Role of Fly Cleaning Behavior on Carriage of Escherichia coli and Pseudomonas aeruginosa

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Abstract

Flies are known to be mechanical vectors of bacterial, viral, and parasitic diseases. Although flies are known to transmit disease, the effects of cleaning behavior have not been well studied. This study quantified the cleaning effectiveness and behavior of three fly species: Sarcophaga bullata, Musca domestica L., and Drosophila virilis. Flies were transferred to plates of Escherichia coli or Pseudomonas aeruginosa and allowed to walk on the bacteria for a total of 5 min. After the flies were contaminated, they were either immediately collected to quantify bacteria or were placed onto sterile plates to clean for 5 or 10 min. After cleaning, flies were placed into tubes with 1 ml of sterile 0.85% saline and were gently shaken for 1 min to remove bacteria. A serial dilution was made and 50-μl spot titers were plated. Cleaning behavior was also monitored and scored for a period of 5 min. Results demonstrate a bacterial reduction for both bacteria on all three fly species. Sarcophaga bullata and D. virilis both showed a significant reduction of both bacteria within 10 min, whereas M. domestica only showed a significant reduction in P. aeruginosa. Cleaning behavior increased significantly in flies that were exposed to bacteria compared to flies that were not exposed to bacteria. This study is important, as it demonstrates that fly cleaning could affect mechanical transmission of disease, and additional studies should look at flies’ abilities to remove other types of microorganisms.

Key words: Sarcophaga bullata, Drosophila virilis, Musca domestica, cleaning behavior, mechanical vector

Flies are known mechanical vectors of disease that are capable of carrying and transmitting bacteria, viruses, and parasites (Graczyk et al. 1999, Calíbre-Hayes et al. 2003, Ahmad et al. 2007). A fly can carry bacteria on its legs, wings, mouthparts, and abdomen. Parisot and Fernier (1934) found one fly to be able to carry over 32 million bacteria on its body, and Graczyk et al. (1999) found a single fly to carry >200 oocysts of Cryptosporidium parvum, while as little as 30 oocysts can cause an infection (DuPont et al. 1995). Oyerinde (1976) found 329 hookworm eggs and 1,485 larvae to be on the legs of a single fly. These studies suggest flies can be grossly contaminated and therefore a significant vector of infectious disease pathogens.

Although flies are known for their ability to transmit disease pathogens, their cleaning abilities are not well-studied. Barber and Starner (1949) found houseflies (Musca domestica L.) spend about 18% of their time cleaning (head, feet, wings, abdomen, and mouthparts). When coated with dust, Drosophila melanogaster Meigen, 1830 begin cleaning within 30 s of being contaminated and tripled their total time spent cleaning (Phillis et al. 1993). Another study by Holloway (1976) done on the hover fly (Diptera: Syrphidae), found that flies contaminated with either pollen or charcoal dust cleaned the pollen or dust from their bodies using leg combing and tapping. The flies were known to ingest pollen, but they also ingested charcoal dust as noted by black feces and charcoal presence in the gut after dissection (Holloway 1976). Lewis and Hughes (1957) noted two mechanisms for removing contaminates from the bodies of the Phormia terraenovae including 1) the removal of particles by forming aggregates and knocking them off in clumps, or 2) by simply “walking” them off. “Walking” them off happens when the contaminants stick to the substrate on which the flies are walking. The amount of particles lost varies by the type of substrate on which the flies are walking.

