*Journal of Medical Entomology*, 58(2), 2021, 518–527
doi: 10.1093/jme/tjaa198
Advance Access Publication Date: 5 December 2020
Behavior, Chemical Ecology



# Oviposition-Site Selection of *Phlebotomus papatasi* (Diptera: Psychodidae) Sand Flies: Attraction to Bacterial Isolates From an Attractive Rearing Medium

**Madhavi L. Kakumanu,[1] Bahjat F. Marayati,[2] Coby Schal,[1] Charles S. Apperson,[1,3] Gideon Wasserberg,[2] and Loganathan Ponnusamy[1,3,4]**

[1]Department of Entomology and Plath Pathology, North Carolina State University, Raleigh, NC 27695,[2]Department of Biology, University of North Carolina at Greensboro, 235 Eberhart Building, Greensboro, NC 27402,[3]Comparative Medicine Institute, North Carolina State University, Raleigh, NC 27695, and [4]Corresponding author, e-mail: loganathan_ponnusamy@ncsu.edu

Subject Editor: Richard Johnson

Received 5 June 2020; Editorial decision 17 August 2020

## Abstract

Phlebotomine sand flies are worldwide vectors of *Leishmania* parasites as well as other bacterial and viral pathogens. Due to the variable impact of traditional vector control practices, a more ecologically based approach is needed. The goal of this study was to isolate bacteria from the most attractive substrate to gravid *Phlebotomus papatasi* Scopoli sand flies and determine the role of bacterial volatiles in the oviposition attractancy of *P. papatasi* using behavioral assays. We hypothesized that gravid sand flies are attracted to bacterially derived semiochemical cues associated with breeding sites. Bacteria were isolated from a larvae-conditioned rearing medium, previously shown to be highly attractive to sand flies. The isolated bacteria were identified by amplifying and sequencing 16S rDNA gene fragments, and 12 distinct bacterial species were selected for two-choice olfactometer bioassays. The mix of 12 bacterial isolates elicited strong attraction at the lower concentration of $10^7$ cells per ml and significant repellence at a high concentration of $10^9$ cells per ml. Three individual isolates (SSI-2, SSI-9, and SSI-11) were particularly attractive at low doses. In general, we observed dose-related effects, with some bacterial isolates stimulating negative and some positive dose–response curves in sand fly attraction. Our study confirms the important role of saprophytic bacteria, gut bacteria, or both, in guiding the oviposition-site selection behavior of sand flies. Identifying the specific attractive semiochemical cues that they produce could lead to development of an attractive lure for surveillance and control of sand flies.

**Key words:** *Phlebotomus papatasi*, sand flies, leishmaniasis, dose–response bioassay, semiochemical

Leishmaniasis is a common neglected disease in tropical, subtropical, and arid parts of the world; it affects millions of people each year, causing thousands of deaths (Reithinger et al. 2007, Alvar et al. 2012, WHO 2016, Torres-Guerrero et al. 2017). According to the World Health Organization, about 700,000 to 1 million new cases occur annually worldwide, making it one of the seven most important tropical diseases (WHO 2020). Leishmaniasis is caused by intracellular trypanosomatid protozoan parasites in the genus *Leishmania*, which are vectored and transmitted to humans by phlebotomine sand flies, primarily *Phlebotomus* and *Lutzomyia* species (Sharma and Singh 2008). While leishmaniasis is largely concentrated in warm tropical, semiarid, and arid environments, it appears to be emerging globally in concert with anthropogenic land-use changes, human migration, climate change, drug resistance, poverty, and HIV coinfection (Ashford 2000, Desjeux 2001, Murray et al. 2005, Alvar et al. 2006).

With few therapeutic options and no available vaccines to protect humans against the etiological agent of leishmaniasis, limiting exposure to sand flies and sand fly control are efficient and generally cost-effective methods to reduce the incidence of the disease (Murray et al. 2005, Antinori et al. 2012). Of several prominent approaches, sand fly control with residual insecticide sprays in human dwellings and animal shelters (Alexander and Maroli 2003, Warburg and Faiman 2011) is the most common; however, the efficacy of this method is highly variable across locations (Ashford 1999, Alexander and Maroli 2003). Moreover, residual

insecticide sprays also can affect a wide range of nontarget insects (Pimentel 1995). Hence, more focused, targeted, and efficient methods are needed for sand fly control (Warburg and Faiman 2011).

An alternative approach to delivery of the insecticide to the vector is to bring the vector to the insecticide using attractants (Hamilton 2008, Simpson et al. 2011, Zeichner and Debboun 2011). Oviposition-site attractants can provide the basis for a novel control and surveillance approach targeting gravid females that are typically responsible for pathogen transmission and population amplification. Oviposition traps have been used for the control of mosquitoes (Ritchie et al. 2008, 2009; Ponnusamy et al. 2010; Day 2016; Paz-Soldan et al. 2016), but no such tool yet exists for the control of sand flies. In mosquitoes, bacteria isolated from breeding sites have been shown to play a key role in the production of semiochemicals that attract females to oviposition sites and stimulate egg laying (Hasselschwert and Rockett 1988; Trexler et al. 2003; Ponnusamy et al. 2008, 2015; Davis et al. 2013; Eneh et al. 2019). In the present work, we applied a similar approach to identify bacterial isolates that produce volatile semiochemicals that guide oviposition site-seeking gravid sand fly females.

There is compelling evidence for sand flies that organic material from various sources elicits oviposition responses. This behavior makes adaptive sense given that larval sand flies are coprophagic (Killick-Kendrick 1999, Ready 2013). For example, volatiles from rabbit and chicken feces were attractive to gravid *Lutzomyia longipalpis* females, a New World sand fly species (Elnaiem and Ward 1992, Dougherty et al. 1995, Wasserberg and Rowton 2011, Peterkova-Koci et al. 2012), and hexanal and 2-methyl-2-butanol were identified as bioactive compounds (Dougherty et al. 1995). Similarly, *P. papatasi* (Schlein et al. 1989, Chelbi et al. 2008, Wasserberg and Rowton 2011, Marayati et al. 2015) and *P. argentipes* (vector of visceral leishmaniasis in India) (Kumar 2013) were shown to be strongly attracted and/or stimulated to oviposit by cow and rabbit feces and by larval rearing substrate.

