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Research article

Special Collection: Filth Fly–Microbe Interactions

Interkingdom Cues by Bacteria Associated with Conspecific and Heterospecific Eggs of *Cochliomyia macellaria* and *Chrysomya rufifacies* (Diptera: Calliphoridae) Potentially Govern Succession on Carrion

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Abstract

Deciphering mechanisms that regulate succession on ephemeral resources is critical for elucidating food web dynamics and nutrient recycling. Blow fly (Diptera: Calliphoridae) colonization and utilization of vertebrate carrion serve as a model for such studies, as they are the primary invertebrates that recycle this ephemeral resource. Initial colonization by blow flies often results in heightened attraction and colonization by competing conspecifics and heterospecifics, thereby regulating associated arthropod succession patterns. We examined the response of *Cochliomyia macellaria* (F.) and *Chrysomya rufifacies* (Macquart) to conspecific and heterospecific eggs. Because *Ch. rufifacies* is facultatively predacious and cannabalistic, we hypothesized that adults would recognize the presence of conspecific and heterospecific eggs, thus avoiding potential predation and competition. Using a Y-tube olfactometer, we measured the residence time response of *C. macellaria* and *Ch. rufifacies* to conspecific and heterospecific eggs of three different age classes (fresh to 9-h-old). Fly responses to surface-sterilized eggs and to an aqueous solution containing egg-associated microbes were then examined. High-throughput sequencing was used to survey egg-associated bacteria from both species. We report that *C. macellaria* and *Ch. rufifacies* exhibit differential responses to eggs of conspecifics and heterospecifics, which appear to be a result of microbial volatile-related odors. These behaviors likely influence predator–prey interactions between species. Preliminary high-throughput sequencing revealed *Ch. rufifacies* had a similar egg-associated fauna as *C. macellaria*, which may serve as a form of camouflage, allowing it to colonize and thereby attract *C. macellaria*, a common prey for its larvae.

Key words: Interkingdom eavesdropping, quorum sensing, forensic entomology, carrion decomposition, succession

Cochliomyia macellaria (F.) (Diptera: Calliphoridae) and *Chrysomya rufifacies* (Macquart) (Diptera: Calliphoridae) larvae exploit carrion, yet temporal variation occurs with adult arrival and oviposition (Eberhardt and Elliot 2008, Biavatim et al. 2010). *Cochliomyia macellaria* acts as a primary colonizer of carrion, arriving within 24 h after death (Brundage et al. 2014), whereas *Ch. rufifacies* is often a secondary colonizer arriving 24–48 h after death (Fuller 1934, Eberhardt and Elliot 2008, Cammack et al. 2010). *Chrysomya rufifacies* larvae are also facultative predators on *C. macellaria* larvae and are known as facultatively cannibalistic (Baumgartner 1993). Arrival time (i.e., temporal sequence of arrival and colonization during the succession process) significantly affects the fitness of both species. When fitness is defined in terms of increased larval survivorship, pupal weight, and adult fecundity, *Ch. rufifacies* are more fit if they arrive after *C. macellaria*, whereas *C. macellaria* are more fit if they arrive before *Ch. rufifacies*. *Cochliomyia macellaria* colonization of carrion within two days of *Ch. rufifacies* significantly lowers *C. macellaria* survivorship (Brundage et al. 2014).

Variation in time of arrival and colonization indicates that cues associated with carrion are used by *C. macellaria* and *Ch. rufifacies*

to locate and assess resources. As with many insects, these cues may be visual (Wallis 1962, Easton and Feir 1991, Collins 1996, Brodie et al. 2015b), auditory (Wertheim et al. 2005, Wicker-Thomas 2007), tactile (Eismann and Rice 1987, Easton and Feir 1991), or olfactory (Crombie 1944, Browne 1960); these may emanate from the resource itself (Wall and Fisher 2001, Zheng et al. 2013b) or from heterospecifics or conspecifics inhabiting the resource (Judd and Borden 1992, Davies 1998, Diaz-Fleischer and Aluja 2003).

Volatiles associated with ovipositing conspecific (Browne 1960) and possibly heterospecific females are also used to locate resources. *Lucilia cuprina* (Wiedemann) (Diptera: Calliphoridae) preferentially oviposit in the presence of other actively ovipositing conspecific females (Browne et al. 1969). This behavior results in larval masses that may accelerate digestion of the resource and enhance larval feeding (Vartib-Browne 1958). Such a process is not unique to blow flies, as the house fly, *Musca domestica* (L.) (Diptera: Muscidae) has also evolved such an ability (Lam et al. 2007). Adult flies are able to assess an environment based on volatile profiles, proving to be important for optimal fitness. House flies ovipositing in conjunction with conspecifics suffered lower levels of predation than those arriving later (Lam et al. 2007). Researchers have suspected that an oviposition pheromone may be present (Browne et al. 1969). However, no such compound has been discovered to date.

Volatiles from bacteria are known to regulate behaviors of many other arthropods ranging from parasitoids to herbivores (Davis et al. 2013), as well as vertebrates (Jojola-Elverum et al. 2001). Volatiles produced by bacteria associated with blow fly larvae are used by conspecific adults to assess suitability of a resource for oviposition (Ashworth and Wall 1994). *Lucilia sericata* (Meigen) (Diptera: Calliphoridae) are attracted to *Pseudomonas aeruginosa*-derived volatiles when searching for oviposition sites (Urech et al. 2004). Similarly, calcium sulfide, calcium carbonate, and sodium sulfide all attract gravid *L. sericata* and *L. cuprina* and are associated with larval bacteria (Ashworth and Wall 1994).

We determined that fly physiology played an important role in response to bacteria exhibiting quorum-sensing-related behaviors (Tomberlin et al. 2012). A chemical analysis of quorum-sensing bacteria revealed differential production of several known fly attractants, indicating that interkingdom "communication," or "eavesdropping," occurs (Ma et al. 2012). Age and nutrition history influenced fly attraction and oviposition responses, with younger flies being more selective toward bacteria exhibiting swarming (Tomberlin et al. 2012). In a related field study, sex and ovarian status were found to influence adult blow fly behavior, with gravid females being more attracted to fresh carrion than male or non-gravid adults, indicating that volatile shifts, potentially explained by microbial succession, regulate these behaviors (Mohr and Tomberlin 2014). This pattern shifted with regard to gravid and non-gravid females as the carrion decomposed (Mohr and Tomberlin 2014, 2015). The level of fly attractancy to carrion resources has not been examined at finer scales with regard to the presence or absence of conspecific eggs of various ages.

