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Microbial effects on the development of forensically important blow fly species

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ABSTRACT

Colonisation times and development rates of specific blow fly species are used to estimate the minimum Post Mortem Interval (mPMI). The presence or absence of bacteria on a corpse can potentially affect the development and survival of blow fly larvae. Therefore an understanding of microbial-insect interactions is important for improving the interpretation of mPMI estimations. In this study, the effect of two bacteria (*Escherichia coli* and *Staphylococcus aureus*) on the growth rate and survival of three forensically important blow fly species (*Lucilia sericata*, *Calliphora vicina* and *Calliphora vomitoria*) was investigated. Sterile larvae were raised in a controlled environment (16:8 h day: night light cycle, 23:21 °C day: night temperature cycle and a constant 35% relative humidity) on four artificial diets prepared with 100 μl of 10^5 CFU bacterial solutions as follows: (1) *E. coli*, (2) *S. aureus*, (3) a 50:50 *E. coli*:*S. aureus* mix and (4) a sterile bacteria-free control diet. Daily measurements (length, width and weight) were taken from first instar larvae through to the emergence of adult flies. Survival rates were also determined at pupation and adult emergence. Results indicate that bacteria were not essential for the development of any of the blow fly species. However, larval growth rates were affected by bacterial diet, with effects differing between blow fly species. Peak larval weights also varied according to species-diet combination; *C. vomitoria* had the largest weight on *E. coli* and mixed diets, *C. vicina* had the largest weight on *S. aureus* diets, and treatment had no significant effect on the peak larval weight of *L. sericata*. These results indicate the potential for the bacteria that larvae are exposed to during development on a corpse to alter both developmental rates and larval weight in some blow fly species.

1. Introduction

Bacteria play a critical role in the life-cycle of necrophagous flies and can have a significant impact on their development and survival [1]. This inter-kingdom interaction is of particular importance in forensic entomology where developmental data for specific blow fly species is utilised to estimate the minimum post-mortem interval (mPMI) [2,3].

Blow flies are commonly the primary colonisers of a human corpse [4,5]. The colonisation interval (the time between death and colonisation) of an exposed corpse can vary from minutes to days. In cases where a corpse has been hidden this could be much longer [6]. Environmental factors such as temperature [7–9], humidity [8,10] and sunlight [8,11] are known to influence colonisation times. Bacteria inhabiting the corpse can also be highly influential in regulating chemo-attraction and choice of oviposition sites [1,12,13]. A recent study, by Richards et al. [14] found that *Calliphora vicina* (Robineau-Desvoidy) larvae develop significantly more slowly on decomposed liver compared to fresh liver, and it has been suggested that temporal changes in the bacteria colonising the liver are responsible for this.

Such bacterial influences on larval development in a corpse environment will have an impact on the interpretation of mPMI calculations. To date there is limited research documenting microbial succession on a corpse. However, recent research indicates that microbial communities change significantly, following a predictable timescale during decomposition [15]. The initial bacterial community varies with the age [16] and health [17] of the victim, the location on the body [18], environmental temperature and humidity [19], and the season [20,21]. Variation also exists within organs, for example, the species of bacteria in the right lobe of the liver (*Escherichia coli*) differ from those in the left lobe (*Bacillus subtilis*) [22]. The bacterial community the emerging blow fly larvae are exposed to can therefore vary. As decomposition continues, the microbial community changes as bacteria move from sites internal to the body to locations on the surface, and are

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transferred from the soil, scavengers and insects [15,17,23]. Different species of bacteria are known to be associated with different stages of the decomposition process. For example *Staphylococcus aureus* has been shown to be the first organism to migrate from the small intestine during decomposition, followed by coliform bacteria and anaerobic bacteria [24]. During the bloat stage, *Ignatzschineria* and *Wohlfahrtimonas* bacteria are commonly found on the skin of the corpse, and anaerobic bacteria including *Lactobacillus* and *Bacteriodes* increase in the abdominal cavity [18]. Microbial communities on a corpse generally become less diverse [23,25] and more similar to each other across body sites [15] with time.

Blow flies are most likely to come into contact with the natural bacterial communities of the human body during the initial stages of decay. Research to date demonstrates that, whilst ingesting the common skin bacterium *S. aureus* has no effect on pupation times, pupal size or survival rate of *Lucilia sericata* (Meigen) [26], certain bacterial strains have been shown to have a negative impact on blow fly larval development. For example, Ahmad et al. [27] isolated four gut mutualistic bacteria (*Providencia* sp., *Escherichia coli* O157:H7, *Enterococcus faecalis*, and *Ochrobactrum* sp.) from the blow fly, *Cochliomyia macellaria* (Fabricius) and demonstrated that cultures containing *Ochrobactrum* sp. and *E. faecalis* supported larval development to a significantly greater extent than those of *Providencia* sp. and *E. coli* O157:H7. This research suggests that the structure of the bacteria community present is an important factor affecting larval development, as well as the specific strains present.