As part of a broader project aimed at developing an oviposition attractive lure for the control and surveillance of *P. papatasi* (a vector of Old World cutaneous leishmaniasis), we applied an interdisciplinary, integrated, hypothesis-driven approach, including behavioral, electrophysiological, analytical, and microbiological investigations to study the chemical ecology of sand fly oviposition behavior. Based on the natural history of *P. papatasi* and previous studies (Wasserberg and Rowton 2011), we hypothesized that larval rearing substrate would constitute such an attractive source. Our strategy was to, first, screen larval rearing media of several larval developmental stages to identify the most attractive source. Second, to isolate and culture bacteria from this source and screen them to identify the isolates that produced most attractive volatiles. Third, to identify and isolate the volatile chemicals, and finally, to behaviorally screen those compounds in order to identify the potent oviposition attractants. Prior to this study, we identified larval rearing substrate conditioned by second–third instar larvae as the most attractive and oviposition-stimulating source material (Marayati et al. 2015). In this paper, we report our findings concerning the second phase of this research. Specifically, our goals in this work were to: 1) isolate and molecularly identify bacteria from this most attractive rearing medium; and 2) behaviorally screen the attraction of gravid females to these bacterial isolates.

## Materials and Methods

### Sand Fly Colony Rearing and Maintenance

We followed the rearing method and maintenance of sand flies (*P. papatasi*) as described in Lawyer et al. (2017). Briefly, sand flies were collected in Akbuk, Turkey in 2004, colonized at the Walter Reed Army Institute of Research (Silver Spring, MD) and a subcolony was maintained at the University of North Carolina in Greensboro and used in this study. The colonies were maintained in incubators (Model: 6030-1, Caron, Marietta, OH) under a 12:12 (L:D) h photoperiod at 26°C and 80% RH. Adult flies were fed on anesthetized mice (UNCG IACUC protocol 14-07 dated 26 February 2015) and the larvae were fed on powdered fermented 1:1 mixture of rabbit feces and rabbit chow.

### Isolation of Bacteria From Rearing Substrate

Recently, we showed that gravid *P. papatasi* females were attracted to the rearing substrate of second–third instar larvae (Marayati et al. 2015). From this material, we isolated bacteria using three different media, including Tryptic Soy Agar (TSA), Nutrient Agar (NA), and Plate Count Agar (PCA). Media were used at 1× and 0.25× concentrations with and without the addition of 1% substrate extract (filter-sterilized extract from 10 g substrate sample in 100 ml sterile water) to the media. Using serial dilution and plating, the rearing substrate suspension diluents were spread onto different media plates, replicated twice, and incubated at 28°C for a week. Multiple bacterial colonies with distinct morphologies were selected and plated on fresh TSA media and restreaked multiple times until pure isolates were obtained. The selected bacterial isolates were maintained on TSA plates for routine work and stored as glycerol stocks at −80°C for long-term storage.

### Molecular Identification of Bacterial Isolates

In total, 18 purified colonies were identified by sequencing a part of the 16S rRNA gene (Lane 1991). The genomic DNA from bacterial isolates was obtained by boiling the purified culture in 50 µl of nuclease-free water and the supernatant was directly used as template for PCR. For isolates that failed to amplify, genomic DNA was isolated from the purified bacterial culture using a Qiagen blood and tissue kit (Qiagen, Valencia, CA), according to the manufacturer's recommendations. The 16S rRNA gene was amplified by PCR with universal primers 27F and 1492R (Lane 1991). PCR amplification and sequencing analysis of the 16S rRNA gene were conducted as described previously (Ponnusamy et al. 2008). The amplicons were directly sequenced using 27F as the sequencing primer at Eton Bioscience sequencing facility (Research Triangle Park, NC). The 16S rRNA gene sequences from all isolates were checked for chimeras using Decipher (Wright et al. 2012) and were identified by comparing to the type strains available in EzTaxon-e database (Kim et al. 2012). Sequences generated in this study have been deposited in the NCBI GenBank.

### Bacterial Growth Conditions

To determine the influence of the bacterial isolates (Table 1) on attraction of gravid sand flies, 12 bacterial isolates were tested individually and in a mixture using two-choice olfactometer bioassays (Marayati et al. 2015). For testing the bacterial mix, individual bacterial isolates were inoculated ($10^4$ cells per ml) in TSB and grown for 48 h at 28°C. At the end of the incubation period, we estimated the cell densities of each bacterial culture using a hemocytometer, and

mixed the 12 cultures at equal cell densities. For testing individual isolates, 48-h cultures of each bacterial isolate were considered 1×, and serially diluted 10-fold with sterile 0.85% saline to achieve final cell densities of $10^7$ to $10^9$ cells per ml for the bacterial mix and $10^6$ to $10^9$ cells per ml for individual cultures. TSA medium without bacteria was also serially diluted using sterile 0.85% saline and used as a control in each bioassay.

## Two-Choice Attraction Assays

A detailed description of the olfactometer is provided in Marayati et al. (2015) (Fig. 1). The olfactometer experiments were conducted in a walk-in environmental room (Hotpack, SP Scientific, Warminster, PA) at the University of North Carolina in Greensboro under standard conditions (28°C and 80% RH). In two-choice behavior assays, four different concentrations ($10^6$, $10^7$, $10^8$, $10^9$ cells per ml) of bacterial cultures served as the treatment and serially diluted blank TSB media served as the control. All treatments and controls were placed in 30-ml graduated cups (Item#: 81772, Moore Medical LLC, Farmington, CT) and placed in opposite chambers located on either end of the olfactometer. For each replication, 20 gravid *P. papatasi* females of same age and stage (8–11 d old, 72-h post-blood meal, previously shown as the stage most responsive to oviposition cues; Marayati et al.

