Sub-additive effect of conspecific eggs and frass on oviposition rate of *Lutzomyia longipalpis* and *Phlebotomus papatasi*

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ABSTRACT: Oviposition behavior is a fairly neglected aspect in our understanding of the biology of sand flies. In this study, we used a comparative approach using both new- and old-world species (*Lutzomyia longipalpis* and *Phlebotomus papatasi*) in choice and no-choice oviposition chambers to evaluate the effect of old sand fly colony remains (frass), conspecific eggs, and their combination on oviposition rates of these sand flies. We also tested the effect of egg washing with de-ionized water on oviposition rates. In both choice and no-choice experiments, sand fly species laid more eggs on a substrate containing frass. The effect of eggs alone was not significant but showed a positive trend. Furthermore, for both sand fly species, the effect of the combined treatment was sub-additive suggesting a potential inhibitory effect of one factor on the other. Egg washing did not have a significant effect. The choice and no-choice experimental designs did not differ in their outcomes suggesting the choice-design could serve as an effective high throughput method for screening oviposition attractants/stimulants. ***Journal of Vector Ecology* 36 (Supplement 1): S138-S143. 2011.**

Keyword Index: Sand fly, oviposition behavior, attractants, stimulants, leishmaniasis, control.

INTRODUCTION

Oviposition behavior is an important target for the control of hematophagous insects because (a) it involves females, the only stage that transmits disease agents in most species, (b) it involves blood-fed females, which are epidemiologically the most important cohort of the adult female population, and (c) the eggs that are oviposited represent the greatest potential amplification of its population. Although much work on the chemical ecology of oviposition has been done with mosquitoes (Takken and Knols 1999, Bentley and Day 1989, Pates and Curtis 2005, Logan and Birkett 2007, Navarro-Silva et al. 2009, Pickett et al. 2010), very little is known on this topic concerning sand flies (Feliciangeli 2004). Gravid sand flies do not lay their eggs indiscriminately but use a range of semiochemical cues indicating an oviposition site with conditions suitable for the larvae (Killick-Kendrick 1999). Based on work with laboratory colonies, it has been shown that for *Lutzomyia longipalpis* (Lutz and Neiva), *Phlebotomus papatasi* (Scopoli), *P. dubosqui* (Neveu-Lemaire), *Sergentomyia schwetzi* (Adler, Theodor & Parrot) and (marginally) *S. ingrami* (Newstead), conspecific eggs act as an oviposition attractant or stimulant (Elnaiem and Ward 1991, Srinivasan et al. 1995, Dougherty and Hamilton 1997, Basimike 1997). The behaviorally active compound found in *Lu. longipalpis* eggs has been identified as dodecanoic acid (Dougherty and Hamilton 1997) and shown to be produced by the accessory glands of the female Dougherty et al. (1992). Basimike (1997) showed that *P. dubosqui*, *S. schwetzi* and *S. ingrami* are also attracted to compounds emanating from conspecific larvae, pupae or adult sand flies but not from non-conspecifics. Elnaiem and Ward (1991) found that the relations between the number of conspecific eggs and *Lu. longipalpis* oviposition rates are non-linear. They obtained a significant oviposition impact only at density of ≥ 80 eggs per rearing pot for *Lu. longipalpis*. They observed an increase in the oviposition rate between 80 to 160 eggs but no difference when increasing to 320 eggs.

Organic matter is the main food source for sand fly larvae (Killick-Kendrick 1999) and therefore is expected to produce oviposition attractants/stimulants. Indeed, gravid females of *L. longipalpis* and *P. papatasi* have been shown to be attracted to organic material of various sources (e.g., chicken, cattle, or rabbit feces, larval rearing medium, frass) (Dougherty et al. 1993, Dougherty et al. 1995, Elnaiem and Ward 1992, Schlein et al. 1989). In addition, it was shown that soil bacteria isolated from natural breeding habitats attract gravid *P. papatasi* (Radjame et al. 1997). For *Lu. longipalpis*, the active chemicals in chicken or rabbit feces were identified as hexanol and 2-methyl-2-butanol (Dougherty et al. 1995, Elnaiem and Ward 1992). These chemicals were shown to function as attractants for these sand flies (Dougherty et al. 1995) but their role as oviposition stimulants was not evaluated. Dougherty and colleagues also showed that the relative effect of egg pheromones and organic matter apneumons (semiochemicals of non-animated origin) did not differ in their relative effect but their combined effect appeared additive (Dougherty and Hamilton 1997, Dougherty et al. 1993). Dougherty et al. (1993) hypothesized that gravid females are first attracted to an oviposition site by the physical and chemical constituents of the substrate and, at close range, are stimulated to oviposit by pheromones of conspecific eggs.

