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CHARACTERIZATION OF MALE AND FEMALE WINGBEAT FREQUENCIES IN THE *ANOPHELES QUADRIMACULATUS* COMPLEX IN MISSISSIPPI

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ABSTRACT. Classifying individual mosquitoes of the *Anopheles quadrimaculatus* species complex to species has always been time consuming and complex, involving genetic analysis and profiling. We characterized the wingbeat frequencies of the 3 species of the *Anopheles quadrimaculatus* complex (*An. quadrimaculatus, An. smaragdinus,* and *An. maverlius*) that occur in Mississippi to determine if this character could successfully distinguish between individuals of the 3 species. Wingbeats of females varied from 320 to 480 beats/sec, but no significant differences in wingbeat frequencies were found among females from 3 populations of the 3 species. Wingbeats of males were higher than those of females (500–770 beats/sec), and the mean wingbeat frequency of *An. maverlius* was significantly different from the means of *An. quadrimaculatus* and *An. smaragdinus,* although overlap occurred at the individual level. Such overlap precludes use of wingbeat frequencies as an identification mechanism, and indicates that, at least for the *An. quadrimaculatus* complex, wingbeat frequencies are not involved in mate recognition.

KEY WORDS Wingbeat, sexual differentiation, species identification

INTRODUCTION

The common malaria mosquito species complex *Anopheles quadrimaculatus* Say consists of at least 5 different sibling species that can be most reliably distinguished by utilizing genetic profiles (Narang et al. 1989a, Lanzaro et al. 1990). Members of this complex are among the most important anopheline mosquitoes in the southeastern USA (Kaiser et al. 1988), and have in the past been important vectors of malaria. With the development of insecticide resistance in many anopheline species (Metcalf 1989) and the resurgence of malaria worldwide (Rogoff 1985), the *An. quadrimaculatus* complex remains a potential threat to human health.

The individual species of the *An. quadrimaculatus* species complex vary in behavior and ecology, and may vary in their competency to vector the malaria parasite. Research on the species complex is hampered by the difficulties in identifying individual mosquitoes to species. At present, genetic profiles based on electrophoresis (Narang et al. 1989a, 1989b; Lanzaro et al. 1990) or DNA molecular markers (Johnson et al. 1992, 1993; Cornel et al. 1996) are required for each individual. In other genera of the Culicidae, wingbeat frequency differences have been used to automate detection of sex and species. Moore et al. (1986) used an optical tachometer to identify *Aedes aegypti* (L.) and *Ochlerotatus triseriatus* (Say) adults to sex and species with 84% accuracy. A trained neural network significantly increased the number of correct classifications (Moore 1991), suggesting that characteristics of the wingbeat frequency spectrum other than the fundamental frequency could be important in identifying species (e.g., relative height or shape of the harmonics). Such a technique could be used to monitor anopheline populations in the field, with the potential for remote, automated species identification. Automated identification could be used in situations where molecular identification procedures would be impractical, but use of the technique would be limited to live adults.

In addition to testing the feasibility of the use of wingbeat tones in automated species identification, we were also interested in the biological factors that determine wingbeat frequency. Wingbeat tones are known to play a role in mating responses of *An. quadrimaculatus* (Roth 1948) as well as other anopheline species (Brogdon 1994, 1998). Tones similar to those produced by the wingbeats of female *An. quadrimaculatus* clearly elicit mating behaviors in receptive males. If wingbeat frequencies played a role in species isolation and were used by males to identify females of the same species, one would expect differences among wingbeat tones of females. In contrast, if males respond to a relatively broad range of wingbeat tones, stabilizing sexual selection might maintain similar wingbeat frequencies in females despite the potential for genetic drift after speciation.

We characterized the wingbeat frequencies of the 3 *An. quadrimaculatus* complex species that occur in Mississippi (*An. quadrimaculatus* Say, *Anopheles smaragdinus* Reinert, and *Anopheles maverlius* Reinert) (Reinert et al. 1997) and determined if these characters can be used to identify species of this complex.

MATERIALS AND METHODS

Anopheles quadrimaculatus mosquitoes were collected with mechanical aspirators on 3 different dates in the Noxubee Wildlife Refuge, Noxubee County, Mississippi (August 13, 1993; September

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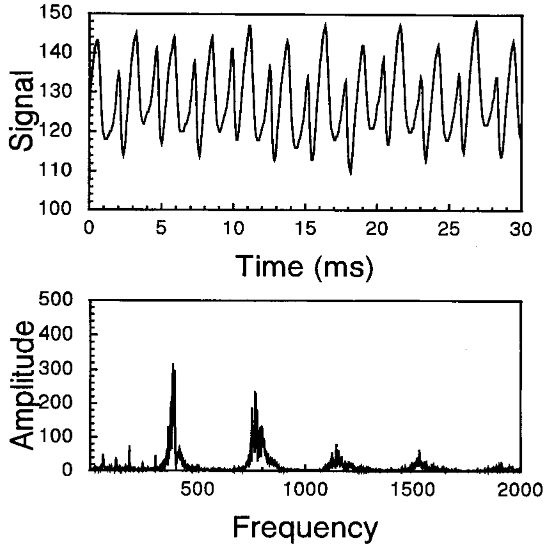

Fig. 1. The signal input from the optical tachometer for an individual mosquito and the result of the fast Fourier transformation showing a peak frequency of approximately 445 beats/sec.