We tested the hypothesis that *C. macellaria* and *Ch. rufifacies* respond to olfactory cues from conspecific and interspecific eggs, and that microbes associated with the eggs partially serve as a mechanism governing their attraction to a resource. We also began preliminary investigations of the bacterial succession associated with *C. macellaria* and *Ch. rufifacies* eggs. Because many representative bacteria associated with these flies are not available in GenBank, we used a phylogenetic approach to classify the bacteria that were not classified using Ribosomal Database Project's naïve Bayesian Classifier. Data generated from this research could lead to a greater explanation of the mechanisms governing succession patterns on decomposing ephemeral resources and the corresponding behaviors of the organisms, both invertebrate and possibly vertebrate (Jojola-Elverum et al. 2001), using ephemeral resources.

Materials and Methods

Y-tube Olfactometer Design

A dual-choice olfactometer adapted from Erler et al. (2006) was used to evaluate the choice of treatments by calliphorid adults. The olfactometer comprised three stacked 2.7-cm-thick sheets of solid Teflon, covered by a removable sheet of glass. The intake port on each arm measured 1.3 cm in diameter and was located at the terminal end. The exhaust port located at the terminal end of the stem measured 1.7 cm in diameter. A 1.6-cm access port for introducing the flies to the olfactometer was located at the base of the olfactometer ~2.5 cm from the terminal end. Air was pulled through the olfactometer with a 50-mm USB-powered computer cooling fan (5VDC Fan, Dc Fans, Thermal Management NMB Technologies Corporation, Chatsworth, CA) mounted to the external surface of the outflow port at the terminal end of the stem. Airflow through the olfactometer at the access port was measured using an anemometer (Testo 435-1, Testo, Inc., Sparta, NJ) to be 0.5 m/s (based on a 90-s average). Preliminary tests sans resource were conducted with colony flies to ensure adequate wind speed and that lighting, temperature, and ambient odors did not induce behavioral responses or bias toward one arm. These "olfactometer integrity checks" were replicated throughout the experiment.

Air flowed through long glass tubes (15 mm in diameter and 14.5 cm in length) containing activated charcoal (Aqua-Tech, Marineland Aquarium Products, Moorpark, CA) and were plugged with polyester filter floss (Aqua-Tech, Marineland Aquarium Products, Moorpark, CA). The columns were attached to plastic chambers (15 by 15 by 12 cm^3; S.C. Johnson & Son, Inc., Racine, WI), which were used to hold the treatments for all experiments. Chambers were attached to olfactometer arms with 7-cm Tygon tubing, and chambers were replaced after the completion of each experiment. Two fluorescent light tubes (60 W) served for overhead illumination. Temperature in the olfactometer room was maintained at 24.0 ± 2.0°C through all experiments.

General Behavioral Bioassay

For both species, ~7-d-old flies were collected from colonies and placed individually in clean glass vials (2-dram, 40-mm height × 17-mm diameter) for sexing before experiments. The olfactometer was cleaned with 80% ethanol and allowed to air dry for 2 min before the introduction of a specimen. Treatment location in relation to the olfactometer arms was rotated after each fly was tested in order to rule out any bias.

For the experiments examining the response of flies to conspecific and heterospecific non-sterile eggs, flies were individually observed for 5 min to determine response to heterospecific and conspecific eggs of various ages. For remaining experiments, the bioassay time was reduced to 2 min, as it was determined to be an appropriate amount of time to determine similar responses as those generated with the 5-min bioassays without statistical significance ($P < 0.05$). Total residence time in each arm was observed and analyzed using Odorifferous (Brundage Inc, Bryan, TX, https://github.com/brundage/odifferous). Residence time outside of either arm was considered a "non-choice" and was removed from the statistical analysis. Flies were removed and killed after each test. Females were dissected and ovarian status recorded following Avancini (1986). Females with fully developed ovarioles were considered "gravid,"

whereas those without ovarioles were considered "non-gravid." A total of 61 experiments, each containing a minimum of 25 individual flies, were conducted between 0800 and 1,800 h each day (Supp. Tables 1–6 [online only]).

Blow Fly Colonies

Laboratory colonies of *C. macellaria* and *Ch. rufifacies* were initiated from specimens collected in Brazos County, TX, during spring and summer of 2009 and 2010. Larvae were reared at a standard density of 100 larvae on 50 g of fresh bovine liver in 3-liter plastic containers. All flies were reared, separated by species, in Rheem Environmental walk-in growth chambers (Ashville, NC) at 27 ± 1°C, 60% RH, and a photoperiod of 12:12 (L:D) h. Dispersing third-instar larvae were transferred to 3-liter containers with autoclaved sand (Town & Country Landscape Supply Co., Chicago, IL) for pupation. Resulting adults were maintained in 30 by 30 by 30 cm^3 cages (Bioquip Products, Rancho Dominguez, CA) held in the Rheem Environmental chamber. Granulated sugar (Imperial Sugar Co., Sugar Land, TX), buttermilk powder (Saco foods Inc., Middleton, WI), and water were provided ad libitum, and 20 g of bovine liver was placed in the cage between 2 and 5 d after emergence for 8 h to induce ovarian development.

Egg Collection

Eggs were collected from colonies by presenting flies with fresh bovine liver for 1 h. Using fresh liver as an oviposition resource allowed for the rapid collection of similarly aged eggs. Once eggs were collected, they were aged in a Rheem Environmental walk-in growth chamber set at 27°C.

Adult Flies

Adult flies used in bioassays were collected from colonies separate from those used for egg collection. Colonies were reared in Rheem Environmental walk-in growth chambers at 27°C and separated by species.

