Supplemental Information: Male accessory gland molecules inhibit harmonic convergence in the mosquito *Aedes aegypti*

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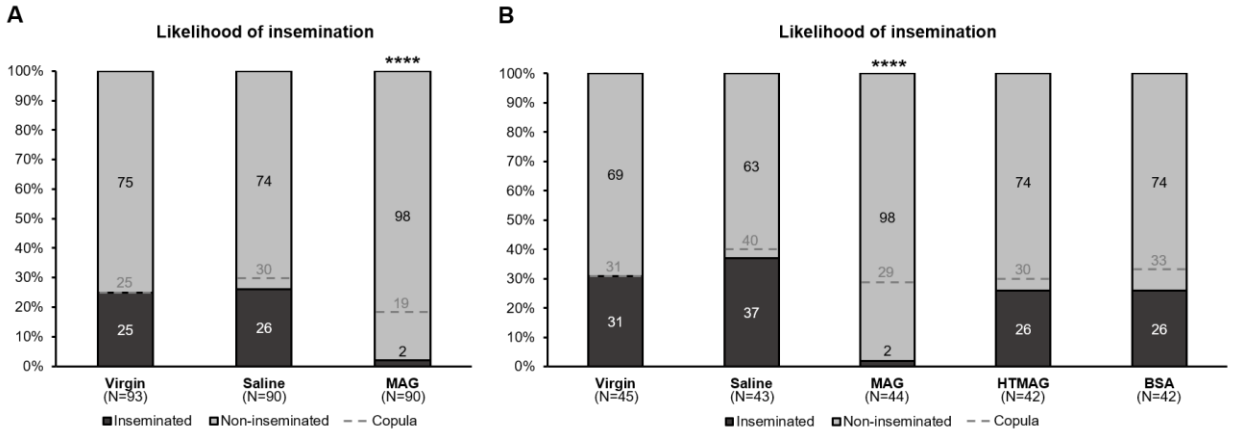

Figure S1. MAG-induced inhibition of harmonic convergence correlates with lower insemination rates. Treatment had a significant effect on the proportion of apparent copulas that resulted in female insemination (χ^2: treatment, $P<0.05$ for both sets of replicates). (A) MAG-injected females (2%; N=90) were successfully inseminated less often compared to virgin (25%; N=93) and saline-injected (26%; N=90) control females. (B) Heat-treated MAG (HTMAG; 26%; N=42) and BSA-injected (26%; N=42) females did not display lower insemination rates compared to virgin (31%; N=45) and saline-injected (37%; N=43) control females, but were more likely to be inseminated than MAG-injected (2%; N=44) females. Treatment did not have a significant effect on apparent copula formation (N=89–93 and N=43–45 pairs per group for replicates 1–7 and 4–7, respectively; χ^2: treatment, $P\geq0.124$ for both sets of replicates) and copula formation did not correlate with HC (χ^2: $P\geq0.268$ for both sets of replicates). Mated groups were excluded from the mating outcome analyses because they were already 100% inseminated prior to testing. Dashed gray lines represent the percent of apparent copulas formed in each group based on our visual analysis. Asterisks denote significance for insemination data compared to the saline control group. **** $P\leq0.0001$.

Treatment	Converging sex								Converging harmonics							
	M		F		B		U		2:1		3:2		4:3		5:3	
	Ratio	%	Ratio	%	Ratio	%	Ratio	%	Ratio	%	Ratio	%	Ratio	%	Ratio	%
Virgin	15/42	36	1/42	2	2/42	5	24/42	57	18/42	43	9/42	21	0/42	0	15/42	36
Saline	12/40	30	-	-	4/40	10	24/40	60	19/40	47.5	6/40	15	2/40	5	13/40	32.5
Mated	5/20	25	-	-	-	-	15/20	75	10/20	50	2/20	10	0/20	0	8/20	40
MAG	5/18	28	-	-	-	-	13/18	72	9/18	50	4/18	22	0/18	0	5/18	28
HTMAG	2/19	11	-	-	-	-	17/19	89	10/19	53	2/19	10	1/19	5	6/19	32
BSA	5/20	25	-	-	-	-	15/20	75	14/20	70	2/20	10	1/20	5	3/20	15
Total	44/159	28	1/159	<1	6/159	4	108/159	68	80/159	50	25/159	16	4/159	3	50/159	31

