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

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Summary

Aedes aegypti mosquitoes transmit pathogens such as yellow fever, dengue, Zika, and chikungunya viruses to millions of human hosts annually [1]. As such, understanding *Ae. aegypti* courtship and mating biology could prove crucial to the success of pest and disease control efforts that target reproduction. Potentially to communicate reproductive fitness [2,3], mosquito males and females harmonize their flight tones prior to mating in a behavior known as harmonic convergence (HC) [4]. Furthermore, after mating or treatment with male accessory gland (MAG) extracts, female *Ae. aegypti* become resistant, or refractory, to re-mating [5]. To test the hypothesis that mating and MAG fluids inhibit a female's ability to induce HC in males, we recorded audio of pre-copulatory flight interactions between virgin males and either virgin, mated, or MAG extract-injected females and analyzed these recordings for the presence or absence of HC. We further assayed HC with heat-treated MAG- and bovine serum albumin (BSA)-injected females to test if potential MAG effects are due specifically to heat-labile molecules. We found that mating and MAG extract, but not heat-treated MAG or BSA, lower HC occurrence by 53% compared to all other controls. Because MAG-injected females formed only nonproductive pseudocopulas and HC was most often accomplished by male tone modulations, these results suggest that mating may inhibit HC indirectly via the broader range of MAG-induced female refractory mating behaviors. Together, our results demonstrate an important new role for MAG molecules in mediating female post-mating behavior.

To test the effect of MAG injection on a female's tendency to elicit flight tone harmonization from courting males, pre-copulatory flight interaction acoustics between virgin males and either non-injected virgin, saline-injected virgin, mated, or MAG-injected females were recorded and analyzed for the presence or absence of HC (Figure 1). Both mating (22%

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Author Contributions

Conceptualization, L.C.H. and M.F.W.; Methodology, L.C.H., M.F.W., and G.P.L.; Validation, G.P.L.; Formal Analysis, G.P.L.; Investigation, G.P.L. and L.L.B.; Resources, L.C.H. and M.F.W.; Writing – Original Draft, G.P.L., L.C.H., and M.F.W.; Writing – Review and Editing, G.P.L., L.C.H., M.F.W., and L.L.B; Visualization, G.P.L., L.C.H., and M.F.W; Supervision, L.C.H. and M.F.W.; Project Administration, L.C.H., M.F.W., and G.P.L.; Funding Acquisition, L.C.H. and M.F.W.

Declaration of Interests

The authors declare no competing interests.

converging pairs) and MAG injection (20%) of females significantly decreased the likelihood of HC compared to both non-injected (45%) and saline-injected (44%) virgin females (Figure 1A; N=90–93 pairs per group; X^2: treatment, $P<0.0001$; $P\leq0.001$ for all pairwise comparisons). Furthermore, injection of MAG at one-quarter MAG-equivalent dose (normal volume transferred during a natural mating [6]) was already saturating for response, as doubling the concentration to a one-half MAG-equivalent dose did not further reduce the likelihood of HC (data not shown; P=0.935). These results demonstrate that a female's receipt of MAG components during mating reduces the likelihood that a subsequent male with harmonically converge with her.

MAGs contain a variety of factors, including potentially heat-labile seminal fluid proteins, that could reduce the likelihood of HC. Additionally, it is possible that injection of proteins in general, rather than MAG proteins in particular, could also mediate this reduction in HC. Therefore, we repeated the experiments with additional female groups injected with heat-treated MAG fluids, to ablate the function of heat-labile MAG molecules, or with a protein (BSA), to test for the specificity of the response, and assayed for HC (Figure 1B). With these experiments, we again found that mating (21% converging pairs) and MAG injection (22%) of females significantly decreased the likelihood of HC compared to both non-injected (47%) and saline-injected (45%) virgin females (Figure 1B; N=43–45 pairs per group; χ^2: treatment, $P\leq0.01$; $P<0.05$ for all pairwise comparisons). However, injecting females with either heat-treated MAG (44%; N=43) or BSA (47%; N=43) did not lower the likelihood of HC compared to virgin female controls ($P\geq0.874$ for all comparisons). These results show that heat-labile MAG molecules specifically, and not protein *per se*, depresses the ability of females to elicit HC in potential male mates.

Upon closer examination of mating and acoustic data during flight interactions, we found that MAG-treated females formed only non-productive pseudocopulas with males that did not result in insemination (Figure S1) and that HC was almost always achieved by the male (Table S1). Together, these findings suggest that declines in convergence associated with mated or MAG-injected females relative to virgin females are correlated with increased female mating refractory behavior and indirect effects on a male's ability to converge with a female.

