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Review

No sexual pheromones in *Anopheles* mosquitoes?

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Swarming behavior is the cornerstone of the anopheline mating system. At dusk, males congregate in monospecific swarms in which females come to find a mate once in their lives. Although many *Anopheles* species coexist in sympatry, hybrids are infrequent, suggesting the existence of strong premating reproductive barriers. Chemical cues, particularly pheromones, often play a crucial role in bringing sexes together in a species-specific manner among insects. While the existence of sexual pheromones in *Anopheles* species has been postulated, only a few studies developed experimental designs to investigate their presence. Here, we discuss the contrasting and debatable findings regarding both long-range and contact sex pheromones in the context of swarm ecology in *Anopheles* species.

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Introduction

In *Anopheles* species, the existence of sexual pheromones has often been postulated or debated but rarely investigated [1–6]. Sex pheromones are important both from an evolutionary point of view and in terms of vector control. Indeed, the evolutionary landscape of *Anopheles* mosquitoes is notably intricate, with sympatric species still undergoing speciation processes, where mating behavior likely plays a pivotal role [7]. Moreover, the reproductive barrier between certain species is permeable, allowing for the occasional formation of hybrids that, although rare, contribute to gene introgressions between closely related species [8,9]. From a vector control perspective, the discovery of sex pheromones could pave the way for numerous advancements in existing techniques or the development of novel ones, like strategies employed against crop pests [10,11]. Unfortunately, research into anopheline mating behavior has been limited, mainly focusing on the *Anopheles gambiae* complex.

Briefly, we know that mating takes place mainly outdoors at sunset and to a lesser extent at sunrise [12], mainly in monospecific swarms containing a few to thousands of males wherein virgin conspecific females seek out a mate [12–18]. While numerous *Anopheles* species coexist in sympatry, they generally exhibit assortative mating, except for the closely related species *Anopheles gambiae* and *Anopheles coluzzii*, which can produce hybrids at variable frequencies across time and space. Some of these hybrids can be viable but generally suffer a reduction in fitness [19]. This suggests that, in *Anopheles* species, reproductive isolation primarily occurs through robust premating reproductive barriers [7,20,21]. In addition, as males seem to swiftly grab females entering swarms, and because females only mate once in their lives [22], entering a nonspecific swarm should be costly and fall under negative selection. Therefore, one would expect the existence of specific cues guiding females to conspecific male swarms or facilitating the discrimination between conspecifics and related species within swarms.

Acoustic, visual, and chemical cues have been explored as potential mating signals. While acoustic signals are involved in interactions between sexes in the swarm [23], their use at long range by females has recently been ruled out, as male swarms can only be heard when females come into contact with the swarm [24]. Furthermore, although flight tones exhibit variations among *Anopheles* species [25], these differences alone fail to explain the mating barrier between some important sympatric species even at close range [3,23]. Visual cues such as swarm markers, which are contrasted landscape features, play a crucial role for both males and females in locating and stabilizing swarms. These cues contribute to spatial segregation among certain sympatric species [12,17,26]. However, in regions characterized by high hybridization rates, this segregation may be incomplete, and although some species use visual markers interchangeably, no mixed swarms have been detected to date [27]. Furthermore, it remains unclear whether mosquitoes possess the ability to differentiate and select among different types of markers to identify species-specific locations or the distance at which these markers can be detected or identified. In this context, semiochemicals appear to be promising candidates, with long-range sex pheromones potentially facilitating the attraction of both sexes while maintaining precopulatory reproductive isolation among sympatric species exhibiting similar swarming behaviors. Additionally, contact pheromones may contribute to upholding reproductive barriers within swarms.

Long-range sex pheromones in *Anopheles* sp.

Historically, only a limited number of studies have been published on experiments aimed at demonstrating the presence of sex pheromones in *Anopheles* species. The first known attempts involved testing female choice using a Y-shaped olfactometer with odors from dead males [28] or introducing either a cage of females or a paper on which 50 females had been crushed into a swarm and observing male behavioral responses [12]. In neither case was behavioral evidence of male or female interest in these stimuli reported. Recently, two studies combining behavioral, electrophysiological, and chemical analysis methods were conducted. However, these studies produced conflicting results. In the first one, Mozūraitis et al. [29] identified five compounds (acetoin, sulcatone, octanal, nonanal, and decanal) emitted in larger quantities by males of laboratory strains of *Anopheles arabiensis* (Dongola and KGB) and *An. gambiae* (Keele) while swarming in a 1-l bottle. They also identified incorporation of stable-isotope-labeled glucose in each of these compounds, suggesting that males synthesize them. A synthetic blend of these five compounds, released in proportions reflective of the quantities emitted by 150 *An. arabiensis* males (1, 4, 13, 50, and 400 $\mu g.ml^{-1}$, respectively), triggered an upwind flight of both males and females of *An. arabiensis* and *An. gambiae* in very similar proportions in a Y-olfactometer. This '*An. arabiensis*-like' synthetic blend also induced increased flight activity in *An. gambiae* males during the swarming period. Finally, it induced a higher proportion of female insemination in five laboratory species, including four belonging to the *An. gambiae* complex (*An. gambiae s.s.* [G3], *An. coluzzii* [COGS], *An. arabiensis* [KGB], and *Anopheles merus* [MAFUS]) and additionally in *Anopheles funestus* (FANG) during semifield experiments.