**Table 1.** List of bacteria isolated from the most attractive sand fly substrate, and their closest match in EzTaxon-e database

| Isolate code | Closest cultured bacteria/sequence | Phylum | Sequence[a] similarity (%) | Included in bioassay | Accession number |
|---|---|---|---|---|---|
| SSI-1 | *Leucobacter holotrichiae* | Actinobacteria | 99.24 | Yes | MT538320 |
| SSI-2 | *Microbacterium sorbitolivorans* | Actinobacteria | 98.94 | Yes | MT538317 |
| SSI-3 | *Sphingobacterium daejeonense* | Bacteroidetes | 99.94 | Yes | MT538315 |
| SSI-4 | *Cellulosimicrobium cellulans* | Actinobacteria | 98.63 | Yes | MT538319 |
| SSI-5 | *Luteimonas padinae* | Proteobacteria | 97.72 | Yes | MT538310 |
| SSI-6 | *Alcaligenes faecalis* | Proteobacteria | 99.09 | Yes | MT538309 |
| SSI-7 | *Brevibacterium sediminis* | Actinobacteria | 99.39 | No | MT538318 |
| SSI-8 | *Alcaligenes faecalis* | Proteobacteria | 99.55 | Yes | MT538305 |
| SSI-9 | *Sphingobacterium* sp. | Bacteroidetes | 96.40 | Yes | MN032123 |
| SSI-10 | *Stenotrophomonas indicatrix* | Proteobacteria | 99.24 | Yes | MT538311 |
| SSI-11 | *Bacillus zhangzhouensis* | Firmicutes | 99.39 | Yes | MT538321 |
| SSI-12 | *Alcaligenes faecalis* | Proteobacteria | 100 | No | MT538306 |
| SSI-13 | *Pseudomonas nitrititolerans* | Proteobacteria | 99.39 | Yes | MT538314 |
| SSI-14 | *Alcaligenes faecalis* | Proteobacteria | 99.85 | No | MT538307 |
| SSI-15 | *Brevundimonas olei* | Proteobacteria | 99.24 | Yes | MT538316 |
| SSI-16 | *Pseudomonas nitrititolerans* | Proteobacteria | 98.94 | No | MT538312 |
| SSI-17 | *Pseudomonas nitrititolerans* | Proteobacteria | 99.10 | No | MT538313 |
| SSI-18 | *Alcaligenes faecalis* | Proteobacteria | 99.54 | No | MT538308 |

[a]~660-bp-long 16S rRNA gene sequences were used for identification.

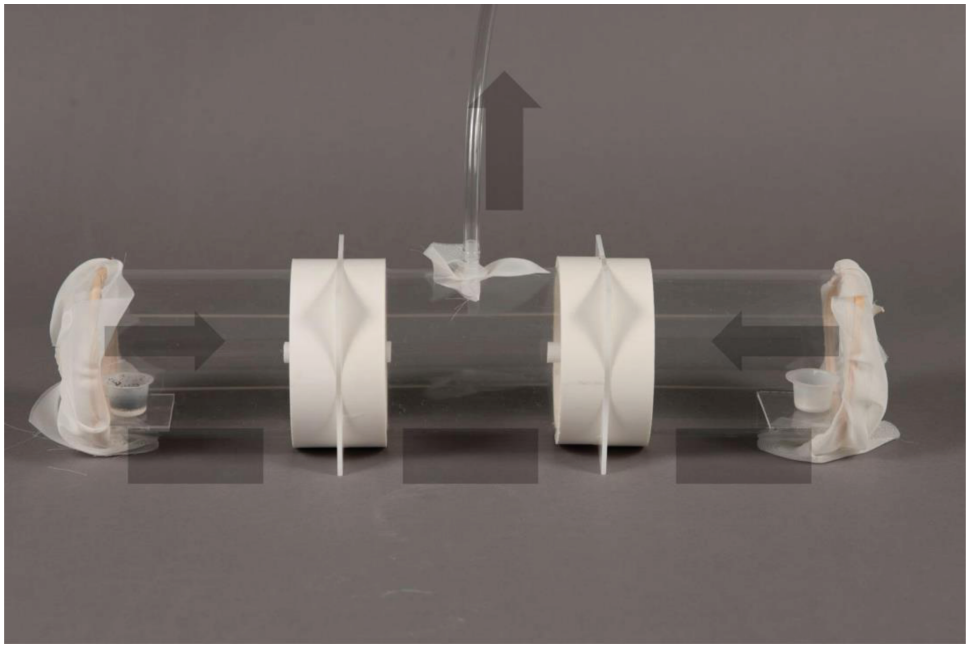

**Fig. 1.** Three-chamber linear olfactometer. The olfactometer was constructed so that the vacuum pump drew air across the treatment and control cups and into the middle chamber, where 20 gravid females were introduced. Cups containing test or control materials were placed on a small shelf at the end of each side chamber. Chambers were connected by 6-cm-long (1-cm-inner diameter) tubes extending 3 cm into both the side chamber and the middle chamber.

2015, Shymanovich et al. 2019b) were transferred to the middle chamber of the olfactometer and given 1 h to acclimate. The middle chamber was then connected for 2 h to a vacuum pump (Air Admiral, Cole-Parmer, Vernon Hills, IL) that had a total volumetric flow of 1.05 liters/min, which delivered the odors to the release (middle) chamber. We recorded the distribution of females in the treatment, control, and middle (nonresponders) chambers after freezing the olfactometer and females at −20°C. We tested all four dilutions concurrently for each treatment. We initially tested sand fly preferences using three replicates for each isolate at each concentration. Those isolates exhibiting directional trends (either attraction or repulsion) were then replicated several more times (maximum $n = 7$) to establish sufficient statistical power.

### Data Analysis

To test for differences in the responses of sand flies to various bacterial isolates, calculations were done based on the proportion of females (responders) in the treatment (bacterial odor) chamber relative to the total flies trapped in the treatment and control chambers of the olfactometer (hereafter 'preference index'). Given that the preference index is a proportion, we analyzed the data using weighted logistic regression, with the total number of flies trapped in the treatment and control traps combined as the weighting factor. Based on this logistic regression analysis, we estimated the mean value of the odds (±95% confidence interval) for each dose of each isolate and determined its significance level. Using a similar weighted regression approach, we also tested for dose effects on sand fly's preference. Results were analyzed with the R program (R Core Team 2013).

