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Biology and Ecology of the Anopheles Quadrimaculatus Sibling Species Complex in Louisiana.

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BIOLOGY AND ECOLOGY OF THE ANOPHELES QUADRIMACULATUS SIBLING SPECIES COMPLEX IN LOUISIANA

A Dissertation

Submitted to the Graduate Faculty of the Louisiana State University and Agricultural and Mechanical College in partial fulfillment of the requirements for the degree of Doctor of Philosophy

in

The Department of Entomology

by

Cynthia Roxanne Rutledge
B. S., Louisiana State University, 1992
M. S., Louisiana State University, 1995
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Abstract

A polymerase chain reaction method for identifying individuals in the *Anopheles quadrimaculatus* Say sibling species complex was validated for wild mosquitoes from Louisiana and Mississippi. This method distinguished *An. quadrimaculatus* species A, B, C, and D by detecting species-specific differences in the 2nd internal transcribed spacer of ribosomal DNA and was 100% specific and 95% sensitive.

A polymerase chain reaction assay that detects differences in the second internal transcribed spacer of ribosomal DNA was tested for its usefulness in identifying all immature stages of two representatives in the *An. quadrimaculatus* sibling species complex. The technique was successful in amplifying DNA from eggs, 1st through 4th instars, and pupae of *An. quadrimaculatus* Say and *An. imundatus* Reinert.

Attempts were made to colonize four of the five sibling species of the *Quadrimaculatus* Complex. Field-collected mosquitoes were brought into the laboratory to use as starting material for colonies. No colonies were established during this study. The approach taken for this research was too broad and a better approach would be to spend more time on one of the species and study a select, small number of natural populations.

Sixteen larval habitats of sibling species of *An. quadrimaculatus* were characterized during 1997-98. Larval mosquitoes were identified using PCR. Habitat descriptions were made along with water analysis for inorganic ions. *Anopheles quadrimaculatus* Say was present in fifteen of the sites. *Anopheles smaragdinus* larvae were not collected from any site. *Anopheles imundatus* was the only species collected.
from one habitat, and was found in association with *An. quadrimaculatus* at another site. *Anopheles maverlius* was collected along with *An. quadrimaculatus* from one larval habitat. The water analysis from the different habitats could not be statistically compared due to a lack of locating a larger number of sites with species other than *An. quadrimaculatus*.

Sixty-two mosquitoes were tested for the presence of the intracellular parasite *Wolbachia pipiens* which causes cytoplasmic incompatibility in some species of insects. Using an established PCR protocol, the rickettsia was not detected in any of the specimens.
Chapter 1

Introduction

Anopheles quadrivaculatus Say, the common malaria mosquito, was originally described by Thomas Say in 1824 (Carpenter and LaCasse 1955). This species has recently been shown to be a sibling, or cryptic species complex of at least 5 morphologically similar species. They were described and named by Reinert et al. (1997) as An. quadrivaculatus sensu stricto (s.s.) Say, An. smaragdis Reinert, An. diluvialis Reinert, An. inundatus Reinert, and An. maverlius Reinert. In an integrated approach to identifying the sibling species, use of all of the following methods have been employed: morphologic, cytologic, genetic, biochemical, and ecologic techniques. These methods along with distribution studies are discussed by Reinert et al. (1997) and listed in Reinert’s (1997) Bibliography of Anopheles quadrivaculatus Say sensu lato (s.l.).

Sibling species, those species that are morphologically similar yet reproductively isolated, are important historically, for showing that species can exist that are morphologically indistinct yet separate species. The discovery that sibling species of mosquitoes have different vector potentials makes the recognition of species complexes an important role in mosquito identification. The An. gambiae complex demonstrates the importance of proper identification. There are six species in the gambiae complex, and only two of these are considered to be important malaria vectors.

One of the classical cases of differentiation of sibling species is the An. maculipennis complex in Europe (Hackett and Missiroli 1935; Bates 1940). This was the first in depth study of a species complex involving an insect of medical importance.
Initially researchers noticed that cases of malaria and the distribution of *An. maculipennis* were not closely correlated. A retired sanitary inspector in Italy published results showing the differences in the patterns of eggs from *An. maculipennis* (Falleroni 1926). Later, studies showed that malaria was associated with females that laid gray eggs and was not present in areas where the females laid dark eggs (Kettle 1990). There are six sibling species and one subspecies recognized within the complex (Knight and Stone 1977). There are differences that exist between members of the *An. maculipennis* complex; one being that three are malaria vectors and three are not. Additionally, one species will mate in a small cage without swarming, another requires a large outdoor cage, and others will not mate in captivity (Mayr 1942).

*Anopheles quadrimaculatus* s.l. is considered the most important vector of malaria in the southeastern United States. By 1875, malaria had reached its peak in the U.S., but remained one of the most important diseases affecting humans in the South until the 20th century (Duffy 1988). Citizens in the U.S. are still at risk for contracting malaria. Risk factors are increased by the rising number of travelers visiting areas where malaria is endemic. If returning travelers are infected with one of the malaria parasites, there is a chance that their illness will be misdiagnosed because a majority of physicians in the U.S. have never treated malaria. Gametocytemic persons (immigrants and native-born U.S. civilians) are present in the U.S. and potentially serve as reservoirs of the parasite (Zucker 1996). This, in addition to the fact that the mosquito vectors are still present in this country, serves as a warning for potential outbreak situations.
In more recent times, *An. quadrimaculatus s.l* has been considered a pest of humans and their companion animals. The range of this species is east of the Rocky Mountains to the East Coast, north to Canada, and south to the Gulf of Mexico (Darsie and Ward 1981). It has been recorded from all 64 parishes in Louisiana (Chapman and Johnson 1986).

The larvae of *An. quadrimaculatus s.l* are found in permanent fresh water of lakes, ponds, flooded rice-fields, and slow moving streams containing emergent vegetation or floating debris. The larval and pupal periods are approximately 12 to 30 d and 2 to 6 d, respectively. The time period is dependent on temperature and available food. The number of generations have been reported to be from 7 to 10 per year. Adults seek shelter during the day in dark corners and search for blood meals at night. The overwintering stage is reported to be inseminated females. Eggs are laid singly on the water surface. Females will lay eggs about 3 d post-emergence if a blood meal has been consumed. Blood hosts of *An. quadrimaculatus s.l* include deer, rabbit, cattle, horse, human, dog, racoon, opossum, and pig. The flight range of the adult varies, perhaps dependent on proximity to blood hosts (Keener 1945). Typical flight range has been reported to be 1 mile or less.

A detailed knowledge of the life history of mosquitoes is often the key to control them and the disease pathogens which they transmit. Previous work reported on *An. quadrimaculatus* has been based on a single species. In light of the discovery of a species complex, some of this research should be revisited. Much information on the ecology of adult and immature stages, maximum geographic ranges, and vector abilities
of the *An. quadrimagulatus* complex is unknown (Reinert 1997). Before reliable research can be done to determine vector capacity for the individual sibling species, laboratory colonies must be established in order to have mosquitoes available when needed for malaria transmission studies. It also is important to have these colonies established for insecticide tolerance bioassays and other studies relating to the individual species.

Foster (1980) reported on the colonization and maintenance of mosquitoes and stressed that the literature on mosquito rearing is diffuse and often redundant. Most published rearing techniques are methods that are successful, not necessarily the most productive and not usually of interest to malariologists. He gives details for rearing mosquitoes, but these methods are not specific for *An. quadrimagulatus s.l.* Gerberg et al. (1994) give details for the rearing of *An. quadrimagulatus s.l.*; however, there is no published literature specific for colonizing individual species of the *An. quadrimagulatus* complex.

Robertson (1997) studied the mating behavior and heritability of wing-beat frequency in *An. quadrimagulatus* sibling species. He concluded that the mosquitoes are poorly adapted to laboratory conditions and those that are captured in the wild often fail to mate in captivity. More often than not, the females will not oviposit unless induced to do so by trauma by removing a wing. He states that the low level of mating activity that takes place in cages would make it necessary to use forced mating. This limitation makes mass rearing difficult.
Perhaps a key to colonizing a species lies in uncovering components in their natural habitats. Specific characteristics in larval habitats and in oviposition behavior may be two of these keys.

Larval habitats are determined by the female in her preference for an oviposition site (Foster 1980). The female An. quadrimaculatus (s.L) lays her eggs in neutral or alkaline ponds (Buxton and Leeson 1949). Oviposition site selection is a result of chemical and physical factors (Bentley and Day 1989), and for many species, the selection is influenced by the presence of chemical substances.

Differences in larval ecology may explain the existence of sympatry in adult sibling species (Charlwood and Edoh 1996). However, according to Laven (1967), new species of Culex were arising by a form of sympatric speciation due to cytoplasmic incompatibility. Yen and Barr (1974) showed that this incompatibility was due to a bacterial symbiont, Wolbachia pipiens, that exists in members of the Cx. pipiens complex. The rickettsiae is transmitted through the cytoplasm of eggs and also are transmitted horizontally between arthropod species (Werren 1997). The presence of W. pipiens in a host can cause cytoplasmic incompatibility between strains and related species. Wolbachia pipiens has been found in over 80 species of insects making it the most abundant of parasitic bacteria (Werren 1997).

Wolbachia pipiens is a fastidious bacterium that is difficult to culture outside the host. O’Neill (1997) published a protocol for the detection of W. pipiens using a PCR technique that amplifies the genes of the bacteria from a mixture of insect DNA. With the advent of this technique, O’Neill predicts that hundreds of thousands of insect
species will be shown to have the agent, including a number of insect disease vectors. There are no published reports on the detection or screening for *W. pipientis* in *An. quadrimaculatus s.l.* to date.

The objectives of this research are: 1) to verify that the molecular technique of PCR is applicable to sibling species of the Quadrimaculatus Complex that are present in Louisiana; 2) to test the PCR assay on immature stages of *An. quadrimaculatus* sibling species; 3) to establish laboratory colonies of those siblings species present in Louisiana and other selected habitats; 4) to characterize larval habitats of the sibling species and 5) to screen wild populations of *An. quadrimaculatus s.l.* for *W. pipientis* using an established PCR protocol.

