ASSORTATIVE MATING IN THE *ANOPHELES GAMBIAE* SPECIES COMPLEX

An Undergraduate Research Scholars Thesis

by

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ABSTRACT

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The *Anopheles (An.) gambiae* species complex includes some of the most significant African malaria vectors, specifically *An. gambiae* sensu stricto (s.s.), *An. coluzzii* and *An. arabiensis*. These mosquitoes are currently primarily controlled via insecticides, but the emergence of insecticide resistance necessitates improved understanding of the mosquito vectors in order to develop novel control strategies. Mating in these mosquitoes occurs in swarms. However, members of the *An. gambiae* species complex exhibit geographic and behavioral differentiation, limiting the occurrence of multi-species mating swarms. Even in such swarms, hybridization rarely occurs. In this study, we attempt to determine the frequency of insemination and interspecific mating in mixed-species cages of *An. arabiensis*, *An. coluzzii*, and *An. quadriannulatus*. Our results demonstrate that swarm composition is not likely to influence female insemination ($p>0.05$). *An. coluzzii* females in mixed swarms showed a strong preference for same-species mating ($p<0.05$). *An. quadriannulatus* females were equally likely to be mated with conspecific or heterospecific males ($p=0.1306$), suggesting no preference for mating partner. Understanding the mating behaviors of these species could help aim vector control strategies and provide insight into other traits such as host seeking and host preference.

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CHAPTER I

INTRODUCTION

Malaria

Malaria is one of the most prominent tropical diseases, causing 216 million cases and 445 thousand deaths in 2016, with 90% of cases occurring in Africa (WHO, 2017). The disease is caused by a protozoan parasite of the genus *Plasmodium*, which requires both a human host and a mosquito vector in order to complete its lifecycle (CDC, 2012). Due to the complexity of the pathogen, no vaccine is currently available, and malaria control strategies focus on the insect vector primarily through the use of insecticide treated bed nets (ITNs) and indoor residual spraying. However, the development of insecticide resistance demands the development of novel vector control approaches in order to continue suppressing disease transmission.

Anopheles gambiae Species Complex

Feeding and Swarming Behavior

Along with the *Anopheles (An.) funestus* species complex, the most important African vectors of malaria are in the *An. gambiae* species complex, which contains eight morphologically indistinguishable species that differ primarily in larval ecology and feeding behavior (Clarkson et al., 2014). *An. gambiae* and *An. coluzzii* are anthropophilic feeders, making them highly significant malaria vectors (Pates et al., 2001). *An. arabiensis*, another prominent vector, is an opportunistic feeder. *An. quadriannulatus* is zoophilic, feeding primarily on cattle, and thus is not considered a prominent malaria vector (Dekker, Takken, 1998). All *An. gambiae* complex species mate in swarms, which typically occur during the evening and are formed when male mosquitoes cluster over high-contrast environmental markers such as vegetation or footpaths (Charlwood et al., 2002). Female mosquitoes then fly into these swarms in order to mate. In order to complete egg development, inseminated females must then obtain a blood meal, potentially transmitting malaria while feeding. Gravid females oviposit directly on water sources such as rice-fields or ditches, and eggs hatch in about three days. Mosquito larvae are aquatic and primarily feed on vegetation suspended in the water. Larvae go through four instars before progressing to pupae, the second aquatic life stage. Pupae are non-feeding and spend about two days developing before emerging as adults. Adult mosquitoes typically mate within a few days of emerging (CDC, 2012).

Hybridization

In nature, hybridization between *An. gambiae* complex species is rare due to prezygotic isolation in the form of reproductive behaviors, such as different mating seasonality and geographic separation (Manoukis et al., 2009). In a study examining naturally occurring swarm compositions in two different locations, Dabire et al. (2013) observed that more than half of these swarms were spatially and temporally segregated between *An. coluzzii* and *An. gambiae*. Of 33 copulae collected from mixed-species swarms, only 4 were mixed, suggesting that even in areas where both of these species co-occur, *An. gambiae* s.s. prefers to mate assortatively.

