Microbial effects on the development of forensically important blow fly species

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 (16:8h day: night light cycle, 23:21˚C day: night temperature cycle and a constant 35% relative humidity) on four artificial diets prepared with 100µl 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.

Keywords

Forensic entomology; Bacteria; Calliphoridae; minimum post-mortem interval; *Calliphora*; *Lucilia*

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 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

Insect culturing

Blow fly colonies were maintained in mesh cages (52 x 58 x 60cm) 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.

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 hours after death [30-31].

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 hours. 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.

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 9ml of phosphate buffered saline (PBS) with one loop (10µl) 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. 100µl 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.

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 (16:8h 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 hours 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.

Statistical analysis

A generalized 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 potential level of interaction that was assessed was the three-way interaction, and nested levels within higher order interactions were not examined. To assess the importance of individual independent variables, a likelihood ratio test was used to compare the full minimal adequate model with models in which the particular independent variable and all the interaction terms including it were omitted. Analyses were performed using the 'R' statistical and programming environment [37] and the 'nlme' package (linear and nonlinear mixed effects models; [38]).

A bootstrap-routine was implemented for each model to obtain estimates of the 95% confidence intervals around model predictions. This involved repeatedly (n= 5000) randomly reordering the model residuals, adding these to the fitted values from the original model, reapplying the model, and obtaining model predictions from the updated model. This resulted in distributions associated with every possible combination of levels of the independent variables from which estimated 95% confidence intervals could be obtained.

Analysis of survival rates to pupation and adult emergence were conducted using Chi Square in R.

3. Results

Weight, length and width of individuals were measured. Below we only present the results of the analyses of weight, both for clarity and because preliminary analysis revealed that the weight measurements had greater resolution of results and showed the greatest variation. However, as length is a metric often used in forensic analysis, and for completeness, we present coplots for both length and width in the supplementary material, along with details of the levels of correlation between length, width and weight. A full analysis was also run on the larva data with length as the dependent variable, as described above for weight as the dependent variable. This resulted in a model with the same structure as for the weight-model with very similar results, as expected given the high levels of correlation between weight and length in all three species (see supplementary material). The visualisation of the model is presented in the supplementary material for comparison with Figure 1.

Statistical analysis of the data showed a significant three-way interaction between species, day and diet (L-Ratio = 231.94, df =4, $p < 0.0001$). A visualisation of the model (Figure 1) shows how the predicted values of larval weight vary for each diet for each species. The relative importance of the three independent variables was estimated using likelihood ratio tests. The L-ratios for day, species and diet were 9380, 3432 and 409 respectively.

Differences in larval weight

Calliphora vomitoria displayed the greatest variation of larval weight between bacterial diets. The biggest difference was seen on day 5 when predicted larval weight relative to the control diet was 23% higher for larvae fed on the mixed diet and 15% higher for larvae fed on the *E. coli* diet. There was no difference for the larvae on the *S. aureus* diet. Predicted weights and

uncertainties for day 5 are shown in Table 1. Larvae reached their peak weight on day 6 with the exception of larvae fed the mixed bacterial diet which attained their peak weight 24 hours earlier. Differences in peak larval weights were significant for both the *E. coli* ($p = 0.040$) and mixed diets ($p = 0.0216$) relative to control diets. On day 7 a greater difference was evident between *C. vomitoria* larvae fed the control diet and the *S. aureus* diet. In this post-feeding stage, larvae on the *S. aureus* diet weighed 22% less than control larvae on day 7, although with a greater degree of uncertainty reflecting greater variance within the data. Indeed larvae on all the bacterial diets weighed less by day 7 compared to the control diet. This may reflect an earlier start of pupation.

Calliphora vicina showed **s**maller differences between bacterial diets than for *C. vomitoria.* The greatest positive larval weight difference relative to the control diet was seen for larvae on the *S. aureus* diet. Larvae reached their peak weight on day 4 on all diets. Differences in larval weight on day 4 are shown in Table 2. Larvae fed the *S. aureus* diet were 6.5% heavier than those on the control diet on day 4. This difference increased to 12% on day 5. Predicted weights and confidence intervals for day 4 are shown in Table 2 and for day 5 in Table 3. By day 6 there was no significant difference in weight between the larvae fed on the different diets.

Lucilia sericata showed no significant differences in larval weights between the different diets.

Influence of bacterial diet on rate of larval growth in C. vomitoria

Calliphora vomitoria larvae fed on the mixed bacterial diet reached their peak weight 24 hours earlier than larvae on the other three diets, suggesting a more rapid growth for *C. vomitoria* larvae (Figure 2). No such difference was observed for *C. vicina* or *L. sericata*.

Influence of diet on pupal survival and weight

Variation in pupal weight was only seen for *C. vomitoria* (Figure 3). Pupae from larvae fed on the *S. aureus* diet weighed 14.6% less than those on the control diet (Table 4). This observation is consistent with the larval weights. Model predictions for day 7 indicate larvae fed on the *S. aureus* diet are 22% smaller compared with the control diet. There were no significant differences for the *E. coli* and mixed diets relative to the control. No differences in survival rate with diet were observed for any species.

Statistically significant but small variations were seen in adult fly weight for *C. vomitoria* only. No differences in survival rate with diet were observed for any species (Table 5).

Chi-square test conducted for survival

Calliphora vomitoria showed the lowest survival rate of all species within the control diet (Table 5). However, there was no significant difference in the survival rates of this species across the diet treatments ($x^2 = 5.63$, d.f. = 3, $p = 0.131$). Notably, the larvae thrived or died *en masse* on a single plate rather than equally across all plates within the control treatment for this species.

