

Bacterial Associations Across House Fly Life History: Evidence for Transstadial Carriage From Managed Manure

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Abstract

House flies (Diptera: Muscidae; *Musca domestica* L.) associate with microbe-rich substrates throughout life history. Because larvae utilize bacteria as a food source, most taxa present in the larval substrate, e.g., manure, are digested or degraded. However, some species survive and are present as third-instar larvae begin pupation. During metamorphosis, many bacteria are again lost during histolysis of the larval gut and subsequent remodeling to produce the gut of the imago. It has been previously demonstrated that some bacterial species survive metamorphosis, being left behind in the puparium, present on the body surface, or in the gut of the emerged adult. We used a combined culture-molecular approach to identify viable microbes from managed manure residue and a wild population of house fly larvae, pupae, puparia, and adults to assess transstadial carriage. All larval (10/10), pupal (10/10), and puparial (10/10) cultures were positive for bacteria. Several bacterial species that were present in larvae also were present either in pupae or puparia. Four viable bacterial species were detectable in 6 of 10 imagoes reared from manure. Of note is the apparent transstadial carriage of *Bacillus sonorensis*, which has been associated with milk spoilage at dairies, and *Alcaligenes faecalis*, which can harbor numerous antibiotic resistance genes on farms. The potential of newly emerged flies to harbor and disseminate bacteria from managed manure on farms is an understudied risk that deserves further evaluation.

Key words: bacteria, larvae, pupae, puparia, adult

Larval house flies (Diptera: Muscidae; *Musca domestica* L.) require live bacteria for successful development and metamorphosis (Schmidtman and Martin 1992, Zurek et al. 2000). While adult house flies do not have this nutritional requirement for bacteria, they tend to associate with microbe rich habitats for reproductive purposes both in domestic and agricultural settings (West 1951). Animal manure is the major source of microbes in house fly developmental habitats at confined animal facilities, such as dairies (Meyer and Shultz 1990). First-, second-, and third-instar larvae of house flies ingest bacteria from manure; consequently, species composition and abundance of these bacteria can vary widely between sites due to variation across host species (i.e., cattle, swine, and poultry) and among individual hosts (Larrain and Salas 2008).

When third-instar larvae cease feeding and prepare for pupation, the undigested contents of the gut, including live bacteria, are subjected to lysis by digestive enzymes (West 1951). As metamorphosis progresses within the puparium, enclosure and histolysis of the larval gut and its contents including waste and residual undigested bacteria (also referred to as “meconium”) begin, as does histogenesis of the new adult gut (Carpenter 1913, Engel and Moran 2013). Bacteria that can withstand, survive, or escape these destructive

processes either colonize the newly formed gut of the imago or are otherwise discarded in the meconium, which is left behind in the puparium or passed by the adult.

The goals of this study were 1) to identify cultivable bacteria associated with a larval developmental substrate (residual managed manure) and life history stages of wild house flies and 2) to assess transstadial carriage of bacteria.

Materials and Methods

Larval substrate containing third-instar larvae of *M. domestica* (Hewitt 1914) was collected from the Kansas State University Dairy Facility (Riley County, Manhattan, KS) in August and October 2014. This natural fly developmental substrate consisted almost entirely of accumulated residue of managed manure from the KSU dairy herd. Approximately 100 g of manure in a sterile 16 oz polypropylene container with larvae ($n = 20-25$) was placed in a secondary plexiglass container (25 by 25 by 20 cm) to minimize contamination and maintained at 28°C under a photoperiod of 14:10 (L:D) h. Manure was observed daily for presence of puparia, which were subsequently removed with sterile forceps, surface

sanitized (described below), and placed in individual sterile 15 ml conical tubes for imago emergence.

For microbe culture, samples (1.0 g manure and 5 third-instar larvae) were collected on day 0, on days 7–8 (five late-stage pupae), and on days 9–10 (five newly emerged adults and matching puparia). Fly stages (larvae, pupae, or adults) were surface sanitized by sequential washes in 0.5% sodium hypochloride, 70% ethanol, and sterile deionized water, 2 min each. Samples were homogenized in sterile phosphate buffered saline and cultured on tryptic soy agar (Fisher Scientific, Atlanta, GA) at 37°C overnight. Total colony forming units (CFU) were enumerated and distinct colony morphotypes were subcultured on tryptic soy agar, after which a single colony was picked for 16S rDNA-polymerase chain reaction (PCR) bacterial identification.

PCR amplification of the 16S rRNA gene was performed using universal eubacterial primers: 8F (5'-AGAGTTTGATCCTGG CTCAG-3') and 806R (5'-CTACCAGG GTATCTAAT-3') (Hugenholtz et al. 1998). PCR products were purified with DNA Clean & Concentrator (ZYMO Research, Irvine, CA) and sequenced by Clemson University Genomics Institute using the same PCR primers. Sequences were manually edited in CodonCode Aligner (version 3.7.1) (CodonCode Corporation) and taxonomically identified by BLAST (Basic Local Alignment Search Tool) search of the NCBI GenBank database (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>).