Cleaning benefits the fly by keeping the wings and appendages free of debris, facilitating better movement and sensory reception (Sutcliffe and Mciver 1974). The olfactory organs of D. melanogaster located on the antennae and the maxillary palps are covered with tiny sensory hairs (Stocker 1994, Shanbhag et al. 1999) and are important for locating food and a place to oviposit. Shaffer et al. (2007) suggested flies were able to remove significant amounts of bacteria when the flies were allowed to clean for various times after being exposed to bacterial contamination.
This current study quantified the cleaning ability of three fly species: Sarcophaga bullata, M. domestica, and Drosophila virilis by monitoring the removal of Escherichia coli and Pseudomonas aeruginosa following contamination, both of which have been shown to be transmitted by two of the three fly species (Greenberg 1971, Forster et al. 2007). The fly species were selected because two are known to transmit disease in humans, S. bullata and M. domestica, while D. virilis is not known to transmit disease pathogens in humans. Sarcophaga bullata belongs to the group of flies called flesh flies (Diptera: Sarcophagidae), which are the largest of the species in this study, measuring 10–15 mm in length (Service 2004). Musca domestica (Diptera: Muscidae) is the medium-sized species measuring between 6–9 mm in length (Service 2004). Drosophila virilis is a species of fruit fly (Diptera: Drosophilidae) that is slightly larger than the more commonly used D. melanogaster. Drosophila virilis is the smallest species used in this study, having a body length of 2.5–4.5 mm (Arnett and Jacques 1981). This species was selected for its size and the potential to serve as a vector of infectious microorganisms in fruits and vegetables (Sela et al. 2005).

Materials and Methods

Fly and Bacterial Stocks
Three fly species were used in this study: M. domestica, S. bullata, and D. virilis. Musca domestica and S. bullata pupae (Carolina Biological Supply Company, Burlington, NC) were transferred to a disinfected container with a paper towel moistened with sterile water and held at room temperature (RT). The flies were left unfed to prevent introducing additional bacteria. Drosophila virilis were obtained from Dr. Kimberly Carlson at the University of Nebraska at Kearney, Kearney, NE. Drosophila virilis were maintained in bottles of pastureurized fly food (standard cornmeal, molasses, and torula yeast medium). All flies were used within 3 d of emergence.

Laboratory strains of E. coli (ATCC: 25922) and P. aeruginosa (ATCC: 27853) were grown on Luria Broth (LB) or Luria Broth Agar (LBA; Thermo-Fisher Scientific, Pittsburg, PA). Escherichia coli and P. aeruginosa were grown on LBA plates at 37°C overnight for stock plates that were maintained at 4°C until use. To create confluent growth (lawns), bacteria from the stock plates were spread over LBA plates using a sterile cotton swab and plates were incubated at 37°C for 24 h to be used the next day for fly cleaning experiments. The lawn plates of bacteria were discarded after each experiment and made fresh for the next day.

Quantification of Fly Cleaning
Before bacterial exposure, flies housed in holding containers were placed in the refrigerator for 5 min to slow their movement. Individual larger flies were randomly selected then transferred using sterile forceps onto a lawn culture plate of each bacterial species. Drosophila virilis were transferred to bacterial lawn plates using an ethanol-disinfected mouth aspirator. Flies were allowed to walk on the plate for a period of 5 min, while the plate was rotated or gently tapped to encourage fly movement and to prevent cleaning. It had been previously shown that blowflies picked up the largest quantity of insecticide during the first minute exposed, so in this study it was decided that we use a little longer time period for bacterial exposure and variation in the size of flies (Lewis and Hughes 1957). Flies that flipped upside down on the bacterial lawns were eliminated from the study due to gross contamination.

Flies were split into three treatment groups: 1) flies that were not allowed to clean, 2) flies that were allowed to clean for 5 and 10 min, and 3) unexposed flies taken directly from their holding container that served as controls. Flies were allowed to clean themselves on sterile petri plates for the allotted time. Flies from each group were transferred to wash tubes containing 1 ml of sterile 0.85% saline (pH 6.6) and were gently shaken for 1 min to remove any surface bacteria (Fig. 1). Serial dilutions were prepared to 10⁻⁶ in 0.85% saline and 50-μl spot titers were plated on LBA. Plates were incubated at 37°C for 24 h. All resulting colonies were counted. Bacterial colony-forming units (CFU) were figured to determine the total contamination of the flies. Control flies were not exposed to bacteria. The control flies were transferred directly from the holding container to the 1.0 ml of sterile 0.85% saline and were gently shaken for 1 min to remove any surface bacteria. Serial dilutions were prepared to 10⁻³ in 0.85% saline, and 50-μl spot titers were plated on LBA. The plates were incubated at 37°C for 24 h. Bacterial colony-forming units were enumerated, and the average CFU of unexposed control fly was subtracted from the bacteria enumerated in the treatment groups prior to statistical testing. The resulting data were log transformed, and linear regression analysis was performed (P ≤ 0.05). All statistical testing was done using GraphPad Prism (La Jolla, CA).