Response to Eggs of Various Ages

Behavioral response of *C. macellaria* and *Ch. rufifacies* to conspecific and heterospecific eggs of different ages was examined. Adult male, non-gravid female, and gravid female flies previously described were examined for their response to eggs aged: 1) <3 h (H3); 2) 3 to <6 h (H6); and 3) 6–9 h (H9). The oldest time treatment was selected, as it was closest to egg hatch (Byrd and Butler 1996, 1997; Boatright and Tomberlin 2010; Flores et al. 2014). Because fresh beef liver was used to induce oviposition, 5,000 eggs (0.5 g) were placed on 1.0 g of fresh beef liver as the treatment, and 1.0 g of fresh beef liver was used as the control, as there was no way to completely remove the possibility of beef liver volatiles being associated with the eggs collected. Preliminary experiments confirmed 0.5 g of eggs induced a behavioral response by the blow fly adults.

Response to Non-Sterile Eggs

Because *C. macellaria* and *Ch. rufifacies* responses to conspecific or heterospecific eggs were not explained by egg age ($P > 0.05$) (Supp. Tables 1 and 2 [online only]), we focused our efforts in this experiment on the H3 and H6 eggs, as they are more biologically relevant with egg development. The H9 eggs were excluded, as they represent eggs preparing to hatch, which could be more indicative of larval development. The H3 and H6 age groups were used to determine if attraction was governed by egg respiration or associated microbes.

Response to Sterile Eggs

As with the previous experiment, we focused our efforts on the H3 and H6 egg classes. Furthermore, we focused on the responses of gravid and non-gravid adults, as their responses are more indicative of aggregation. This same design was used with all subsequent experiments. Eggs of appropriate ages were surface-sterilized (Brundage et al. 2016) and presented to individual gravid and non-gravid females in the olfactometer. Eggs were separated and placed on a sterile Millipore 20-µm membrane filter (Millipore, Billerica, MA), and placed in a 25-mm stainless steel Luer Lock filter holder (Millipore, Billerica, MA). The filter holder was attached to a sterile glass Luer Lock syringe (Thermo Fisher Scientific, Waltham, MA), loaded with 20-ml Professional Lysol antibacterial all-purpose cleaner concentrate (undiluted; Reckitt Benckiser. Parsippany, NJ). Further, 10 ml of Lysol was washed through the filter, thereby submerging the eggs in the disinfectant. Eggs were soaked for 10 min, and then rinsed in 20 ml of sterile insect saline (Pringle 1938) to remove the residual Lysol. Surface-sterilized eggs were transferred on filters to sterilized plastic chambers (15 by 15 by 12 cm^3; S.C. Johnson & Son, Inc.; Table 4.1). Sterile Millipore 20-µm membrane filters without eggs were subjected to the same sterilization procedure and used as controls for this experiment.

Response to Surface Microbes

We focused our efforts in this experiment on microbes associated with the H3 and H6 egg age classes as with the previous two experiments. In order to decouple fly response to microbes associated with the liver and those with the fly eggs, a sterile liver and agar mixture, adapted from Sherman and Tran (1995), was used to collect eggs from colonies. Further, ~20 g of fresh bovine liver was placed at 37°C in a Rheem Environmental Chamber for 24 h to induce putrefaction. A putrefied liver was pureed and mixed with 20 g of nutrient agar. The mixture was autoclaved, and 30-ml aliquots were partitioned into sterile plastic cups (Bio-Serv, Frenchtown, NJ). Oviposition "troughs" (5 mm in length, 2 mm in width, and 5 mm in depth) were cut into the solidified mixture to provide oviposition locations. Cups were presented to laboratory colonies for 1 h to collect the requisite 5,000 eggs.

To determine if microbes were responsible for the behavioral response of the flies, eggs from the described age categories were macerated using a tissue homogenizer. Eggs were aseptically removed and placed in sterile 25-ml centrifuge tubes (Thermo Fisher Scientific, Waltham, MA), mixed with 9-ml sterile phosphate-buffered saline, and homogenized using a PolyTron handheld tissue homogenizer (Kinematica AG, Nurnberg, Germany). The resulting liquid was filtered through a sterile, low-protein-binding Millipore 0.22-µm membrane filter (Millipore, Billerica, MA) to collect any microbes and other substances associated with the eggs. The filter with collected microbes was transferred to a sterile plastic chamber as described above and used for behavioral assays as previously described.

DNA Extraction and Preliminary 454 FLX Pyrosequencing

All extraction methods and bioinformatics analyses were adapted from (Zheng et al. 2013a) and are available in the supplemental section of this publication.

Statistical Analysis

Behavior data (residency time) for fly (gravid, non-gravid, and/or male) response to eggs (ages H3, H6, and/or H9) were analyzed

with PROC GLM (SAS 2011). A least significant difference test was used to test for significant differences between means ($P < 0.05$). *P* test (Martin 2002) as implemented in FastUniFrac (Hamady et al. 2009) was performed with 1,000 tree permutations to test significance of pairwise and overall FastUniFrac-based clustering of bacterial communities.

Results

Response to Non-Sterile Eggs

Cochliomyia macellaria responses to conspecific and heterospecific eggs of different ages are presented in Supp. Table 1 (online only). Species of egg was a significant predictor of fly attraction (df = 1, 1,010; $F = 6.87$; $P = 0.009$) and there was a significant interaction of egg age, fly sex, and physiological state on *C. macellaria* response to the presence or absence of conspecific eggs (df = 4, 1,010; $F = 2.48$; $P = 0.043$). Gravid *C. macellaria* adults were significantly more attracted to conspecific eggs over the liver control regardless of the egg age (df = 1, 1,010; $F = 6.87$; $P = 0.008$) (Fig. 1a). In contrast, the response of *C. macellaria* to heterospecific *Ch. rufifacies* eggs of different ages compared with the control was not significantly different ($P > 0.05$). *Chrysomya rufifacies* responses to conspecific and heterospecific eggs of different ages were not significant ($P > 0.05$) (Supp. Table 2 [online only]).

Response to Sterile Eggs

Cochliomyia macellaria responses to conspecific and heterospecific surface-sterilized eggs of different ages are presented in Supp. Table 3 (online only). There was a significant (df = 2, 306; $F = 5.84$; $P = 0.016$) interaction between ovarian status and species of egg. Non-gravid *C. macellaria* adults were significantly (df = 1, 306; $F = 2.75$; $P = 0.006$) more attracted to the liver control over conspecific eggs (Fig. 1b).