In addition to behavioral incompatibilities, there are some postzygotic barriers as well. Crawford et al. (2015) noted several genomic barriers to hybridization, including divergent regions of the X chromosome. Slotman et al. (2003) also found that hybridization between *An. gambiae* and *An. arabiensis* resulted in male sterility and occasionally inviability due to incompatibilities between the *An. gambiae* X chromosome and regions of *An. arabiensis* autosomes. Despite these barriers to hybridization, selective pressure can drive introgression between species. Following a widespread ITN distribution in Mali, a region of the *An. gambiae* chromosome 2, including a knockdown resistance (kdr) allele, was found to have introgressed into and persisted in *An. coluzzii* populations (Norris et al., 2015). This study illustrates that, while rare, interspecies mating could have profound consequences on the dispersal of important alleles such as kdr throughout these species. A greater understanding of the mating and hybridization behavior of these species could lead to alternative control strategies, as well as inform existing control methods.

CHAPTER II

METHODS

Mosquito Rearing

Anopheles arabiensis (Dongola strain), *Anopheles quadriannulatus* (Sangwe strain), and *Anopheles coluzzii* (Mopti strain) were reared at 28° Celsius and 80-90% humidity with an LD 12:12 hour photoperiod. Larvae were reared in plastic bins filled with distilled water. Bins were split regularly to maintain a relatively constant population size in each. Larvae were fed ground TetraPro Tropical Crisps® fish food daily. Pupae were removed from the bins using a vacuum pump and placed in plastic cups within the adult cages to eclose. Adults were given a 5% sucrose diet by placing a cotton ball soaked in the sugar solution at the top of the cage. Adults were blood-fed for thirty minutes twice a week using defibrinated sheep blood (HemoStat Laboratories). Moist filter paper was provided after blood feeding in order to collect eggs, which were then placed in new larval bins filled with distilled water.

Cage Setup

Pupae of each species were sexed using the *Methods in* Anopheles *Research, 4th edition* manual (Benedict, 2014) and the sexes were placed in separate cages to eclose. Once eclosed, adults were aspirated into experimental cages. For the control cages, 50 females and 50 conspecific males were aspirated into a cage, while 50 females and 50 heterospecific males were grouped in a separate cage. For the competition cages, 50 females of one species and 25 males of each species were grouped in a cage. Cages were given constant access to sucrose solution but were not blood fed. Mosquitoes were allowed approximately five days to mate before being collected.

Spermathecae Dissection

Dissection methods were adapted from Tripet et al., 2001. Female mosquitoes were killed by being placed at -20 °C for about ten minutes and were stored in 70% ethanol to dehydrate. Dehydration allows for coagulation of the proteinaceous fluid in the spermatheca. Mosquitoes were rehydrated for about three days prior to dissection. Mosquitoes were placed ventral side up in a drop of distilled water on a glass slide. Under a dissecting microscope, a dissection needle was used to pull off the last segment of the abdomen, removing the spermatheca as well. The spermatheca was then viewed under a compound microscope at 40X to 400X magnification to determine whether or not the mosquito was inseminated. If the female mosquito was from a competition cage and was inseminated, then the spermatheca was cleared of as much maternal tissue as possible and collected in 20 microliters of deionized water.

Species Identification

DNA Extraction

Samples were ground using sterile pestles in 20 microliters of deionized water and centrifuged at 12,000 rpm for three minutes. 6% InstaGene Matrix was mixed using a stir plate, and 200 microliters of InstaGene Matrix was added to each sample. Samples were incubated for 30 minutes at 56 °C, vortexed for ten seconds, then placed in a 90 °C water bath for ten minutes. Samples were then vortexed for another ten seconds and then centrifuged at 12,000 rpm for three minutes. The supernatant was removed and stored at -20 °C.

