A NOVEL TECHNIQUE FOR FEEDING AND CONFIRMING UPTAKE OF BACTERIA IN LARVAE OF THE SOUTHERN HOUSE MOSQUITO, *CULEX QUINQUEFASCIATUS* (DIPTERA: CULICIDAE)

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ABSTRACT

Aquatic macroinvertebrates play important roles in freshwater ecosystems. The larvae of the vector mosquito *Culex quinquefasciatus* Say (Diptera: Culicidae) primarily reside in standing water rich in organic matter; these conditions are also suited for growth of microbial biofilms. As microbes serve as a food source for larvae, the study of interactions between microbial biofilms and *C. quinquefasciatus* can aid in understanding the routes by which aquatic larvae can acquire pathogens, and the role such larvae may play in environmental persistence of microorganisms. Studies in butterflies, nematodes, and ticks have described interactions with bacteria acquired by the oral route, including arthropods that play a role in dissemination of bacteria on beet plants. In pursuit of these goals, this manuscript describes a new protocol for investigating larval mosquito feeding on microbial biofilms. Fluorescent microscopy and GFP expressing bacterial strains were used to show larval acquisition and midgut localization of bacteria from both planktonic and biofilm sources. PCR testing for the GFP plasmid confirmed presence of the test strain in fed larvae. The results of microscopy and PCR assays demonstrate that *Culex quinquefasciatus* larvae will feed on microbial biofilms in a laboratory environment. The efficiency of studying microbial fate through common microscopic and molecular techniques, in combination with an easily maintained vector insect colony, means this approach can be used to investigate a multitude of research questions relating to microbial effects on larval physiology, fitness, and conditioning.

Key Words: *Pseudomonas*, *Culex,* mosquito, biofilms, GFP

RESUMEN

Los macro-invertebrados acuáticos desempeñan un papel importante en los ecosistemas de agua dulce. La larva del mosquito vector, *Culex quinquifasciatus* (Diptera: Culicidae), reside principalmente en aguas estancadas, ricas en materia orgánica, condiciones que apoyan el crecimiento de películas biológicas microbianas. Debido a que los microbios proporcionan una de las fuentes de consumo de las larvas, el estudio de las interacciones entre películas biológicas microbianas y el *C. quinquefasciatus* puede ayudar a entender las rutas por las cuales las larvas acuáticas adquieren los patógenos y el papel que desempeñan las larvas en la persistencia de microorganismos en el medioambiente. Investigaciones de mariposas, nematodos, y garrapatas han descrito interacciones con bacteria adquerida por la ruta oral, incluyendo artropodos que desempeñan un papel en la diseminación de bacteria en plantas betabeles. Para lograr estos objetivos, este manuscrito describe un nuevo protocolo para investigar la alimentación de las larvas del mosquito con películas biológicas microbianas. La microscopía fluorescente y cepas bacterianas que expresan proteína verde fluorescente (GFP) fueron utilizadas para mostrar la adquisición de larvas y la localización de bacterias, de fuentes planctónicas y de películas biológicas, en el intestino medio. Además, la Reacción Polímera Estandar en cadena (PCR) fue utilizada para analizar los plásmidos GFP y se confirmó la presencia de la variedad examinada en larvas alimenticias. Los resultados descritos en estos experimentos apoyan que el *C. quinquefasciatus* se alimentará de películas biológicas microbianas en un ambiente de laboratorio. La eficiencia de estudiar el destino microbiano a través de técnicas microscópicas y moleculares comunes, en combinación con una colonia vector de insectos, fácilmente mantenida, significa que este método puede ser utilizado para investigar una multitud de preguntas de investigación asociadas con los efectos microbianos en la fisiología de la larva, su formación y su condicionamiento.

Translation provided by the authors.