Quantification of Bacterial Transfer
To determine the amount of the physical removal of bacteria that could be transferred, 18 M. domestica were allowed to walk on the bacteria plates for a period of 5 min. After the allotted time, they were transferred to sterile 2.0-ml centrifuge tubes to clean for 5 min. The fly was then removed from the tube, and 1.0 ml of sterile 0.85% saline was added. The tube was vortexed for 1 min to remove any bacteria that were physically removed by the fly through grooming behavior. Serial dilutions were prepared to 10⁻⁴ in 0.85% saline, and 50-μl spot titers were plated on LBA. Plates were incubated at 37°C for 24 h. Bacterial colony-forming units were enumerated to determine the contamination of the flies. A Student’s t-test (P ≤ 0.05) was used to test for statistical differences in the removal of each bacterium.

Fly Weight Measurement
The average weight of each fly species was calculated by weighing 50 individuals of each fly species using an analytical balance (Mettler-Toledo, Columbus, OH). The sex of the individual flies was not determined. The number of bacteria per gram body weight was determined by dividing the amount of bacteria (CFUs) removed from the fly by the average weight of the fly species. Statistical analysis was performed using a Kruskal–Wallis one-way analysis of variance (P ≤ 0.05) followed by a Dunn’s post hoc test for nonparametric data.

Fly Cleaning Behavior
Changes in cleaning behavior were quantified using D. virilis, which cleaned the best of the three species, was exposed to E. coli, which is a fecal coliform and serves as a model organism for numerous infectious bacteria known to be spread by mechanical transmission. Twenty-two flies were aseptically removed from their containers and placed on a 24 h lawn of E. coli. In order to increase the likelihood of extended cleaning, E. coli was used because it was slightly more difficult to remove than P. aeruginosa based upon cleaning quantification. Flies were allowed to walk around for a period of 5 min. Controls were not exposed to bacteria but immediately placed into a sterile Petri plate to score cleaning. Flies were removed after 5 min and placed into sterile Petri plates. The cleaning behavior...
of the individual flies was observed with a dissecting microscope (Bausch and Lomb, Rochester, NY) for a period of 5 min in the sterile petri plate. Specific cleaning behaviors were scored from adaptation of Szopenyi (1969): prothoracic legs to mouth, prothoracic legs to head, prothoracic legs together, prothoracic legs cleaning mesothoracic legs, mesothoracic legs cleaning prothoracic legs, mesothoracic legs cleaning metathoracic legs, metathoracic legs cleaning wings, metathoracic legs cleaning body, metathoracic legs rubbing together, and metathoracic legs cleaning mesothoracic legs were counted. The total number of actions were counted over 5 min and means figured. A Student’s t-test ($P \leq 0.05$) was used to test for statistical differences in cleaning behavior between exposed and unexposed flies.

### Results

#### Quantification of Fly Cleaning

Three species of flies were exposed to *E. coli* or *P. aeruginosa* for 5 min and then allowed to clean for up to 10 min. Bacterial reduction was seen for 5 and 10 min for both bacteria on all three fly species (Figs. 1 and 2). All three species of flies were able to reduce bacterial contamination over time. After 10 min, *M. domestica* were able to remove 86% of *E. coli* and 92% of *P. aeruginosa* (Table 1). Sarcophaga bullata were able to remove 76% and 86%, respectively, and *D. virilis* 83% and 97% (Table 1). Linear regression analysis was used to test for significant reduction in bacterial numbers...
Table 1. Percent bacterial reduction

|            | E. coli | P. aeruginosa |
|------------|---------|--------------|
|            | 5 min   | 10 min       | 5 min   | 10 min       |
| M. domestica | 84%     | 86%          | 93%     | 92%          |
| S. bullata  | 43%     | 76%          | 72%     | 86%          |
| D. virilis  | 87%     | 83%          | 73%     | 97%          |