Table S1. Harmonic convergence is most often achieved by males at the female second and male first harmonics. Converging sex and converging harmonics data presented for each treatment group. Most often the converging sex shifted its flight tones too early in an interaction to determine which sex's flight tone modulations achieved HC (U, unknown: 68% of converging pairs). However, when either sex's flight

tone modulations resulted in HC in a detectable manner, HC was almost always achieved by the male alone (M, male: 28%) rather than the female alone (F, female: <1%) or both sexes (B, both: 4%). The sex responsible for convergence did not vary between treatment groups (N=18–42 and N=9–21 per group for trials 1–7 and 4–7, respectively; χ^2: P≥0.601 for both sets of replicates). Convergence occurred most often by the merging of the female second and male first harmonics (2:1; 50%) and at similar harmonic combinations across all replicates (N=18–42 and N=9–21 per group for replicates 1–7 and 4–7, respectively; χ^2: P≥0.731 for both sets of replicates). Data for each treatment group were pooled across replicates 1–7 for trials including virgin, saline, mated, and MAG groups or across replicates 4–7 for trials containing additional HTMAG and BSA groups. M, male; F, female; B, both; U, unknown. 2:1: Female second and male first harmonics; 3:2: Female third and male second harmonics; 4:3: Female fourth and male third harmonics; 5:3: Female fifth and male third harmonics.

Supplemental Experimental Procedures

Mosquito rearing and maintenance

Aedes aegypti mosquitoes (Thai strain) originated from collections in Bangkok, Thailand (15°72'N, 101°75'E) and have been maintained in colony with annual supplementation of F_1 eggs since 2009. Mosquitoes were reared in an environmental chamber as described previously [1] to obtain uniform, medium-body-sized adults (male wing lengths: 2.21 ± 0.08 mm; females: 2.87 ± 0.12 mm) [2]. Briefly, eggs were vacuum hatched and 200 larvae were placed in plastic trays containing 1 L of distilled water and 4 fish food pellets the following day (Hikari Cichlid Gold, Hayward, CA, USA). Male and female pupae were visually separated by size and females were isolated in test tubes to confirm their sex and ensure their virgin status prior to transfer into 8 L plastic buckets, where they fed on a 10% sucrose solution *ad libitum*.

MAG extract preparation, mosquito injections, and antennae removal

A total of fifty MAGs were dissected from males at two-to-three days post-eclosion and pooled at a 1:1 ratio in a 50 µl solution (one-quarter MAG-equivalent injections) of modified phosphate-buffered saline (PBS; pH 6.9) or at a 2:1 ratio in 25 µl PBS (for one-half MAG-equivalent injections). Standard injections were performed at the one-quarter MAG-equivalent dose as this concentration is effective for inducing a complete refractory mating response in *Ae. aegypti* [3] and corresponds approximately to a single male ejaculate [4]. The buffer was comprised of 133.58 mM sodium chloride (Fisher Scientific, Pittsburgh, PA, USA), 2.63 mM potassium chloride (Fisher), 9.75 mM sodium phosphate dibasic (Fisher), 3 mM potassium phosphate monobasic (Fisher), and 2 mM calcium chloride dihydrate (MilliporeSigma, St. Louis, Missouri, USA) in Milli-Q water (MilliporeSigma). Buffer was filtered through a Durapore Membrane Filter (0.22 µm pore size; MilliporeSigma). Dissected MAGs were then homogenized, sonicated for 30 s, and centrifuged at 4 ˚C and 14,500 rcf for 15 min, and the resulting supernatant was used for MAG injections.

Injections were performed on female mosquitoes at two-to-three days post-eclosion using a Nanoject III Programmable Nanoliter Injector (Drummond Scientific Company, Broomall, PA, USA) and finely pulled glass capillary needles to inject liquids

into the lateral thorax at the anepisternal cleft. MAG contents were injected at a one-quarter MAG-equivalent concentration, or 250 nl injections of the 1 MAG/µl PBS solution, and in the case of the MAG dosage experiment, at one-half MAG-equivalent concentration, or 250 nl of 2 MAG/µl PBS solution. For injection of heat-treated MAG contents, MAG homogenate was heated for 5 min at 95 ˚C in a Bio-Rad C1000 Thermal Cycler (Bio-Rad Laboratories, Hercules, CA, USA) [1]. For experiments comparing BSA and MAG injections, protein concentrations were verified for equivalency using a Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA). For BSA injections, we injected BSA protein powder dissolved in PBS at approximately 1 µg/µl concentration, as this is close to the average one-quarter MAG-equivalent homogenate concentration of 1.03 ± 0.27 ug/ul (N=8 samples). To control for potential needle injury effects, we included a cohort of females injected with 250 nl of PBS alone (saline treatment).

All injected females as well as non-injected controls, including mated females (males added at a 1:1 male to female ratio), were placed in 2 L wax-lined cardboard recovery cages and transferred to an environmental chamber under the conditions referenced above. Mosquitoes were held for two days prior to audio recordings and were provided with 10% sugar-soaked cotton pads. Males were removed from the mated group cage 24 h prior to recordings.