MAG contents are known to induce mating refractory behavior as part of the female *Ae. aegypti* post-mating response [5]. Here, we expand our knowledge of the role of MAG fluids by showing that female mating decreases the likelihood that HC will occur with subsequent males, and that this phenotype is fully recapitulated in virgin females by injecting them with MAG extracts (Figure 1). Because MAG-induced reduction of the likelihood of HC was lost upon heat treatment of MAG extracts prior to injection (Figure 1B), we conclude that this effect is mediated by heat-labile MAG components, such as seminal fluid proteins, which have been implicated in long-term mating refractory behavior in female mosquitoes [5]. In *Drosophila*, seminal fluid proteins affect such diverse processes and behaviors as mating refractoriness, ovulation, sleep, feeding, longevity, and immunity [7]. In Lepidoptera, mating inhibits pheromone production, and hence female attractiveness [8], while in Orthoptera, mating can suppress female locomotor activity in response to male calling (but not courtship

songs) [9]. Ongoing studies will identify specific MAG components, protein or otherwise, that cause the refractory behavior we document here.

Although the MAG-mediated effects we observed primarily impacted females, MAG-induced changes to female physiology likely have secondary effects on males. This is supported by our observations that MAG contents reduce a female's ability both to permit successful insemination (Figure S1) and to induce HC in males (Figure 1), who were most often responsible for HC when it occurred (Table S1). MAG may either affect a female's ability to produce signals that induce male convergence or alter her perception and behavior such that her ability to detect males and elicit convergence is impaired. Because HC has previously been linked to reduced female rejection behavior and is more likely to occur in pairs that form a productive mating copula [2], we hypothesize that the reductions in harmonic converge we observed were due to MAG-induced female mating refractory behavior that in turn diminish the female's ability to induce male convergence responses. More detailed examinations of potentially affected female sensory modalities, such as those mediated by the antennae, as well as other behaviors in MAG-treated females during courtship, for example female kicking [2], could further delineate the roles of female refractory behavior in determining HC outcomes.

Mosquito-borne diseases pose an increasing public health threat in tropical and subtropical zones around the world [1]. As HC plays a critical role in both sex recognition [10] and mate choice [2,3], these findings advance our understanding of mosquito courtship, mating, and post-mating behavior and can help uncover novel approaches for controlling mosquito populations by interfering with their reproductive biology.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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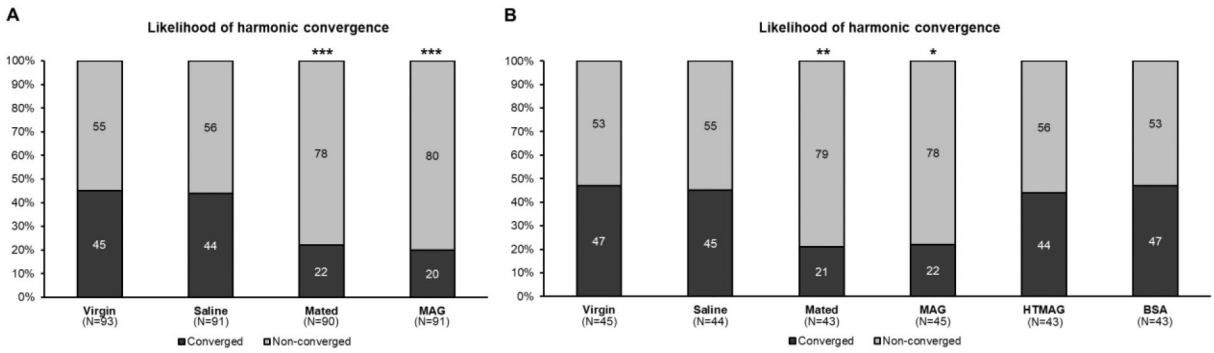

Figure 1. Mating inhibits harmonic convergence via heat-labile male accessory gland molecules.

(A) Mated (22%; N=90) and MAG-injected (20%; N=91) females elicited HC about half as often as virgin (45%; N=93) and saline-injected (44%; N=91) females. (B) Heat-treated MAG- (HTMAG; 44%; N=43) and BSA-injected (47%; N=43) females were as likely to elicit HC in males as virgin (47%; N=45) and saline-injected (45%; N=44) control females, but were more likely to induce HC than mated (21%; N=43) and MAG-injected (22%; N=45) females. Asterisks denote significance compared to the saline control group. * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$.