## Results

### Bacteria Isolated From the Larvae-Conditioned Rearing Medium

The colonies of purified bacterial isolates were identified by sequencing a fragment of the 16S rRNA gene. The bacterial isolates represented diverse bacterial phyla, including *Actinobacteria*, *Bacteroides*, *Firmicutes*, and *Proteobacteria*. Sixteen of the 18 bacterial isolates had ≥98% sequence identity to type strains in the EzTaxon database (accessed 27 May 2020), and one isolate had ≤96% sequence identity. Based on sequence analysis, we selected 12 unique bacterial species (Table 1) for behavioral assays with gravid sand flies.

### Responses of Gravid Sand Flies to Volatiles From Bacterial Mix

There was a significant negative dose–response relationship between bacterial cell density and *P. papatasi* preference for the bacteria-containing cup (Fig. 2). Females were significantly attracted to the mix of 12 bacterial isolates at low cell density ($10^7$ cells per ml), they exhibited neutral responses at an intermediate cell density, and were significantly repelled at the highest concentration ($10^9$ cells per ml).

### Responses of Gravid Sand Flies to Volatiles From Individual Bacterial Isolates

In behavioral assays with individual bacterial isolates, gravid *P. papatasi* females were differentially attracted to the isolates and their responses also varied across concentrations of the same isolate (Table 2).

#### Bacteria isolates with mixed effect

Six out of 12 bacterial isolates—namely, SSI-1, SSI-2, SSI-3, SSI-9, SSI-11, and SSI-13—elicited mixed responses by *P. papatasi* females. That is, the volatiles from the same bacteria elicited both attractancy and repellency depending on the cell densities. We therefore grouped our bacterial isolates as follows:

a) Bacterial isolates that were attractive at low cell densities (negative dose–response): The volatiles from three bacterial isolates (SSI-2, SSI-9, and SSI-11) were highly attractive to gravid females at low cell densities, but significantly repellent at higher cell densities (Table 2; Fig. 3). The most attractive was isolate SSI-9 at the density of $10^6$ cells per ml, with odds of 7.35 ($P < 0.0001$) for sand flies being found in the treatment side compared with the medium control (>80% trapped in the chamber containing SSI-9). The responses to SSI-9 gradually decreased with higher bacterial cell densities, and then finally became highly repellent at the highest density (Fig. 3B). Isolates SSI-2 and SSI-11 were also highly attractive at low cell density (odds = 4.5; $P < 0.0001$) and gradually became repellent at higher concentrations (Fig. 3A and C).

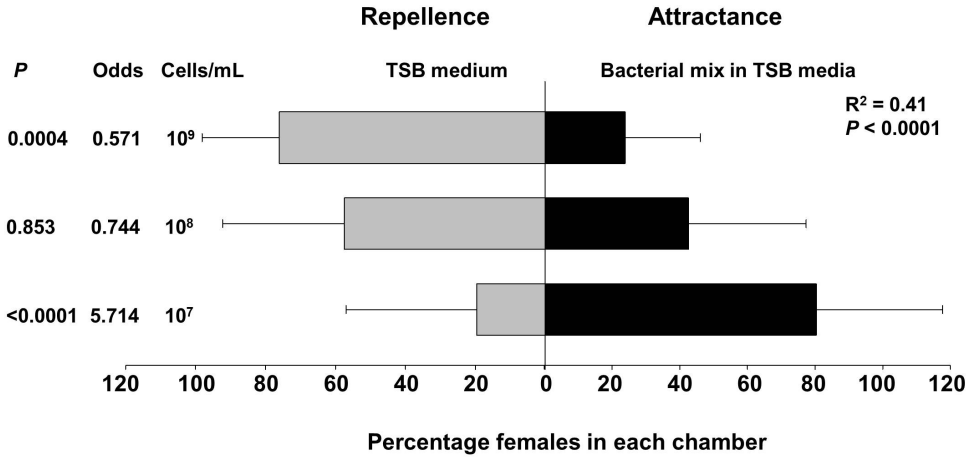


**Fig. 2.** Results of two-choice olfactometer bioassays in which a mixture of 12 bacterial isolates in TSB medium was tested against plain TSB medium for attraction of gravid *Phlebotomus papatasi*. Bars show the mean percentage of gravid females trapped in each of the two end chambers of the olfactometer. Error bars represent standard deviation. Each assay consisted of 20 gravid females, and 4–5 assays were conducted at each bacterial cell density.

**Table 2.** Summary of two-choice olfactometer bioassays in which the attraction of *P. papatasi* to different bacterial isolates at different cell densities was tested against control TSB medium, as illustrated in Fig. 1

| Isolate | Number of replicates | Bacterial cell density (cells per ml) | | | |
|---|---|---|---|---|---|
| | | $10^6$ | $10^7$ | $10^8$ | $10^9$ |
| Mix | 4–5 | ND | +++ | NS | −−− |
| SSI-1 | 3 | −− | − | NS | ++ |
| SSI-2 | 4–6 | +++ | +++ | − | −−− |
| SSI-3 | 3–4 | ++ | NS | NS | NS |
| SSI-4 | 3–4 | NS | NS | NS | NS |
| SSI-5 | 3–4 | NS | NS | NS | NS |
| SSI-6 | 6 | NS | NS | NS | NS |
| SSI-8 | 3 | − | −− | NS | NS |
| SSI-9 | 6–7 | +++ | NS | NS | −−− |
| SSI-10 | 3 | NS | − | NS | NS |
| SSI-11 | 6 | +++ | + | NS | −− |
| SSI-13 | 3 | − | −− | NS | ++ |
| SSI-15 | 6 | NS | NS | NS | NS |

NS, no significant attraction or repellency; ND, not determined.
Attraction: $+P < 0.05$; $++P < 0.01$; $+++P < 0.001$.
Repellency: $-P < 0.05$; $--P < 0.01$; $---P < 0.001$.

b) Bacterial isolates that were attractive at high cell densities (positive dose–response): Isolates SSI-1, SSI-3, and SSI-13 were significantly repellent at the lowest bacterial cell density, with SSI-1 being the most repellent (odds = 0.269; $P < 0.002$) (Fig. 4). Preferences for SSI-1 and SSI-13 increased and finally switched to significant attraction at the highest cell density (Fig. 4A and C). Sand fly responses to SSI-3 also changed gradually from significant repellence at low cell density toward greater (but nonsignificant) attraction at the highest cell density (Fig. 4B).