The second study, by Poda et al. [30], focused on recently colonized *An. coluzzii* and *An. gambiae*. They used large plexiglass boxes of 432 l in which males could swarm freely at dusk and assessed the behavioral activity of their headspace on females through dual-port olfactometer assays. However, females exhibited no discernible interest in the airflow emanating from the swarms. They also collected swarm headspace in a 125 l plexiglass box with various methods and adsorbents, placed odor traps (Twister) directly within a natural swarm, and made solvent extracts of swarming males. However, none of these extracts revealed a single compound specifically emitted by males during swarming. They also replicate the extraction method used by Mozūraitis et al. [29] with solid-phase microextraction in a 1-l bottle but with different controls, yet none of the five compounds could be specifically attributed to male odor or to the swarming period. Electroantennographic experiments were also conducted, testing swarm headspace on females' antennae, but once again, no notable physiological response was recorded. They concluded that while absence of evidence is not evidence of absence, their findings support the absence of long-range sex pheromones emitted by male swarms. These results are in stark contrast with the conclusions of Mozūraitis et al. [29], which were that acetoin, sulcatone, octanal, nonanal, and decanal were aggregation pheromones emitted by males during swarming, which not only attract both males and females but also increase the insemination rate of females, which actually would make them aggregation-sex pheromones [31].

Why long-range pheromones may not exist in *Anopheles* sp.?

These conflicting findings are puzzling and warrant careful consideration. First, the actual function of the five compounds identified in Mozūraitis et al. [29] deserves examination. Their biological activity is particularly interesting, especially given their apparent effectiveness across multiple *Anopheles* species, suggesting the potential for developing a broadly effective tool for vector control. At the same time, the broad activity of these compounds raises doubts regarding their classification as pheromones in closely related and sympatric species. The strict definition of a pheromone stipulates that it is emitted by one organism and triggers a behavioral or physiological response in another individual of the same species [32,33]. Moreover, sex pheromones are expected to facilitate the location, identification, contact, and mating of the two sexes in a species-specific manner, thereby preventing hybrid formation and ensuring reproductive isolation among closely related species [34,35]. While closely related species may often share similar compounds, their relative proportions typically display high species specificity [35,36]. Of course, some species may exploit the chemical signals of others for fitness benefits (e.g. predation, parasitism, or aggregation to enhance survival) [35], but what would be the benefit for sympatric *Anopheles* species when the result of heterospecific gathering in swarms could result in the formation of hybrids suffering fitness disadvantages? The absence of divergence in compound composition could be conceivable only if cross-attraction is not costly, or if alternative mechanisms exist to ensure reproductive isolation [35].

Second, we might ask whether long-range sex pheromones are useful in the anopheline reproductive system and why they may not have been selected during the evolution of *Anopheles* species. If they exist, long-range sexual pheromones could attract more females from a greater distance and indicate the identity of the swarming males. This is common in substrate-lekking insects such as tephritid fruit flies in which males are gathered on leaves and females can easily identify the emitting male [37,38]. However, in aerial lekking species, that is, swarming species, the role of emitted chemicals remains poorly understood and generally emitting individuals have a stationary flight during swarming [4,37]. This is a major difference compared with anopheline swarms in which a large number of males fly quickly in a limited airspace, making it challenging for females to track a pheromonal plume to its male [39]. Thus, long-range sexual pheromones in *Anopheles* mosquitoes would be of collective interest but would be costly at an individual level, as pheromone production might impose a considerable energy burden for a hypothetical benefit. Under certain circumstances, such a system could also favor the emergence of 'cheaters' that produce little or no pheromones, thereby exploiting the signal of other males [39]. Furthermore, because the odor plume of males flying downwind and away from swarms would be easier to track, females could be more likely attracted to those males before they enter swarms. Taken to their extreme, these possibilities could cause evolutionary instability of long-range sex pheromones (or even swarming behaviors altogether) due to selection for such males, further shedding doubt on a dominant role for such pheromones.

What about contact sex pheromones?