Chrysomya rufifacies responses to conspecific and heterospecific eggs that were surface-sterilized are presented in Supp. Table 4 (online only). The response of *Ch. rufifacies* to conspecific eggs was significant (df = 1, 306; $F = 16.27$; $P = 0.001$), with flies exhibiting more attraction toward the liver with eggs rather than the control (Fig. 1c). Age of the eggs and ovarian status of the fly were not significant ($P = 0.573$ and 0.532, respectively) predictors of fly response. In regard to *Ch. rufifacies* response to surface-sterilized *C. macellaria* eggs, there was a significant (df = 2, 290; $F = 6.23$; $P = 0.$

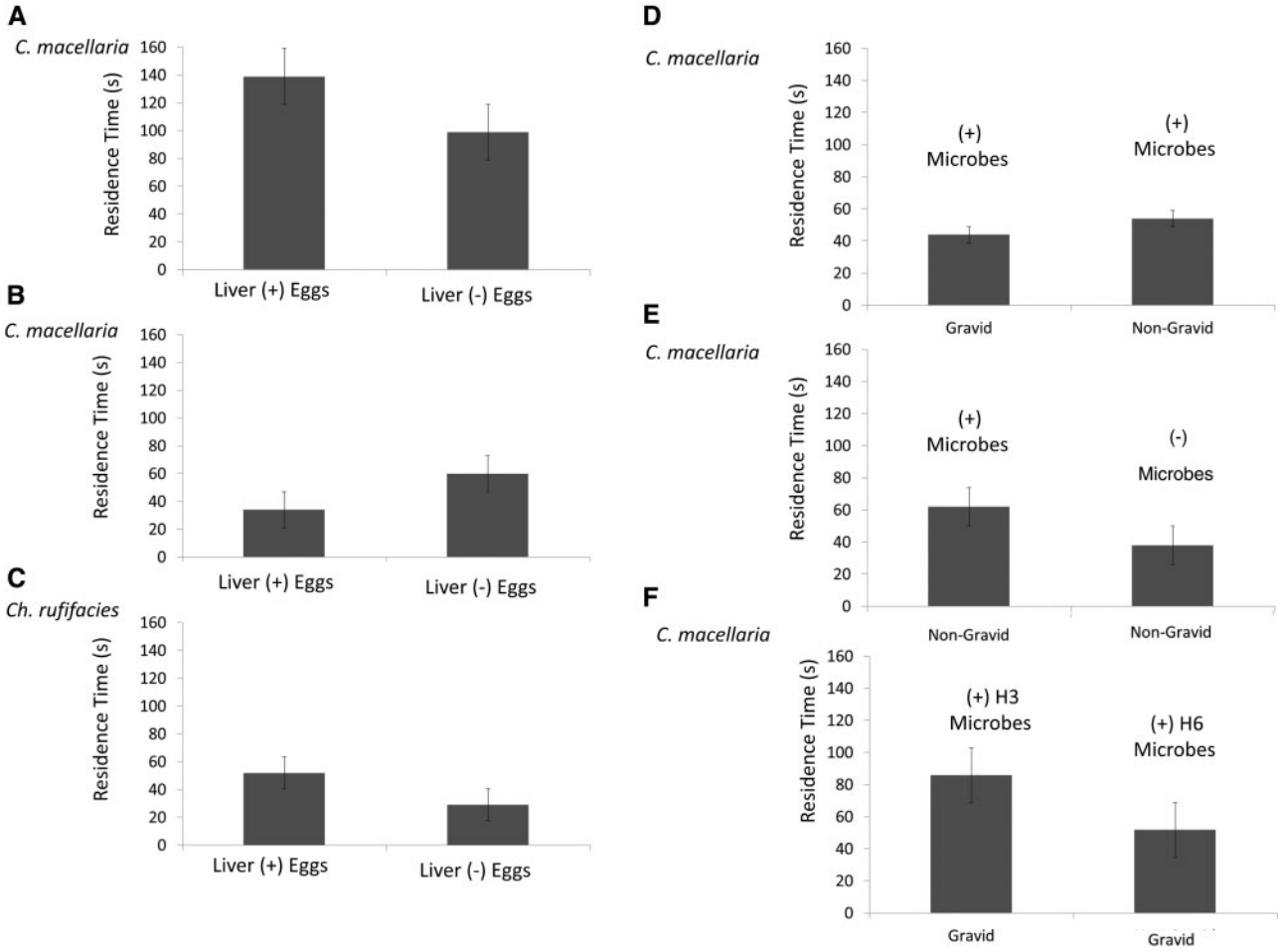

Fig. 1. Residence time (s) response ±SE in a y-tube olfactometer of (**A**) gravid adult *C. macellaria* ($n = 158$) to liver with and without 0.5-g non-sterile conspecific eggs (0- to 9-h-old); (**B**) non-gravid adult *C. macellaria* ($n = 68$) to liver control with and without 0.5-g sterile conspecific eggs (0- to 9-h-old); (**C**) gravid adult *Ch. rufifacies* ($n = 155$) to liver with and without 0.5-g sterile conspecific eggs (0- to 9-h-old); (**D**) gravid ($n = 135$) and non-gravid ($n = 108$) adult *C. macellaria* to microbes isolated from conspecific and heterospecific eggs (0- to 3- and 3- to 6-h-old) on liver; (**E**) non-gravid adult *C. macellaria* ($n = 35$) to liver with and without microbes isolated from conspecific eggs (0- to 3- and 3- to 6-h-old); (**F**) gravid *C. macellaria* ($n = 84$) to liver with microbes isolated from heterospecific eggs 0- to 3-h-old (H3) and 3- to 6-h-old (H6).

013) three-way interaction (sex, species, and egg age), with fly response being greater (~60.5%) toward liver with *C. macellaria* eggs.