### Bacterial isolates with negative effect

Two of the 12 isolates, SSI-8 and SSI-10, were significantly repellent at the low doses, and their effect on gravid sand flies was neutral at higher cell densities (Fig. 5).

### Bacterial isolates with a neutral effect

Four isolates (SSI-4, SSI-5, SSI-6, and SSI-15) did not have any effect on the preference for the sand flies' side at any bacterial cell density (Fig. 6).

We observed variation in the percentage of nonresponding gravid females among the bacterial isolates (e.g., >60% nonresponding sand flies to SSI-10, whereas <30% with SSI-3 and SSI-13). We

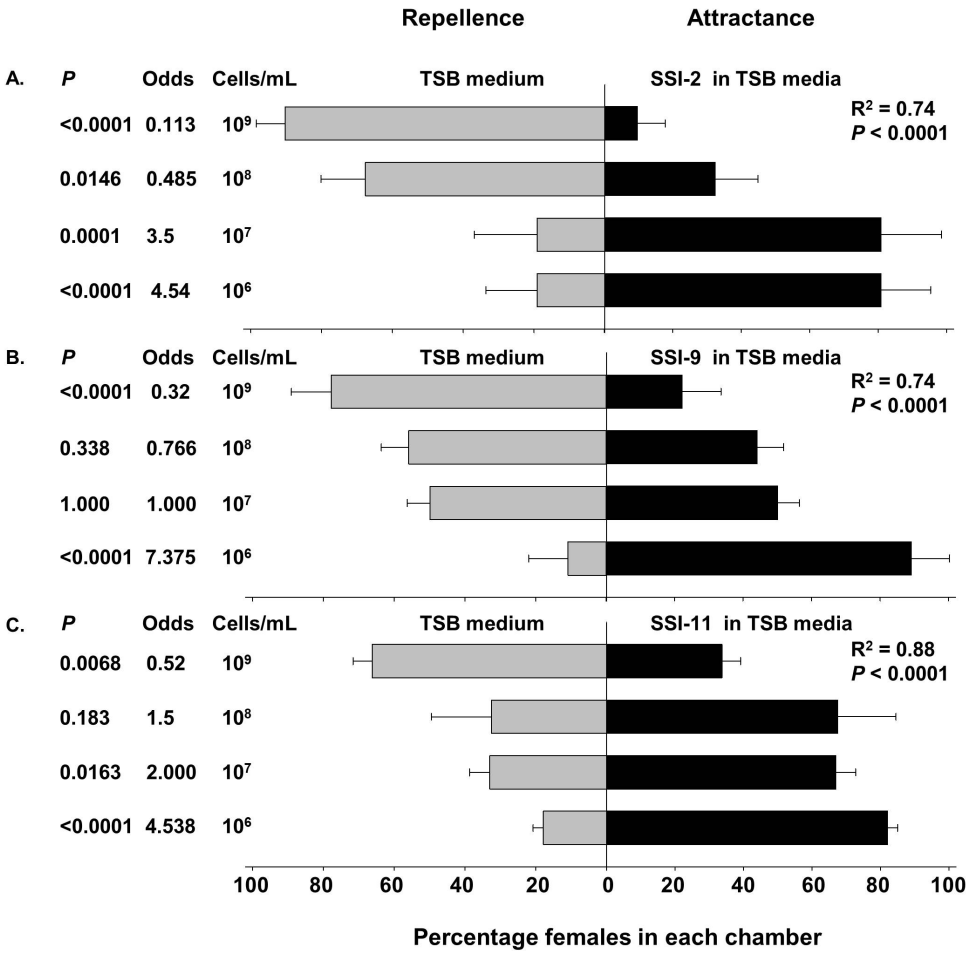


**Fig. 3.** Results of two-choice olfactometer bioassays, showing results for individual bacterial isolates (A) SSI-2, (B) SSI-9, and (C) SSI-11 that elicited significant attraction in gravid *Phlebotomus papatasi* females at lower cell densities and repellency at higher cell densities. Bacterial isolates were in TSB medium and tested against plain TSB medium. Bars show the mean percentage of gravid females trapped in each of the two end chambers of the olfactometer. Error bars represent standard deviation. Each assay consisted of 20 gravid females, and 4–7 assays were conducted at each bacterial cell density.