Microbial life forms the base of the food chain in many environments. In turbulent or nutrient scarce environments, microbes typically enter a biofilm state. A biofilm forms when planktonic (suspended or free-living) microorganisms bind to a substrate. After a period of time this attachment becomes irreversible and the organisms grow and replicate while excreting a thick, protective extrapolymeric matrix. As the biofilm matures other microbial species (both eukaryotic and prokaryotic) begin to join and the biofilm gains diversity, becoming a community of organisms (Rickard et al. 2003). When the biofilm reaches the terminal point in its growth, cells are released in a dispersal event and may eventually form new biofilms (Hall-Stoodley et al. 2004). The biofilm state allows microorganisms to resist disruption by turbulent conditions, and to concentrate nutrients in microenvironments (Freeman et al. 1995). Microorganisms such as those found in biofilms play a role in breaking down plant detritus and animal wastes in freshwater environments, converting the carbon within these sources to a form more readily available to aquatic invertebrates. They also are a primary food source for many aquatic insect larvae (Merritt et al. 1992). Much of the information on microbial roles in aquatic invertebrate ecology has been gained through studies on planktonic phase bacteria-larval interaction. The interaction between microbial biofilms and aquatic larvae has to date not yet been well studied. However, many environmental pathogens, such as *Legionella* species, exhibit specific virulence factor expression based on phase (planktonic vs. biofilm, stationary vs. exponential) that affect interaction with a host (Lüneberg et al. 1998). Therefore, bacteria presented in a biofilm to a grazing host may have different effects and fates than those presented as planktonic phase.

Culex quinquefasciatus Say (Diptera: Culicidae) larvae inhabit freshwater environments in a cosmopolitan distribution ranging from the southeast United States to the southern tip of Africa. Its close relative, *C. pipiens*, ranges as far north as Norway and Sweden (White 1989). Extensive work has been done regarding the feeding habits of larval mosquitoes, revealing a diet largely composed of microorganisms (Merritt et al. 1992). Although these habits are known, the effects of larval feeding on microbial communities is not well understood, nor is much known regarding the potential for the acquisition of pathogens from such sources. There may be significant variability between species and individuals in clearance of larval midgut bacteria during metamorphosis (Moll et al. 2001), although it is unclear as to whether this rule holds for comparisons of different species of *Culex*, or different species, strains or phases of bacteria. Given the vector potential of *Culex* spp., it would be advantageous to model how larval mosquito–microorganism interactions shape both freshwater bacterial communities, as well as the mosquito life cycle.

Laboratory studies of bacteria-insect interactions have often used intrahemocoelic injection of microorganisms to determine the virulence effects on host physiology and immune response (Vonkavaara et al. 2008; Aperis et al. 2007; Hillyer et al. 2004). While these studies have been informative regarding insect antimicrobial immunity and identification of bacterial virulence factors, the most likely route of entry for many pathogenic microorganisms to invade larval mosquitoes is by ingestion (Rodcharoen & Mulla 1995). The literature suggests that bacterial acquisition by the oral route in particular uniquely impacts both parties. For example, oral uptake of *Yersinia pestis*, the causative agent of bubonic plague, results in biofilms formed in the flea foregut (allowing for transmission to a mammalian host). This biofilm is instrumental in the regurgitation action which leads to pathogen transfer to new hosts. The same genes governing flea biofilm formation are also used as an environmental biofilm strategy to avoid predation by nematodes (Darby et al. 2002; Hinnebusch & Erickson 2008). A 1997 study suggests that caterpillars play a role in dissemination of *Pseudomonas fluorescens* populations on sugar beet plants through oral acquisition and fecal deposition (Lilley et al. 1997). Thus, a method that could reliably present mosquito larvae with various microorganisms for ingestion would be highly advantageous for more specific and efficient studies on the natural interactions between larvae and environmental microbes. Such a method would bypass the disadvantages related to intrahemocoelic injection of microorganisms into arthropods and more closely mimic a natural route of microbial uptake in the environment in a controlled laboratory setting. The most successful protocols would be easily and rapidly evaluated for microbial uptake and fate, and transferable to other systems, thereby facilitating studies of insect-microbial interactions. Here, we describe such a system in which larval mosquito ingestion of plasmid-bearing bacteria is scored and validated by use of fluorescent microscopy and PCR. The method is intended to be widely applicable to several species of larval mosquito and a variety of microbial species.

Materials and Methods

Composition and Preparation of Moderately Hard Water (MHW)

MHW was used as the liquid medium for all insect rearing and biofilm environments unless otherwise noted. MHW is a mixture of ultrapure water with the following concentrations of 4 salts:

4.0 mg per liter KCl, 60 mg per liter each of $MgSO_4$ and $CaSO_4$, and 96 mg per liter $NaHCO_3$. Salts were dissolved over a 48-h period. Water was autoclaved and filter sterilized before use in laboratory procedures.