Response to Microbes Isolated From Eggs

Cochliomyia macellaria responses to microbes and associated components on filters of conspecific and heterospecific microbes from eggs of different ages are presented in Supp. Table 5 (online only). There was a significant (df = 1, 214; $F = 9.75$; $P = 0.002$) interaction of fly sex and treatment (fly species from which microbes originated). Gravid flies responded significantly (df = 1, 214; $F = 8.01$; $P = 0.007$) less to the treatment than non-gravid flies (Fig. 1d). More specifically, non-gravid flies also responded significantly (df = 1, 214; $F = 8.63$; $P = 0.002$) more to the filter with the conspecific microbes rather than the filter without conspecific microbes (Fig. 1e).

There was also a significant (df = 1, 270; $F = 3.87$; $P = 0.050$) interaction of egg age, sex of the individual responding, and the treatment (age of the *Ch. rufifacies* eggs) in the *C. macellaria* response to microbes from *Ch. rufifacies* eggs. In particular, gravid female response to microbes from H3 versus H6 *Ch. rufifacies* eggs was significantly (df = 2, 270; $F = 2.99$; $P = 0.003$) greater (Fig. 1f).

Chrysomya rufifacies responses to microbes isolated from conspecific and heterospecific eggs of different ages are presented in Supp. Table 6 (online only). A significant (df = 1, 274; $F = 3.93$; $P = 0.047$) three-way interaction was identified, suggesting the age of eggs from which the microbes were isolated, sex of the fly examined, and treatment (microbes versus control) impacted their attraction to microbial odors. In addition, there was a significant (df = 1, 284; $F = 18.25$; $P < 0.001$) interaction of microbe age (corresponds to egg age), sex of the fly examined, and treatment (microbes versus control) in *Ch. rufifacies* responses to microbes from *C. macellaria* eggs.

Taxonomic Distribution of 454-Sequences

This study used 454-sequence of 16S rRNA gene to perform a preliminary survey of the bacterial community associated with different age group eggs of *Ch. rufifacies* and *C. macellaria*. In this study, we obtained 6,602 (average length = 293 base pair [bp]) and 7,620 (average length = 309 bp) sequences from *Ch. rufifacies* and *C. macellaria*, respectively (see Supp. File 1 [online only] for more information on taxonomic distribution). Overall, Firmicutes and Proteobacteria constituted more than 93% of the bacterial phyla identified on *Ch. rufifacies* and *C. macellaria* eggs (Fig. 2 and 3, Supp. Table 7a and b [online only]). In *Ch. rufifacies*, Proteobacteria gradually increased over time, whereas this was not true in case of *C. macellaria* eggs (Fig. 2 and 3, Supp. Table 7 and b [online only]).

The heat map of the bacterial genera associated with different age groups of eggs is clustered based on an unrooted NJ tree of genera and groups (see Supp. Methods [online only]). FastUniFrac-based clustering of different age group eggs shows that bacterial genera associated with different egg age groups are significantly clustered on the tree (*P*-test $P \leq 0.001$) both in *Ch. rufifacies* and *C. macellaria*. Although H6 and H9 eggs in *Ch. rufifacies* and H3 and H9 eggs in *C. macellaria* shared the most number of bacteria genera, the bacterial genera associated with each egg age group in both fly species were significant different (*P*-test $P \leq 0.001$).

A Venn diagram (Fig. 4) shows major sharing (30) of bacterial genera between *Ch. rufifacies* and *C. macellaria* eggs. In general, *C. macellaria* eggs have more unique bacteria than *Ch. rufifacies* eggs. Except for a few, the majority of unique genera associated with eggs of these fly species are in very low percent-relative sequence abundance (<0.5%). Overall, *Lactobacillus* and *Vagococcus* constituted almost half of all bacterial genera associated with eggs of both *Ch. rufifacies* and *C. macellaria*. Eggs of both fly species shared *Lactococcus* and *Myroides*; however, their percent-relative sequence abundance was much higher in *Ch. rufifacies* eggs than in *C. macellaria* eggs (Fig. 4; Supp. Table 8a and b [online only]). Eggs of both fly species shared *Carnobacterium* and *Morganella*, but the percent-relative sequence abundances were much higher in *C. macellaria* eggs than in *Ch. rufifacies* eggs (Supp. Table 11a and b [online only]). *Escherichia/Shigella* was the most dominant genus of H6 eggs of *C. macellaria*, although it was not detected in either H3 or H9 eggs (Fig. 4; Supp. Table 11b [online only]). However, these data are only a glimpse into the potential bacterial community associated with *C. macellaria* and *Ch. rufifacies* eggs. Additional replicates are needed to determine if the data generated are truly informative, and thus these data presented should be applied with caution.

Discussion

Response to Egg Age

The age of both *C. macellaria* and *Ch. rufifacies* eggs influenced adult attraction, which was compounded by the physiological state and the sex of the adult. *Chrysomya rufifacies* tended to be attracted to both conspecfic and heterospecific eggs, whereas *C. macellaria* tended to be attracted to only conspecific eggs. *Chrysomya rufifacies* were more attracted to heterospecific, rather than conspecfic, eggs as they aged. Although group larval feeding may impart some benefit upon members of the larval mass in the form of increased exodigestion efficacy (Fenton 1999, Rivers et al. 2011), the threat of cannibalism may select for larvae of similar ages to mass, rather than masses formed of larvae of varying ages. This pattern of adult regulation of cannibalistic larvae was observed in the house fly by Lam et al. (2007). Adults were found to respond to conspecific, egg-associated cues that initially induced conspecific oviposition, and then inhibited oviposition after 24 h. This selection for induction/inhibition results in larval masses of similar ages, and reduces the likelihood of younger larvae being cannibalized by older larvae. The origin of the cues in the house fly system is bacteria found on the chorion of conspecific eggs (Lam et al. 2007). *Cochliomyia macellaria* adults were attracted to conspecific eggs, but mostly H3 and H6 eggs. This trend appeared to continue for H9 eggs, and although significance was not technically reached ($P = 0.051$), the response approached significance and may simply have failed because of insufficient statistical power.

Olfactory responses to semiochemicals are an important part of the suite of behaviors (i.e., tactile, visual, olfactory, and thermal), resulting in blow flies exploiting resources (Hall 1995, Chapman 2003). However, olfaction may simply result in the activation of searching or oviposition behavior (Chapman 2003), and further stimuli are required to continue through with actual oviposition. *Cochliomyia macellaria* possibly require additional sensory input before being engaged to seek out oviposition sites (Dunn 1918; Baldridge et al. 2006) and ovarian status of the females significantly affects olfactory response (Chaudhury et al. 2014).