Table 1. Identification of bacterial isolates from cow manure and house flies based on 16S rDNA sequencing (August 2014)

Sample	Positive cultures	Identification	% ID	Length (bp)		
Manure		<i>Homoserinomonas</i> sp.	97	716		
		<i>Pseudochrobactrum</i> sp.	96	686		
Third instars	5/5	<i>Aeromonas</i> sp.	100	709		
		<i>Enterococcus</i> sp.	100	737		
		<i>Escherichia</i> sp.	99	716		
		<i>Leucobacter</i> sp.	96	704		
		<i>Microbacterium</i> sp.	99	701		
		<i>Providencia stuartii</i>	100	716		
		<i>Providencia</i> sp.	99	720		
		<i>Serratia marcescens</i>	100	693		
		<i>Acinetobacter</i> sp.	98	534		
Pupae	5/5	<i>A. faecalis</i>	99	682		
		<i>Arthrobacter</i> sp.	99	690		
		<i>Brevundimonas</i> sp.	98	676		
		<i>Myroides</i> sp.	99	718		
		<i>Sphingobacterium</i> sp.	98	726		
		<i>Acinetobacter</i> sp.	98	675		
Puparia	5/5	<i>Agrobacterium vitis</i>	98	651		
		<i>A. faecalis</i>	99	718		
		<i>Arthrobacter</i> sp.	100	202		
		<i>Arthrobacter</i> sp.	97	698		
		<i>Brevundimonas</i> sp.	99	658		
		<i>Brevundimonas</i> sp.	98	615		
		<i>Microbacterium</i> sp.	99	700		
		<i>Pseudomonas</i> sp.	98	709		
		<i>Pseudomonas</i> sp.	99	739		
		<i>Pseudomonas</i> sp.	98	722		
		<i>Pseudomonas</i> sp.	98	710		
		<i>Staphylococcus saprophyticus</i>	99	742		
		Adults	2/5	<i>B. sonorensis</i>	99	680

Results and Discussion

Culture-Based Estimate of Bacterial Density

Total CFU recovered from manure on day 0 were 8.0×10^7 CFU/g for August and 2.6×10^8 CFU/g for October. All larval cultures (5/5 for each collection) displayed bacterial growth ranging from 1.30×10^5 to 2.25×10^6 CFU in August (mean: $7.94 \pm 9.85 \times 10^5$ CFU per larva) and 2.43×10^4 to 3.0×10^5 in October (mean: $1.39 \pm 1.34 \times 10^5$ CFU per larva). Variation observed across larval samples and collection dates could be attributed to feeding versus nonfeeding third-instar larvae as well as the abundance and diversity of bacteria in the manure. The abundance of bacteria in pupae and emerged adults also varied considerably. All pupae had bacterial growth (10/10), ranging from 4.58×10^3 to 4.10×10^4 CFU per pupa for August (mean: $2.22 \pm 1.82 \times 10^4$ CFU per pupa) and 1.55×10^5 to 2.78×10^6 CFU per pupa for October collection (mean: $1.66 \pm 1.32 \times 10^6$ CFU per pupa). Of the 10 teneral adults cultured, 6 had recoverable amounts of bacteria ranging from 5.0 to 1.5×10^1 CFU per adult in August (mean: $1.00 \pm 0.707 \times 10^1$ CFU per adult) and 7.25×10^2 to 1.03×10^3 CFU per adult in October (mean: $8.98 \pm 1.56 \times 10^2$ CFU per adult). All 10 puparia corresponding to these adults were positive for bacteria, with concentrations ranging from 1.88×10^2 to 1.31×10^5 CFU per puparium in August (mean: $3.64 \pm 6.02 \times 10^4$ CFU per puparium) and 1.85×10^2 to 2.98×10^5 CFU per puparium in October (mean: $1.02 \pm 1.36 \times 10^5$ CFU per puparium).

Isolation and Identification of Bacteria

Distinct morphotypes of these isolates were subcultured and sequenced for taxonomic identification (Tables 1 and 2). Interestingly, bacterial taxa in larvae were not present in manure and vice versa. While this may be a result of the limitations of culture-based

Table 2. Identification of bacterial isolates from cow manure and house flies based on 16S rDNA sequencing (October 2014)