Flies were exposed to E. coli or P. aeruginosa for 5 min and then not allowed to clean or allowed to clean for 5 or 10 min. Bacterial reduction was then calculated by dividing the average bacterial measure for 5 min or 10 min by the average bacterial measure for no cleaning and multiplying by 100.

over time. Sarcophaga bullata were able to significantly reduce the number of both E. coli (F = 7.59, df = 1, 26, P = 0.011) and P. aeruginosa (F = 7.991, df = 1, 14, P = 0.014), while M. domestica were not able to significantly reduce the number of E. coli (F = 1.419, df = 1, 26, P = 0.245) but were able to significantly reduce the number of P. aeruginosa (F = 4.446, df = 1, 26, P = 0.045). Drosophila virilis was able to significantly reduce both the E. coli (F = 9.115, df = 1, 29, P = 0.005) and P. aeruginosa (F = 4.497, df = 1, 25, P = 0.044) within 10 min.

Quantification of Bacterial Transfer
To determine the amount of bacteria that could be transferred to the flies’ surroundings during the grooming process, M. domestica were allowed to clean and the amount of bacteria physically removed was quantified. Eighteen M. domestica were exposed to either E. coli or P. aeruginosa, placed into an enclosed tube, and allowed to groom for 5 min. Flies were removed and bacteria remaining in the tube were quantified. Control flies were not exposed to bacteria, but placed in the enclosed tubes for 5 min. Control flies transferred an average of 2.7 × 10^6 CFU, while flies exposed to E. coli transferred 1.2 × 10^7 CFU and to P. aeruginosa 1.1 × 10^7 CFU. Musca domestica were able to transfer statistically the same amount of both E. coli and P. aeruginosa to the surrounding surface during grooming (P = 0.7112).

Fly Species Comparison (of Bacteria on the Surface)
In order to compare the fly cleaning ability between species, 50 flies of each species were weighed and an average fly weight (Fig. 3) was used to standardize for the number of bacteria per gram weight of fly (Table 2). The average weight of S. bullata was 0.045g, 0.007 g for M. domestica, and 0.002 g for D. virilis. Bacterial contamination for all three flies at each time point (0, 5, and 10 min) was then compared using an ANOVA on Ranks. At time 0, all three flies carried roughly the same number of E. coli or P. aeruginosa per gram weight. The same result was found for the 5- and 10-min time points. The only significant difference was seen with D. virilis, which picked up a significantly higher amount (P < 0.05) of P. aeruginosa (7.57 × 10^7 CFU/g) than E. coli (1.68 × 10^8 CFU/g).

Cleaning Behavior
In order to distinguish changes in cleaning behavior, D. virilis were exposed to E. coli bacterial lawns for 5 min. Flies exposed to bacteria showed a significant difference (P < 0.05) in cleaning activity compared to the nonexposed control group with the exception of the prothoracic legs cleaning the metathoracic legs and the metathoracic legs cleaning the mesothoracic legs (Table 3). The flies appear to clean by a rubbing motion, balling up debris and flinging it off of their legs. Bacteria were also removed from the wings by simply flapping the wings or “flying-in-place.” These cleaning behaviors would leave spots or specks of the debris on the top or bottom on the sterile petri dish housing the fly. Flies were also observed to significantly increase the movement of their prothoracic legs to their proboscis.

Discussion
This study has quantified the ability of three fly species to remove bacteria after surface contamination, and contributes to our understanding of how flies can serve as mechanical vectors of bacterial diseases. Our results show that D. virilis and S. bullata are able to remove a significant amount of both E. coli and P. aeruginosa. Surprisingly, M. domestica was only able to remove a significant amount of P. aeruginosa, but not E. coli. Differences in cell size are unlikely to account for the difference in bacterial removal as E. coli (1.1–1.5 μm × 1.5–5.0 μm) and P. aeruginosa cells (0.5–1.0 μm × 1.5–5.0 μm) are of similar size. Pace et al. (2017) also found a difference in the amount of bacteria that flies deposit. They found no difference in the amount of Salmonella enterica that blow flies (Phorina regina and M. domestica) acquired, but P. regina was able to deposit more of the bacteria onto lettuce leaves compared to M. domestica. These data suggest that S. enterica may adhere more strongly to M. domestica, which is consistent with our current study showing a reduced ability of M. domestica to remove E. coli.