Response to Surface-Sterilized Eggs

Gravid female response was reduced when the eggs were sterilized. Egg-associated microbes are known attractants in some fly species. Lam et al. (2007) found that gravid house flies were positively attracted to microbes cultured from the surface of conspecific eggs, although he did not identify which microbes were responsible for this

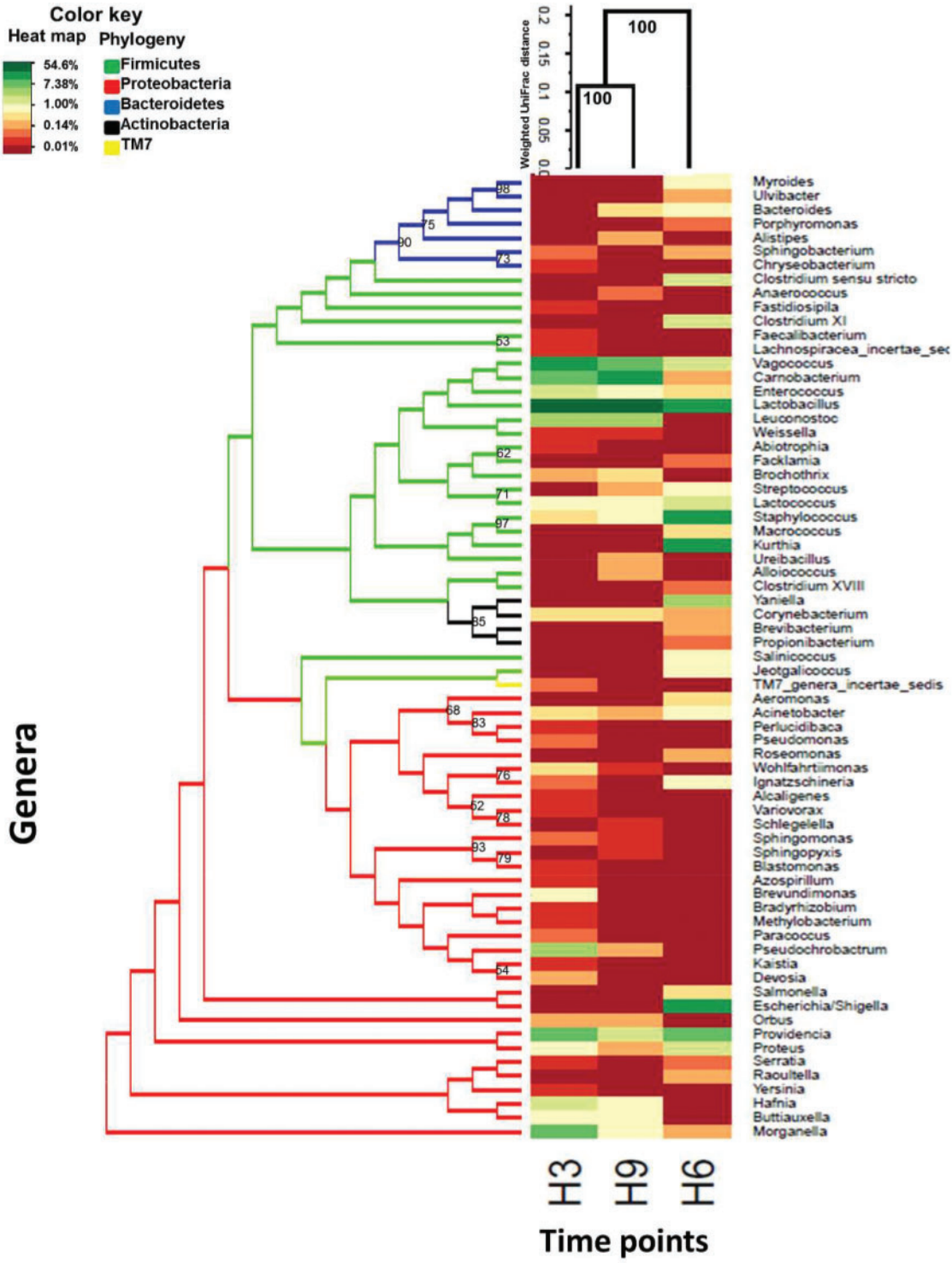

Fig. 2. Heat map of percent-relative sequence abundances of bacterial genera associated with egg stage of Ch. rufifacies. Heat maps displayed in natural log-transformed abundance profiles where the 0% values were converted into 0.01% for log transformation. Y-axis cluster is based on unrooted neighbor-joining tree of the classified genera and X-axis cluster is based on the FastUniFrac-based clustering of different age group eggs. Only nodes with bootstrap support ≥50% are shown on the NJ tree. Numbers on the FastUniFrac tree are jackknife support for each clade.

attraction. Lam et al. (2007) determined that the house fly laid 75% more eggs on oviposition sites dosed with egg-associated microbes compared with those without egg-associated microbes (Lam et al. 2007). This response in another higher fly with similar biology to Calliphoridae, along with the reduced attraction found in this experiment suggests that egg-associated microbes or their semiochemicals (message-bearing chemicals) associated with larval or adult activity are used as an oviposition cue. Similar results have been determined for adult attraction to resources with larval (Yang and Shiao 2012) or adult (Brodie et al. 2015a) blow fly feeding.