Audio recording and analysis of pre-copulatory flight interactions

Females were tethered using nail glue (L.A. Colors, Ontario, CA, USA or similar product) at the dorsal mesothorax by a human hair attached to an insect pin [5]. Similar to previously described recording methods [7], tethered females were placed approximately 3 cm away from either a particle velocity microphone or an omnidirectional microphone (NR-21358 and FG-23329-C05, respectively; Knowles, Itasca, IL, USA) attached to a custom amplifier [6], in a 20 x 14 x 10 cm plastic recording arena. Upon initiation of female flight, audio recordings using Audacity 2.1.3 and 2.2.2 software (https://www.audacityteam.org/, accessed May 23, 2017 and updated April 13, 2018) were initiated and three virgin males were added into the arena. The recording was stopped after the first male-female pre-copulatory flight interaction lasting > 1 s was recorded and the female either persistently rejected or formed an apparent mating copula with the male. A flight interaction was defined as a male approaching a female in flight and rapidly modulating his flight tones in an attempt to secure, harmonically converge, and mate with the female. To be considered a copula, the couple had to be connected for > 8 s, as this is the minimum amount of time necessary for insemination to occur [8,9]. After audio recordings, female spermathecae were dissected to verify insemination status. If an apparent copula led to successful insemination, the copula was considered a mating copula; if not, it was considered a pseudocopula [10,11]. All recordings were performed at 28.04 ± 1.75 ˚C and 49.63 ± 6.96% RH.

To determine whether the male and female wing beat harmonics converged during flight interactions, audio files were analyzed as spectrograms using Raven Pro 1.5 software (Bioacoustics Research Program, Cornell Laboratory of Ornithology, Ithaca, NY, USA). Potential instances of convergence were systematically tested at all harmonic combinations below 3,000 Hz, including the female second and male first

harmonics (2:1), the female third and male second harmonics (3:2), the female fourth and male third harmonics (4:3), the female fifth and male third harmonics (5:3), and the female fifth and male fourth harmonics (5:4). Because convergence was never detected at 5:4, this harmonic combination was excluded in all analyses. To be considered a true instance of convergence, male and female harmonics had to be within 5 Hz of each other for at least 1 s [12]. The sex responsible for HC was defined as the individual, male or female (or both), who shifted his or her flight tone during a courtship interaction to achieve convergence. If HC occurred too early in an interaction to determine which sex's flight tone shift achieved convergence, the sex responsible for convergence was deemed unknown. Average flight tone frequencies during male-female interactions across treatment groups were similar for most replicates (N=90–93 and N=43–45 per group for replicates 1–7 and 4–7, respectively; two-way ANOVA: treatment, $P>0.05$ for nine out of eleven replicates), with males flying at a fundamental frequency of 841.82 ± 56.59 Hz and females flying at 476.81 ± 36.11 Hz across all treatment groups and replicates. This, together with the converging harmonics data (Figure S1), suggests that the differences in ability to induce male convergence between mated or MAG-injected females and virgin females are caused by subtle (that is, ≥ 5 Hz tone shifts), rather than drastic changes in courtship flight tone frequencies.

Statistical analysis

All data were analyzed using IMB SPSS Statistics software (SPSS version 24, IBM Corp., Armonk, NY). For all *post hoc* pairwise comparisons, significant differences were identified only for those comparisons with P values that were less than 0.05 and were less than their Benjamini-Hochberg critical values with a false discovery rate of 0.25 [13–15]. All means are presented ± standard deviation (SD). HC, copulation, and insemination data were analyzed using a binary logistic generalized linear model, with treatment and replicate effects tested using Wald Chi-Square (χ^2) test. When comparing proportions of insemination events, mated groups (which had 100% inseminated outcomes) were excluded from the analyses to run the binary logistic model, as the 95% confidence intervals as calculated by 3/N (where N = the total number of outcomes across all replicates) for these groups were <0.1 [16]. Fundamental flight tone data were analyzed with a two-way ANOVA test and copulation-convergence correlation, converging harmonics, and converging sex data were analyzed using a χ^2 test.

For HC data, after testing for replicate effects and finding none ($P=0.810$), we combined the seven replicate experiments conducted with virgin, saline, mated, and MAG groups. Four of the seven experiments also contained additional heat-treated MAG and BSA controls and were tested for replicate effects ($P=0.362$), combined into a single analysis, and analyzed separately. Data from all experiments on copulation and insemination rates ($P>0.05$ for three out of four sets of replicates), fundamental flight tone frequency ($P>0.05$ for nine out of eleven replicates), as well as converging harmonics and converging sex data ($P>0.05$ for all twenty-two replicates), were likewise combined into the aforementioned replicate groupings to ensure robust sample sizes in all analyses.

Supplemental References

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