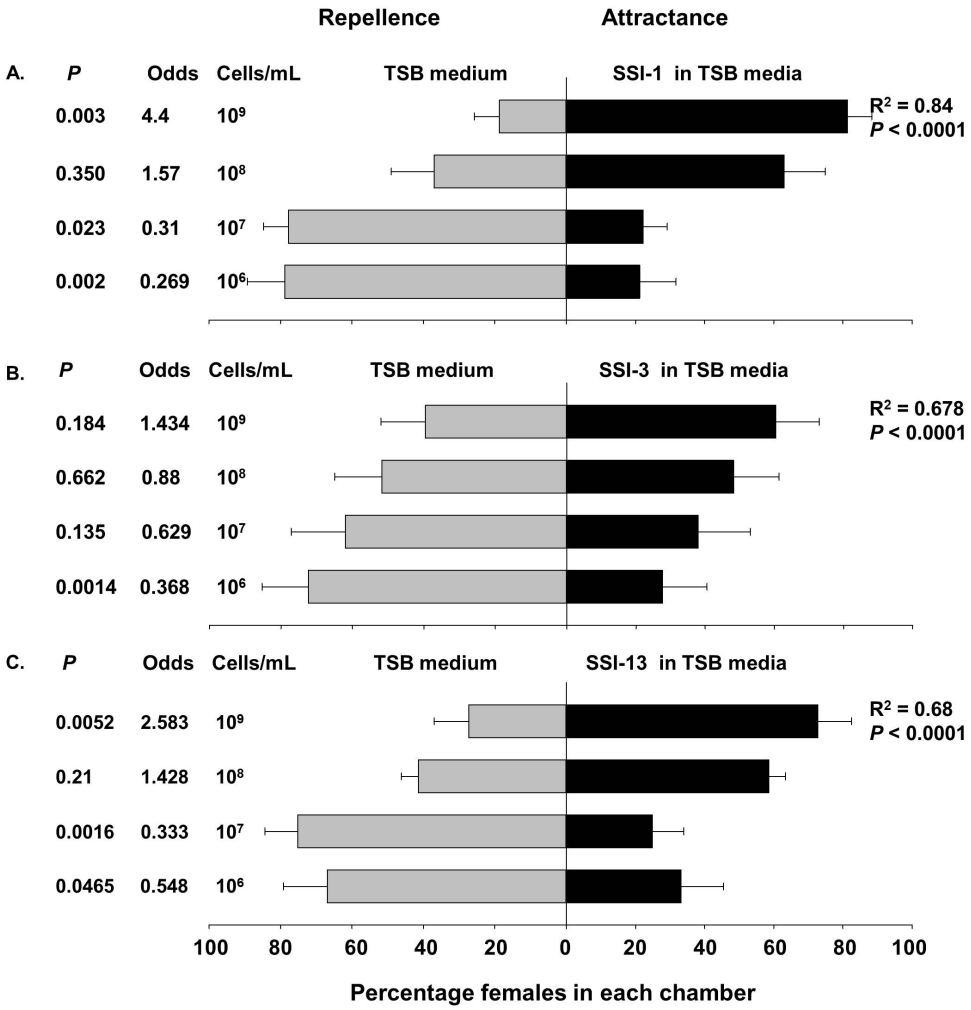


**Fig. 4.** Results of two-choice olfactometer bioassays, showing results for individual bacterial isolates (A) SSI-1, (B) SSI-3, and (C) SSI-13 in TSB medium tested against plain TSB medium. These isolates elicited significant repellency in gravid *Phlebotomus papatasi* females at lower cell densities and attraction at higher cell densities. Bars show the mean percentage of gravid females trapped in each of the two end chambers of the olfactometer. Error bars represent standard deviation. Each assay consisted of 20 gravid females, and 3–4 assays were conducted at each bacterial cell density.

observed no clear pattern, however, in the percentage of the females that failed to respond in relation to either level of attractancy or to bacterial cell density.

## Discussion

Oviposition-site selection of gravid sand fly females is a complex process involving a range of cues, including chemosensory, visual, and tactile (Elnaiem and Ward 1992; Dougherty et al. 1993, 1995; Dougherty and Hamilton 1997; Radjame et al. 1997; Feliciangeli 2004; Marayati et al. 2015; Shymanovich et al. 2019a; Kowacich et al. 2020). Moreover, responses to environmental cues are often affected by photoperiod and the female's physiological state (Shymanovich et al. 2019a). A fundamental assumption underlying oviposition-site selection in sand flies (and for insects in general) is that natural selection should favor oviposition behavior that optimizes offspring performance (Jaenike 1978). Therefore, given the coprophagic diet of larval sand flies, it is reasonable to hypothesize that gravid females should be attracted to cues indicative of the presence of decomposing fecal organic matter. Indeed, enhanced oviposition in response to fecal material of various sources has been demonstrated for several sand fly species (Schlein et al. 1989, Dougherty et al. 1995, Wasserberg and Rowton 2011, Peterkova-Koci et al. 2012, Marayati et al. 2015). For example, hexanal and 2-methyl-2-butanol from rabbit feces were shown to enhance oviposition response in *L. longipalpis* (Dougherty et al. 1995). Nonetheless, the source of production of these and other semiochemicals remained unknown. Although it is reasonable to assume that these semiochemicals are of bacterial origin, little evidence is available in support of this idea. In one study, Radjame et al. (1997) showed that gravid *P. papatasi* females were attracted to soil bacteria isolated from potential breeding sites. More recently, Peterkova-Koci et al. (2012) showed that *L. longipalpis* females laid 85% more eggs in an unsterilized rabbit feces substrate compared with sterilized feces. Yet, neither of these studies separated attraction to volatile cues from contact-based oviposition stimulation. In the present study, we isolated and identified the bacterial species responsible for the attractiveness of larval-conditioned rearing substrate previously shown to be highly attractive to gravid *P. papatasi* females (Marayati et al. 2015). Although the natural oviposition sites of sand flies support complex assemblages of bacteria and fungi, we limited our current investigation to bacteria.