Sample	Positive cultures	Identification	% ID	Length (bp)
Manure		<i>Arthrobacter</i> sp.	97	716
		<i>Vibrio</i> sp.	98	716
Third Instars	5/5	<i>A. faecalis</i>	99	721
		<i>Bacillus cereus</i>	99	728
		<i>Providencia</i> sp.	99	711
		<i>Providencia</i> sp.	99	711
		<i>Staphylococcus</i> sp.	100	723
		<i>Wohlfahrtiimonas</i> sp.	97	722
Pupae	5/5	<i>Wohlfahrtiimonas</i> sp.	97	730
		<i>A. faecalis</i>	99	715
		<i>Enterococcus</i> sp.	99	687
		<i>Providencia</i> sp.	100	670
		<i>Providencia</i> sp.	100	666
		<i>Rummeliibacillus stabekisii</i>	100	727
Puparia	5/5	<i>A. faecalis</i>	99	717
		<i>Bacillus pumilus</i>	99	731
		<i>Providencia</i> sp.	99	715
		<i>Pseudochrobactrum</i> sp.	100	662
Adults	4/5	<i>Sphingobacterium alimentarium</i>	99	738
		<i>A. faecalis</i>	99	716
		<i>Micrococcus luteus</i>	99	700
		<i>Pseudochrobactrum asaccharolyticum</i>	98	671

techniques and visual selection of morphotypes, this phenomenon is also attributable to digestive processes within the larvae. Bacterial taxa present in manure but not in larvae indicate ingestion and digestion of the microbes. Similarly, it follows that species detected in larvae and pupae, but not detectable in manure, were not digested within the larvae and instead accumulated in the gut, as has been previously suggested (Su et al. 2010). Some of these taxa have been reported previously as being associated with manure (Banjo et al. 2005), larvae, or wild-caught flies (Zurek et al. 2000, Nazni et al. 2005, Gupta et al. 2011).

Taxonomic Identification of Isolates

Several bacterial genera present in pupae and puparia were not detected in emerging adults including *Alcaligenes faecalis*, *Arthrobacter* sp., *Brevundimonas* sp., and *Acinetobacter* sp. in August and October (Tables 1 and 2). *Providencia* sp. also was detected in larvae (Table 2). Bacterial species present in larvae and pupae but not cultured from either puparia or the emerged adults presumably were destroyed by the histolytic and proteolytic processes occurring during metamorphosis or were undetectable due to very low abundance.

Four taxa were cultured and identified from emerging adults: August collection, *Bacillus sonorensis* from two adults, and October collection, *Micrococcus luteus*, *A. faecalis*, and *Pseudochrobactrum* sp., where the last two species were both isolated from one adult (Tables 1 and 2). *B. sonorensis* previously has been isolated from dairies and is a potential contaminant during milk production processes (Buehner et al. 2014). While the ability of flies to disseminate *B. sonorensis* was not determined, sanitary practices in the dairy parlor potentially can be impacted if newly emerged flies harbor this microbe. *A. faecalis* has been isolated from dung, soil, and flies (Agersø and Sandvang 2005, Su et al. 2010, Resende et al. 2014). Because *A. faecalis* has the potential to harbor antibiotic resistance genes (Agersø and Sandvang 2005, Resende et al. 2014), transstadial carriage in house flies and possible dissemination from manure should be of concern in the design of manure and fly management practices. *Pseudochrobactrum* has been detected in flies (Su et al. 2010); this bacterium is typically associated with soil and can cause opportunistic infections in humans (Kämpfer et al. 2006).

Carriage of bacteria across stages of house fly life history has been previously demonstrated. Using artificial rearing media (wheat bran and calf manure), Su et al. (2010) showed carry-over of several bacterial taxa through larval stages to newly emerged adult house flies using a molecular-based approach. Transstadial carriage of bacteria also has been demonstrated in experimental feeding trials. Tebbutt (1912) showed variable, low abundance carryover of pathogenic bacteria from larvae to emerged adult flies. Similarly, Radvan (1960) showed survival of pathogenic bacteria to house fly pupal stages but no significant transstadial carriage to adults. Greenberg (1959) showed that persistence of pathogens across house fly life history was species specific, at least for *Salmonella* spp. *Salmonella paratyphi*, but not *Salmonella typhi*, survived through metamorphosis in some flies and was cultured from emerged adults, even when larvae are reared in medium naturally contaminated with other ubiquitous microbes. More recent studies determined persistence of bacteria through metamorphosis using bacterial monocultures. Studies where house fly third-instar larvae were fed *Escherichia coli* via culture plate showed 100% of puparia and 78% of emerging adults harbored bacteria (Rochon et al. 2005). However, this number dropped to 66% when flies were previously rinsed, indicating that some of the emerged adults primarily harbored bacteria on their

external surfaces. Using a traceable bioluminescent strain of *E. coli*, Schuster et al. (2013) showed dose-dependent transstadial carriage of bacteria in flies reared from larvae on sterilized manure inoculated with various concentrations (0, 3, 5, and 8 log₁₀ CFU/ml).

While the approach used in this study had probable limitations of detection, our aim was to identify only viable bacteria. In contrast, while using solely a molecular-based approach (i.e., PCR of DNA extracts from our samples) is more sensitive, it cannot distinguish viable from nonviable bacteria (Gupta et al. 2011). Future studies can be improved by growing cultures at several temperatures on both selective and nonselective media, which would allow for a more extensive survey of viable bacterial species richness. Nonetheless, we showed evidence of transstadial carryover of several species of bacteria in flies that emerged after rearing in a natural developmental substrate (managed manure residue). The potential of newly emerged flies to harbor and disseminate bacteria, even with manure management strategies in place, warrants further study.

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