Species-Diagnostic PCR

The species identities of the samples were determined using the species-diagnostic ribosomal DNA polymerase chain reaction protocol established by Scott et al. (1993) and Fanello et al. (2003), except that the number of cycles was increased to 40 and the amount of primer per reaction was increased to 0.6 microliters. The PCR reaction uses species-specific nucleotide sequences in the intergenic spacers (rDNA IGS) and the 28S coding region of the ribosomal DNA. The protocol uses a universal primer, an *An. arabiensis* specific primer (315 bp PCR product), an *An. quadriannulatus* specific primer (153 bp PCR product), and an *An. coluzzii* specific primer (390 bp). PCR results were visualized using gel electrophoresis.

Statistical Analysis

The proportion of inseminated females from each cage was calculated, along with a 95% confidence interval. For each species of female, a two-proportion z-test was used to compare the proportion of inseminated females in heterospecific and mixed-species cages against the insemination rates of females in conspecific cages, and a sequential Bonferroni correction for multiple tests was applied. A one proportion z-test was used to determine the significance of the proportion of heterospecific matings in competition cages against the null hypothesis H_0=0.5 (no male preference).

CHAPTER III

RESULTS

Insemination

Insemination was determined for female mosquitoes in swarms of conspecific, heterospecific, and mixed-species cages. The insemination rate in conspecific cages was compared against insemination in heterospecific and competition cages via a two-proportion z-test, and the p value was adjusted using a sequential Bonferroni correction.