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One common problem with many medical entomology studies is the lack of methodological consistency which often hampers generalization (Dye 1992). This problem also affects the topic of oviposition attractants where different investigators use different methods and different sand fly species thereby making it difficult to discern whether differences in results are meaningful or stem from different methodology.

In this study, we used a comparative approach using both new- and old-world species (*Lu. longipalpis* and *P. papatasi)* in choice and non-choice oviposition chambers to evaluate the relative oviposition effect of frass (aqueous filtrate of old colony remains) versus conspecific eggs and whether the combined effect is additive or multiplicative.

MATERIALS AND METHODS

Phlebotomus papatasi, originated from females collected in Jordan (hereafter, PPJO), and *Lu. longipalpis,* from females originated from Jacobina, Brazil (hereafter, LLJB), were reared and maintained in the Division of Entomology at Walter Reed Army Institute of Research (Modi and Rowton 1999, Lawyer et al. 1991). Newly emerged sand flies were kept in a 30x30x30 cm polycarbonate mating/feeding cage containing an approximately equal ratio of males and females for three days to allow for mating. They were then blood-fed on anesthetized mice and left undisturbed for another day. In each experiment, 125 ml rearing pots (Nalgene™ Model 81063, polypropylene jars, six cm diameter, six cm height) were used. In the bottom of each, a one cm radius hole was drilled to allow for water absorption. Pots contained 40 cc (ca. one cm height) Plaster-of-Parris and were placed in a tray with water for two hours prior to the introduction of the sand flies to ensure substrate saturation. Pots were sealed with a fine mesh screen secured with a pair of rubber bands. In all experiments, we used 20 females per pot.

The four treatments used in all experiments comprised conspecific eggs (hereafter, EGGS), aqueous filtrate of old conspecific colony remains (hereafter, FRASS), eggs plus frass, and water (control). Conspecific eggs were obtained one day before from rearing pots used for colony maintenance. Eggs (approximately 240 eggs per vial) were kept refrigerated (4° C) in a glass vial (20 ml) containing de-ionized water. Frass extract was obtained from old larva-rearing pots (at least eight-week-old pots that stopped producing any new adults). We created an aqueous suspension of 3.5 gram frass in 10 ml deionized water. The suspension was manually stirred for 10 min., in room temperature, and allowed to settle for 5 min. The supernatant was then filtered through standard medical gauze and transferred to another 20 ml vial. We used two experimental designs: choice and non-choice pots. In both designs, we scratched the plaster substrate into four equal-sized quarters. Active (non-anesthetized) blood-fed female sand flies were transferred using a mouth aspirator into the experimental oviposition pots and left for five days in an incubator (26° C, 85% RH, total darkness) to lay their eggs while supplying them daily with sugar meal source (cotton wad soaked in a 10% glucose solution). After five days, surviving and dead females were removed and a photograph of the substrate was taken. Using a magnified color print of each pot substrate we counted the total number of eggs. Subtracting the initial number of eggs from the total counted provided the net number of new eggs.

In the choice experiment we allocated one treatment to each quarter and compared the number of new eggs laid in each quarter (Figure 1). In the FRASS treatment, we applied eight drops of the frass filtrate (seven uniformly placed along its sides at least 3 mm from the edge and one drop in the center) using a glass pipette. In the EGGS treatment we applied a drop of water containing approximately ten eggs in the center of the quarter and added seven water drops along its sides to ensure consistency of amount of water between treatments. In the EGGS+FRASS treatment we placed a drop containing approximately ten eggs in the center of the quarter and added seven drops of frass filtrate along its sides. In the control, we added seven drops of de-ionized water along the sides and one in the center of the quarter (Figure 1). Treatments associated with frass were located on opposing sides of the pot to minimize potential effects of spill-over. For both species, we repeated this experiment three times with total sample size of 32 for PPJO (10, 12, and ten replicates in sessions 1, 2, and 3, respectively) and 27 for LLJB (5, 10, and 12 replicates for sessions 1, 2, and 3, respectively) .