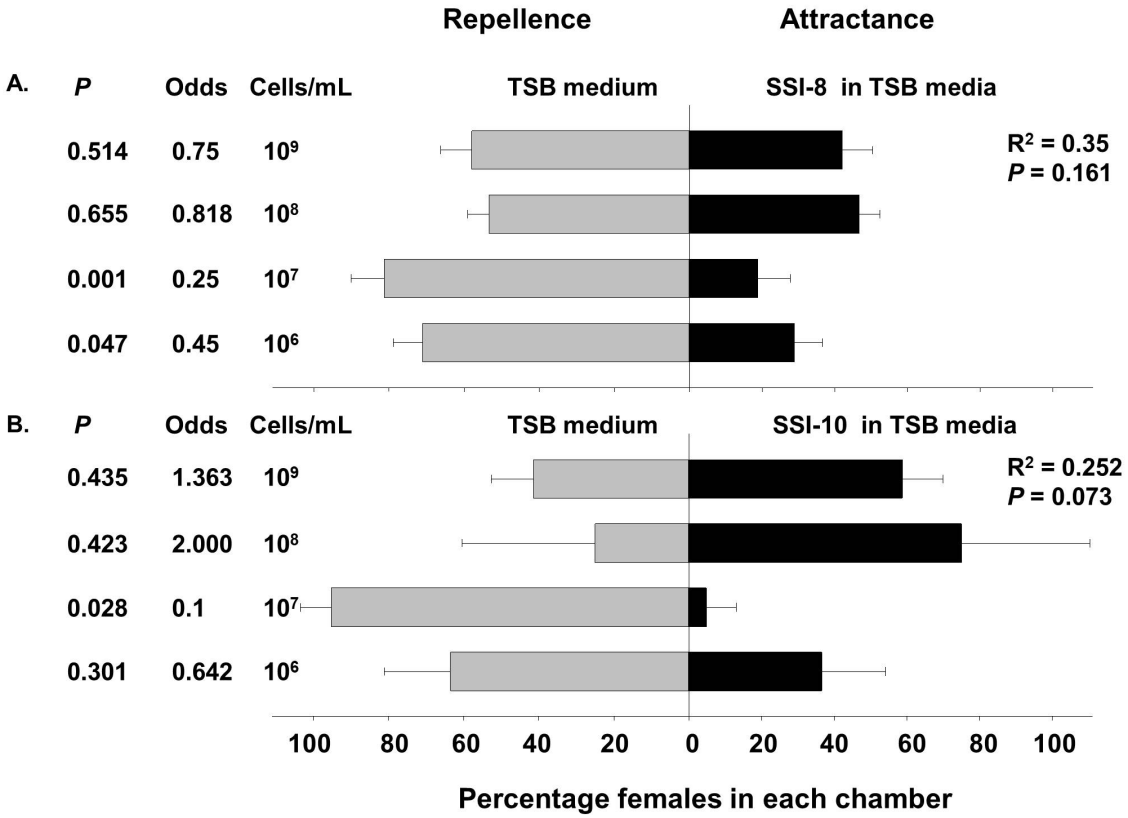


**Fig. 5.** Results of two-choice olfactometer bioassays, showing results for individual bacterial isolates (A) SSI-8 and (B) SSI-10 that elicited significant repellency in gravid *Phlebotomus papatasi* females at lower cell densities and no significant preferences at higher cell densities. Isolates were in TSB medium and tested against plain TSB medium. Bars show the mean percentage of gravid females trapped in each of the two end chambers of the olfactometer. Error bars represent standard deviation. Each assay consisted of 20 gravid females, and three assays were conducted at each bacterial cell density.

We isolated, purified, and identified 18 bacterial isolates from larvae-conditioned rearing substrate, including both gram-negative (13 isolates) and gram-positive (five isolates) bacteria. These bacteria might have originated from the rabbit gut microbiome and its feces, or the *P. papatasi* ovipositor-associated or larval gut microbiome, or they might represent saprophytic environmental bacteria that were established in the substrate. Of the 18 bacterial isolates, five had the highest similarity to species in the genus *Alcaligenes*, three to *Pseudomonas* spp., and two were *Sphingobacterium* spp. These bacteria commonly reside in the intestinal tracts of vertebrates and act as opportunistic pathogens (Hurst 2018). *Alcaligenes faecalis* was previously isolated from the gut of *P. papatasi* (Hassan et al. 2014), *Pseudomonas* sp. from *P. duboscqi* (Volf et al. 2002), and *Pseudomonas* sp. from *P. argentipes* (Hillesland et al. 2008). *Sphingobacterium daejeonense* was isolated from *L. longipalpis* (Sant'Anna et al. 2012) and *Sphingobacterium spiritivorum* from *P. duboscqi* (Volf et al. 2002). Other bacteria belonging to the genera *Brevundimonas*, *Micrococcus*, and *Bacillus* were previously detected in the guts of wild and lab-reared sand flies (Akhoundi et al. 2012, Mukhopadhyay et al. 2012, Fraihi et al. 2017, Karakuş et al. 2017). The bacteria that we isolated from sand fly rearing media have been found in a variety of natural environments that support development of hematophagous insects. For example, some of these bacteria were found in *Aedes aegypti* mosquito oviposition sites, suggesting that similar bacterial volatiles may guide oviposition behavior in a broad range of hematophagous insects (Hasselschwert and Rockett 1988; Trexler et al. 2003; Ponnusamy et al. 2008, 2015).

We applied a bioassay-guided fractionation approach, using a two-choice linear olfactometer, first to evaluate the attractiveness of a mixture of our 12 bacterial isolates with each isolate represented equally in the mix. Later, we also screened individual isolates in a dose–response manner in order to identify the isolates that most likely drive the attraction of sand flies to the larvae-conditioned rearing medium. Identifying the most attractive bacterial strains and the cell density in which they were most attractive would allow us in the future to 1) identify the volatile attractive compounds they produce, and 2) produce a highly attractive bacterial blend. Gravid *P. papatasi* females were attracted to the bacterial mixture at a low bacterial cell density ($10^7$ cells per ml), but repelled by high cell density ($10^9$ cells per ml). These results suggest either that some bacterial isolates in the mix inherently produced volatile repellents, or that some isolates were attractive at low bacterial density but became repellent at higher cell density.

The 12 bacterial isolates were highly variable in their dose-dependent effects on gravid *P. papatasi* females, suggesting possible quantitative and qualitative changes in volatiles affecting *P. papatasi* response. Based on the sand fly responses in the olfactometer, we categorized the isolates into four general groups. The first group, represented by SSI-2, SSI-9, and SSI-11, had a similar pattern of bioactivity as the 12-isolate mix, exhibiting a negative dose-dependent effect on attraction, being highly attractive at low bacterial cell density and highly repellent at high cell density. Isolates in the second group, represented by SSI-1, SSI-3, and SSI-13, exhibited a positive dose-dependent effect on attraction, being repellent at low bacterial cell density, and gradually becoming highly attractive at high cell