Culex quinquefasciatus Colony Rearing Techniques and Characterization

A laboratory colony of *C. quinquefasciatus* was established from 1,000 larvae obtained from Benzon Research (Carlisle, PA), and supplemented with additional larvae after several generations. Larvae were reared in white enamel pans in aerated water (approximately 500 per pan) at 27-29°C, 60-80% relative humidity, and a 14:10 (L:D) photoperiod. Pupae were transferred to rearing cages, and cages observed for adult emergence. Adults were immediately provided pads soaked in 10% honey solution. Blood meals were provided to 8-12 d old adults. Blood was presented in 50-mL glass beakers with tops covered in Parafilm that had been rubbed on human skin. Beakers were placed upside down on the wire-mesh top of the cage. Two rice filled socks heated in a microwave for 10 min were placed in contact with the beakers to heat the blood and induce females to feed. Heparin treated cow's blood was obtained from the Clemson University Godley-Snell animal research center. Oviposition containers were provided the next day and egg rafts were collected and transferred to enamel pans. To determine the relative efficacy of this approach, several metrics were observed after establishment of the assay. One hundred adult females and 100 adult males between 8 and 12 d old were collected from the lab colony and placed in an empty rearing cage supplied with honey pads. After a 24-h acclimation period, a blood meal was presented as described previously. Twelve hours post-blood meal 2 cups filled with MHW were placed in the cage as oviposition substrate, and 48-h post blood meal egg rafts were collected, counted, and placed in enamel rearing pans with MHW. The number of rafts laid per 100 females was recorded for 2 replicates and mean rafts per 100 females were calculated from the data. Emerged larvae were fed with 1 mL of UV sterilized dog biscuit slurry consisting of 1.5 uncolored dog biscuits blended with 50 mL of MHW every other day. Larvae were counted once they had reached the 2nd instar. Pupae were counted, placed in cups with MHW and placed in rearing cages daily. Pupation rate (total pupae / total larvae at 2nd instar) was calculated for each of 2 replicates. Eclosion rate (total adults/ total pupae) and sex ratio were calculated from pooled adults from both replicates. This process was repeated on an additional 100 males and 100 females. All rearing took place on a photoperiod of 14:10 (L:D) at 27-29°C and 60-80% relative humidity.

Pseudomonas aeruginosa Culture Conditions

Pseudomonas aeruginosa PAO1 containing a green fluorescent protein (GFP) expressing plasmid (obtained from Dr. Matt Parsek, University of Washington) was grown in LB Broth (Fisher Scientific) or on LB agar plates (Difco). Media were supplemented with 300 µg/mL carbenecillin to retain the GFP plasmid. Bacterial incubations were performed at 37°C with 5% CO_2. Bacteria were centrifuged and resuspended in sterile MHW before presentation to mosquitoes.

Growth of *Pseudomonas aeruginosa* Biofilms

Pseudomonas aeruginosa PAO1 expressing GFP was used in all biofilm feeding experiments. Bacteria were inoculated in 10 mL of medium to give an OD_{600} of 0.3 and added to a plastic container containing 3 sterile glass slides and 40 mL medium supplemented with 300 µg/mL carbenecillin. Containers were incubated for 18 h at 37°C to allow for biofilm establishment and maturation. The supernatant was removed and slides rinsed twice with 10 mL of MHW to remove any unattached (non-biofilm) bacterial cells. Fifty milliliters of fresh MHW was added to the chamber, and the chamber equilibrated at 27°C for 24 h before use in biofilm feeding experiments.

Larval Feeding on *Pseudomonas aeruginosa* Biofilms and Planktonic Cultures

Third instars were collected from the lab colony, starved in sterile MHW at 27°C for 24 h, and separated into 3 treatment groups. Control larvae were placed in a beaker containing 50 mL of sterile MHW with 100 µL of sterile dog biscuit slurry. Larvae exposed to planktonic bacteria were placed in a beaker containing 50 ml MHW consisting of 47 mL sterile MHW and 3 mL of *P. aeruginosa* GFP preparation (OD 0.5 in MHW). Larvae exposed to biofilms were presented with biofilms in containers established as described above. All larvae were allowed to feed for 24 h at 27°C, then examined as below.

Larval Imaging after Bacterial Feeding

Larvae from each experimental group were placed in separate wells of a 24-well plastic tissue culture plate and immobilized in a solution of 50% glycerol in water with 0.5% agarose. The solution was allowed to sit for approximately 15 min prior to imaging. Images were captured under bright field and FITC epifluorescence modes on a Nikon AZ100 epifluorescence microscope with a Nikon Ri-1 color camera. Images were subsequently examined in Nikon Elements Software (Basic Research vers. 3). The bright field images were converted to grayscale and the green chan-

nel from FITC images was overlaid on the gray-scale images to form a composite image of GFP localization in sampled larvae. All images were acquired at the Jordan Hall Imaging Facility at Clemson University.