In the no-choice experiment, we used a single treatment per pot and compared the number of new eggs laid per pot between the treatments. In each experimental session, we used 12 oviposition pots with four treatments and three replicates per treatment. We conducted five and seven replicate sessions for PPJO and LLJB, respectively, resulting in sample sizes of 15 and 21 for each species, respectively. In this experiment we used two levels of initial egg number. Low level comprised ca. 20 eggs per pot (two replicate sessions

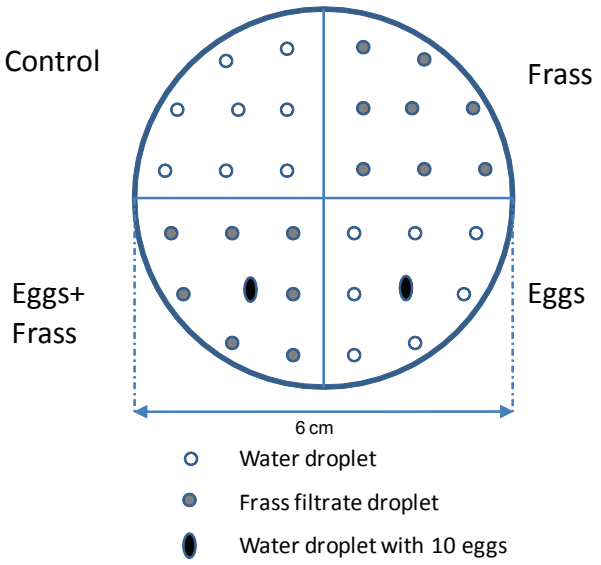

Figure 1. Choice experimental design.

for PPJO and LLJB) and high level comprised ca. 40 eggs per pot (three and five replicate sessions for PPJO and LLJB, respectively). Within a pot, we distributed the treatment equally among the four quarters. For the FRASS treatment, we applied eight frass filtrate droplets per quarter in each of the four quarters. In the EGGS treatment, we distributed equal amount of eggs in the center of each quarter and added seven drops of water in the margins of each quarter. In the EGGS+FRASS treatment, we distributed equal amount of eggs in the center of each quarter and added seven drops of frass filtrate in the margins of each quarter. In the control we added 8 droplets of water in each quarter.

Eggs used in the experiments described above were collected the previous day from the lab colony and kept refrigerated in de-ionized water overnight. To ascertain that this method of egg storage did not bias the sand fly's oviposition response, we conducted a choice-experiment in which CONTROL, FRASS, WASHED EGGS, and UNWASHED EGGS treatments were allocated to each quarter pot as described above (see, choice experiment) (N = 12). UNWASHED eggs were obtained directly from the colony's oviposition pots and were transferred with a fine brush into the experimental pot. Treatments associated with eggs were located on opposing sides of the pot.

Because the response variable in this study (number of eggs laid) is a count variable, we applied a generalized linear model approach using Poisson regression. Since in the no-choice experiment a high degree of overdispersion was detected (Dispersion parameter = 97.77) we used a quasipoisson regression which adjusts the standard error for such overdispersion (McCulagh and Nelder 1989). In all models, factors were treated as dummy variables (McCulagh and Nelder 1989). In the choice experiment, observations on treatment effects cannot be considered independent since they are clustered within a pot. Therefore, to test for the effect of treatments after controlling for heterogeneity among pots we used a random-intercept poisson regression, with 'pot' as the random intercept (Rabe-Hesketh and Skrondal 2008). Analysis was conducted using STATA software (StataCorp., College Station, TX).

RESULTS

The effect of frass, eggs, and their combination on sand fly oviposition

In both experiment types and for both species, the strongest oviposition response was in treatments associated with FRASS (Figure 2). In the no-choice experiment, *Lu. longipalpis* laid highest number of eggs in the FRASS and FRASS+EGGS treatments ($t>2.635$, $P<0.004$). The effect of the EGGS treatment was not statistically different from the control ($t = 1.075$, $P=0.285$). For *P. papatasi*, only the effect of FRASS was statistically (albeit marginally) significant ($t = 1.693$, P(one-sided) = 0.046). The effects of EGGS and FRASS+EGGS were not significant. However, it is worth pointing out that while the EGGS treatment was slightly

