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Emergent acoustic order in arrays of mosquitoes

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The yellow fever mosquito *Aedes aegypti* forms aerial swarms that serve as mating aggregations [1]. Despite lacking the remarkable collective order of other animal ensembles, such as fish and birds [2], the kinematic properties of these swarms bear the hallmark of local interaction and global cohesion [3,4]. However, the mechanisms responsible for collective behaviour in mosquitoes are not well understood. Mosquitoes use their antennae as hearing organs to locate and interact with one another via the frequencies of sounds generated by their beating wings [5]. Acoustic detection and recognition are known to mediate copula formation in opposite-sex pairs [6], but have not been investigated in larger groups. By recording the flight tones of multiple tethered male *Ae. aegypti*, we test the hypothesis that acoustic signalling is a feature of swarm morphology and present the first compelling evidence that flight tone interactions between males drive observed group coherence in the frequency domain. We find that group size critically affects collective and individual acoustic traits: cohesive acoustic behaviours emerge in groups of more than six male mosquitoes, occurring to a greater degree than predicted in the absence of interaction. Importantly, acoustic interactions between multiple males differ from those reported previously for pairs [7,8]. Our findings enable future research targeting key behavioural and reproductive aspects of the biology of mosquitoes of epidemiological importance.

We simultaneously recorded the individual flight tones of up to $N = 8$ tethered male *Aedes aegypti* in a linear arrangement using a custom-built microphone array (Figure 1A and Supplemental Information). Extraction of each mosquito's fundamental wing beat frequency was performed using Hilbert spectral analysis [9], yielding high resolution spectra in both time and frequency (Figure 1B).

To test whether the mosquitoes' acoustic emissions changed with the number of males present we calculated the differences between the flight tones of all pairs of males. Sorting these frequency differences for each male according to their closest, second closest, etc. flight tone match at each time point yielded a series of

distributions that became progressively sharper, left-skewed and clearly separated with increasing group size (Figure 1C, left column and Figure S1A, details in Supplemental Information). Consequently, as groups became larger, individuals' wing beat frequencies were more regularly spaced, and the average flight tone separation between them decreased (Figure 1D, black line). The instantaneous standard deviation of the group's collective wing beat frequencies (the "group spread", Supplemental Information) also varied with ensemble size. Small- and intermediately-sized groups exhibited a broader range of flight tone dispersals than larger arrays, for which the group spread was narrowly distributed (Figure 1C, right column, grey shaded area). In larger groups ($N = 7, 8$), individuals were tightly bound to the acoustic average: the median spread of flight tones in these arrays was comparable to the smallest ($N = 3$), and was much lower than at intermediate sizes ($N = 4 - 6$, Figure 1E, black line).

We hypothesise that acoustic interactions between males drive emergent phenomena in the frequency domain. To test this, we used permutation sampling to construct arrays of non-interacting individuals selected at random from recordings of different groups, or from recordings of males flying alone (labelled respectively "random group" and "random lone", detail in Supplemental Information). For all group sizes both the median frequency separation and group spread were always lower for mosquitoes flown together than in the non-interactive arrays (Figure 1D,E), but only significantly so (in >95% permutations) in the largest groups ($N = 7, 8$). The summary statistics of non-interactive groups also changed with their size: for combinations of lone males this relationship was roughly linear, while the properties of randomly sampled grouped individuals fell between the live groups and lone permutations (Figure 1C, right column; 1D,E). Crucially, this result demonstrates that being part of a group modifies an individual's acoustic behaviour. In effect, mosquitoes that experienced collective flight tones exhibited wing beat frequency characteristics that were distinct from males recorded in isolation. Moreover, the spectral structure of collective flight tones was affected by group size. In arrays containing more than six individuals, flight tone structuring and group

homogenisation thus emerged as a result of active interactions between males, occurring to a substantially greater extent than in non-interactive random permutations.