This study investigates the impact of two bacterial species commonly associated with the human body (*E. coli* and *S. aureus*) alone and in combination, on the development of three blow fly species of forensic importance, *Calliphora vomitoria* (Linnaeus), *C. vicina* and *L. sericata*. There is a particular emphasis on the early larval stages where ingestion of bacteria is likely to have the greatest effect. An improved understanding of the interactions between bacteria and larvae is likely to lead to more accurate interpretation of development-based mPMI calculations.

2. Materials and methods

2.1. Insect culturing

Blow fly colonies were maintained in mesh cages ($52 \times 58 \times 60$ cm) at the University of Derby at 23.5 °C ± 1.5 °C, under a light:dark 16:8 h photocycle. Flies were fed sugar and water ad libitum. Fresh porcine liver was added to the mesh cages in the morning as an oviposition substrate, with oviposition typically taking place within 4–6 h.

2.2. Representative bacterial species

Gram-positive *Staphylococcus aureus* (ATCC 25923) and Gram-negative *Escherichia coli* (NCIMB 11866) were chosen as representative species that larvae may potentially be exposed to and therefore, consume in the corpse environment. Adult blow flies oviposit in natural body orifices. Staphylococci are abundant in the oral cavity, upper respiratory tract and genital regions of the human body [28], with *Staphylococcus aureus* being found in nasal passages of 30% of the human population [29] and *Escherichia coli* is commonly found in the rectum area [28]. Both bacterial species have also been isolated from corpses during the first 72 h after death [30,31].

2.3. Sterilisation of eggs

Blow fly eggs were collected from the oviposition substrate and sterilised according to Barnes and Gennard [32]. The absence of bacteria was confirmed by spreading an aliquot of each egg batch onto agar plates and incubating at 37°C for 24–48 h. Hence the only bacterial species the larvae were exposed to during the experiment were those introduced through the artificial diet. Sterile eggs were left in the laboratory at 23.5 °C for 16 h overnight to hatch.

2.4. Artificial diet

Larvae were reared on a blood-yeast agar diet prepared as described in Barnes and Gennard [32]. Bacterial solutions were prepared by inoculating 9 ml of phosphate buffered saline (PBS) with one loop (10 μl) of bacteria from a stock plate of nutrient agar (Oxoid Ltd). This was then diluted to give a concentration of 10^5 colony forming units (CFU)/ml. 100 μl of the appropriate bacterial solution was spread evenly over the agar surface. All plates were then incubated at 37 °C for 18 h to initiate bacterial growth. The artificial diets were as follows: *E. coli; S. aureus; Mixed* (containing 50% *E. coli* and 50% *S. aureus)* and a control diet with no added bacteria.

2.5. Insect development

Live sterile 1st instar larvae (20 per plate) were transferred to the artificial diets via a sterile paint brush. There were 100 larvae per diet. The larval stage was chosen over the egg stage to ensure that each diet treatment had the same starting number of larvae, and to allow time to ensure that larvae were sterile (see egg sterilisation). The petri dishes were transferred onto a thin layer of sawdust (pupation substrate) within propagators and put in a controlled environment (16:8 h day: night light cycle, 23:21 °C day: night temperature cycle and a constant 35% relative humidity) in an Insect Growth Chamber (Fitotron SGC120).

Measurements of individual width (mm), length (mm) and weight (g) were taken every 24 h for ten randomly selected individuals per diet (2 individuals were randomly selected from each of the five agar plates per treatment). Measurements were carried out within a dedicated insectary with positive pressure using sterilised equipment and minimum exposure time to keep exposure to other microbes at a minimum.

Metrics were then collected for ten randomly selected pupae (length/width and weight) and for ten adults (length and weight) following emergence. The number of surviving insects for each diet was also recorded at pupation and adult emergence. The experiment was repeated a minimum of 6 times for each of the 3 blow fly species. Each time a new caged population of flies were sampled.

2.6. Statistical analysis

A generalised least squares (GLS; [33,34]) statistical mixed modelling approach was used to model larval development, treating species, day and diet as dependent variables. A GLS framework was preferred over linear regression (using transformed data), because it retains the structure of the data while accounting for unequal variance in the variance–covariate matrix.

In each case, as a first step, a linear regression model was fitted. Model validation showed no evidence of nonlinearity but there was evidence of unequal variance among the explanatory variables. The GLS framework was then adopted in order to model this heterogeneity of variance. The most appropriate random structure was found by examination of AIC scores in conjunction with plots of fitted values versus residuals using restricted maximum likelihood (REML, [35]). The fixed component of the model was refined by manual backwards stepwise selection using maximum likelihood (ML) and the minimum adequate model was presented using REML. Following Underwood [36], the highest