injecting larvae with *E. coli* at 0.88 OD_{600}

Replicates of pupae were also microinjected with the two bacterial concentrations (0.67 OD_{600} and 0.88 OD_{600}): 21 pupae were selected from the stock, microinjected as above, and placed in separate vermiculite-filled Stendor dishes.

Hemolymph extraction: All larvae survived microinjection. At 24 hrs post-injection hemolymph was recovered for assay: about 1µl of anticoagulant (i.e., 0.392gm NaOH, 0.853 gm NaCl, 0.632 gm EDTA, 1.206gm citric acid, and 100ml ddH_2O) was microinjected into test larvae at the original site of injection to facilitate hemolymph recovery at the site. Pressure was applied to the larva until hemolymph appeared at the wound. Using a micropipette, 1µl of hemolymph was withdrawn and expelled into an individual well previously formed in the agarose plate. In some cases, due to the insufficient amount of hemolymph, larval tissues were crushed to expel hemolymph.

Twenty-one samples of larval hemolymph were pipetted into pre-formed wells on each agarose plate and 1µl of anticoagulant was placed into two negative control wells, without hemolymph or bacteria, for a total of 23 wells. This procedure was repeated using pupal hemolymph. Inoculated agarose plates were inverted in an incubator at 37°C for 24 hrs.

Inhibition Zones: Incubated plates were removed after 24 hrs. Each zone of inhibition was measured as to circumference, diameter, and length of zone, using Image Pro™.

Statistical Methods: Multivariate statistical procedures were conducted using Matlab™ to determine the test variable(s) with the greatest contribution to total primary variation:

E. coli concentration; circumference, diameter and length of zone of inhibition; and rank age. A principal component analysis (PCA) and a discriminant function analysis (DFA) were performed on the data. A Kruskal-Wallis non-parametric test was applied to the PCA scores. Univariate treatment effects (ANOVA) were assessed using a General Line Model (one response model), with four treatments, in Minitab™.

RESULTS

The first principal component (PC1) accounted for 99.6% of the total variation in the data set, indicating a significant size vector. The GLM, with four levels of treatments (combinations of *E. coli* concentration and instar development), was highly significant for group effects at $p < 0.0001$ (3.7×10^{-9}). For all groups, within-group variation accounted for 59% of the total variation and between-group variation accounted for 41%. The alternate trial densities of bacterial challenge (0.67 vs. 0.88 OD_{600}) differed only marginally in a means-comparison. The Kruskal-Wallis results of PCA scores for 0.67 and 0.88 OD_{600} was 12.52 and 14.50 respectively ($N = 56$, $p = 0.054$, $H = 3.71$) with the range of values at the lower concentration falling within the range of the higher concentration. The higher concentration of bacteria also had a higher variance structure for inhibition response. Circumference of zone of inhibition was the best predictor of effect. The ANOVA, by stage, was highly significant ($N=56$, $p=0.004$, $F=9.05$), and the Kruskal-Wallis test of PC scores by stage was significant ($N= 56$, $p=0.014$, $H=6.01$). The DFA (Fig. 1A) and the box plots (Fig. 1B) indicated that the greatest response to *Escherichia coli* challenge is at the point when *Calliphora vicina* is just entering the pupal stage, when it cannot defend itself mechanically by moving away from a challenge, nor fully tanned so as to structurally protect itself from an attempted challenge.

DISCUSSION

Cecropins are likely plesiomorphic chemicals, manufactured for the protection against infection (Moore et al. 1996). They are conserved in organisms as divergent as *Bombyx*, *Drosophila*, *Sarcophaga*, and even porcines (Boman 1995).

In insects, the immune system is considered to be strongest at times of greatest vulnerability, when external barriers are weakest (Dunn 1986). The cuticular envelope of insects, including *Calliphora vicina*, is thinnest just after each ecdysis and prior to cross-polymerization and tanning of the cuticle (Dennell 1946, Vincent and Hillerton 1979, Chapman 1982). The thicker and highly tanned pupal chrysalis acts as an enhanced barrier to mechanical insult by pathogens.

Our data indicates that the protective function of cecropin B in *C. vicina* varies according to developmental stage. Organisms that had just pupated, and were not fully tanned, exhibited the greatest response to the administered insult by *E. coli*. The exhibition of this high immune response is due in part to the high concentrations of cecropin (and related proteins) manufactured at this instar of the fly's life cycle. It may also vary across a broad range of bacterial densities. Examination of this possibility will be left for future work to determine if the diptera exhibit a graded immune response as suggested by Gillespie *et al.* (1997).