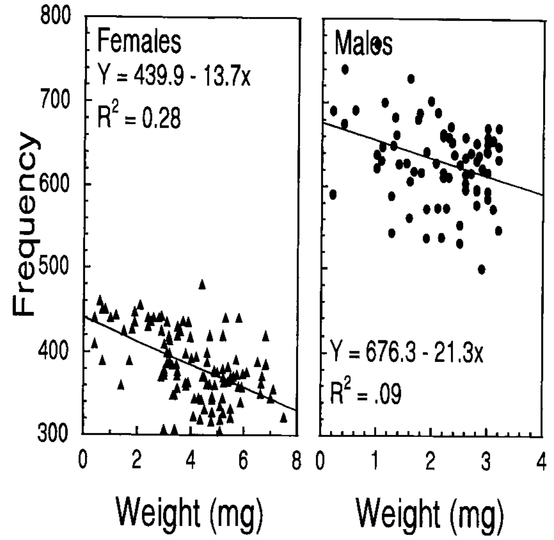

Fig. 2. Regression of weight on wingbeat frequency for both males and females of the 3 species of *Anopheles quadrimaculatus* found in Mississippi (A, B, and D). The regression coefficients (−21.3 beats/mg for males and −13.7 beats/mg for females) were not significantly different from each other but were significantly different from 0.

2, 1993; and July 7, 1995) and twice from Tishomingo County, Mississippi (August 23, 1993, and September 20, 1995). Samples were returned to the laboratory and wingbeat frequencies were estimated after 12 h and within 48 h. The waiting period allowed young males to mature because wingbeat frequencies of male *An. quadrimaculatus* rise for 1–2 days after eclosion (Roth 1948, Moore et al. 1986, Robertson 1997).

Wingbeat frequencies were measured by placing each insect in a scintillation vial and flying the insect for approximately 2 sec between a light source and an optical tachometer (Moore et al. 1986). All measurements were made at 26°C. The output of the tachometer was recorded on tape and later digitized using a 16-bit sound card on an IBM PC compatible computer. The sound was recorded at 14,000 samples/sec and 0.5 sec of each sample was selected for analysis. The digitized sound was then analyzed with the fast Fourier transform function in MathCad (Fig. 1; MathSoft Inc., Cambridge, MA.). Wet weights were recorded for individual insects to determine if wingbeat frequency was correlated with weight.

Statistical analysis: Data were analyzed using analysis of covariance, with weight as a covariate and sex and species as categorical effects. In preliminary analyses the covariate by categorical effect term was included to test for the assumption of homogeneity of slopes across each category (Wilkinson et al. 1996).

Insect identification: After wingbeat frequency measurements, insects were stored at −80°C and identified to species by electrophoresis. Individuals were homogenized in 0.05 M Tris, 1 mM ethylenediaminetetraacetic acid, and 0.2% mercaptoethanol and run on 12% starch shells (Sigma Chemical, St. Louis, MO) using a CA-8 buffer system (Steiner and Joslyn 1979). Gels were run for 16 h and stained for the following enzyme systems: isocitrate dehydrogenase (IDH 1.1.1.42, 2 loci), glutamate oxaloacetate transaminase (GOT 2.6.1.1, 2 loci), mannose phosphate isomerase (MPI 5.3.1.8, 1 locus), glucose-6-phosphate isomerase (GPI 5.3.1.9, 1 locus), malic enzyme (ME 1.1.1.40, 1 locus), malate dehydrogenase (MDH 1.1.1.37, 2 loci), and hydroxyacid dehydrogenase (HAD 1.1.1.30, 1 locus). Individuals were identified to species by genetic profiles (Narang et al. 1989a, 1989b; Lanzaro et al. 1990).