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**Darsie, R. F., Jr., and R. A. Ward.** 1981. Identification and geographical distribution


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CHAPTER 2

Validation of an Ribosomal DNA-Polymerase Chain Reaction Species Diagnostic Assay for Members of the Common Malaria Mosquito (Diptera: Culicidae) Sibling Species Complex

Introduction

A POLYMERASE CHAIN reaction (PCR) method for identifying individual mosquitoes in the *Anopheles quadrimaculatus* Say sibling species complex was developed by Cornel et al. (1996). This method, which detects species-specific differences in the 2nd internal transcribed spacer (ITS2) of ribosomal DNA (rDNA), is capable of distinguishing *An. quadrimaculatus* species A, B, C, and D. Distinguishing species C	ext{1} from species C	ext{2} currently is not possible with this assay. The original development and testing of the rDNA—PCR assay were based on specimens collected only in Florida. Because populations of the same species can vary, it is important to test the new assay across the range of the species. We conducted the following study to test the ability of this assay to correctly identify members of the *An. quadrimaculatus* complex from other parts of the range of this species. Starch gel electrophoresis (SGE) of allozymes has been established as a reliable method of identification for all 5 of the sibling species in this complex (Kaiser et al. 1988; Lanzaro et al. 1988; Narang et al. 1989, 1990).

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Materials and Methods

In a survey conducted from May 1993 to September 1995, adults of the *An. quadrivaculatus* sibling species complex were collected throughout Louisiana and parts of Mississippi. Species A adults were collected from inside a garage in east central Louisiana (St. Landry Parish) and from a daytime resting station (Weathersbee and Meisch 1988) in a rice field in southwest Louisiana (Jefferson Davis Parish). Species B adults were collected resting in chicken coops (St. Tammany Parish) and underneath a bridge in the Joyce Wildlife Management Area (Tangipahoa Parish) in Louisiana. Species C were captured from a resting station at the edge of a swamp near the Vermilion River in Vermilion Parish. Species D adults were aspirated from underneath a bridge in Tishomingo County, Mississippi. All adults of the *An. quadrivaculatus* sibling species complex were transported alive to the mosquito research laboratory at the Louisiana State University Agricultural Center and subsequently were stored at -70°C before being used for isozyme SGE and PCR.

SGE was used to identify members of the complex based on isozymes (Narang et al. 1989). Methods were described previously by Steiner and Joslyn (1979) with some modifications by Rutledge (1995). Reference mosquitoes (*An. quadrivaculatus*, species A) from the USDA—ARS, Medical and Veterinary Entomology Research Laboratory (Gainesville, FL) were F₁ progeny reared from eggs of strain Q2, which contained a biochemical marker.

Before grinding the mosquitoes in buffer for SGE, 1 leg was removed and placed in a 1.5-ml Eppendorf tube with a code number corresponding to the tube that held the
remainder of the mosquito body. The code number was assigned to each specimen so that blind PCR identifications could be correlated with isozyme identifications. Tubes containing the legs were returned immediately to the ultracold freezer. After a sufficient number of identifications were made with SGE, a shipment consisting of 1 mosquito leg from each of 10 specimens from each of the 4 species (40 individual tubes) was mailed overnight on dry ice to the Centers for Disease Control and Prevention, Division of Parasitic Diseases, for PCR identification.

DNA was extracted from each of the frozen mosquito legs according to the standard crude mosquito DNA extraction protocol described in Collins et al. (1987), except that before ethanol precipitation, yeast transfer RNA (tRNA) was added to the DNA-containing solution to a concentration of 15μg/μl. The precipitated DNA was resuspended in 10μl of sterile water.

The PCR reactions were carried out as described in Cornel et al. (1996) with reagents and buffers provided in the Perkin-Elmer Cetus GeneAmp PCR reagent kit (Perkin-Elmer Cetus, Norwalk, CT). Each 25-μl reaction contained 1μl of the 10μl of DNA extracted from the single leg, 2.5μl of the 10x PCR reaction buffer with MgCl₂, 200μM of each dNTP, 2mM of extra MgCl₂, 0.625 units of AmpliTaq polymerase, and the following molar amounts of each of the diagnostic primers: UNAQ at 1.6 pmol, AQA at 1.0 pmol, AQB at 4.6 pmol, AQC at 2.3 pmol, and AQD at 5.4 pmol. The species diagnostic bands were resolved by electrophoresing the PCR products through a 2% agarose gel containing ethidium bromide. The bands were visualized on an UV light transilluminator and photographed using Polaroid black and white Type 55 film.
Results and Discussion

Thirty-eight of the 40 specimens identified by isozyme electrophoresis were identified to the same species by the PCR products in the rDNA-PCR assay for a specificity of 100% and a sensitivity of 95% (Table 1). Two of the specimens failed on 2 consecutive attempts to produce a PCR product. Fig. 1 shows a typical assay result which unambiguously identified all specimens.

Table 1. Results of isozyme SGE and PCR assays.

<table>
<thead>
<tr>
<th>Species</th>
<th>SGE</th>
<th>PCR (+)</th>
<th>PCR (-)</th>
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<tbody>
<tr>
<td>A</td>
<td>10</td>
<td>9</td>
<td>1</td>
</tr>
<tr>
<td>B</td>
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<td>10</td>
<td>0</td>
</tr>
<tr>
<td>C</td>
<td>10</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>D</td>
<td>10</td>
<td>9</td>
<td>1</td>
</tr>
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</table>

There could be several reasons why there was no DNA amplification in 2 specimens. One could be attributed to improper DNA leg extractions. Another possibility may be that there is some minor subpopulation variation in 1 of the species. There also may be the chance that there is another species within the *An. quadrimaculatus* complex. If identifications of 100% of specimens are critical, then for those few (5%) specimens where the PCR assay on legs did not provide an identification, the remainder of the mosquito carcass could be identified electrophoretically.

The PCR assay evaluated previously (Cornel et al. 1996) only identified specimens from Florida. The assay reported here correctly identified field-collected
Fig. 1. Photograph of a 2% agarose gel showing fragments amplified by PCR. Lanes 1 and 10 (left to right): 100-bp ladder DNA standard. Lanes 2-5: specimens from Louisiana and Mississippi. Control specimens from Florida (lanes 6-9) represent PCR amplifications of DNA extracted from whole mosquitoes.

sibling species from 6 geographically distinct populations in Louisiana and Mississippi, further validating this assay. The assay separated the species in their extreme eastern and western ranges which is in agreement with Mitchell et al. (1992) and Perera et al. (1995), who reported on the conservation of the rDNA in all the species.

Although all of the methods previously reported for the identification of species of the *An.quadrimaculatus* complex are useful, some advantages and disadvantages between PCR and SGE were discovered during our evaluation. Identifications are faster with PCR. With SGE, the total time involved in gel and mosquito preparation, processing the gel, and then staining the gel for identification is ~ 2 d. In a normal procedure, 38 mosquitoes are identified on each gel. With the PCR assay, as many as 100 specimens can be identified in one day (Cornel et al. 1996).

In addition, the PCR assay works well on a small amount of material (e.g., 1 leg), eliminating the necessity of killing the mosquito for identification. This technique will be
valuable in rearing operations and blood-feeding experiments when it is necessary to
know the species identification while research on the live mosquito continues. For the
sake of establishing a colony or genetically pure line, identifications from a leg of a wild-
caught mosquito would be most useful. Mosquitoes are still able to lay eggs with 1 leg
missing. Blood-fed females can be identified during the time it takes for oviposition.
Therefore, the F1 generation has been identified immediately without waiting for the
female to lay eggs or for the F1 generation to emerge. Identification of the parental
generation ensures the identification of laboratory colonies without sacrificing
individuals who continue to mate and produce eggs. If wild mosquitoes are used to
assess host blood-feeding preferences, the females can be identified and the rest used in
blood meal identification. Also, the remainder of the mosquito body can be saved for
age grading and parasite detection.

In summary, the PCR assay of Cornel et al. (1996) was 100% in agreement with
SGE in the identification of field-collected An. quadrivaculatus species A, B, C, and D
from Louisiana and Mississippi. The PCR technique will increase efficiency of species
identification in the An. quadrivaculatus sibling species complex by increasing the
number of individuals that can be identified in 1 d. Combined with methods previously
developed for the sibling species identification, researchers working with this complex
will have another option to choose from when making identifications.

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Chapter 3

PCR Assay to Identify All Immature Stages of Two Species of the *Anopheles quadrimaculatus* Sibling Species Complex (Diptera: Culicidae)

Introduction

*Anopheles quadrimaculatus* Say is a sibling species complex that includes at least five sibling species. Reinert et al. (1997) published a comprehensive taxonomic treatment describing *An. quadrimaculatus sensu stricto* (s.s.) and four new species. What were formerly known as species A, B, C1, C2, and D are now known, respectively, as *An. quadrimaculatus* Say, *An. smaragdimus* Reinert, *An. diluvialis* Reinert, *An. inundatus* Reinert, and *An. maverlius* Reinert.

Additional studies that have added to the general knowledge of these five species include work on species genetics, adult identification, species distribution, filarial susceptibility and host preferences. These works, along with others on *An. quadrimaculatus sensu lato* (s.l.) are listed in Reinert’s (1997) bibliography of *An. quadrimaculatus* (s.l.).

There remains, however, a lack of detailed descriptions on the larval habitats of these sibling species. In general, the larval habitats of *An. quadrimaculatus* (s.l.) include permanent fresh water in sluggish streams, canals, ponds, and lakes (Carpenter and LaCasse 1955). Larvae can be found in emergent vegetation or floating debris that offers protection from wind and predators. Open, unprotected waters are not favorable habitats for *An. quadrimaculatus* (s.l.) larvae.
In an effort to further characterize the larval habitats of this complex of mosquitoes, we encountered a need for a quick, reliable method to identify the immature stages soon after collections were made. Starch gel electrophoresis (SGE) of isozymes to identify individual adult mosquitoes has been used in the past; however, this method is limited to the adult stage of the mosquito and is not reliable for individual specimens of the immature stages of *An. quadrinaculatus* (s.l) (Janet McAllister, personal communication).

We tested the polymerase chain reaction (PCR) of Cornel et al. (1996) for its ability to amplify ribosomal DNA from each immature stage of *An. quadrinaculatus* sibling species. In this assay, five oligonucleotide primers were combined in a single PCR reaction to identify members of the *An. quadrinaculatus* complex. Comparisons were made between SGE and PCR as an identification tool for adults.

**Methods and Materials**

Adult mosquitoes were collected from natural resting sites that were located during a 2-year distribution survey of *An. quadrinaculatus* sibling species in Louisiana (Rutledge and Meek 1998). Blood-fed females from these collections were placed in individual mosquito breeders (BioQuip, Gardena, CA). Prior to placement into the mosquito breeders, one wing was removed from each female to induce oviposition. After the eggs were laid, each adult female was frozen and stored at -70°C for subsequent identification using SGE. The eggs were placed into white enamel larval rearing pans with tap water for further immature development. A portion of the eggs, 1st, 2nd, 3rd and 4th instar larvae, and pupae were removed as they developed in the emergence containers. The
immature stages were stored individually in 1.5 ml Eppendorf microtubes at -70°C for later identification using PCR. Larvae in the rearing pans were fed daily with ground dog food. The mosquitoes were maintained in a rearing room at 25°C, 75% R.H., and a photoperiod of 12:12 (L:D).