The change in attraction and repellency of both fresh and aged eggs to gravid adults owing to surface-sterilization implies that

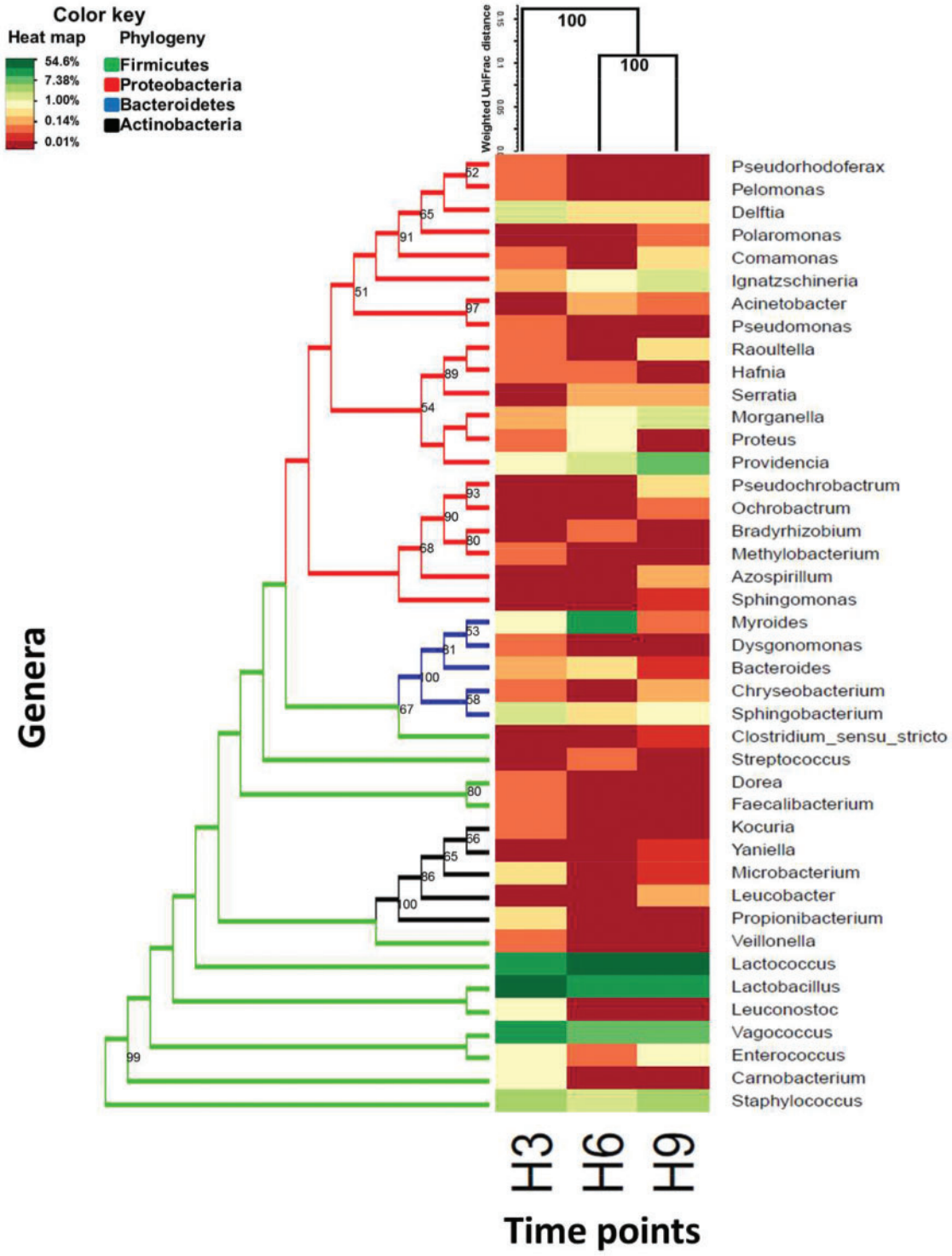

Fig. 3. Heat map of percent-relative sequence abundances of bacterial genera associated with egg stage of *C. macellaria*. Heat maps displayed in natural log-transformed abundance profiles where the 0% values were converted into 0.01% for log transformation. Y-axis cluster is based on unrooted neighbor-joining tree of the classified genera and X-axis cluster is based on the FastUniFrac-based clustering of different age group eggs. Only nodes with bootstrap support ≥50% are shown on the NJ tree. Numbers on the FastUniFrac tree are jackknife support for each clade.

surface-associated substances have an effect on adult behavior. During oviposition, eggs are laid in groups or clutches on the oviposition medium. The clutches are covered with a layer of mucoprotein that may prevent dehydration and adheres the egg clutch to the substratum (Peterson 1991). The external surface of fly eggs are contaminated with bacteria (Mohd Masari et al. 2005), and the mucoprotein layer may be responsible for the adhesion of bacteria to the egg surface. Although the mucoprotein layer itself or other unknown substances deposited during oviposition may be responsible for the observed patterns of adult attraction, the known attraction of microbial volatiles to adult Calliphoridae is a more likely explanation (Yang and Shiao 2012).

