Journal of the American Mosquito Control Association, 19(2):163–165, 2003

SCIENTIFIC NOTE

DETERMINATION OF *ANOPHELES GAMBIAE* LARVAL DNA IN THE GUT OF INSECTIVOROUS DRAGONFLY (LIBELLULIDAE) NYMPHS BY POLYMERASE CHAIN REACTION

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ABSTRACT. We examined the predator–prey relationship between larvae of the malaria mosquito *Anopheles gambiae* and nymphs of the dragonfly (Libellulidae). Studies were conducted to determine whether polymerase chain reaction (PCR) can be used to detect DNA of *An. gambiae* in the gut of libellulid nymphs, and to determine how long after feeding on *An. gambiae* that mosquito DNA remains detectable by PCR. Total DNA was extracted from the gut contents of libellulid nymphs by using 2 types of DNA extraction methods. The target sequence for the diagnostic PCR was the intergenic spacer regions of the ribosomal DNA gene locus. These sequences were analyzed by using *An. gambiae* complex–specific primers. After analyzing nymphal gut contents with PCR at regular postfeed intervals, a 390–base pair product could be amplified. The presence of mosquito larvae was visually confirmed for up to 40 min after feeding. Regardless of the number of mosquito larvae ingested, libellulid gut contents could be amplified or visually seen up to 1 h of digestion. This result indicates the nymphs have a high rate of digestion and that PCR with *An. gambiae* complex primers will be best utilized within 1 h after feeding as a detection system. This study confirmed that dragonfly nymphs feed well on anopheline larvae, and that mosquito DNA, although rapidly digested, can be successfully recovered and detected from within nymphal digestive tracts.

KEY WORDS Predator–prey interactions, *Anopheles gambiae*, polymerase chain reaction, gut-content analysis, dragonfly nymphs

The main malaria vector mosquitoes in sub-Saharan Africa include *Anopheles funestus* Giles and some members of the *Anopheles gambiae* complex. Minakawa et al. (1999) described larval habitats of *An. gambiae* Giles sensu stricto and *Anopheles arabiensis* Patton of the *An. gambiae* complex in western Kenya. In addition, we recently completed a survey of mosquito larval habitats located along the Kenyan coast (Mbogo et al., unpublished data). In both of these studies, dragonfly nymphs were found to be abundant invertebrate predators in larval habitats of *An. gambiae*. Libellulidae is the largest and most familiar family of dragonflies in southern Africa (Scholtz and Holm 1986). Depending on their developmental stage, dragonfly nymphs can consume up to 28 4th-stage mosquito larvae or more than 100 2nd-stage larvae per day (Urabe et al. 1986). The larval ecology of African malaria vectors relative to the potential impact of predator–prey interactions with libellulid nymphs has not been studied. One reason for a lack of studies on the invertebrate predators of *An. gambiae* could be the difficulties faced in examining and identifying gut contents. Traditional methods of detecting gut contents of invertebrate predators have relied on the use of biochemical markers, such as isozymes (Giller 1986), and serological techniques with antibodies developed against the prey (Sunderland 1988, Greenstone 1996). However, production of monoclonal antibodies is expensive and complex, and these techniques have failed to achieve desired levels of specificity and sensitivity (Greenstone 1996). Despite these drawbacks, there have been some successes. Service (1977) used precipitin tests to serologically confirm the prey of mosquito predators.

More sensitive and specific methods are needed to understand the predator–prey dynamics of dragonfly larvae and larval *An. gambiae*. Thus, this study concentrated on determining whether polymerase chain reaction (PCR) can be used to detect DNA of *An. gambiae* in the gut of libellulid nymphs, and how long DNA can be detected after feeding on *An. gambiae*. This PCR-based analysis requires knowledge of a unique region in the genome of the prey that is not conserved in the predator species. Therefore, we chose the assay developed by Scott et al. (1993), which utilizes the intergenic spacer (IGS) region of ribosomal DNA. Ribosomal DNA is present in hundreds of tandemly repeated copies, so a very small amount of tissue provides a sufficient template for PCR. In addition, the IGS is variable enough to distinguish among members of the *An. gambiae* complex, but is too variable to be conserved in the Libellulidae. To ensure that dragonfly nymphs would prey upon and

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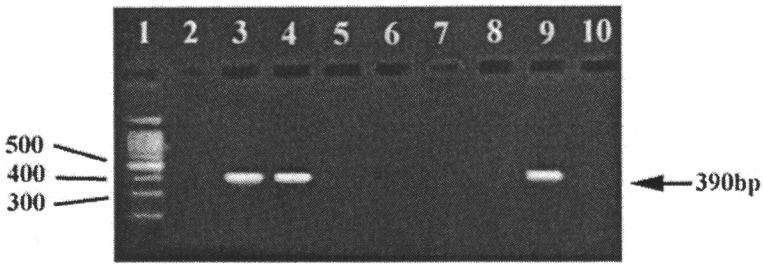

Fig. 1. Polymerase chain reaction products with primers GA and UN of Scott et al. (1993) fractionated on a 2% agarose gel. (1) Molecular size standard in base pairs; (2) starved dragonfly nymph negative control; (3–8) DNA extracted from the gut of dragonfly nymphs fed on G3 larvae of *Anopheles gambiae* at 0, 40, 80, 120, 160, and 200 min after feeding; (9) *An. gambiae* positive control; and (10) water negative DNA control.

eat larval *An. gambiae,* dragonfly nymphs were collected from permanent ponds in City Park, New Orleans, LA, in October 2001. Nymphs were collected by benthic drags along pond edges with a meshed-metal cage (30 × 30 × 30 cm). Captured predators were thoroughly rinsed and then separated into 1-liter plastic containers for transport to the laboratory. Mosquito larvae were obtained from a G3 strain laboratory colony of *An. gambiae* sensu stricto maintained at Tulane University. Several dragonfly nymphs were individually placed in a 15-cm petri dish with 3–5 3rd- to 4th-stage larvae of *An. gambiae* to observe predation.