RESULTS AND DISCUSSION

Wingbeat frequencies between males and females were significantly different (F = 45.38, df = 1,180, $P < 0.001$), so subsequent analyses were performed separately for each sex. Regressions of weights on wingbeat frequencies were significant for each sex (Fig. 2), but preliminary analysis of covariance including the species by weight interaction was not significant for either sex (males: F = 1.23; df = 2, 71; $P = 0.296$; females: F = 1.671; df = 2,109; $P = 0.193$), suggesting that the assumption of homogeneity of slope across species for the analysis of covariance was not violated (Wilkinson et al. 1996). The mean effect of weight on the wingbeat frequency trait was a reduction in wingbeat frequency of 21.3 beats/mg for males and

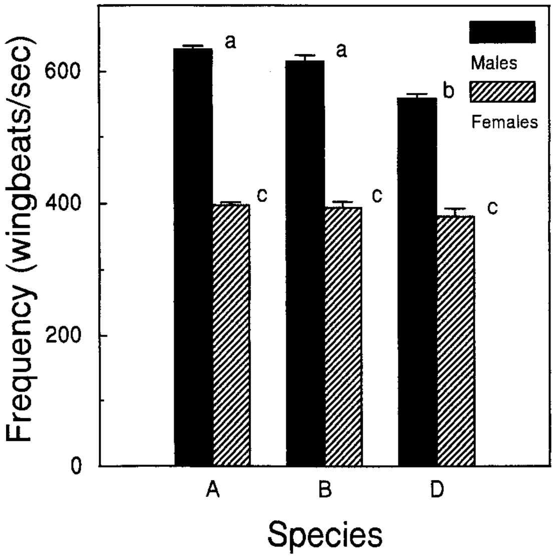

Fig. 3. Mean wingbeat frequencies for males and females (±SEM) for each of the 3 species of the *Anopheles quadrimaculatus* complex that occur in Mississippi. Bars with the same letter do not differ significantly from each other.

13.7 beats/mg for females. The slopes of the regressions of weight versus sex were not significantly different, as indicated by the lack of significance of the sex by weight interaction (F = 2.53; df = 1,180; P = 0.113).

Mean wingbeat frequencies of females did not differ significantly between the 3 *An. quadrimaculatus* species found in Mississippi (Fig. 2; F = 0.972; df = 2,111; P = 0.382). The measured frequencies corresponded well with the range (320–480 beats/sec) of response of receptive *An. quadrimaculatus* males to tuning forks (Roth 1948). Significant differences were found among the mean frequencies of the males of the 3 species (Fig. 2; F = 8.91; df = 2,73; $P < 0.001$). Using a Bonferroni adjustment for multiple pairwise comparisons (Wilkinson et al. 1996), the mean wingbeat frequency of males of *An. maverlius* was found to be significantly lower than the means for males of *An. quadrimaculatus* and *An. smaragdinus,* whereas no significant difference was found between the latter 2 species (Fig. 3). However, significant overlap of individual wingbeat frequencies occurred among the 3 species. This overlap precludes use of the fundamental wingbeat frequency alone as a method to identify individual mosquitoes to species, although other characteristics of the wingbeat frequency spectrum possibly could improve species classification (Moore 1991).

The overlap in wingbeat frequencies also suggests that it is unlikely that wingbeat frequencies could be related to species isolation in the *An. quadrimaculatus* species complex. Although females conceivably could use the differences among mean wingbeat frequencies of male mating swarms as a location cue, females have never been demonstrated to respond to male wingbeat tones. Females possibly use the same visual or spatial cues to locate appropriate swarming sites that males seem to use. Wingbeat frequencies of both males and females differed significantly among closely related species in the *Anopheles gambiae* complex, in contrast to the results reported here. These differences may be in part due to the isolating mechanisms in each complex as well as potential differences in mating behavior. *An. gambiae* species form evening swarms (Charlwood and Jones 1980, Marchand 1984). We have been unable to find any reports of similar swarming behavior in the *An. quadrimaculatus* complex and have had no success in locating such swarms. Our results suggest that further research on the mating behavior in the *An. quadrimaculatus* complex is necessary.

Variation in wingbeat tone among the species may be related to differences in selection on this trait. If female wingbeat tones are used by males to identify potential mates from close distances, stabilizing selection could maintain wingbeat frequencies of females within a limited range. In contrast, lack of similar selection on wingbeat tones of males would allow differentiation among species because of genetic drift during or after speciation if the wingbeat trait in males and females is not completely governed by the same or tightly linked genes. This is supported by heritability estimates that suggest higher levels of additive genetic variation are maintained in males than females (Caprio, unpublished data).

Although the perception of auditory stimuli plays an important role in culicine reproductive biology (Roth 1948), these data suggest that substantial phenotypic variation occurs in wingbeat patterns among individuals and overlap occurs between individuals of the different species of the *An. quadrimaculatus* species complex. This overlap limits the potential use of wingbeat patterns as a species-specific mate-location stimulus, although the use of wingbeat patterns as a potential general mate or swarm location stimulus is not negated.

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