The blood-fed females that laid eggs in the mosquito breeding containers were identified using SGE, prior to larval identification with PCR. This method (SGE) has been used for identifications of adult members of the An. quadrimaculatus sibling species complex for several years. The protocol and stain recipes have been published elsewhere (Narang et al. 1989, Rutledge 1995).

The immature stages of An. quadrimaculatus (s.s) and An. inundatus were tested using the primers developed by Cornel et al. (1996) which amplify the second internal transcribed spacer region of ribosomal DNA using a universal primer (UNAQ) and four species specific primers (AQA, AQB, AQC, and AQD) in a single reaction mixture. The fragment size generated for each species specific primer combination is as follows: An. quadrimaculatus (species A) - 319 bp; An. smaragdinus (species B) - 227 bp; An. diluvialis and An. inundatus (species C) - 293 bp; and An. maverlius (species D) - 141 bp. The primers do not distinguish between An. diluvialis and An. inundatus.

Eggs, 1st through 4th instar larvae, pupae and adults of An. quadrimaculatus (s.s) and An. inundatus were examined using the PCR assay. DNA was extracted according to Collins et al. (1990). Reagents used were from the Perkin Elmer GeneAmp PCR core reagents kit (Hoffman-La Roche Inc., Branchburg, NJ). For each 25 µl reaction, the following volumes were used: 2.5 µl Buffer I, 2.5 µl MgCl₂, 0.5 µl each dNTP, and
0.173 µl Ampli-Taq polymerase, 0.5 µl UNAQ, 0.4 µl AQA, 1.4 µl AQB, 0.7 µl AQG, 1.9 µl AQD and 1 µl target DNA. For each run, positive controls of known species A, B, C (in this experiment, we used C2, *An. imundatus*), and D were used. The two negative controls utilized were *An. franciscanus* McCracken and sterile water.

Twenty-five µl reactions were placed in a thermocycler (Perkin Elmer GeneAmp PCR System 2400) (Hoffman-La Roche Inc., Branchburg, NJ) with a denaturing temperature of 94°C, annealing at 50°C, and extension at 72°C for 25 cycles. After the reaction was complete, the product along with a loading dye (bromophenol blue) was electrophoresed through a 2% agarose gel containing ethidium bromide. When the dye had migrated approximately 2.5 cm from the loading wells, the electrophoresis was stopped. Electrophoresis was conducted at 55 milliamps for about 0.5 h. The fragments were visualized using ultraviolet light, and black and white Polaroid photographs were taken of each gel.

**Results and Discussion**

Eggs, 1st - 4th instar larvae, pupae and adults of *An. quadrimaculatus (s.s.*) and *An. imundatus* were identified using PCR. The species specific primers designed by Cornel et al. (1996) succeeded in producing diagnostic bands for all mosquito life stages for the two sibling species evaluated in this study. Fig.2 shows a gel representing each immature stage of *An. quadrimaculatus (s.s.*) using this assay.

The primary strength of using PCR over SGE is that mosquitoes can be identified during any of the life stages. The benefit derived is that larvae can be immediately frozen or identified as soon as they are brought into the lab after collection. The SGE technique
Fig. 2. Photograph of a 2% agarose gel showing amplified DNA of *An. quadriraculatus* sibling species. Left to right: Lanes 1, 9, 10, and 20: 1kb DNA ladder; lanes 2-6: *An. quadriraculatus sensu stricto* eggs, individual specimens of 1st, 2nd, 3rd, and 4th instar larvae, and a single pupa; lanes 8, 9, 12, and 13: *An. quadriraculatus sensu stricto* individual specimens of 1st, 2nd, 3rd and 4th instar larvae; lanes 14-17 positive control *An. quadriraculatus, An. smaragdinus, An. imundatus,* and *An. maverlius*; lanes 18-19: negative controls *An. franciscanus* and sterile water.

requires adult specimens, which means larvae collected in the field must be brought into the laboratory and reared to the adult stage. In this procedure, larval mortality may be high because of the change in environment from the natural habitat to a laboratory setting, and no identifications can be made.

With the SGE protocol developed for this species complex (Narang et al. 1989), only the adults can be identified to species. The diagnostic enzymes used are not detectable using material from the immature stages employing this technique (Janet McAllister, personal communication).
A disadvantage of using SGE includes problems with the gels. Potato starch gels are rather flimsy and easily fall apart when they are being sliced or during the transfer from the slicing table to the gel staining dish. However, the agarose gel used in PCR is very firm and is easily removed from the electrophoresis chamber and transferred to the light table where it is visualized and photographed. Additionally, the SGE procedure requires that the electrophoresis be done in a refrigerator to keep the gels from melting and the proteins from being destroyed. With the PCR, electrophoresis is done on the bench top without the need for temperature control.

The SGE requires that at least three gel slices be readable in order to assess seven diagnostic enzymes for the sibling species. In contrast, PCR needs only one gel, no slicing is required, and there is only one product (1 diagnostic band) per mosquito. The PCR technique saves time when scoring gels because the bands are easily distinguished.

Utilizing SGE, a maximum of 40 mosquitoes could be identified at one time and the total time for processing was about 20 hours. With PCR, a maximum of 90 mosquitoes could be processed in about 7 hours. This time includes extracting the DNA from the specimens. It may be possible to skip this step as noted by Cornel et al. (1996) and Scott et al. (1993). If this is the case, it would reduce processing time by 1-2.5 h.

An advantage of using PCR is the amount and integrity of the material left after the identifications. With SGE, about one half of the homogenate is used when loading it on to the gel. Only half of the material remains available for error corrections or other types of analyses, and the material is susceptible to degradation with a change in temperature. With PCR, there is such a small amount of the extracted DNA (1 - 5 µl) used in the
reaction that there is excess material remaining (approx. 45 μl) for future use and other
types of analyses. This DNA is stable for a long period of time when stored at -70°C.

The PCR method does have disadvantages regarding implementation and continuous
usage. The initial expense of a thermocycler and the on-going costs associated with the
reagents, in particular, the polymerase, may prove to be too financially burdensome for
small labs. The major expense involved in SGE is the electrical power source for
electrophoresis of the gels.

In conclusion, PCR is a highly sensitive technique that can be used to identify
immature stages as well as adults of the *An. quadrimaculatus* sibling species complex.
This technique also serves as a considerable time saving alternative to SGE for
biochemical identification of mosquitoes.

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species diagnostic assay for *Anopheles quadrimaculatus* cryptic species (Diptera:

a new member of the *Anopheles quadrimaculatus* species complex: a biochemical

(*Anopheles*) *quadrimaculatus* complex of sibling species (Diptera: Culicidae) using
morphological, cytological, molecular, genetic, biochemical, and ecological


Chapter 4

Attempts to Colonize Sibling Species of Anopheles quadrimaculatus

Introduction

The colonization of specific mosquito species or local varieties is often an integral part of investigations relating to the study of mosquito-borne disease dynamics, culicid bionomics, physiology, genetics, and toxicology in order to develop better mosquito management programs (Foster 1980). By maintaining stocks of live mosquitoes, researchers are provided with a supply of subject material at all times. The need for a ready supply of a known species is evident, for example, in determining susceptibility to selected chemicals for control. It is important to have mosquitoes available that are the same age to reduce variability in the results due to a mixed age population.

Understanding the life history of a species is critical in establishing a colony (Foster 1980). Different species of mosquitoes may utilize different water qualities and aquatic food sources, whereas the adults of selected species may display unique mating habits, require specific blood hosts, and exhibit distinct oviposition behaviors. The endeavor to colonize individual species often reveals details of the life history that were previously unknown. Foster (1980) states that the different formulae that researchers use to rear and colonize mosquitoes are continually evolving, and many successes and failures have most likely never been published.

There are several references to colonizing An. quadrimaculatus and anophelines in general. These publications include Boyd et al. (1935), Colluzi (1964) and Gahan and Smith (1964), and Gerberg et al. (1994) and arose from a need to understand the malaria
transmission cycle and the need to control the mosquitoes to prevent the further spread of this disease in the United States. While malaria is no longer considered endemic in the U.S., it was once considered a “major scourge in the United States from colonial times until well into the twentieth century” (Duffy 1988).

Until recently, *An. quadrimaculatus* Say was thought to be a single species being widely distributed east of the Rocky Mountains. The discovery that *An. quadrimaculatus* was not a single species, but a complex of sibling species, poses a dilemma regarding the reliability of previous research. It also presented a whole new set of challenges in establishing colonization techniques of the newly described mosquito species. The Quadrimaculatus Complex is described in detail by Reinert et al. (1997) and consists of five morphologically similar, reproductively isolated sibling species, which are often found in sympatric populations. The species were tentatively designated as species A, B, C1, C2 and D and are now known, respectively, as *An. (Anopheles) quadrimaculatus* Say, *An. (Anopheles) smaragdinus* Reinert, *An. (Anopheles) diluvalis* Reinert, *An. (Anopheles) inundatus* Reinert, and *An. (Anopheles) maverlius*.

Because the distinguishing morphologic characters often require the use of pristeen specimens, it is recommended by Reinert et al. (1997) that several techniques be used in an integrated approach to species identification. These include morphologic keys, chromosome analysis, molecular tools, genetic and biochemical evaluation, and ecologic approaches. One or more of these techniques then becomes a required step in the colonization process. There is no published information on colonization of any species belonging to the Quadrimaculatus Complex.

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Robertson (1997) studied the mating behavior and heritability of wing-beat frequency in *An. quadrimaculatus* sibling species. He concluded that the mosquitoes are poorly adapted to laboratory conditions, and those that are captured in the wild often fail to mate in captivity. More often than not, the females will not oviposit unless induced to do so by trauma. He states that the low level of mating activity that takes place in cages would make it necessary to use forced mating. This limitation makes mass rearing difficult.

The objective of this study is to colonize four sibling species of the Quadrimaculatus Complex using field-caught specimens as starting material.

**Methods and Materials**

Adult mosquitoes were collected from sites in Louisiana and Mississippi previously identified as *An. quadrimaculatus* sibling species habitats (Rutledge 1995). At that time, mosquitoes were identified using starch gel electrophoresis of isoenzymes. Additionally, mosquitoes were collected from Stuttgart, Arkansas.