Given the insufficient evidence for long-range pheromones, let us consider whether close-range chemical cues could instead regulate reproductive barriers. Contact sex pheromones, such as cuticular hydrocarbons (CHCs), waxy molecules deposited on the surface of the insect cuticle, are known for their role in mate identification in some insects [40,41]. Profiling CHCs within the *An. gambiae* complex showed differences in relative abundance between species but has not shown any to be species specific [42,43]. Furthermore, CHCs showed greater variation depending on geographic origin rather than species identity [43]. Unfortunately, the available techniques for demonstrating causal roles of CHCs in behavior are rather crude and involve 'painting' insects with either CHCs extracted from other insects using organic solvents or, when available, commercially synthesized compounds dissolved in hexane. To date, no investigations have extracted CHCs from individuals of different *Anopheles* species and applied them to others to test whether this changes their mating success with conspecifics. It is consequently not understood whether CHCs contribute to species recognition in mating. Instead, some efforts focused on whether mating itself alters CHC profiles. Polerstock et al. [44] observed that mated *An. gambiae* females showed differences in their CHC profiles compared with unmated females (while males had no differences after mating). They investigated whether insemination rates of unmated females painted with CHC extracts from mated females were reduced. However, these experiments were inconclusive because extracts from mated females decreased insemination rates, but so did extracts from males or unmated females. Perhaps a cue for receptivity to mating is not useful in anophelines since females are unlikely to appear in mating swarms unless seeking a mate.

On the other hand, there is some evidence that cuticular cues convey signals to females about male fitness. Although females are thought to mate rapidly upon entry into a swarm, *Anopheles* females can exhibit rejection behavior [45,46], suggesting that they can discriminate among males. This was supported by a recent study by Wang et al. [47]. First, they showed that silencing a gene (*desat1*) involved in CHC biosynthesis resulted in the reduction of the CHC heptacosane in *Anopheles stephensi* males. Second, females caged with those males had reduced insemination rates. Silencing this gene also decreased flight activity, so the decrease in insemination rates here may be in part due to reduced swarming behavior, but they ultimately showed that 'painting' males with heptacosane alone also increased insemination rates in females, lending more credence to its role in mating. In another study by Adams et al. [48], *An. coluzzii* males found *in copula* with females in natural mating swarms had a higher abundance of 13 CHCs compared with other swarming males. Interestingly, heptacosane, though identified in the study, was not among the more abundant CHCs, perhaps due to species differences between *An. coluzzii* and *An. stephensi*. Nevertheless, these results suggest that CHCs may have a role to play in anopheline mating behavior.

Like the production of volatiles, contact pheromone production is likely to be energetically costly but may benefit the individual in two distinct ways. First, they are more likely to yield direct rewards to the individual insect in terms of securing a mate; and second, CHCs may directly convey fitness benefits, as these molecules also seal the cuticle against water loss [49,50], and slow the penetration of insecticides across the cuticle [51,52]. In the *An. gambiae* complex, higher total CHC content has been correlated with dry season conditions, increased insecticide resistance, and increased mating success [48,51,53–55]. It is thus interesting to consider which traits evolved first; has selection for CHCs in mating increased cuticular insecticide resistance as a by-product? Or, in environments where insecticide pressure leads to increased CHC abundance, do males that have evolved these traits mate with increased frequency? Perhaps, it is also only under certain environmental conditions, such as arid environments with insecticide pressure, that the costs of producing more CHCs are outweighed by the combined adaptive benefits in mating, desiccation tolerance, and insecticide resistance. It will be interesting for future explorations to untangle the relationships between mate selection and environmental pressures like insecticides and aridity on CHC composition and abundance, as well as fitness costs and benefits associated with these traits.

Conclusions

The validation of long-range sex pheromones would present promising opportunities for vector control. However, unequivocally proving their existence seems to be challenging. Alternative mechanisms for long-range attraction to swarming spots may exist. While auditory cues from swarms have been ruled out [24], swarm visual features such as wing interference patterns [56,57] could be considered. Additionally, environmental cues, such as swarm markers used by both sexes [12,26], would be more straightforward [6], yet their specificity and range of attraction remain unknown, necessitating further investigation.

Contact pheromones could instead provide some signal of specificity contributing to a reproductive barrier, while at the same time playing a putative role in mate choice among conspecifics, although there is not yet sufficient evidence for this. Unfortunately, close-range cues are not strong vector control targets, as they would not attract mosquitoes at a distance. However, the roles of contact pheromones in mating still bear importance for control strategies that would depend upon the ability of sterilized and/or transgenic laboratory-derived males to mate competitively in wild swarms.

Data Availability

No data were used for the research described in the article.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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