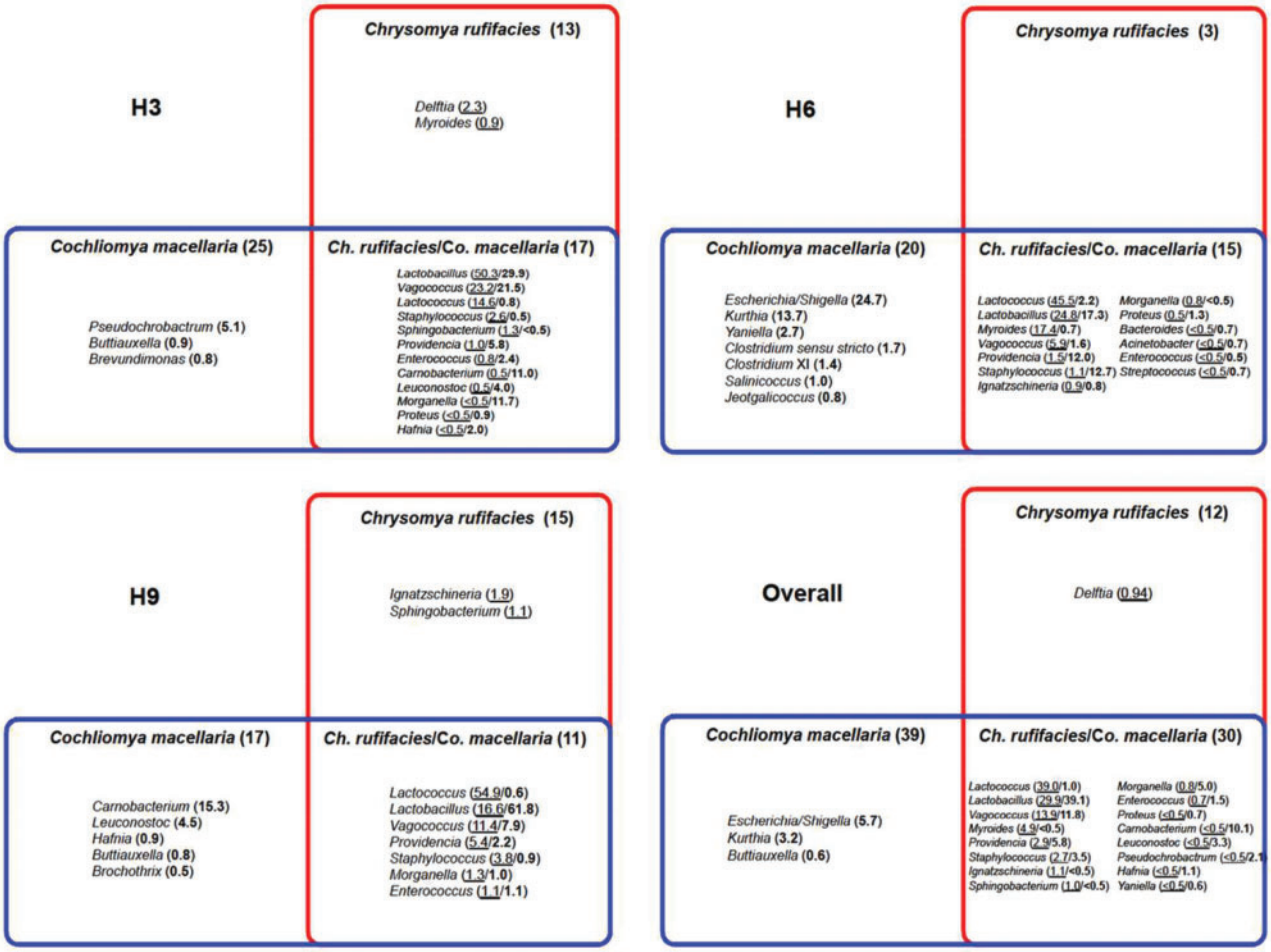

Fig. 4. Venn diagram showing overlap of bacterial genera associated with *Ch. rufifacies* and *C. macellaria* eggs. Values in parentheses of the fly name indicate total unique/shared bacterial genera in two fly species. Number in underline indicates percent-relative sequence abundance of *Ch. rufifacies* eggs, whereas the number in bold indicates percent-relative sequence abundance of *C. macellaria* eggs. Only those genera that were present at ≥0.5% relative sequence abundance are shown in the Venn diagram.

Microbes Associated With Eggs

Much more work has been done regarding bacteria serving as a mechanism governing attraction of blow flies to hosts (Browne 1960, Eddy 1975, Emmens 1982). Emmens (1982) found *Pseudomonas* sp. degrades wool and produces sulphurous compounds, which attract female *L. cuprina*. Browne (1958) noted *L. cuprina* increased oviposition in response to indole and ammonium carbonate, which are products of bacterial metabolism. Bovine blood inoculated with bacteria is significantly more attractive to the primary screwworm *Cochliomyia hominivorax* (Coquerel) (Diptera: Calliphoridae) than uninoculated blood (Eddy 1975, Chaudhury et al. 2010). More recent research has determined that swarming (quorum sensing response) by *Proteus mirabilis*, a bacteria species commonly associated with decomposing vertebrate remains (Barnes et al. 2010), elicits attraction and oviposition by *L. sericata* (Ma et al. 2012). This response indicates ecological eavesdropping, as it appears that blow fly responses are exhibited in conjunction with bacterial responses to resource quality. Recent work indicates that bacterial volatiles indicate the presence of essential amino acids in resources, and such volatiles may be used by dipteran colonizers as an indicator of resource quality. (Liu et al. 2016).

Changes in microbial communities may explain changes in attraction behavior as eggs age. Our analysis of bacterial community structure was a preliminary survey to determine bacterial taxa present and the authors understand that it must be replicated to confirm any similarities and differences in bacterial associates. Although the discussion will focus on dominant bacteria, understand that bacteria present in minor proportions may strongly produce semiochemicals that could influence insect behavior and the dominant species may not. Further in-depth analyses with appropriate replication would be necessary for conclusive evidence of semiochemical production and influences.

With these limitations in mind, our survey showed that the bacterial community changed significantly as the eggs of each species aged. This change in bacterial community structure may affect the attraction of *Ch. rufifacies* adults to eggs. *Chrysomya rufifacies* were attracted to both conspecific and heterospecific fresh eggs, and *Lactobacillus* was the dominant species on those eggs, indicating that *Lactobacillus* may act as an attractant for *Ch. rufifacies*. As conspecific eggs became dominated by *Lactococcus sp*., it may be that *Lactococcus* is a repellant for *Ch. rufifacies*.

Cochliomyia macellaria showed no obvious patterns in their attraction when compared with microbial change during the time course chosen for analysis in this study. Other time points need to be explored. Attraction to eggs by *C. macellaria* may be governed by a complex system of volatiles emanating from the egg itself or from

a combination of the bacterial community and the developing embryo. Investigation into the volatile production of both *C. macellaria* and *Ch. rufifacies* eggs and associated microbes is necessary to determine the extent to which the volatiles may govern attraction and repellency.

In conclusion, consepecific and heterospecific eggs appear to be major mediating factors in the oviposition timing of both *Ch. rufifacies* and *C. macellaria*. The attraction changes over time, implying the ability of ovipositing adults to assess egg age and alter behavior in response to these cues. In addition, the major sources of attraction cues appear to be derived from egg-associated bacteria, which undergo substantial community change as the eggs age. Further, each species may be using these cues to differentiate between conspecific and heterospecific eggs, thereby directly affecting offspring fitness. Future work should attempt to identify the specific attractant responsible for both conspecific and heterospecific attraction, and investigate if these data are observed in the field and the laboratory.

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Supplementary Data

Supplementary data are available at *Annals of the Entomological Society of America* online.

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