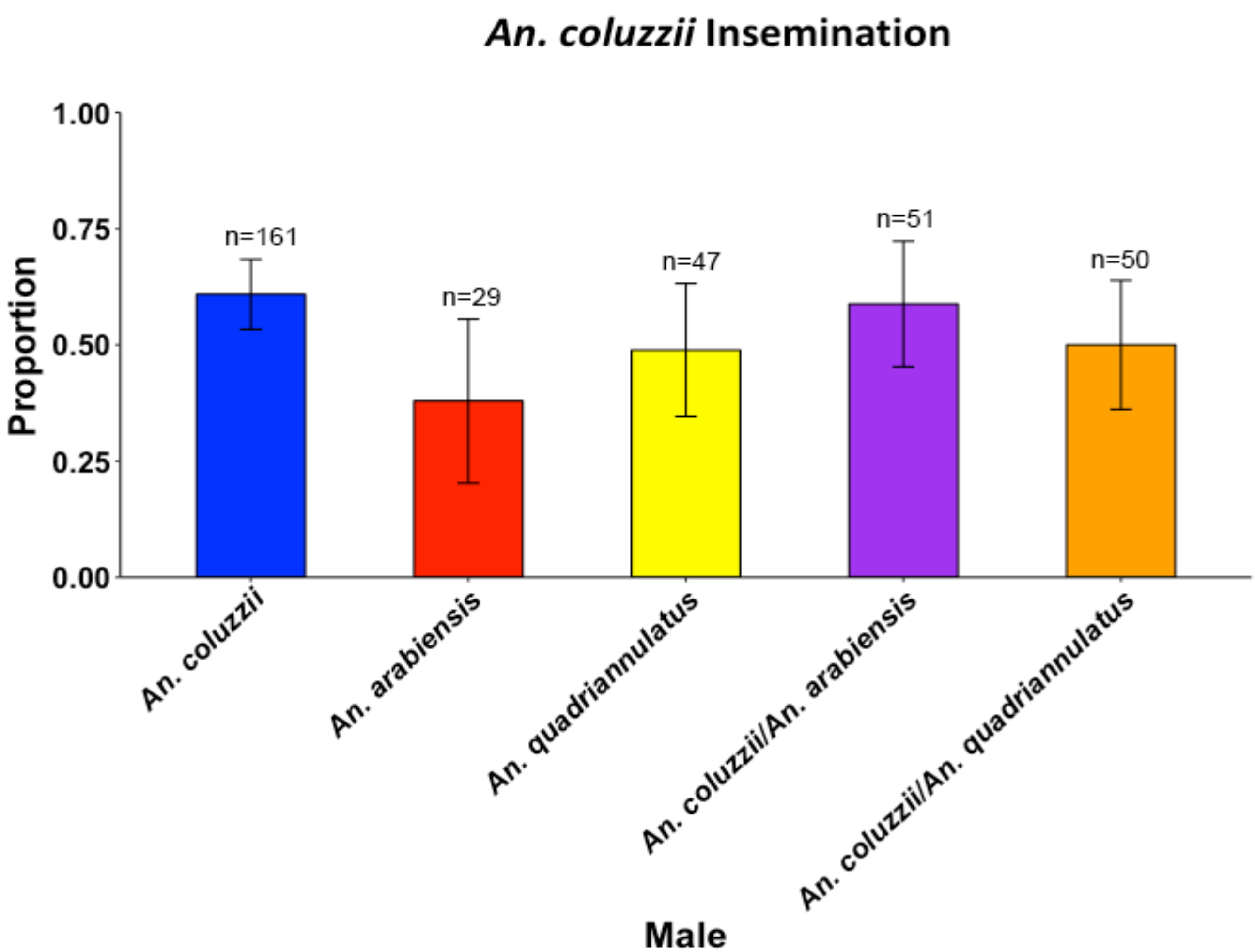

Figure 1: Insemination of *An. coluzzii* females. Male swarm composition is indicated on the x-axis. N represents the total number of females analyzed.

An. coluzzii insemination was analyzed with conspecific males, *An. arabiensis* males, *An. quadriannulatus* males, and in cages with multiple species of males. No significant differences could be detected between the proportions of inseminated *An. coluzzii* females in different male swarms (p>0.05); however, insemination was highest in *An. coluzzii* male swarms (60.87% of females inseminated) and lowest in *An. arabiensis* male swarms (37.93% of females inseminated).