Mosquitoes were collected using a battery-powered aspirator (Perdew and Meek 1990) into a 0.45 liter paper can (WLMA Inc., Newark, NJ). The bottom of each paper can had been removed and replaced with 16-mesh aluminum screen with the opposite end open to receive a lid once the mosquitoes had been collected. Collections were made from natural resting sites such as tree holes and hollow tree trunks, and from daytime resting stations (DRS) (Weathersbee and Meisch 1988), and black boxes (BB) (Service 1976). The paper cans containing the adult mosquitoes were stored temporarily in ice-lined insulated chests for transport to the laboratory. In the laboratory, the paper
cans were placed momentarily into a freezer to immobilize the mosquitoes to allow rapid sorting of the anophelines from the other mosquito species.

Adult females that were blood engorged upon collection were placed individually into a mosquito breeder (BioQuip Products, Gardena, CA), and provided with water for oviposition. The breeders are 21 cm high and 12 cm in diameter and constructed of 2 clear, 0.95 liter styrene containers. There is a plastic lid in between the two sections that contains a vinyl funnel through which the adult mosquito flies after emergence. The top portion has aluminum screening to provide ventilation. After the female laid her eggs on the water surface, she was removed from the container and subsequently identified using the polymerase chain reaction (PCR) method of Cornel et al. (1996). The progeny from individual females were kept separate from other broods until the identities were known to prevent mixing of the sibling species.

Unengorged anopheline females from the same site were placed together in 30.5 cm³ collapsible aluminum screen cages (BioQuip Products, Gardena, CA). These cages are constructed of an aluminum frame with 16 × 18 mesh screen on 3 sides, and a surgical stockinette sleeve on the front of the cage to allow access to the inside. The tops of the cages are equipped with a nylon feeding hammock that can hold a small animal for blood meal feeding. The cages were covered with an easily removable plastic sheet to aid in maintaining the proper humidity within the cage. Several larger cages were employed during this study to provide the mosquitoes with a greater area for swarming/mating. The cages used were of the same design, but were 46 cm³ and 61 cm³.
The caged adult mosquitoes were provided with a carbohydrate source. Dental wicks were placed into a 250 ml glass media bottle and then filled with a 10% sugar-water solution. One bottle was placed inside each cage. Wicks and the sugar-water were changed every 3 days to reduce bacterial growth and other potential contamination.

The female anophelines were offered blood meals from guinea pigs and/or humans. Twenty-four hours before offering a blood meal, the sugar-water source was removed from each cage. A colony of guinea pigs was maintained at the laboratory annex of the LSU School of Veterinary Medicine located in the same building as the insectary. This animal holding facility was accredited by the American Association for Accreditation of Laboratory Animal Care and exceeded the animal care standards set by the USDA. This part of the project was approved by the Animal Use and Care Committee, protocol A96-29. Guinea pigs were transferred to the insectary where the mosquito colonies were maintained. The underside of each guinea pig belly was shaved to enhance the blood feeding opportunity of mosquitoes. One animal was placed on top of the adult mosquito cage for about 20 minutes to allow blood feeding by the female mosquitoes.

Mosquitoes were observed from the screened sides of the cages. If females made no attempt to feed after 20 minutes, the animal was removed from the cage. A human blood meal was offered to the mosquitoes by inserting a bare arm into the cage. If no feeding was attempted on the arm, blood meals were offered again the next day. Daily attempts to blood feed continued until the females imbibed a meal, or, in some cases, until the mosquitoes died without having fed. Upon taking a blood meal, each female anopheline mosquito was removed from the screened cage and placed into a mosquito
breeder. The same handling procedure was followed for collecting eggs as described previously for those females that were blood engorged from the field collections.

The insectary was located in the Mosquito Research Lab of the Department of Entomology, Louisiana State University, in the Life Sciences Building. The room has one door and no windows. It is supplied with a source of water and is connected to the Life Sciences Building heating and air-conditioning system. A humidifier (Bionaire Environmental Air Products, Denver, CO, Model W-6) was used in the room to maintain the humidity between 60% - 75% R.H. The light cycle was kept at 12:12 (D:L) and a sunrise-sunset simulator was used to attempt to provide a dusk and dawn period for the mosquitoes. This simulator consisted of two blue and two green light bulbs that were connected to a timer so that they would come on individually at 15 min intervals at or near natural sunrise and go off individually at 15 min. intervals at or near sunset.

Larvae were fed daily with finely ground Gaines® Cycle Light dog food. The dog food was ground in a standard kitchen blender and then passed through a No.100 sieve. The amount of food varied with the water conditions in the pans and with the size and number of larvae present. If the water began to show signs of food build up, bacterial growth, or scum, the amount of larval food was reduced. When necessary, the larvae were removed to a new pan of clean water. All pans were covered with screened frames to exclude oviposition by adult mosquitoes that might be free flying in the rearing room. Pupae were harvested daily and placed in a bowl of water which then was placed inside the aluminum screened cages to await adult emergence.
Mosquitoes were identified using PCR as described in detail in Chapter 2. Briefly, DNA is extracted from the mosquito following the extraction procedure of Collins et al. (1987). The PCR is performed using species-specific primers based on differences in the sequence of the ITS2 region of nuclear ribosomal DNA and a universal primer derived from a sequence in the 5.8S coding region. The species-specific primers can distinguish four of the five sibling species and produce different fragment sizes for each of the species.

Species C1 and C2 (*An. diluvialis* and *An. imundatus*) could not be distinguished from each other using this protocol. Based on the distribution of these two siblings, bands that were diagnostic for species C collected in Louisiana were assumed to be C2, *An. imundatus*. This assumption was made based on previous collections from the area and subsequent identification by chromosome analysis by Paul Kaiser of the USDA Center for Medical and Veterinary Entomology (CMAVE) Laboratory in Gainesville, FL. *Anopheles diluvialis* has not been reported outside of collections from Florida (Reinert et al. 1997).

Table 2 lists the sibling species collected together with the date and sample number, life stage and site name, and the type of resting site from which the samples were taken.

**Results**

The fate of each of the samples collected for colonization purposes was as follows:

1-96: *An. quadrimaculatus ss*. Blood engorged females: Three females laid eggs that hatched and survived to the 2nd instar, 100% mortality in the larvae.
Table 2. Sibling species of the Quadrimaculatus Complex collected for colonization purposes.

<table>
<thead>
<tr>
<th>Sibling species</th>
<th>Date collected</th>
<th>Site</th>
<th>Resting/Collection Site</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>quadrimaculatus ss inundatus</em></td>
<td>23 May 1996 1-96</td>
<td>Esther, LA</td>
<td>DRS*, tree cavity.</td>
</tr>
<tr>
<td></td>
<td>2-96</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>30 May 1996 3-96</td>
<td>Esther, LA</td>
<td>DRS, tree cavity.</td>
</tr>
<tr>
<td></td>
<td>4-96</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>11 July 1996 5-96</td>
<td>TWMA, LA</td>
<td>DRS, under bridge.</td>
</tr>
<tr>
<td><em>maverlius</em></td>
<td>18 July 1996 6-96</td>
<td>Tishomingo, MS</td>
<td>In buttresses of trees, under bridge.</td>
</tr>
<tr>
<td></td>
<td>25 July 1996 7-96</td>
<td>TWMA, LA</td>
<td>DRS.</td>
</tr>
<tr>
<td><em>quadrimaculatus ss inundatus</em></td>
<td>19 Aug 1996 11-96a</td>
<td>LA 717, LA</td>
<td>DRS. Tree cavity.</td>
</tr>
<tr>
<td></td>
<td>11-96b</td>
<td>Esther, LA</td>
<td>DRS.</td>
</tr>
<tr>
<td></td>
<td>12-96</td>
<td>Pleasant, LA</td>
<td>DRS.</td>
</tr>
<tr>
<td><em>quadrimaculatus ss inundatus</em></td>
<td>29 May 1997 2-97</td>
<td>P829, LA</td>
<td>DRS.</td>
</tr>
<tr>
<td><em>quadrimaculatus ss</em></td>
<td>9 June 1997 4-97</td>
<td>Pecan Island, LA</td>
<td>Barn. Abandoned shed.</td>
</tr>
<tr>
<td><em>quadrimaculatus ss inundatus</em></td>
<td>24 June 1997 7-97a</td>
<td>Esther, LA</td>
<td>Tree cavity.</td>
</tr>
<tr>
<td></td>
<td>7-97b</td>
<td>LA 717, LA</td>
<td>DRS.</td>
</tr>
<tr>
<td></td>
<td>8-97</td>
<td>Elton, LA</td>
<td>BB.**</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Pleasant, LA</td>
<td>DRS.</td>
</tr>
</tbody>
</table>

(Table 2 continued)
<table>
<thead>
<tr>
<th>Sibling species</th>
<th>Date collected</th>
<th>Site</th>
<th>Resting/Collection Site</th>
</tr>
</thead>
<tbody>
<tr>
<td>quadrimaculatus ss</td>
<td>30 June 1997</td>
<td>Esther, LA</td>
<td>Tree cavity.</td>
</tr>
<tr>
<td></td>
<td>10-97a</td>
<td>Hwy 82, LA</td>
<td>Buttresses of cypress tree.</td>
</tr>
<tr>
<td></td>
<td>10-97b</td>
<td></td>
<td>DRS.</td>
</tr>
<tr>
<td></td>
<td>10-97c</td>
<td>Pleasant, LA</td>
<td>Under bridge.</td>
</tr>
<tr>
<td></td>
<td>10-97d</td>
<td>Whiskey Bay, LA</td>
<td>BB</td>
</tr>
<tr>
<td></td>
<td>10-97e</td>
<td>Hwy 3150, LA</td>
<td></td>
</tr>
<tr>
<td></td>
<td>15 July 1997</td>
<td>Ponchatoula, LA</td>
<td></td>
</tr>
<tr>
<td></td>
<td>11-97</td>
<td></td>
<td>Under bridge.</td>
</tr>
<tr>
<td>smeragdimus</td>
<td>17 July 1997</td>
<td>Yellow Creek, AL</td>
<td>Tree holes, tree cavities, and BB.</td>
</tr>
<tr>
<td></td>
<td>12-97</td>
<td></td>
<td>Utility shack.</td>
</tr>
<tr>
<td>maverilus</td>
<td>23 July 1997</td>
<td>Stuttgart, AR</td>
<td>Horse barn.</td>
</tr>
<tr>
<td></td>
<td>14-97a</td>
<td>Stuttgart, AR</td>
<td>Under steps.</td>
</tr>
<tr>
<td></td>
<td>14-97b</td>
<td></td>
<td></td>
</tr>
<tr>
<td>quadrimaculatus ss</td>
<td>27 Aug 1997</td>
<td>Esther, LA</td>
<td>Tree cavity.</td>
</tr>
<tr>
<td>inundatus</td>
<td>15-97</td>
<td></td>
<td>DRS.</td>
</tr>
<tr>
<td></td>
<td>16-97</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>18 Sept 1997</td>
<td>Murphy, LA</td>
<td>Rotten base of tree.</td>
</tr>
<tr>
<td>quadrimaculatus ss</td>
<td>1 May 1998</td>
<td>Pleasant, LA</td>
<td>DRS.</td>
</tr>
<tr>
<td>inundatus</td>
<td>1-98</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*DRS = Daytime Resting Station (Weathersbee and Meisch 1988)

**BB = Black Box (Service 1976).
2-96: *An. inmundatus*. Blood engorged females: One female laid eggs on 25 May 96. All eggs sank in the oviposition cup. Seven of the blooded females were gravid, but died without laying eggs.