PCR Verification of Bacteria in Larval Homogenates

Three larvae from each group were pooled and examined by PCR for validation of microscopy. Larvae were washed in sterile ultrapure water and homogenized via motorized pestle in sterile 1.5-mL microcentrifuge tubes. The homogenates were transferred to 25 mL of medium with 300 µg/mL carbenecillin to allow for enrichment of the target bacteria. The homogenate broth was incubated at 37°C with shaking at 100 rpm overnight. Plasmid DNA was isolated from broth cultures with the Zyppy™ Plasmid Miniprep Kit (ZYMO RESEARCH) and used for PCR analysis. Primers were designed in Lasergene (DNAStar) to amplify a 155 bp region of the GFP gene in the pMRP9-1 plasmid carried by *P. aeruginosa*. Primer sequences were 5'-GACCATGTGGTCTCTCTTTTCGTTGT and 5'-AGACACAACATTGAAGATGGAAGCGT.

Results

Egg Raft and Larval Pupation and Eclosion Data from Membrane Feeding Assay

The need for live blood feeding to maintain mosquito colonies inhibits many researchers from establishing their own colony. To circumvent this difficulty, we developed and verified the use of a membrane feeding assay to maintain *C. quinquefasciatus*. In Table 1, we show the outcomes of our laboratory membrane feeding and colony maintenance protocols. The membrane feeding protocol presented here yielded an average of 60 egg rafts per 100 females. In our colony, pupation rate of the larvae hatched from collected rafts was approximately 19.0% and eclosion rate for the pupae was 71.5%. The blood feeding technique and protocols as described in materials and methods are easy to perform and have allowed for continuous rearing of *Culex quinquefasciatus* for 10 generations.

Epifluorescence Microscopy Demonstrates Oral Acquisition of GFP Expressing *P. aeruginosa* by *C. quinquefasciatus* Larvae

Molecular techniques that fluorescently label bacteria facilitated quick and precise identification of infected mosquito larvae. This identification assists in eliminating variation in protocols due to the contamination of samples by larvae that have not ingested the microorganism being tested. Micrographs of control larvae (Figs. 1A-C) demonstrate only slight auto fluorescence in larvae fed solely on sterile biscuit slurry. However, larvae fed for 24 h on biofilm (Figs. 1D-F) or planktonic (Figs. 1G-I) *P. aeruginosa* GFP demonstrate significant fluorescence within the gut tract, verifying uptake of the bacteria. A distinct localization of fluorescence can be observed in the crop and gut of the larvae from the biofilm and planktonic groups, while it is absent in control larvae.

PCR Confirms Target Bacteria Presence in Larval Homogenates

We have observed auto- and background fluorescence in some invertebrates (Turnbull et al., personal observations). A corroborative approach to validate microscopy can be helpful. Here, we used a simple PCR to ensure that fluorescence observed in a group of larvae is due to the presence of a particular bacterial strain, rather than false positive. Fig. 2 demonstrates representative results from PCR with primers specific to a 155-bp region in the GFP expression gene of the plasmid in *P. aeruginosa* and bacterial enrichments from larval homogenates. Minipreps from bacterial enrichments of both biofilm- and planktonic-fed larvae yielded 155-bp amplimers, suggesting that the specific strain of GFP expressing *Pseudomonas* was present in the larvae. Bacterial enrichment from control larvae, fed solely on sterile slurry, did not yield a band, confirming the absence of the GFP plasmid and therefore the orally acquired *Pseudomonas* strain. These molecular data thus confirm the epifluorescence signal as true positive.

TABLE 1. A RECORD OF DEVELOPMENT, SURVIVORSHIP AND FECUNDITY IN AN ARTIFICIAL MEMBRANE FED LABORATORY COLONY OF *CULEX QUINQUEFASCIATUS*. EGG RAFTS PER 100 FEMALES AND PUPATION RATE REFLECT RESULTS FROM 2 REPLICATES WHILE ECLOSION RATE AND SEX RATIO WERE CALCULATED FROM POOLED DATA FROM 2 REPLICATES.