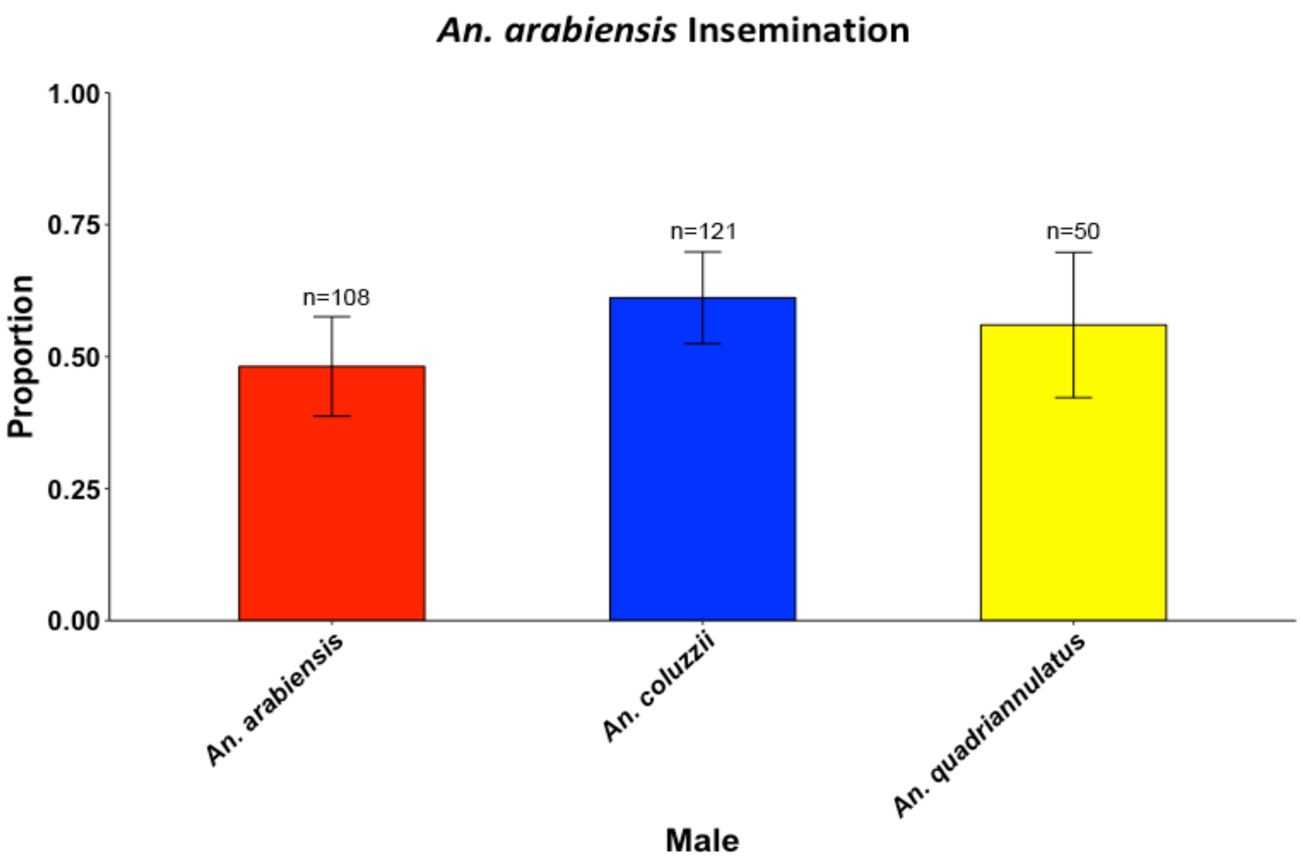

Figure 2: Insemination of *An. arabiensis* females.

An. arabiensis insemination was tested with conspecific males, *An. coluzzii* males, and *An. quadriannulatus* males. No significant differences were detected in *An. arabiensis* female insemination in different swarms (p>0.05), but insemination was highest in *An. coluzzii* male swarms (61.16%) and lowest in *An. arabiensis* male swarms (48.15%).