Houseflies are domestic filth flies that breed in animal manure and human excrement (Greenberg 1971, Levine and Levine 1991), where one would expect to find large amounts of E. coli (Guber et al. 2005). Escherichia coli expresses a variety of adhesins (e.g., pili, fimbriae) that may enable it to more strongly adhere to the housefly (Kalita et al. 2014). It has been demonstrated that pili on the surface of E. coli use a roll and stick adhesion mechanism and are, not only resistant to shear force, but activated by it (Thomas et al. 2004). The bacterium may also stick more strongly to the pulvillus, which is the sticky substance released by the glandular hairs of filth flies (Gracyzk et al. 2001). In a study by Tan et al. (1997), it was shown that increased viscosity can make it more difficult for the house fly to remove materials adhering to the legs. It is possible that the E. coli create a more viscous solution that adheres to the fly bristles and hairs more strongly than other bacteria. The strain used in the study presented here (E. coli ATCC 25922) encodes a limited number of adhesins including papG (DR76_1632), fimH (DR76_2431), sfaD (K758_20486), and csgA (DR76_3880) (Minogue et al. 2014). Future studies will be needed to define the roles for E. coli ATCC 25922-encoded adhesins, as well as additional adhesins encoded by other pathogenic E. coli strains.
Flies were exposed to E. coli or P. aeruginosa for 5 min and then not allowed to clean or allowed to clean for 5 or 10 min. Bacterial numbers per gram weight were calculated by dividing the average bacterial load by the average weight of the fly. Asterisks indicate there was statistically significant greater amount of P. aeruginosa picked up by D. virilis than E. coli (P ≤ 0.05).

Table 3. Cleaning behavior of D. virilis

| Cleaning behavior                  | Control flies | Experimental flies |
|-----------------------------------|---------------|--------------------|
| Prothoracic legs to mouth         | 0.6 ± 0.2     | 6.3 ± 1.9          |
| Prothoracic legs to head          | 4.1 ± 1.1     | 9 ± 1.2            |
| Prothoracic legs to prothoracic legs | 6.8 ± 1.1   | 17.5 ± 2.1         |
| Metathoracic legs to metathoracic legs | 0.6 ± 0.18   | 0.8 ± 0.2*         |
| Metathoracic legs to mesothoracic legs | 3.9 ± 0.82   | 4.5 ± 0.93*        |
| Mesothoracic legs to wings        | 2.9 ± 1.1     | 9.7 ± 0.85         |
| Mesothoracic legs to body         | 3.0 ± 0.74    | 5.6 ± 0.86         |
| Mesothoracic legs to body         | 8.6 ± 1.6     | 18.4 ± 1.5         |
| Mesothoracic legs to metathoracic legs | 0            | 2.9 ± 0.78         |

Twenty individuals were observed. Data report the mean number of behaviors and standard error observed over 5 min. For every behavior, experimental flies demonstrated significantly higher rates of cleaning behavior than control flies, except those indicated by an asterisk (P ≤ 0.05).

Qin et al. 2013, McWilliams and Torres 2014) in adherence to the various fly species including M. domestica.

The three species of flies tested were selected for their size variation. It was found that the flies did not pick up significantly different amounts of bacteria/gram weight. After allowing time for cleaning, they also were able to remove similar amounts of bacteria, as the 5 and 10 min cleaning times were not significantly different for the three fly species. In a study by De Jesús et al. (2004), the number of E. coli that M. domestica could pick up from food and carry on their body were quantified to be up to 5.5 log_{10} CFU/fly. Our study indicates that the number of E. coli cells adhering to M. domestica flies was highly variable (~5.5 log_{10}8 log_{10} CFU/fly). However, our results show that flies pick up similar amounts of bacteria when fly size is taken into account (Table 3). Another interesting finding was that D. virilis picked up significantly more P. aeruginosa than E. coli. Pseudomonas aeruginosa is a known pathogen of D. virilis that relies upon the twitching pathway to be pathogenic (D’Argenio et al. 2001). Twitching is a locomotor mechanism which relies upon type IV pili (Sennler et al. 1999). It is possible that the type IV pili could be used for attachment to the fly. It is not known why it would be higher in one species of fly, but there were differences in M. domestica’s ability to remove E. coli as well. We may find bacterial adaptations for fly species that are yet unknown.