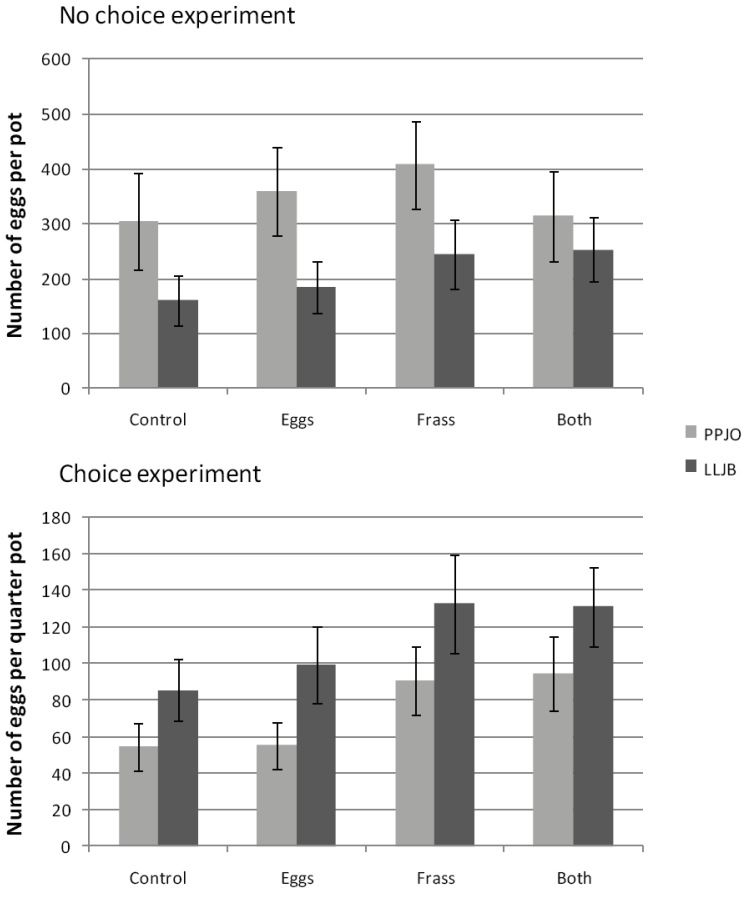

Figure 2. The effect of frass, conspecific eggs, and both on the number of new eggs laid in the no-choice and multi-choice experiments. Error bars represent 95% CI.

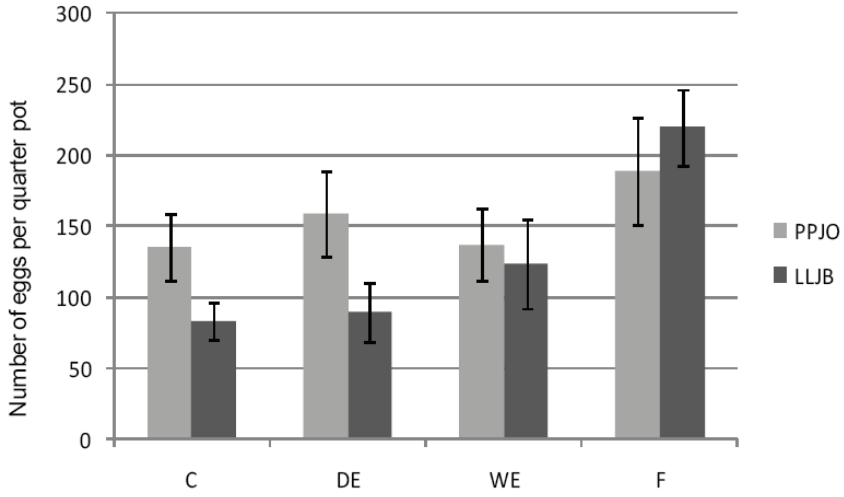

Figure 3. The effect of frass (F), conspecific un-washed eggs (DE), conspecific washed eggs (WE), and control (C) on the number of new eggs laid in the multi-choice experiment. Error bars represent 95% CI.

higher than the control (t=1.061, P=0.293) the combined effect (EGGS+FRASS) was indistinguishable from the control (t = 0.025, P = 0.98) (Figure 2). Sand fly species did not differ significantly in their egg production although a marginally significant trend suggests *P. papatasi* to be more productive than *Lu. longipalpis* (t = 1.753, P = 0.082) (Figure 2).

In the choice experiment, treatments associated with frass (FRASS, FRASS+EGGS) exhibited the strongest oviposition response (Figure 2). For *Lu. longipalpis*, both of these effects were highly significant (z>3.60, P<0.0001) whereas the effect of EGGS was not significant (z = 0.93, P=0.35). Similarly, for *P. papatasi* the effect of both FRASS and FRASS+EGGS treatments was highly significant (z>5.28, P<0.0001) whereas the effect of EGGS was not (z = 0.48, P=0.628). In this experiment, *Lu. longipalpis* was significantly more productive (z = 3.21, P = 0.001).