Replicate	Egg Rafts per 100 Females	Pupation Rate (%)	Eclosion Rate (%)	Sex Ratio (M:F)
1	75	21.5	—	—
2	45	7.4	—	—
Pooled	—	—	71.5	113:144

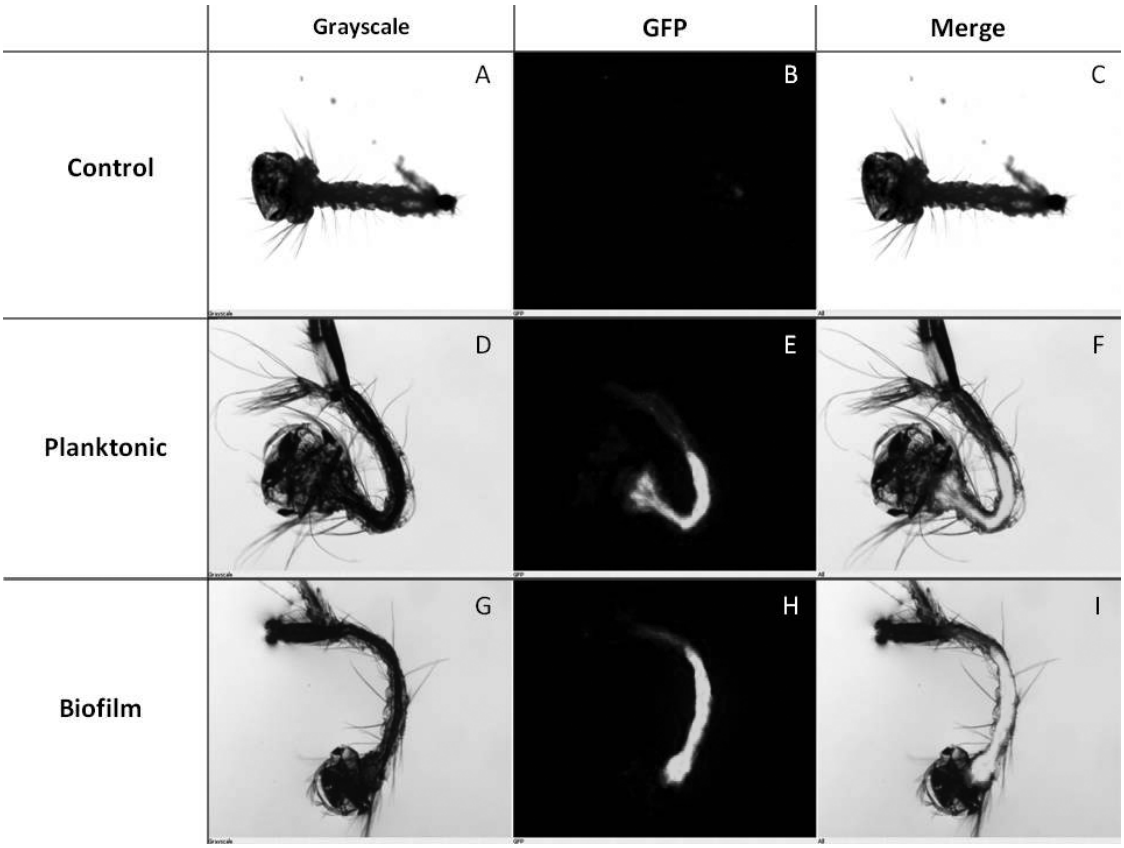

Fig. 1. Microscopy of larvae fed for 24 h on a non-bacterial biscuit slurry control diet (A-C), or GFP-expressing *Pseudomonas aeruginosa* planktonic (D-F), or biofilm (G-I) culture.