3-96: *An. quadriraculatus ss*. Blood engorged females laid eggs; larvae survived for 2 instars, all died in 2nd or 3rd instar.

4-96: *An. inmundatus*. Blood engorged females: Half of the blooded females were given tap water for an oviposition medium. The other half were provided with water from the swamp adjacent to the adult resting site (larvae had been collected from this swamp). Nine females laid eggs in swamp water, all of which subsequently sank. Eight females laid eggs in tap water, all of which subsequently sank.

5-96: *An. quadriraculatus ss*. Unfed females that were collected would not take guinea pig or human blood meal.

6-96: *An. maverlius*. Blood-engorged females: Eggs were laid in tap water and survived to emerge as adults on 16 Aug 96. Progeny were offered blood meals from 18 Aug 96 - 2 Sept 96. No attempt was made to feed on either guinea pigs or humans. On 2 Sept 96, all adults were dead.

7-96: *An. quadriraculatus ss*. Unfed females: Would not blood feed on guinea pig or human.

8-96: *An. quadriraculatus ss*. Unfed females were collected. No blood meals were taken.
9:96: *An. maverlii.* Unfed females were collected. No blood meals were taken.

11-96(a): *An. quadrimaculatus* ss. Blood-engorged females: Two females laid eggs that hatched and survived to the 4th instar. High larval mortality. Adults emerged and were offered guinea pigs and humans blood meals until 26 Sept 96. Three females fed on 22 Sept 96. On 29 Sept 96, no eggs had been laid, and all adults were dead.

11-96(b): *An. quadrimaculatus* ss. Blood engorged females: Two females laid eggs that subsequently sank. One female laid eggs that hatched. Larval mortality was high and three adults emerged, one male and 2 females.

12-96: *An. imundatus.* Blood meals were offered from human and guinea pig, none were taken. All adults were dead by 12 Sept 96.

2-97 *An. quadrimaculatus* ss. Blood fed females laid eggs that survived to adult. Few fed on guinea pig, more fed on human. No progeny.

4-97: *An. quadrimaculatus* ss. Blooded females: Twenty females laid eggs. Larvae survived. High mortality in pupal stage. Few adults emerged. Adults would feed on human blood meal, but no eggs were laid.

6-97: *An. quadrimaculatus* ss. Unfed females and males were collected. No blood meals were taken.

7-97a: *An. quadrimaculatus* ss. Blood fed females: No eggs were laid.


10-97a, b, c, d, e: *An. quadrimaculatus ss.* Blooded females: Eggs hatched, few consumed blood meals, high mortality in all immature stages, adult mortality after F1.

11-97: *An. smaragdinus*. Blood engorged females: Eggs were laid and adults emerged. Blood meals (human and guinea pig) were offered for 9 days. One female took a human blood meal. No eggs were laid. All wild caught adults died. Unfed females: Two females fed on a human blood source. Eggs were laid - but none hatched.

12-97: *An. maverlius*. Blood fed females: Thirty eight out of forty gravid females would not lay eggs. After 3 days, half were traumatized by wing removal, still no eggs.

13-97 a, b, c, d, e, f: *An. quadrimaculatus ss.* No blood meals were taken and no mating was observed to occur.

14-97 a, b: *An. quadrimaculatus ss.* Blood engorged females: Eggs were laid and hatched and survived to the adult stage. On 22 Sept 97, three adults took human blood meals and three took guinea pig blood. No eggs were laid. All adults died by 4 Oct 97.

15-97: *An. quadrimaculatus ss.* Blood fed females were collected from Esther, LA on 27 Aug 97. Eggs were laid and hatched and larvae survived to the adult stage.

16-97: *An. imundatus*. On 21 Sept 97 - 10 Oct 97, approximately 100 female adults were offered blood meals from human and guinea pigs. None fed. On 7 Oct 97, five females fed on guinea pig blood. On 19 Oct 97, no eggs had been laid and all adults were dead.

17-97: *An. quadrimaculatus ss.* Blood engorged females: Eggs were laid. High mortality in larval stages. No adults emerged.
18-97: *An. inundatus*. Blood engorged females were collected from Esther, LA on 18 Sept 97. Eggs were laid, hatched, and the larvae survived though the adult stage. Blood meals from human and guinea pig were offered from 6 Oct 97 - 19 Oct 97. Twenty-three females fed on human blood. No eggs were laid and all adults were dead on 30 Oct 97.

1-98: *An. quadrimaculatus ss*. Blooded females: No eggs were laid.

2-98: *An. maverlii*. Blooded females: Eggs hatched, all larvae died.

Unfed females: Consumed blood meal, laid eggs, all larvae died.

3-98 a, b, c: *An. quadrimaculatus ss* and 4-98a, b: *An. inundatus*. From all collections on this date: Females laid eggs. Larvae survived through the 3rd instar. High mortality after 3rd instar. Few adults emerged. No adults would take a blood meal.

Two separate collections (2-96 and 4-96) of sympatric *An. quadrimaculatus s.s.* and *An. inundatus* from the same site yielded some females which laid eggs that failed to hatch. Eggs that sank or failed to hatch were cursorily examined and found to have damaged floats or apparently absent floats. Many appeared to be empty eggs cases or contained amorphic material.

Several collections of mosquitoes were separated and placed into different sized cages. None of the larger sizes proved to be large enough to allow the formation of swarms for mating, if indeed that was part of the problem. The results were similar on all attempts, in that occasionally the females would take a blood meal, but would not lay eggs.
Discussion

Three major factors affecting laboratory colonization of mosquitoes were considered. Those factors are 1) mating, 2) oviposition substrate and 3) feeding behavior of the larvae and the adult stages of the mosquito. The failure to get any of the sibling species into an established colony may be due to one of these factors, or possibly several acting together.

Gahan and Smith (1964) warn that in colonizing a laboratory strain of insects, the colony will have been established from abnormal insects that may behave differently from the largest part of the population present in natural settings. For this reason, one must be careful in drawing conclusions about natural populations based on laboratory populations. It is possible that the females that did lay eggs in the lab, and those that did mate in captivity (of which there were only a few) are the abnormal members of the populations sampled. The females that would not lay eggs, (which was much greater than those that would), or would not mate in captivity, were perhaps reflective of the normal population of wild mosquitoes which may be very sensitive to oviposition substrate and mating conditions.

In many of the collections, there were at least 30 gravid females that were collected from one site and then only 1 or 2 out of the group would lay eggs. If those progeny were successfully colonized, the resulting information from 1 or 2 mosquitoes in the entire population would be limited and general conclusions may not be able to be drawn about the population as a whole. Many individuals from a population would be required
to start a colony, and periodically add representatives from a wild population from the same area when studying aspects of their behavior and vector potential.

There were several occasions where caged females would take blood meals, but never lay eggs; perhaps because they would not mate in the small cages. Foster (1980) gives two reasons that mosquitoes in confinement may refuse to mate. One is that there is an inability to form swarms due to insufficient space, lack of swarm sites, or inappropriate lighting conditions. The second is that there is insufficient space for copulation as a pair falls from the swarm.

Several collections were separated into sub-samples to use as a test of the cage size. Increasing the size of the cages by 1.0 x and then again by another 1.5 x, did not increase the likelihood of females laying eggs. Perhaps an even bigger cage is required to provide enough room for swarming and mating.

Larger cages are available and may have provided sufficient space to allow for the formation of swarms, and thus mating. However, the larger cages would not fit into the insectary that was used during this research. Starting with large cages and selecting for mosquitoes with subsequent reduction of cage size can result in the colonization of a species that does not require a swarm for mating. If this is to be done, however, it would prove necessary to select in this fashion every time wild material was introduced into the colony.

Induced mating is always an option; however, in maintaining several colonies, costs due to labor and time are often a limiting factor. Optimally, a self-sustaining colony is the ultimate goal. Gahan and Smith (1964) indicate that a common problem with An.
*quadrimaculatus s.l.* collected from the wild is that they will lay fertile eggs in the laboratory, but that the adults of the F1 generation do not mate readily. They state that only a minute proportion of *An. quadrimaculatus s.l.* in the natural population will adapt to laboratory conditions.

Successful rearing of *An. barbirostris* by Soelarto et al. (1995) followed the general guidelines of Gerberg (1970) and included the use of natural light from windows and skylights. The temperature and humidity were not controlled, but left at ambient conditions. They used clumps of grass in well water in their larval rearing pans. They reported that mortality was high for first-generation larvae and pupae from wild adults, but that beyond the F2 generation, there was minimal mortality. However, they do not mention if wild material was subsequently introduced into the colony to keep variety in the gene flow. This type of natural light exposure was not feasible in the laboratory utilized in this study. While we did use a sunrise-sunset simulator, this may be too artificial, and natural light may be more important in inducing swarm formation and/or blood feeding. Perhaps that should be a consideration in the future in order for mating to occur, for blood feeding to commence and for oviposition to take place.

It is plausible that the eggs that sank or failed to hatch (which were laid by females that were collected from the same site on separate dates) were perhaps those that were the result of a mating between two different sibling species. Another potential explanation is that perhaps those mosquitoes were infected with the *Wolbachia pipiensis* parasite which induces cytoplasmic incompatibility if infected males mated with
uninfected females. In such instances, the females lay eggs that are not viable. Sixty-two specimens were examined for the parasite and is the topic of Chapter 6.

It appears that the *An. maverlius* mosquitoes are particularly sensitive to the oviposition substrate. Females (collection 12-97) that were blood fed and gravid would not oviposit on tap water, water brought into the lab from the natural habitat, or water that had been adjusted to a more acidic pH such as the natural water. Water sampled at this site had a pH value of 6.0 (Chapter 5). This pH is considerably lower than the average reported for *An. quadrimaculatus s.l.* After several days when none of the gravid, blood fed females had laid eggs, a portion of them were traumatized by removing one wing. This procedure has been used in the laboratory in other research to induce oviposition; however, this technique did not induce oviposition and all of the specimens died without laying eggs.