It is known that as humans age, their immune system becomes less protective (Miller 1996). However, comparable studies in insects are rare, but it appears that senescence in adult *Lucila sericata* (the green bottle fly) also affects the immune response. As flies age, the amount of cecropin B produced in response to the bacterial challenge significantly decreases (Knight and Ashley 1999). It is possible that a general

demographic pattern exists in which there are developmental windows of increased response of immune systems. Pursuing studies of antibacterial proteins in a diversity of organisms may resolve the magnitude, scope, and pattern of occurrence.

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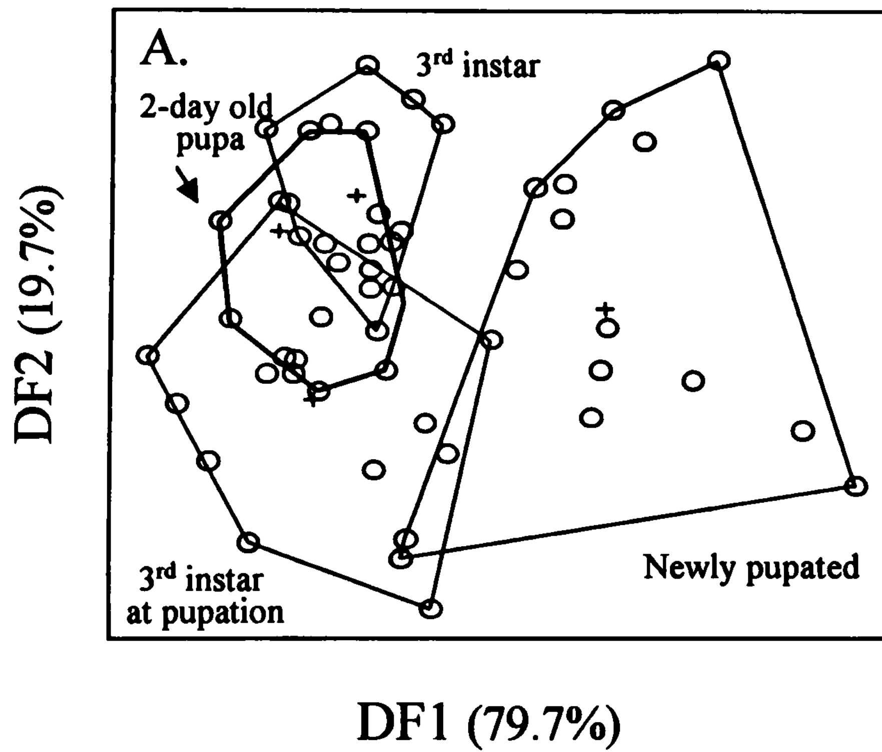

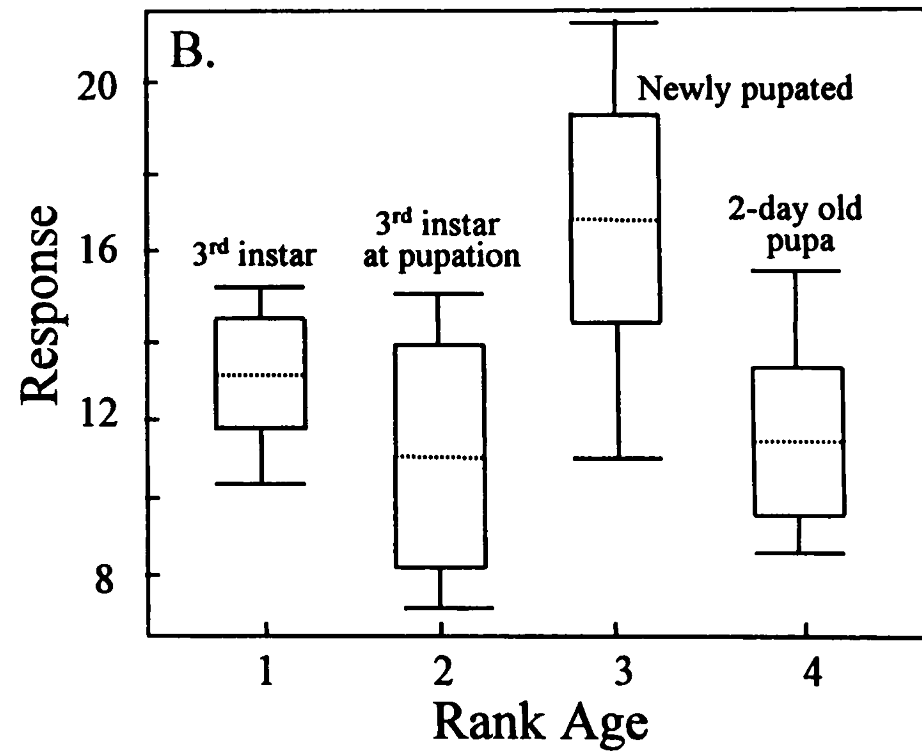

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