Lewis and Hughes (1957) reported two primary mechanisms that flies use to remove fine particles: “walking” them off of the tarsal spines and forming clumps of particulate that could be knocked off. Szebenyi (1969) characterized cleaning behavior in D. melanogaster as sweeping and rubbing that moved particles off the body, while Holloway (1976) observed that hover flies contaminated with pollen used tapping, combing, and eating to remove the pollen. In this study, flies that were exposed to bacterial contamination increased their cleaning behaviors significantly. They also used the combing or rubbing behavior to fling bacteria in aggregates off their bodies (Table 3). This action could be contributing to bacterial transmission, but it was also noted that they significantly increased the movement of the legs to the mouth. This would suggest that the flies could be eating the contaminating bacteria, although it was not quantified in this study. A recent publication by Nayduch and Burrus (2017) described the interaction of house flies and bacteria. Larval house flies eat bacteria as a nutritional requirement. This could be an important means of reducing bacterial loads on fly surfaces.

In order to elucidate the contribution of fly cleaning to environmental contamination, we quantified the bacteria that got transferred from the fly to the surrounding surface during the cleaning process. Musca domestica was used for two reasons: it demonstrated the lowest level of bacterial removal and it was used in another study by Pace et al. (2017) for comparison. It was demonstrated that M. domestica was able to remove similar amounts of E. coli (1.2 × 10^6 CFU) and P. aeruginosa (1.1 × 10^6 CFU) and transfer them to the surrounding surfaces. Pace et al. (2017) reported that blow flies and house flies deposited similar amounts of E. coli O157:H7 on lettuce leaves. Both of these studies suggest that fly grooming can contribute to mechanical transmission of bacterial pathogens.

The cleaning behaviors measured in our study are likely important for fly survival and success. Yap et al. (2008) found a significant reduction in bacteria from the wings of M. domestica when inoculated with Vibrio cholerae. Flight was also found to significantly reduce the amount of bacteria on flies; however, the proportion of bacteria retained on individual flies varied greatly. This study also reported that M. domestica was capable of removing 86% of both E. coli and P. aeruginosa, although the removal was only significant for P. aeruginosa due to the high level of variability for E. coli. The importance of cleaning seems supported by the fact that flies removed 86% of both E. coli and P. aeruginosa within 10 min of cleaning. However, flies in our study failed to remove all of the plate-acquired bacteria, which may shed insights into the ability of M. domestica to mechanically spread infectious agents for up to 8 d (Graczyk et al. 1999, Nayduch et al. 2002). Moreover, ingested bacteria are greatly reduced in the gut of the fly after 3–4 d but still can remain viable and be transferred via defecation (Kobayashi et al. 1999, Sasaki et al. 2000). This suggests a period of time where fly cleaning could greatly influence pathogen transmission, as bacteria would be expelled from the gut and transmitted again in their food, feces, or from fly-to-fly transmission.

This study is the first like it to demonstrate that cleaning behavior can significantly change bacterial contamination of flies. All three species of flies were able to reduce bacterial contamination on their bodies with increased cleaning activity, yet the cleaning process was also shown to contaminate the surrounding surfaces. Therefore, the length of time from exposure to the bacterial contamination and location of a host may be significant in the transmission of bacterial pathogens. Additional studies are necessary to quantify the effects of cleaning on other infectious microorganisms transmitted by flies.
including larger parasites and bacteria that produce very small endo-
spores. These studies will help us to elucidate the real effects cleaning
may have on the mechanical transmission of microbial pathogens.

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