The effect of egg washing and frass on sand fly oviposition

For both species, strongest oviposition response was in the FRASS treatment (Z>9.93, P<0.0001) (Fig. 3). For *Lu. longipalpis*, the effect of WASHED EGGS was highly significant (z=9.62, P<0.0001) while the effect of UNWASHED EGGS was marginally significant (z=1.80, P=0.072). The difference between the WASHED and UNWASHED eggs treatments was marginally significant with slightly higher oviposition response at the WASHED EGGS treatment (paired-t = 1.795, P = 0.078). For *P. papatasi,* neither of the egg treatments had a significant effect (z<1.74, P>0.083). Similarly, the difference between the WASHED and UNWASHED eggs treatments was not significant (paired-t = 1.795, P = 0.14).

DISCUSSION

Results of previous studies indicated that conspecific eggs and organic matter (of various sources) function as oviposition attractants/stimulants for sand flies (Dougherty and Hamilton 1997, Dougherty et al. 1992, Dougherty et al. 1993, Dougherty et al. 1994, Dougherty et al. 1995, Elnaiem et al. 1991, Elnaiem and Ward 1991, Elnaiem and Ward 1992, Radjame et al. 1997, Schlein et al. 1989, Srinivasan et al. 1995). Results of our study are partially consistent with those observations. In all the experiments conducted in this study, both sand fly species had the strongest oviposition response in the FRASS treatment. However, the effect of conspecific eggs was, in most cases, not significant although a positive trend was suggested (Figures 2 and 3) and in the case of *Lu. longipalpis* in the egg-washing experiment the WASHED EGGS treatment had a significant effect. In contrast with our expectation of synergistic effect of FRASS and EGGS (Dougherty and Hamilton 1997, Dougherty et al. 1993) the combined effect of frass and eggs was not statistically different from the effect of frass alone. Furthermore, *P. papatasi* in the no-choice experiment laid fewer eggs in the combined treatment (Figure 2). These results contrast with the observations of Dougherty et al. (1993) who found no difference between the individual effect of eggs pheromone and rabbit food but did find a significant synergistic effect of the combined treatment. This pattern suggests an inhibitory effect of one factor with respect to the other resulting in suppression of the net oviposition response. The main difference between the two studies is that Dougherty et al. (1993) used a known, purified, egg-derived oviposition attractant and stimulant and a non-specific diethyl ether extract of rabbit food. In our study, we used whole eggs and an aqueous filtrate of frass. In that sense, our experiment provides a closer approximation of natural settings. Eggs and frass could, potentially, contain some inhibitory factors that were removed in Dougherty's egg purification or food extraction process. A possible explanation is that due to crowding stress some inhibitory pheromones are produced by adult females or juvenile stages and are presented on the egg or in the frass, respectively. Thus, when presented together the effect of one factor could partially mask or negate the effect of the other. This finding of negative interaction between eggs and frass with respect to sand fly oviposition response is novel and warrants further investigation.

The EGGS treatment was, as mentioned above,

non-significant in most cases. One possible explanation is that egg density used was too low. For example, using *Lu. longipalpis*, Elnaiem et al. (1991) showed a positive response occurred only at 80 eggs per pot (0.84 eggs/cm^2) or above. In our experiment, highest level used was 40 eggs per pot. However, we used smaller pots. In fact, egg density used in our experiment (1.414 eggs/cm^2) was actually higher than the threshold density observed by Elnaiem et al. (1991). Another possible explanation is that our mode of egg storage (kept refrigerated overnight in de-ionized water) might have washed away the attractant pheromone (possibly dodecanoic acid). However, as our experiment showed, no significant difference in the oviposition response was found between washed- and unwashed eggs (Figure 3). Furthermore, Elnaiem et al. (1991) showed that removal of attractance occurred only following intense treatment with an organic solvent (Hexane). Nevertheless, in most of our treatments a positive trend of EGGS was suggested.

In summary, the overall effect of conspecific eggs, frass filtrate, and the combination of both was consistent among species and experimental designs. One of our original concerns was that in an experiment where the experimental pot contains four different treatments the mixture of volatiles within the pot might disorient the sand flies and result in higher variation and reduced resolution. However, our study demonstrated that the choice design was at least as effective as the no-choice design. This is an important finding, because such a design may provide the basis for a high through-put method for screening a wide range of potential oviposition attractants or stimulants. Improving our understanding of the egg-laying behavior of sand flies have significant implications in terms of improving sand fly colony productivity, surveillance, and potentially control by developing lethal ovitraps such as those used successfully with mosquitoes (Ritchie et al. 2008, Williams et al. 2007).

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