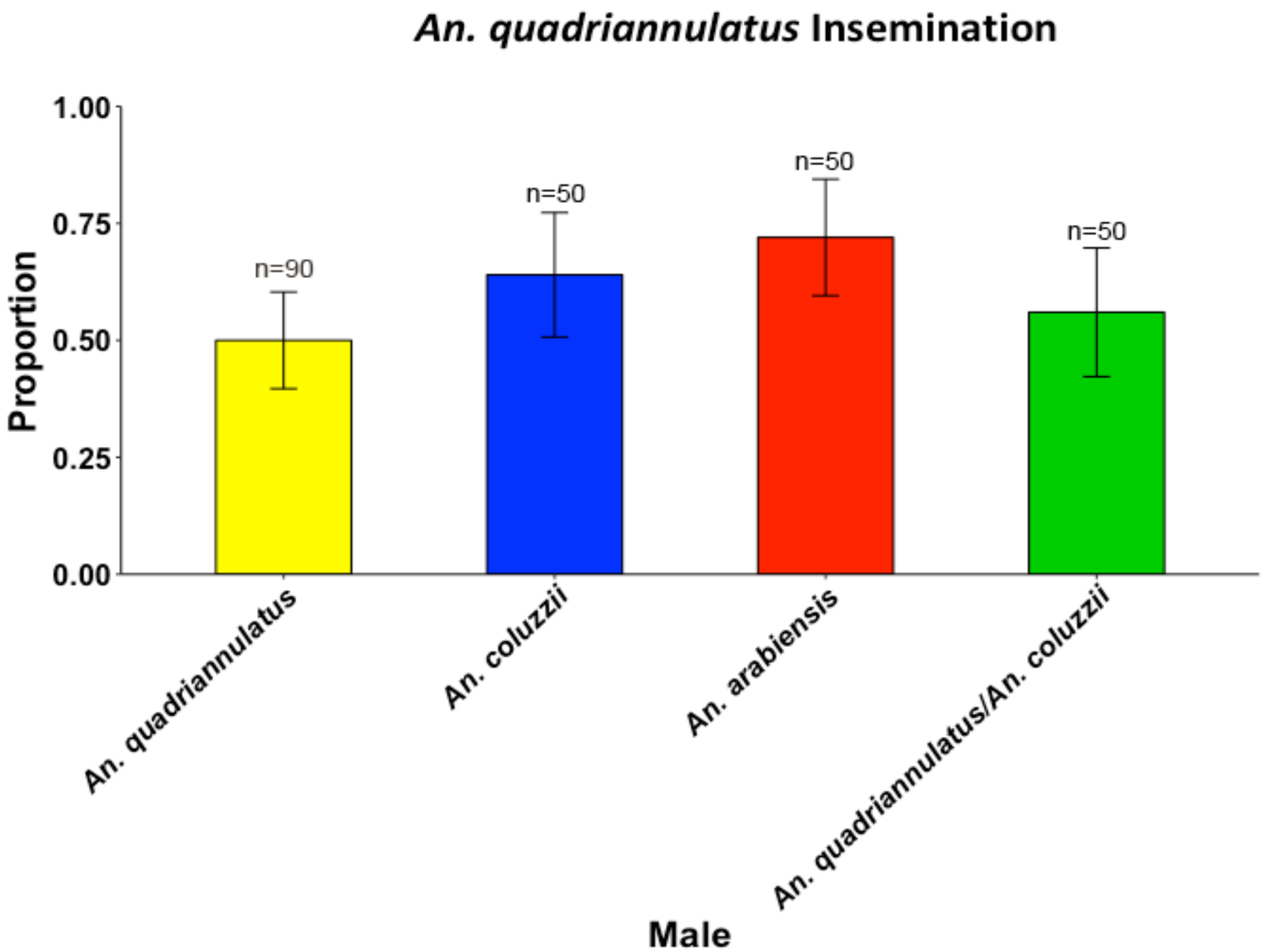

Figure 3: Insemination of *An. quadriannulatus* females.

An. quadriannulatus insemination was analyzed with conspecific males, *An. coluzzii* males, *An. arabiensis* males, and in one cage of multiple species of males. No significant differences were detected in *An. quadriannulatus* female insemination ($p>0.05$). However, insemination was highest in *An. arabiensis* male swarms (72%) and lowest in *An. quadriannulatus* male swarms (50%).

Mate Choice

Inseminated females from cages with mixed-species males were analyzed to determine the species identity of the female's mating partner. The proportion of heterospecific matings in each cage were compared to the null hypothesis $H_0 = 0.5$, which would indicate random mating.