4. Discussion

This study shows that the bacteria blow flies are exposed to in their diet has a significant impact on their development. There were marked differences in the dynamics of larval weight through time between the three species of blow fly (Figure 1), with peak larval weight for *C. vicina*, *C. vomitoria* and *L. sericata* occurring on days 4, 6 and 5 respectively. Although patterns of expected mean development within species and across diets were generally consistent within species, the role of bacteria in the diet was found to have a significant impact. This was supported by the likelihood ratio tests indicating that diet was playing a significant but relatively minor role in affecting larval weight compared with the day and species factors.

The pattern of larval development was altered for *C. vomitoria* on the mixed bacterial diet causing a peak in the larval weight of *C. vomitoria* a day earlier (day 5) compared with the other diets. Interestingly, also with *C. vomitoria*, larvae had clearly reached peak weight and started to decline with all diets except the control diet. Thus, although being relatively minor, there were differences in the effects of diet across species, with *C. vomitoria* showing effects, and the other two species showing no marked effects. The effects of diet on *C. vomitoria* indicate that the microbial community on the corpse may alter larval development rate enough to show significant differences in weight across a 24 hour period.

One notable observation is that *Calliphora vomitoria* larvae thrived or died *en masse* within a single plate when on the control plates. One hypothesis to explain this is that in the absence of bacteria the larvae rely on proximity and numbers to break down the food into nutrients. It

is possible that larvae that move or are placed further apart on the agar plate die due to lack of available nutrients on the sterile plates.

The reasons for the differences in response to bacteria by the different blow fly species are not clear. They may represent the consequences of a separation of environmental niches instantiated in different metabolic interactions with the microbial community. Microbial communities vary spatially on the human body [28], and if there are species differences in preferences for sites to lay eggs, the developing larvae will be exposed to differing microbial communities. Thus different blow fly species may have evolved different relationships with the range of microbial species found on the corpse. Examples of such relationships have been demonstrated in previous studies, including species variation in levels of antimicrobial activity against specific bacteria [39] and ovipositional response to different bacterial species [40-41]. This relationship would extend to the temporal dimension as well, with microbial communities being dynamic through time [15, 42] and species of blow flies laying eggs on the corpse at different times. For example, Erzinçlioğlu [42] found that *C. vicina* preferred decomposed to fresh remains, therefore these flies potentially colonize a corpse later in the decay sequence than *C. vomitoria*. These micro-environmental variations in interactions are likely to be further driven by variation in distributions between species of blow fly that occur at the larger landscape level [42-45].

The mechanisms by which microbial communities can affect the development of blow flies are also unclear. The increased growth rate in *C. vomitoria* found here on the mixed diet compared with the other diets, is consistent with previous studies where multi-species bacterial communities have been found to be favourable to larval development for the blow fly *Cochliomyia macellaria* [27], as well as flies of the *Muscidae* family [46-50]. The blow flies may gain direct nutritional benefits through the ingestion of microbes. They may also benefit from microbes facilitating nutrient breakdown of flesh prior to ingestion. Increasing bacterial diversity has been shown to lead to enhancement of metabolic activity over and above purely additive effects [51], and this offers a potential explanation of the faster developmental rate of *C. vomitoria* occurring with the mixed diet. However, we would expect effects of increased metabolic activity in bacteria on larval development to be potentially positive, negative or neutral, depending on the physiological consequences. This may explain the variation in developmental responses between the three blow fly species used in this experiment. It should be noted that there will be multiple such mechanisms

functioning in the real world, both positive and negative in their impact on blow fly development, including competition and pathogenicity.

Notably, *L. sericata* showed no effects in relation to the two, common and widespread bacterial species used in this study. Previous work has demonstrated that this blow fly is not effected in terms of pupation times, pupal size or survival to adulthood when exposed to *S. aureus* or reared in a sterile environment [26]. However, in terms of development rate, further work will need to be done to see if this species remains robust to a wider range of bacteria.

From the perspective of forensic entomology it is important to note that although diet was part of a significant interaction term with species and time, it played a relatively minor role in terms of overall effect on larval development, and effects were mainly confined to *C. vomitoria*. However, this should be regarded within the context that just two microbial species were used here, whereas there will be significantly more species on a corpse. The greater number of microbial species dramatically increases the number of potential interactions and effects both within the microbial community and between the microbes and blowflies. Thus, it will be important in future work to establish if the presence of larger bacterial communities broadens the effects on different blowfly species or whether there are limited dominant interaction pathways. This will have ramifications in establishing if heterogeneity in microbial communities between corpses is likely to increase variation in blow fly development rates, or whether these rates are likely to be relatively robust and predictable. Potential effects of such heterogeneity could extend to the level of genotype within bacterial species. This is of consequence as, although the expected larval weight was fairly consistent across diet treatments within blow fly species, there was substantial variation in larval weight within species-time-diet treatments, and this extensive amount of variation extended to the pupal weight (Figure 3).

5. Conclusion

This study has shown that in a very limited bacterial community there can be significant effects on larval developmental rates in *C. vomitoria* and on larval weight in both *Calliphora* species tested. These results indicate that the microbial community present on a corpse has potential to alter fly-based mPMI estimations based on both developmental rate and larval metrics. However, it is unclear whether, in a more complex bacterial environment, such effects are likely to stabilise rates of development or to increase the inherent variability in

developmental rates. Establishing which of these occurs under more realistic conditions will be important in establishing levels of reliability in using forensic entomology to estimate mPMI.

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