After confirming nymphal predation on larvae with the local New Orleans species of dragonfly, young nymphal *Nannothemis bella* were obtained from a laboratory culture (Carolina Biological, Burlington, NC). We obtained these cultures to ensure uniform nymphal age and physiological state. The insects were maintained and fed in a controlled temperature room at 28°C and 80% relative humidity under a 12:12 h light : dark regime at all times. Twenty dragonfly nymphs were individually placed in a 15-cm petri dish with 200 ml of distilled water and were fed larval *An. gambiae* for a week. Before feeding, the dragonfly nymphs were starved for 36 h to ensure maximum prey consumption (Thangam and Kathiresan 1994). After this interval, 2 dragonfly nymphs were killed by freezing in dry ice and then placed at −70°C as starved controls. The remaining nymphs were moved to 200 ml of fresh distilled water, given 10 live mosquito larvae, and allowed to feed for a maximum of 30 min. After this period, the number of mosquito larvae ingested was recorded. The number of mosquitoes eaten by the predators averaged 5.5. Three predators were killed, by freezing at intervals of 0, 40, 80, 120, 160, and 200 min after feeding.

Anatomical dissections of the dragonfly nymphs were carried out to minimize nontarget amplification of predator DNA. The fore-, mid-, and hindguts were removed and placed in a sterile 1.5-ml microcentrifuge tube. Two different DNA extractions were tried. The 1st DNA extraction was modified from that of Collins et al. (1987). All the volumes in this DNA extraction were doubled. The other DNA extraction used was the Proteinase K extraction method followed by Wilkerson et al. (1993). All pellets were resuspended in 50 μl of sterile tris-ethylenediaminetetraacetic acid. The DNA concentrations were determined spectrophotometrically.

The DNA extractions were diluted 1:10 and 1:20. Amplification by PCR was performed in 25.0-μl reaction volumes containing 2.5 μl of 10× reaction buffer, 1,200 μM of each deoxynucleoside triphosphate, 1 mM $MgCl_2$, 0.625 unit of Taq polymerase (Promega, Madison, WI), 6.5 ng of *An. gambiae*–specific GA primer (5′ CTG GTT TGG TCG GCA CGT TT 3′), and 12.5 ng of universal primer (5′ GTG TGC CCC TTC CTC GAT GT 3′) (Scott et al. 1993). The PCR was carried out with 30 cycles of denaturation at 94°C for 30 sec, annealing at 52°C for 30 sec, and extension at 72°C for 30 sec (Scott et al. 1993). The entire content of each reaction tube was electrophoresed at 100 V through a 2.0% agarose gel containing ethidium bromide. In samples containing mosquito DNA, these primers yielded a PCR product of 390 base pairs (bp), but did not yield any product in the pure libellulid nymph DNA control or the negative DNA control (Fig. 1).

Both of the DNA extraction methods yielded quality DNA, as indicated by the positive (*An. gambiae* only) DNA control. Amplification of mosquito DNA by PCR was never observed after 1 h after feeding, regardless of the number of mosquitoes eaten by the predator or the DNA extraction method used (Table 1 and Fig. 1). Amplification failures were common when undiluted DNA was used and samples could be amplified only when DNA was diluted 1:10 or 1:20 (data not shown). Foregut contents of dragonfly nymphs were analyzed visually to determine prey presence. However, no remains of mosquito larvae were detected in any specimens after 1 h after feeding. Examination of our results suggests that digestion in the Libellulidae occurs at a relatively fast rate and inhibits detection beyond 1 h after feeding.

The target DNA sequence of 390 bp in length

Table 1. Time intervals and polymerase chain reaction results when using *Anopheles gambiae* complex primers, which gave a product of 390 base pairs. The DNA extraction methods were that of Collins et al. (1987) (method 1) and Proteinase K (method 2). A plus (+) indicates an observable band and a minus (−) indicates no observable bands. Three nymphs were used for each time point.

	Time (min)					
Method	0	40	80	120	160	200
1	+	+	−	−	−	−
2	+	+	−	−	−	−

apparently was quickly degraded by the predator and rendered undetectable by PCR. Although previous studies by Zaidi et al. (1999) and Agusti et al. (1999) used different predator and prey species, their use of smaller target sequences (146 and 254 bp) allowed them to detect prey DNA beyond 1 h after feeding. Zaidi et al. (1999) showed that PCR of prey DNA provides a practical method for detecting prey remains in insect predators after more than 24 h and potentially covers the interval between setting up nocturnal traps and collection of the predator the next morning. Future PCR-based detection studies of this predator–prey system should focus on the use of shorter target DNA sequences.

We thank Clara B. Ocampo and Bryan Foy for their advice in this project. This study was supported by National Institutes of Health grants U19 AI45511, D43 TW01142-02S1, and U19 AI45511–03S1, and National Science Foundation grant DEB-0083602.

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