The larval habitats of this sibling should be analyzed in more detail to give us a better understanding of what factor(s) attract the females and what factor(s) stimulate oviposition. Once these natural factors have been identified, it could be possible to develop a substrate that would be attractive to the females and stimulate them to lay eggs.

Colluzi (1964) indicates that food standardization is one of the major problems in maintaining laboratory colonies of mosquitoes. Because of bacteria, protozoa, and fungi that may result from improper feeding, he states that experience is much better than any rule when it comes to adopting a feeding procedure. Therefore, maintaining a rigid schedule of a strict diet may prove to be deleterious. Flexibility in a feeding program
seems to be the appropriate mode of operation in the laboratory rearing of selected mosquito species.

Most anopheline larvae cannot survive in water with a film or scum on the surface (Bates 1949). Larvae in each pan were fed daily according to experience. Larval habitats are usually determined by the female by her oviposition site preference (Foster 1980). The female *An. quadrimaculatus (s.l.* lays her eggs in neutral or alkaline ponds (Buxton and Leeson 1949). Oviposition site selection is a result of chemical and physical factors (Bentley and Day 1989) and for many species, the selection is influenced by the presence of chemical substances. There was no specific ratio to the amount of food provided for each larvae due to a range of variables that might be introduced into the laboratory. These include unclean pans, high larval mortality - which adds to the formation of the surface film, contaminated hands of lab personnel, contaminated dog food (bacteria).

The approach taken in this research was broad ranging in an attempt to get all the sibling species into colony during the study. Perhaps a better approach would be to spend more time on one of the species and study a select number of natural populations. Mosquitoes were brought in from several habitats at once, and this did not allow for sufficient detail in studying any one population.

References Cited


Chapter 5

Characterization of Larval Habitats of *Anopheles quadrimaculatus* Sibling Species

Introduction

Mosquito larvae of different species are suited for living in different kinds of water and at different sites in the water. Given the adaptations possessed by mosquito larvae, adult oviposition behavior, including general habitat preference, is directly connected to the larva’s chances of survival (Foster 1980). The larvae develop through four instars, each one ending in the molting of the cuticle to allow for growth. Anopheline larvae are unique in that they lack a breathing siphon at their posterior end with the respiratory spiracles being located on the 8th abdominal segment of the dorsum. They attach to the surface film of water by palmate hairs and remain horizontal when at rest at the surface. They feed at this layer, occasionally diving down to feed on submerged food (Foster 1980).

Oviposition preference by female mosquitoes is one of the key factors in determining the survival of the eggs, larvae, and pupae into the adult stage. According to Foster (1980), oviposition often has a characteristic site as well as time of day that it occurs, and the site is determined by chemical, visual, and tactile stimuli.

Oviposition site selection is a result of chemical and physical factors (Bentley and Day 1989) and for many mosquito species, the selection is influenced by the presence of chemical substances. Females of *An. quadrimaculatus s.l.* lays their eggs in neutral or alkaline ponds (Buxton and Leeson 1949). Substances in the aquatic habitat may include pheromones produced by eggs, larvae, and pupae already present at the site, fecal...
material, and microbial metabolites. Additionally, inorganic salts, fatty acids, phytochemicals, and insecticides may influence oviposition behavior in the laboratory and in the field. Bentley and Day (1989) concluded that microanalysis of the chemical composition of field sites should be the focus of future investigations.

Inorganic ions are a major component of the natural oviposition medium and are evaluated by the females before the eggs are laid (Vrtiska and Pappas 1984). Results from analysis of tree hole mosquitoes showed that 7 species could be divided into two groups based on potassium levels in the water.

The objective of this study was to characterize larval habitats where sibling species of the Quadrimaculatus Complex were collected, and in the process to identify any distinguishing characteristics that might be attributable to one or more species.

**Methods and Materials**

Larvae were collected from the water using aquatic dippers constructed with a 400 ml diameter white plastic collection cup attached to a 1 meter long, wooden dowel (BioQuip Products, Gardena, CA). Habitats were selected based on previous research on the distribution of the Quadrimaculatus Complex (Rutledge 1995, Seawright et al. 1992). A minimum of 30 dips per site was made.

Larval habitats were characterized using criteria developed by the Smithsonian Institution. This information was recorded on the Smithsonian Institution Medical Entomology Project collection form (Appendix C).

Water samples were taken from each site where *Anopheles* larvae were collected, filtered through a 0.45 μm filter, and then acidified using trace metal grade nitric acid.
These samples were kept refrigerated until analyzed by inductively coupled plasma atomic emission spectrometer (ICP-AES) by the LSU Agronomy Department, LSU Agricultural Center. The lab follows standard laboratory quality control practices and for each sample set, a calibration was performed. A quality control standard that was not used in the calibration was also run as a check on the calibration. Recalibration was performed if the instrument drifted more than 2% for Mn, Ca, and Cu. The samples were analyzed for a total of 26 inorganic ions using standard methods.

Larval stages of sibling species of the Quadrimaculatus Complex were identified using PCR, and the methodology is described in detail in Chapter 3. Briefly, DNA is extracted from the whole organism following the extraction procedure of Collins et al. (1987). The PCR is performed using species-specific primers based on differences in the sequence of the ITS2 region of nuclear ribosomal DNA and a universal primer derived from a sequence in the 5.8S coding region. The species-specific primers can distinguish 4 of the 5 sibling species and produces different fragment sizes for each of the species. Species C1 and C2 (An. dihuvialis and An. imundatus) could not be distinguished using this protocol. Based on the distribution of these two siblings, bands that were diagnostic for species C collected in Louisiana were assumed to be C2, An. imundatus. This assumption was made based on previous collections from the area and subsequent identification by chromosome analysis by Paul Kaiser of the USDA CMAVE Laboratory in Gainesville, FL. Additionally, An. dihuvialis has not been reported outside of collections from Florida (Reinert et al. 1997).
Results

Sixteen larval habitats were characterized during 1997-98. A total of sixty-three aquatic habitat were sampled. Variables that were noted for each larval habitat are reported below using designations from the collection form of the Smithsonian Institution. The habitats were given subjective designations of A, B, C, D, or mixed, based on the species identification after PCR was completed. (A = *An. quadrimaculatus* s.s., B = *An. smaragdinus*, C = *An. imundatus*, D = *An. maverlii*, mixed = any combination of more than one species). The designations are subjective because so few Anopheline larvae were collected from the habitats. Even if only one species was collected and identified, the possibility can not be ruled out that other species were in the area.

BPP is a type A habitat located in St. Landry Parish, LA, within the Thistlewaite Wildlife Management Area (TWMA). Larvae were collected on June 17, 1998. It is a small pond exposed to full sun during the day. This fresh water pond had permanent, stagnant, clear, green water, with a pH of 8.47 on the date sampled. There was some emergent and submerged grassy aquatic vegetation and a mud bottom. The larvae were collected along the grassy areas of the banks, most often by placing the dipper against some type of aquatic vegetation.

LPP is a type A habitat also located in St. Landry Parish within the TWMA. This small pond is located in full sun. Larvae were collected on June 17, 1998. This fresh water pond had permanent, stagnant, clear green water, with a pH of 7.07 on the date...
sampled. There was some emergent and submerged grassy vegetation and a mud bottom. The larvae were collected along the grassy areas of the banks.

YC larval habitat is located in Iuka, MS, in Tishomingo County, and is a large, natural, fresh water swamp with a mud bottom. This site was sampled on July 18, 1997. The water is temporary, stagnant, and clear, with a pH of 6.00 on the date sampled. There was some emergent grassy vegetation. The larvae were collected in shaded areas that had very little sunlight coming through the canopy. Most often, the larvae were collected from the around the bases of bald cypress trees or in the middle of parrot-feather vegetation. This habitat is designated as mixed. *An. quadrimaculatus* s.s. and *An. maverlus* were identified from the collections.

Hwy 75 DAR is a small ditch off the side of the road in the Darrow Community of Ascension Parish, LA. On July 9, 1997, this larval habitat had temporary, slow moving, clear water that was located in full sun. There was scarce emergent grassy aquatic vegetation. The fresh water had a pH of 6.68 on the date sampled. The only sibling species identified from this ditch was *An. quadrimaculatus* s.s. It is designated as habitat type A.

Paden is designated as habitat type A. This site was located in Tishomingo County, MS, within the Tishomingo State Park, on July 17, 1997. It is a medium sized fresh water pond located in full sun. The water is clear, permanent, and stagnant, with a mud bottom. There was scarce submerged and emergent grassy vegetation and the water pH was 6.59 on the day the larval collections were made. All larvae were taken from close to the banks of the pond, in the protection of the emergent grasses.
Tenn-Tom is a large, fresh water swamp located in full sun. This site was located in Tishomingo County, MS along the Natchez Trace on July 17, 1997. It had permanent, stagnant, clear water, with a pH of 6.19 on the date of sampling. It had a mud bottom with some emergent grassy vegetation. The majority of the cypress located in this swamp were dead. All larvae were collected in the grassy areas on the edge of the swamp. This habitat is designated as habitat type A.

Aberdeen is a larval habitat designated as type A and was located in Monroe County, MS, at the Aberdeen Lock and Dam on July 18, 1998. This location was a small ground pool at the edge of a swamp. The water was polluted with an oily substance and trash from human influence. The semi-permanent, stagnant fresh water was located in partial shade and had a pH of 6.11. The bottom was mud and there was no aquatic vegetation.

Noxubee is a large fresh water stream with a sandy bottom located in the Noxubee Wildlife Management Area in Oktibbeha County, MS. Collections were made on July 18, 1997. The water was permanent, slow moving, clear and had a pH of 6.29. The site was located in partial shade. There was no aquatic vegetation. There were trees along the banks of the stream whose limbs extended out over the water. Larvae were collected only from the areas where the limbs and leaves extended down to the water surface. This habitat is designated as type A.

Pleasant is a small ditch located in Vermilion Parish, LA in the Palmetto Point Swamp. Larvae were collected on July 28, 1997. This habitat was located in partial shade with temporary, stagnant fresh water with a pH of 6.09. There was scarce
emergent grassy vegetation. The larvae were collected among floating debris that consisted mainly of small sticks and leaves. This habitat is designated as type A.