Discussion

Epifluorescent imaging and PCR are practical and common molecular techniques that can be used to confirm successful uptake of bacteria by larvae. The use of such protocols allows both the rough locality and simple presence of bacteria ingested to be determined. It is essential for follow on experiments, such as real-time PCR analysis, Western blotting or two-dimensional gel electrophoresis, to know that all larvae processed contained the bacteria of interest. The ability to verify this simply eliminates a level of variation which could affect significance of results in such assays. In addition, larvae can be sorted based on fluorescence and dissections of fed larvae can be performed to separately analyze larval organs for bacterial presence. While fluorescence microscopy is a useful technique for determining presence of bacteria in larvae, its use is more limited in the analysis of pupae and adults due to a high level of autofluorescence in untreated adults and pupae (McNealy et al., personal observations). Currently cryosection techniques for analysis of bacterial presence in pupae and adults exposed to fluorescently labeled bacteria as juveniles are being optimized. We are optimistic regarding the retention of orally acquired bacteria through the *Cx. quinquefasciatus* molt as seen in the study by Moll et al. (2001), which demonstrated that *Cx. pipiens* exhibited a higher bacterial load after molting than mosquitoes from the genera *Aedes* and *Anopheles*. The results of the Moll et al. (2001) study raise questions regarding how the choice of mosquito species, bacterial species, and mosquito developmental stage impact bacteria-mosquito interactions. However, bacteria need not persist past the larval stage to have an impact on later life stages in holometabolous insects. It has been shown that conditions during the larval-stage can induce effects on later developmental stages. A study on the effects of different stressors on *Bicyclus anynana* butterflies showed that food stressors encountered by larvae impacted adult body size and fecundity (Bauerfeind & Fischer 2005). The protocols presented here enable a more quantitative process to analyze such interactions.

PCR is a simple method that can be used to identify both the false negatives and false posi-

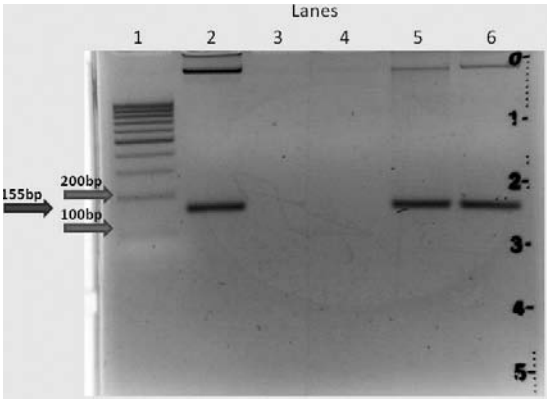

Fig. 2. PCR of GFP specific reaction separated on a 2.5% agarose gel. Lane 1: 100-bp exACTGene DNA ladder (Fisher), 2: *Pseudomonas aeruginosa* plasmid miniprep (positive control), 3: water (negative control); 4: plasmid DNA from bacterial enrichment of control, untreated larvae; 5: plasmid DNA from bacterial enrichment biofilm fed larvae; 6: plasmid DNA from bacterial enrichment planktonic fed larvae.

tives that can occur when using fluorescent imaging. Additionally, we have observed a propensity of false positives due to cross-amplification in *C. quinquefasciatus* using bacterial-specific primers, presumably to endogenous microbial flora (McNealy et al., personal observations). An advantage of targeting a fluorescent protein expressed from a bacterial plasmid is a drastic reduction in this cross-reactivity to either the normal flora of the larval gut or environmental contaminants. The same green fluorescent protein (GFP) gene can be used with most bacteria after subcloning into bacterial specific plasmids, which allows the same highly specific primers to be optimized and used in other experiments. A weakness of this technique is the difficulty in application to the testing of large panels of environmentally sampled bacterial isolates, such as the communities of bacteria found in container habitats or sewer water. The use of our technique is not currently possible for these systems due to the inability to culture the vast majority of bacterial species in the environment, a prerequisite for transformation with a GFP labeled plasmid. However, a number of bacterial-mosquito interactions using model bacteria, can make use of this technique and allow wide, if not ubiquitous use of the protocol.

Future work in this area will include the use of our protocol with other species of pathogenic and non-pathogenic bacteria, including those from the genera *Francisella* and *Legionella*. Together with *Pseudomonas*, these genera represent a continuum across biofilm formation ability, growth requirements (host cell vs. extracellular as well as nutritional) and ability to cause human disease. Species from the genus *Pseudomonas* have very general nutritional requirements, rarely cause human disease, and are among the most proliferate of biofilm formers. *Francisella* species require a host for cell growth, have high pathogenicity even in healthy individuals, and are poor biofilm formers. *Legionella* species form a middle ground between these opposites with their ability to grow on a base of dead bacterial cells, pathogenicity at a level between *Pseudomonas* and *Francisella*, and a modest ability to form biofilms. The use of species from these three genera will allow us to compare and contrast interactions and effects on larvae across a spectrum of biofilm forming ability, pathogenicity, and nutritional niche. Other studies will also adapt this protocol to examine the effects of larval-bacterial associations on fitness and adult behavior, especially oviposition.

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