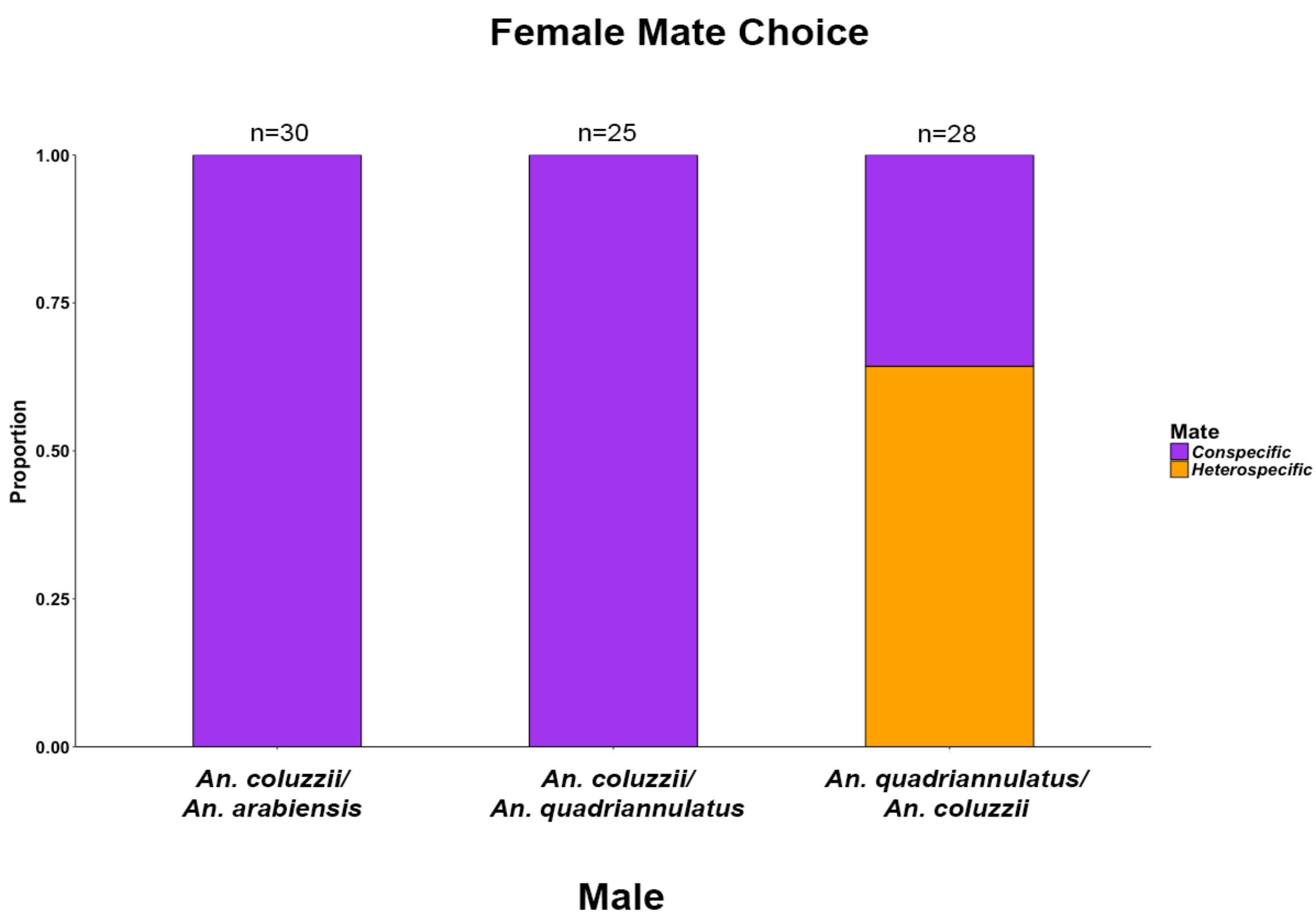

Figure 4: Mate choice of females in mixed male swarms. The first two bars represent *An. coluzzii* females' mate choice, and the third bar represents *An. quadriannulatus* females. N represents the total number of inseminated females analyzed.

For both cages with *An. coluzzii* females, all inseminated females were found to have mated with conspecific males ($p<0.05$). *An. quadriannulatus* females, in contrast, mated with heterospecific males 64.3% of the time, about what would be expected with random mating ($p=0.1306$).

CHAPTER IV

DISCUSSION

Insemination

In all species, female insemination in heterospecific and mixed male cages did not differ significantly from insemination in conspecific male cages, which indicates that female mosquitoes are equally likely to mate in all swarms, regardless of the composition of males. Interestingly, this also suggests that females with a strong male preference, such as *An. coluzzii*, will mate with heterospecific males if nothing else is available. In the future, conditions affecting this behavior could be tested. For example, keeping a small number of conspecific males nearby but inaccessible for mating may influence the likelihood of females to be inseminated by accessible heterospecific males. If female mosquitoes are able to detect even unavailable conspecifics they may be less likely to mate with heterospecifics. Additionally, mosquitoes in this experiment were given five days to mate, but when exactly females were inseminated was unknown. It is possible that species with a strong male preference may take longer to mate with heterospecifics than with conspecifics. In future replicates, samples could be dissected at earlier time points, as opposed to the five days used in this experiment, in order to determine when female mosquitoes mate in different swarms.

Finally, sample sizes in some replicates are currently small (such as *An. coluzzii* females with *An. arabiensis* males, where n=29), which limits statistical power. In the future, increasing the sample sizes for these replicates will be necessary to determine if current trends in the data are significant.