Pleasant (a) is designated as a type C habitat and located within the Palmetto Point Swamp in Vermilion Parish, LA. Larvae were collected on July 10, 1998. It is a large fresh water swamp located in partial shade. The water was semi-permanent, stagnant, and clear with a pH of 6.93. There was some emergent and submerged grassy aquatic vegetation. The larvae were collected from the interior of the swamp and most often at the base of bald cypress trees.

Esther is a large fresh water swamp with semi-permanent, stagnant water located in Vermilion Parish, LA, and exposed to partial shade. The clear water had some emergent, floating and submerged aquatic vegetation. The larvae were collected from around the bases of bald cypress trees and in the midst of floating debris of small sticks and duckweed. Collections were made on July 18, 1997. This habitat had a lot of human influence in the way of trash on the margins and partially in the interior of the swamp. The habitat type for this location is A.

Esther (a) is a medium sized larval habitat that is the freshwater margin of a swamp with semi-permanent water. It was located in Vermilion Parish, LA, on July 10, 1998. It was exposed to full sun and there was abundant emergent and submerged vegetation. The habitat has a mud bottom is designated as type A.

The Audubon habitat is type A and was located in Vermilion Parish, LA. On July 28, 1997 larvae were collected from this small ditch which was located in partial shade and had temporary, slow moving fresh water. The water pH was 7.20 and there was
scarce emergent grassy vegetation. Larvae were collected only in the grassy vegetation along the banks of the ditch.

P733 Swamp is a large fresh water swamp in Vermilion Parish, LA that contained semi-permanent water. Collections were made on June 24, 1997. The area is shaded throughout the day and is dense with bald cypress and oak. The clear, stagnant water had a pH of 6.27 and contained some submerged and emergent grassy vegetation. This site is designated as mixed. *An. quadrimaculatus* s.s. and *An. inundatus* were identified from the collections.

LA 717 is a type A habitat that is located at the margins of Lake Arthur in Vermilion Parish, LA. It is a large fresh water swamp with semi-permanent water that had a pH of 6.66. There was some submerged and emergent grassy aquatic vegetation. Larvae were collected on July 10, 1998, from the bases of bald cypress trees.

LA 717 (a) is a type A habitat was located in a flooded rice field on June 24, 1997. It had clear stagnant fresh water with a pH of 7.21. The water was abundant with emergent vegetation (rice) and exposed to full sun. It is a medium sized habitat with temporary water. The larvae were collected close to the emergent plants and not in the open water.

The results from the water analyses for detection of twenty-eight elements are given in Table 3.

**Discussion**

Sampling of Anopheline larvae generally produces very few larvae per dip due to their random distribution and inaccessibility to many areas of a swamp. In contrast,
Table 3. Water analysis of *Anopheles quadrimaculatus* s.l. larval habitats characterized in 1997-98.

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53
larval collections from a septic ditch containing *Culex quinquefasciatus*, may result in more than 100 larvae per dip. In a flooded cypress swamp, it is not uncommon to collect as few as zero to one anopheline larva for every 20 dips taken. Using indicators such as cypress trees, stagnant or slow moving water, lake margins and flooded swamps, locating larval habitats for *An. quadrimagulatus s.l.* was relatively easy during the 1997 mosquito season. Although the field survey began in May and continued through August, larvae were located only during June and July of 1997. The number of larvae collected per site, however, were less than desirable.

During 1998, there were fewer larval habitats located, and again only during June and July. Rainfall for the summer of 1998 was less than average and many areas in Louisiana were considered to be in a drought. Locating larval habitats proved to be difficult. Many of the sites that were located in 1997 were revisited and found to be dry, or muddy, but with no standing water. Lakes were low, and the water that previously flooded the shady areas of the edges and the swamps, had receded. The edges of lakes were, at the time of the study, in areas that were in the middle of the lake in previous years. With the water being this low, the lake margins no longer had the emergent vegetation, cypress trees, and shaded protected areas where larvae had been located in 1997. This is probably a contributing factor in not finding as many breeding places during 1998.

One of the habitats that appears to be unique when compared to the others is the Noxubee habitat in Mississippi, a fresh water stream with a sandy bottom. However, the only sibling species identified from this habitat was *An. quadrimagulatus s.s.* So, while
it was unique in its description, it is just another item to add to the list of potential breeding sites for *An. quadrimaculatus s.s.*. This species seems to be ubiquitous and can tolerate a wide range of environmental variables.

Bradley (1932) studied the factors associated with breeding of anophelines in Mound, LA and concluded that the range of hydrogen-ion concentration in breeding and non-breeding waters was about the same, and that all were alkaline. The reported mean for pH in breeding waters was 7.42. The range of pH measurements found in the larval habitats characterized during this study was 6.00 - 8.47. *An. quadrimaculatus s.s.* was found in every habitat excluding one, and exhibits a wide range of tolerance for pH. We cannot conclude that pH is a limiting factor in choice of oviposition sites for the female except, perhaps, at the extreme ends of the pH scale.

The habitat with the lowest pH, considered acidic at 6.00, was YC, which produced *An. quadrimaculatus s.s.* and *An. maverlius*. This was the only site where we were able to collect *An. maverlius* larvae, and it is possible that pH might play a role in the choice of oviposition site by the females of this species. However, several more habitats for this species would need to be located in order to make a valid comparison.

*An. inundatus* was found in two separate bodies of water, one having a pH almost neutral (6.93), and the other being acidic at 6.27. Again, there were not enough habitats located for this species to make a statistical comparison. It is also possible that this species may prefer pH’s that are neutral or somewhat acidic.

In the laboratory, Lund (1942) studied the choice of a medium for oviposition by *An. quadrimaculatus* by looking at hardness of water, concentration of calcium ions,
muddiness, presence of algae, salinity, phosphorus content, ammonia content, tannic acid content, pH, presence of ferric chloride or aluminum chloride, and the presence of sucrose in the medium. He concluded that none of the factors provided statistical evidence for a preference for an oviposition medium. It is not clear which of the sibling species Lund was examining, as the Quadrimaculatus Complex was undiscovered at that time. Studies such as these should be done with each of the 5 species in the complex to determine if pH and other elements play a role in the choice of an oviposition medium and to determine if there is a preference.

There were not enough different larval habitats found for any species to make statistical comparisons regarding the elements present in the water (personal communication, Dr. J. Geaghan, LSU Dept. of Experimental Statistics). However, a few observations were notable. Of the twenty-eight elements, those present in the amounts of greater than one part per million (ppm) for the majority of the habitats were calcium (Ca), iron (Fe), potassium (K), magnesium (Mg), manganese (Mn), sodium (Na), sulfur (S), and silicon (Si), and zinc (Zn).

The presence of Ca, Fe, K, Mg, Na and S in the Yellow Creek mixed habitat was generally lower than the numbers reported for the majority of the type A habitats. This cannot be statistically analyzed, but there does appear to be a pattern with regard to those six elements. Further analysis should be conducted with respect to chemicals. It is possible that *An. maverlius* cannot tolerate high levels of those elements in the larval habitats, or conversely, that the other sibling species that were not collected from that
habitat will not lay eggs in this water because the elements are not present at levels that the female can detect.

Two habitats that were characterized were not included in the water analysis; P733 Swamp and LA 717(a). It was noted that beryllium (Be) was not detected in the analysis of water from any of the larval habitats.

For all the type A habitats, the range of Ca was 4.98 ppm - 69.99 ppm and for Na, 0.9 ppm to 150.3 ppm. This also indicates that *An. quadrimaculatus s.s.* is opportunistic as seen by the tolerance for a wide range of elements in the water.

Additional larval habitats need to be found so that statistical comparisons can be made. The results of this research should serve as a reference for future work with the aquatic habitats of the Quadrimaculatus Complex.

References Cited


CHAPTER 6

PCR Screening for Wolbachia pipiensis in Anopheles quadrimaculatus Sibling Species

Introduction

The genus Wolbachia includes bacteria (rickettsiae) that are cytoplasmically inherited and are found in the ovaries and testes of their host. These bacteria are capable of manipulating reproduction in the host through cytoplasmic incompatibility (CI). The incompatibility arises when males of certain strains and females of other strains are mated. The mating and insemination appear normal in the female and the eggs are fertilized, but no karyogamy occurs (Yen 1975).

Wolbachia spp. have been found in over 16% of insect species (over 80 different species) in each of the major insect orders (Werren 1997). Most rickettsiae cannot be cultured outside their host, however, Wolbachia can now be grown in insect cell cultures (Werren 1997).

Historically, the detection of the presence of Wolbachia in it’s host involved antibiotic curing where the females from a cured strain were incompatible with males that were infected with the rickettsiae. Currently, detection is enhanced by the use of the polymerase chain reaction (PCR) which uses 16S rDNA sequences unique to the Wolbachia as primers.

Wolbachia pipiensis was first described in the mosquito Culex pipiens (L.) (Hertig 1936) and the infection appears to be present in 100% of wild-caught mosquitoes in the Cx. pipiens complex (Sinkins et al. 1995).
There are two subdivisions of *Wolbachia* (A and B). *Wolbachia pipiensis* belongs to the B subdivision. There are differences in isolates of *Wolbachia* from different hosts; however, the nomenclature and taxonomic issues have yet to be resolved (Werren 1997). For now, the different isolates are referred to as strains within the *Wolbachia*.

According to Werren (1997), *Wolbachia* have implications for evolutionary processes because of their wide distribution and the host effects. The primary host effect that has been looked at is cytoplasmic incompatibility (CI). Yen and Barr (1974) showed that this incompatibility was due to a bacterial symbiont, *W. pipiens*, that exists in members of the *Cx. pipiens* complex. When large numbers of *Wolbachia* were found beneath the micropyle of the eggs, it was suspected as the cause of CI (Yen 1975).

The mechanisms that have been proposed to cause the incompatibility is 1) *Wolbachia* in the male produces a product that interferes with the processing of sperm in the egg and 2) *Wolbachia* in the male bind a product that is necessary for processing of sperm (Werren 1997).

*Wolbachia* induces CI in two different manners: unidirectional and bidirectional incompatibility. In unidirectional incompatibility, sperm infected with *Wolbachia* are incompatible with uninfected eggs. In bidirectional incompatibility, males and females are infected with different strains of *Wolbachia* and these are mutually incompatible.

One result of unidirectional CI is that infected females have a reproductive advantage over uninfected females in a mixed population of infected and uninfected males. Because *Wolbachia* are maternally inherited, a rapid increase in the proportion of infected hosts in an interbreeding population is expected to occur (Sinkins et al. 1995).
It is of interest in sibling species complexes to consider whether a symbiont or parasite may be the cause of the divergence of the species. While it would be difficult to determine whether the symbiont was present before or after the split, it is possible that it’s presence played an important role in the divergence. Bidirectional incompatibility induced by *Wolbachia* is a possible mechanism for rapid speciation in arthropods (Werren 1997). There have been no investigations for the presence of *Wolbachia* in the *Quadrimaculatus* Complex.