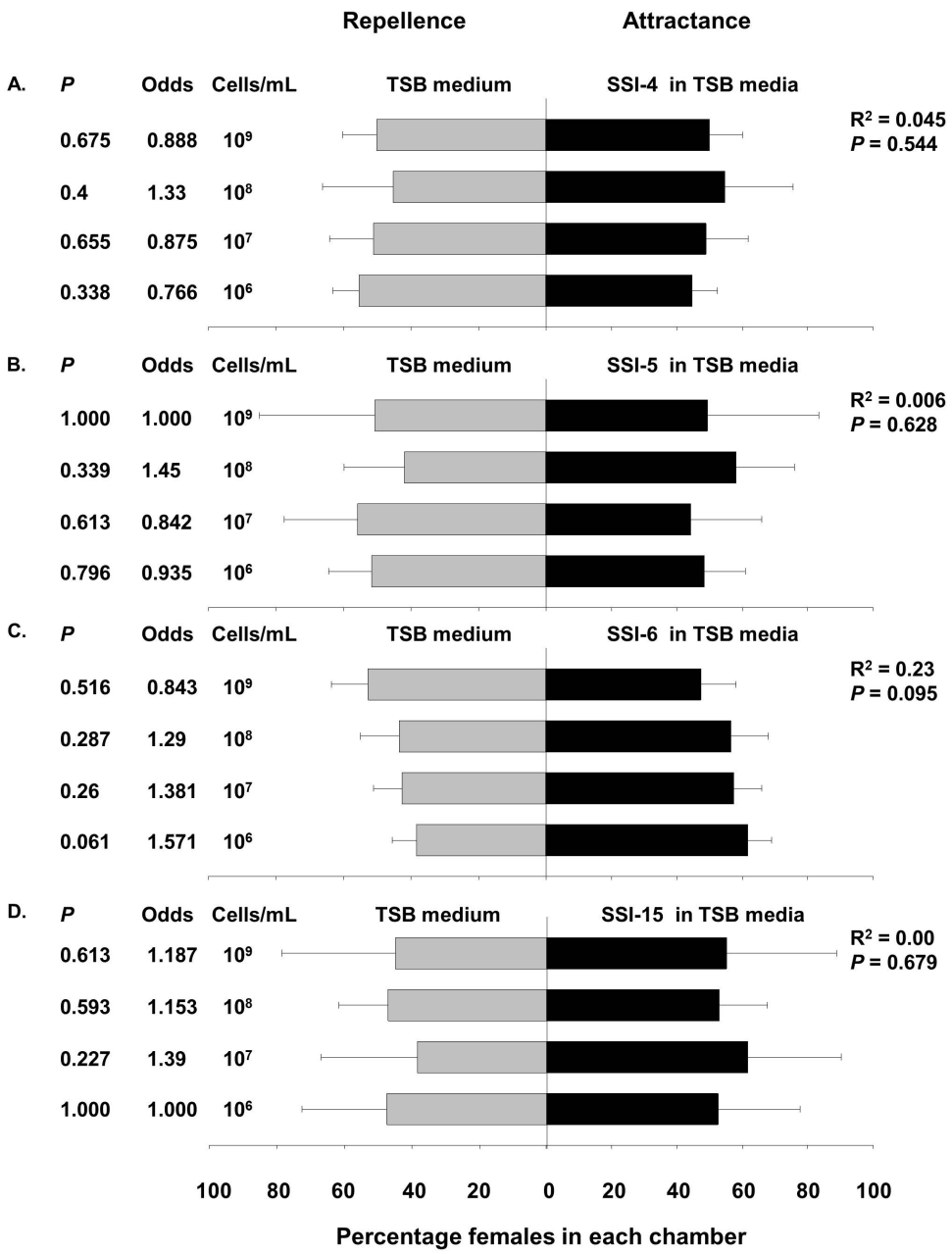


**Fig. 6.** Results of two-choice olfactometer bioassays, showing results for individual bacterial isolates (A) SSI-4, (B) SSI-5, (C) SSI-6, and (D) SSI-15 that elicited no significant preferences in gravid *Phlebotomus papatasi* females at any cell density. Isolates were in TSB medium and tested against plain TSB medium. Bars show the mean percentage of gravid females trapped in each of the two end chambers of the olfactometer. Error bars represent standard deviation. Each assay consisted of 20 gravid females, and 3–6 assays were conducted at each bacterial cell density.

density. The third group of bacterial isolates, represented by SSI-8 and SSI-10, exhibited a strong response (either attraction or repellence) at some cell concentrations but did not exhibit a significant dose–response. Finally, the fourth group, represented by SSI-4, SSI-5, SSI-6, and SSI-15, did not elicit significant responses from sand flies. The underlying olfactory mechanisms in group 1 isolates could involve quantitative changes in specific odorants that are attractive at low concentration and repellent at high concentration or qualitative changes in the volatiles emitted at different cell densities. The responses of *P. papatasi* to group 2 isolates are more complicated. It is unlikely that repellent compounds at low concentration would become attractive at high concentrations. Therefore, we suspect that qualitative changes occur in the suite of emitted compounds at high cell density. Similar variation in attraction and oviposition behavior in response to various bacterial species and either cell densities or odor concentrations have been documented in mosquitoes (Hasselschwert and Rockett 1988, Trexler et al. 2003, Ponnusamy et al. 2015) and other insects (Leroy et al. 2011, Zheng et al. 2013).

The significant attraction of oviposition site-seeking females to bacterial volatiles supports the idea that microbial volatiles play pivotal roles in the selection of breeding sites by *P. papatasi*. Bacteria in the oviposition sites not only cue the gravid females to an appropriate site, and the bacteria themselves might be an important food resource for growing larvae (Merritt et al. 1992,

Peterkova-Koci et al. 2012). It remains to be determined whether microbial volatiles are fully responsible for the odors that mediate this attraction, or if other sources of attractive apneumonal odorants are involved. Consistent with the 'Preference–Performance Hypothesis' which posits that natural selection should favor oviposition behavior that optimizes offspring performance (Jaenike 1978), the dynamic nature of the behavioral responses of gravid *P. papatasi* females to different bacterial isolates and different concentrations might represent an adaptive response to the quality of the breeding site. For example, low bacterial density might indicate fresh, sparsely populated growth medium, whereas high-density bacterial medium might indicate a densely populated medium. Such a density-dependent shift in oviposition-site attraction was recently observed with respect to density of conspecific eggs (Kowacich et al. 2020). Alternatively, bacterial succession at the breeding site might result in attractive bacterial strains occurring in earlier phases, which are then gradually replaced by repellent strains as medium conditions gradually deteriorate.

Our findings strongly support an important role of microbial volatiles in *P. papatasi* selection of breeding sites. They also highlight the complexity, dynamic nature, and even subtlety in the composition of semiochemical cues that mediate the olfactory responses of sand flies to oviposition sites. Our data show that some of the 12 cultured bacterial isolates have great potential to be combined into lures for the trapping or surveillance of sand fly. Efforts to identify some of the bacterial volatiles that attract gravid *P. papatasi* females are in progress, and they should facilitate the development of synthetic lures for sand fly monitoring and control.

## Acknowledgments

This work was supported by the North Carolina Biotechnology Center Multidisciplinary Research Grant (MRG) number 1104 and the National Institute of Allergy and Infectious Diseases, NIH (1R01AI123327-01).

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