Mate Choice

An. coluzzii females in mixed-species cages exhibited a strong preference to mate with conspecific males, with 100% of inseminated females having mated with *An. coluzzii* males. This finding supports observations of mating behavior in the field, where mixed copulae in *An. coluzzii* and *An. gambiae* swarms occurred infrequently (Dabire et al., 2013). This finding also supports the idea that mosquitoes are able to recognize conspecifics at close range, and while the exact mechanism for this mate recognition is not precisely known, the genetic basis of this preference has been shown experimentally. Aboagye-Antwi et al. (2015) found that *An. gambiae* and *An. coluzzii* mosquitoes could be induced to mate with heterospecifics when an island of divergence on the X-chromosome was swapped between these species, demonstrating that this region of the genome influences mate choice. It has also been hypothesized that mosquitoes may modulate wing beat frequencies in order to recognize potential mates at close range (Pennetier et al., 2010; Sanford et al., 2011). This matching of flight tones, referred to as harmonic convergence, ostensibly minimizes the incidence of mixed-species mating and has been proposed as a mechanism for assortative mating in this species complex, although further research is needed in this area.

An. quadriannulatus females, in contrast to *An. coluzzii*, did not exhibit significant male preference in a mixed cage of *An. coluzzii* and *An. quadriannulatus* males: 64.3% of inseminated females mated with *An. coluzzii* males. This suggests that *An. quadriannulatus* females mate randomly under these circumstances, potentially due to an inability to distinguish conspecific from heterospecific males. It is possible that, in the wild, *An. quadriannulatus* swarms do not co-occur with other species' swarms, making a robust close-range mate recognition mechanism unnecessary in *An. quadriannulatus* females. By contrast, *An. coluzzii* has been documented to co-occur in swarms with other species such as *An. gambiae* and so is presumably under a greater selective pressure to develop methods of identifying conspecifics at short range (Dabire et al., 2013). However, as there has been little investigation into the swarming behaviors of *An. quadriannulatus*, this explanation is speculative, and more research is needed to fully explain this finding. It is also possible that *An. quadriannulatus* females in the wild do exhibit assortative mating, but that rearing conditions in the lab have changed this behavior. In a study examining the survival and mating success of *An. coluzzii* Mopti strain, Paton et al. found that lab strains reared indoors lost the ability to distinguish between conspecific and heterospecific males, highlighting the importance of rearing conditions on mating behavior (2013). The authors also noted that this experiment was performed with a relatively easy to colonize strain; this loss of assortative mating preference would presumably be more severe in difficult to colonize species such as *An. quadriannulatus*. This may explain why, in our results, *An. coluzzii* retained a strong assortative mating preference but *An. quadriannulatus* did not. Due to its zoophilic feeding behavior, *An. quadriannulatus* has been regarded as largely insignificant to malaria transmission; however, geographic overlap with other species potentiates gene flow between these species, which could result in the dispersal of resistance alleles if this random mating occurs in the wild (Coetzee et al., 2000; Norris et al., 2015). It may therefore be important to consider the mating behavior of *An. quadriannulatus* when implementing vector control strategies.

Future replicates should include *An. arabiensis* in mixed-species cages in order to determine male preference with both *An. coluzzii* and *An. quadriannulatus* males. Evaluating *An. quadriannulatus* females in cages of conspecific and *An. arabiensis* males is also of interest to determine if females continue to show random mating under these conditions; if *An. quadriannulatus* and *An. arabiensis* swarms co-occur more frequently in nature than *An.*

quadriannulatus and *An. coluzzii* swarms, then it is possible that *An. quadriannulatus* females may discriminate between conspecifics and *An. arabiensis* males. Additional trials could also test the extent of *An. coluzzii* male preference by altering the proportion of male mosquitoes in each cage such that conspecifics are outnumbered by heterospecifics.

Our results indicate that there is little difference in female insemination in different swarm compositions, but that *An. coluzzii* females prefer to mate with conspecific males in mixed swarms while *An. quadriannulatus* females show no preference for male species. However, additional work is needed to determine the behavior of *An. arabiensi*s females in mixed swarms, and to test conditions affecting mate choice.

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