The objective of this study is to screen wild populations of mosquitoes belonging to the *An. quadrimaculatus* complex for infections of *W. pipientis*.

**Methods and Materials**

Sixty-two *An. quadrimaculatus s.l.* adult mosquitoes were tested for the presence of *W. pipientis*. The mosquitoes were collected from the following areas: nineteen specimens from Abbeville, LA, ten specimens from Tishomingo, MS, six specimens from Lake Arthur, LA, eleven specimens from Jennings, LA, and sixteen specimens from Gainesville, FL.

Anopheline mosquitoes were collected from areas that had been determined in a previous survey to have Anopheline populations (Rutledge 1995). Adults were aspirated from resting areas such as barns, tree holes, underneath bridges, and from resting boxes placed in the area by mosquito control district personnel (Vermilion parish Mosquito Control). The mosquitoes were transported to the Mosquito Laboratory at Louisiana State University Agricultural Center, and subsequently sorted to species and frozen at
-70°C until PCR was performed at a later date. These mosquitoes were collected from May - Aug 1996, May - Sept. 1997, and May - July 1998.

_Culex quinquefasciatus_ mosquitoes were collected for DNA extractions to provide a positive control for _Wolbachia_. Larvae were collected from a septic ditch in East Baton Rouge parish, Louisiana, in the Sharon Hills subdivision. The larval containers were placed into screened mosquito cages (BioQuip, Gardena, CA). As adults emerged, they were aspirated from the cages and subsequently frozen at -70°C.

The screening for _Wolbachia_ in Anopheline mosquitoes was conducted utilizing PCR and the protocol published by O’Neill (1997). DNA extractions were done according to Collins et al. (1987) and as follows: Individual mosquito abdomens were homogenized in a 1.5 μl eppendorf tube with a tissue grinder in 100 μl of grinding buffer which was made as follows: 1.6 ml 5M NaCl, 5.48 g sucrose, 170 μl 0.5M EDTA (pH 8.0), 2.4 μl 1M Tris (pH 8.0), 2.5 ml 20% SDS, 1.21 g Tris HCl, and sterile water qs 100 ml. The buffer was filtered through a 0.2 micron filter and heated to 65°C for 1 hr. The stock solution was stored at -20°C.

The homogenized mosquito was incubated at 65°C for 15 m. Fourteen μl of 8M K-acetate was added to each tube and mixed well. This homogenate was then placed on ice for 30 m prior to spinning in a refrigerated centrifuge for 10 m at 14000. The supernatant was transferred to a new sterile microtube. Two-hundred μl of cold 100% ethanol was added to each tube and mixed well. After 10 m, this mixture was spun in a centrifuge for 20 m at 14000. The supernatant was discarded and 100 μl of cold 70% ethanol was added to rinse the remaining pellet. The 70% ethanol was then discarded.
and 100 μl of cold 100% ethanol was added. The 100% ethanol was discarded and the tubes were either placed upside down to air dry, or were placed into a vacuum for 5 m. The pellet was then dissolved in 50 μl of sterile TE and 1 unit of RNase. The DNA extractions were then frozen at -70°C until PCR was conducted.

Three sets of PCR primers were used throughout the screening experiment. The first set of primers were utilized for quality control to assure that the DNA extractions were done properly. The primers amplify from any insect and is designated as 12S rRNA with an expected product size of approximately 400 bp. The sequence of the primer pair is as follows:

12SA1: 5'-AAACTAGGATTAGATACCCTATTAT-3'
12SBI: 5'-AAGAGCGACGGGCGATGTGT-3'

The second set of primers used amplify from the 16S region of *W. pipiens* and are considered diagnostic for *W. pipiens*. The expected product size is approximately 900 bp and the sequence of the primer pair is as follows:

99F: 5'-TTGTAGCCTGCTATGGTATACT-3'
994R: 5'-GAATAGGTATGATTTTCATGT-3'

The third set of primers employed are sensitive for *W. pipiens* and termed ftsZ. The expected product size is approximately 700 bp and the sequence is as follows:

494F: 5'-CCGTATGCAGCGATTTCACTTTG-3'
1262R: 5'-TCGCCATGAGTCATTTGGCT-3'

Primers were made at the GeneLab, Louisiana State University School of Veterinary Medicine.
The master mix for each PCR reaction was 13.5 μl of ddH₂O, 2 μl of 10X reaction buffer, 2 μl of 25 mM MgCl₂, 0.5 μl of 10 mM dNTP mix, 0.5 μl of each primer at 20 μM, and 1 U of Taq polymerase.

Nineteen microliters of the master mix plus one microliter of DNA sample were thermocycled at conditions of 95°C for 3 minutes for initial denaturation followed by 35 cycles of 95°C for 1 m, 55°C for 1 m, and 72°C for 1 m. The samples in each tube were layered with one drop of mineral oil to prevent evaporation during the thermocycling process. Ten microliters of the completed reactions plus 2 microliters of tracking dye were placed on a 2% agarose gel containing ethidium bromide in 0.5X TBE buffer and electrophoresed for 1.5 h at 50 V. On either end of each gel, a 50 - 1000 bp DNA marker was loaded for comparison of size fragments of the sample DNA. Products were visualized by placing the gel on a UV light table and were subsequently photographed using Type 55 black and white Polaroid® film.

Results

*Wolbachia pipiensis* was not detected in any of the sixty-two specimens examined from six *An. quadrimaculatus* adult populations. Insect DNA was amplified in all specimens indicating that the DNA extractions were done properly. The positive control, *Wolbachia*-infected *Cx. quinquefasciatus*, produced visible bands on the agarose gel, indicating that the primers for *W. pipiensis* were working.

Discussion

This preliminary screening of wild populations of *An. quadrimaculatus s.l.* is not an indicator that members of this complex are not infected with the rickettsia. It only shows
that *W. pipiens* was not detected in any of the sixty-two specimens that were examined. No generalizations should be made from this small sample. More mosquitoes from throughout the geographic range should be screened for the parasite.

**References Cited**


Conclusion/Summary

With the discovery of the Quadrimaculatus Complex, a new set of challenges have arisen. Significant research with *An. quadrimaculatus s.l.* in the past may need to be revisited regarding which of the sibling species were involved in the work. There is a lot of information on the adult habitats and morphologic descriptions of the five species. Additionally, several tools are available for identifying the individual species. It is important, now, to use the techniques available and fill in the gaps that are present concerning larval habitats and female oviposition behavior.

In summary, the PCR assay tested on all life stages of field caught *Anopheles quadrimaculatus s.l.* mosquitoes, is a reliable tool for identifying the different sibling species. It is necessary, though, to consider the geographic location of the mosquitoes when species C1 or C2, *An. inundatus* or *An. diluvialis*, is involved.

None of the four sibling species present in Louisiana were colonized during this research effort. Several different techniques were attempted in the laboratory, including use of increasingly larger cages, two blood host choices, and variations in the larval food. It would be beneficial to work with perhaps one or two populations in depth, in order to determine the specific requirements of the different sibling species.

Sixteen larval habitats of the *An. quadrimaculatus s.l.* were characterized. No statistical analysis could be employed because 15/16 of the habitats contained one species, *An. quadrimaculatus s.s.* However, habitat descriptions and analysis of water from each site are reported here and provide a baseline for future research on this group of mosquitoes.

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Wolbachia pipiens was not detected by PCR in sixty-two An. quadrimaculatus s.l. mosquitoes. This parasite commonly is present in the southern house mosquito, Cx. quinquefasciatus and has been found in some sibling species of other insects. It is reportedly a potential cause of rapid speciation. The small sample size in this study warrants additional screening for the parasite within the mosquitoes of the Quadrimaculatus Complex although these limited number of mosquito adults came from six populations in Louisiana, Mississippi, and Florida.
Appendix A

Letter of Request

JUL-20-1998 12:55  ENTOMOLOGY

Louisiana State University
Agricultural Center
Louisiana Agricultural Experiment Station

Journal of Medical Entomology
ESA
9301 Annapolis Road
Lanham, MD 20706-3115

20 July 1998

I am requesting permission to include a publication from the Journal of Medical Entomology in my dissertation for Louisiana State University. The article is:

Validation of a Ribosomal DNA-Polymerase Chain Reaction Species Diagnostic Assay for the Common Malaria Mosquito (Diptera: Culicidae) Sibling Species Complex.


November 1996, Volume 33, No. 6, Pages 952-954.

If this is acceptable, please send a letter to me so that I can include it in my dissertation.

Thank you,
Roxanne Rutledge
LSU, Dept. of Entomology
402 Life Sciences Building
Baton Rouge, LA 70803

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Appendix B

Letter of Permission

Dr. Roxanne Rutledge
Department of Entomology
Louisiana State University
402 Life Sciences Building
Baton Rouge, LA 70803

Dear Dr. Rutledge:

Permission is hereby granted for you to include the material outlined in the attached correspondence, dated July 20, 1998 (see attached).

This permission is granted with the understanding that (1) the material will not be used in any other manner and (2) will be used only in this one project and (3) will not become part of any Internet-based distribution system.

ESA requires that you give credit to the publication from which the material is reprinted, including the full title of the publication, volume number, page numbers, date of issue (if known), author citation, and the title of the article from which the material is taken. You must note that the material is copyrighted by the Entomological Society of America.

Sincerely,

[Signature]
Ray Evermann
Director of Communications

August 19, 1998
Appendix C

Smithsonian Institution Medical Entomology Project Form

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**Remarks:**

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Vita

Cynthia Roxanne Rutledge was born and raised in Birmingham, Alabama. She graduated from Berney Points Baptist High School in 1981. She began her undergraduate studies in Alabama, and completed a bachelor of science degree in Environmental Science in 1992 at Louisiana State University. She obtained a master of science degree in Entomology under the guidance of Dr. C. L. Meek in 1995. Ms. Rutledge continued her graduate studies with Dr. Meek and is currently a candidate for the degree of Doctor of Philosophy in Entomology with a minor in Epidemiology and Community Health. She currently resides in Baton Rouge and shares her home with three lovely dogs, Buddie, Annie, and Sophie.
Candidate: Cyu:hia Roxanne Rutledge

Major Field: Entomology

Title of Dissertation: Biology and Ecology of the *Anopheles quadrimaculatus* Sibling Species Complex in Louisiana

Approved:

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EXAMINING COMMITTEE:

[Signatures]

Date of Examination:

October 16, 1998