It has been suggested that, to facilitate hearing, male mosquitoes within swarms seek to reduce the acoustic interference they create for one another by dividing into local clusters with unique flight tones [10]. Our data reveal the formation of frequency-domain clusters (between individuals within audible range of one another, see Supplemental Discussion) in all group sizes recorded, but with greatly increased prevalence in the largest arrays. Groups containing seven or eight mosquitoes exhibited significantly more clustering than groups constituted from randomly sampled individuals (Figure 1F). Being intrinsically linked to motor function, we suggest that flight tones are an interactive medium through which inter-individual coupling and movement co-ordination [3] take place.

The wing beat frequency characteristics of male mosquito groups are fundamentally different to those flying alone or in pairs, whose flight tones were reported to diverge [7]. We explored the behavioural mechanisms behind the emergent acoustic properties observed here using individual-based models (see Supplemental Information), and found that models based solely on flight tone repulsion yielded group properties akin to models that excluded any individual interactions (Figure S2). Rather, our models suggest that group dynamics are driven by the interplay between frequency attraction and repulsion. Revealing proximate and ultimate causes for such collective acoustics provides a new and necessary angle to existing work on animal collective motion [2].

Our understanding of male-male communication and competition within mosquito swarms is still limited. The findings presented here open up enticing possibilities for the study of swarm formation and cohesion, and highlight the importance of acoustically-mediated interactions in these processes. As the arena in which

mate-seeking and competition take place, an increased knowledge of swarms is vital if we are to develop tools for their control, disruption, or attraction. Acoustics offer a practical means to manipulate swarm activity, which – in view of the importance of swarming for mosquito reproductive biology – may enable us to interfere with the mechanisms that directly support their disease-spreading capacity.

Supplemental Information

Contains experimental/methodological procedures, additional discussion, and two figures.

Author contributions

Conceptualization, A.A., D.R., A.C. and M.H. Methodology, A.A., N.W.F.B. and D.R. Formal Analysis and Investigation, A.A. and N.W.F.B. Writing – Original Draft, A.A. Writing – Review and Editing, A.A., D.R., N.W.F.B., A.C. and M.H.

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Figure legends

Figure 1 (A) Schematic of recording array. The flight tones of individuals were recorded on separate microphones passed through an integrating sound amplifier. Coloured dots indicate the fixed positional orientation of individuals in the array. (B) Example time-frequency spectra selected for single recordings of group sizes $N = 3$ and $N = 8$ individuals, showing the fundamental flight tone component of each male. Colours give the position of males in the array as shown in (A). (C) Distributions of sorted pairwise wing beat frequency differences (left) and group spread (right) for live and non-interactive arrays. Non-interactive array are composed either of randomly combined males recorded in separate groups (“random group”), or randomly combined males recorded alone (“random lone”, full details provided in Supplemental Experimental Procedures). Metrics are calculated at each sampled time point per recording and aggregated over all individuals for a given group size in all replicate trials. Data presented for array sizes $N = 3$ and $N = 8$. For non-interactive groups, distributions shown are from a single iteration of the random sampling procedure (SI). (D) The median flight tone separation between an individual male and his nearest neighbour in the frequency domain, aggregated over all individuals for a given group size. (E) The median aggregated group spread of wing beat frequencies. (F) Clustering: the proportion of recording time for which an individual has at least two partners within 20Hz of his own wing beat frequency. Plotted is the mean cluster fraction taken over all males at a given group size. (D)-(F) Shaded region indicates the mean test statistic (\pm standard deviation) obtained from 1000 iterations of the random sampling procedure used to generate non-interactive groups (SI). Each iteration contained a set of groups equal in number to those recorded during experiments for each array size $N = 3$ to $N = 8$ (replicates for $N = 3$: 14; $N = 4$: 17; $N = 5$: 17; $N = 6$: 13; $N = 7$: 14; $N = 8$: 15). For actual groups, the shaded region gives the mean (\pm standard deviation) taken from 1000 bootstrap iterations of the data.