

Ohba et al.: Predators of *Anopheles* Larvae

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**Predators of *Anopheles gambiae* s.l. (Diptera: Culicidae) Larvae in Wetlands,  
western Kenya: Confirmation by PCR Method**

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**Abstract.** Polymerase chain reaction (PCR) analysis was performed to determine whether mosquito predators in wetland habitats feed on *Anopheles gambiae* sensu lato (s.l.) larvae. Aquatic mosquito predators were collected from six wetlands near Lake Victoria in Mbita, Western Kenya. This study revealed that the whole positive rate of *An. gambiae* s.l. from 330 predators was 54.2%. The order of positive rate was the highest in Odonata (70.2%), followed by Hemiptera (62.8%), Amphibia (41.7%), and Coleoptera (18%). The study demonstrates that the PCR method can determine whether aquatic mosquito predators feed on *An. gambiae* s.l. larvae if the predators have undigested *An. gambiae* s.l. in their midgut or stomach.

**KEY WORDS** Anura, aquatic insect, Coleoptera, Hemiptera, Odonata

Despite a long history of efforts to control malaria, malaria remains a major threat to human health. Since the development of a vaccine for malaria has been slow, and parasite resistance to anti-malaria drugs is developing rapidly, vector control is considered the most practical method for reducing malaria transmission in developing countries (Trape et al. 2002, Fillinger et al. 2004, Killeen et al. 2004). Common measures for malaria vector control include bed nets and indoor residual spray using DDT or pyrethroids (Kouznetsov et al. 1977, Lindblade et al. 2006). However, there is concern about toxicity to non-target organisms (Henry and Kishimba 2006) and vector resistance to chemical insecticides (Corbel et al. 2003, Etang et al. 2003, Hargreaves et al. 2003, Casimiro et al. 2006). Because chemical insecticides also kill predators of mosquitoes (Service 1977) the reduction of predators may increase vectors; therefore, the use of chemical insecticides requires caution.

Among the various natural ecological forces controlling vector populations, predation on immature *Anopheles gambiae* sensu lato (s.l.) appears to be a major factor controlling population size. Predation on *An. gambiae* larvae contributes considerably (between 13.4% and 84.5%: Chandler and Highton 1977) to overall larval mortality (between 92.6% and 97.1%: Service 1971, 1973, 1977). However, little research has been devoted to the effects of larval predation on mosquito population structure and ecology,

possibly because of difficulties in identifying and quantifying the impact of the most common predators in the field.

Using the serological method, Service (1973, 1977) found that predators of *An. gambiae* s.l. include amphibians as well the insects from the orders Araneida, Odonata, Hemiptera, Hymenoptera, Diptera, and Coleoptera. Schielke et al. (2007) showed that *An. gambiae* s.l. DNA can be detected after ingestion by members of the families Lestidae (Odonata), Libellulidae (Odonata), and Notonectidae (Hemiptera). Although the authors fed single laboratory-raised *An. gambiae* sensu stricto (s.s.) to the predators under laboratory conditions, they recommended that the polymerase chain reaction (PCR)-based assay will be useful for future studies of *An. gambiae* larval ecology, particularly in detecting larval predators in natural breeding sites.

However, no studies have applied the PCR analysis to detect predators of *An. gambiae* larvae in natural habitats. Therefore, in the present study, we evaluated the feasibility of detecting predators of *An. gambiae* s.l. in natural habitats using PCR. Specifically, we were interested in whether this method can detect a variety of predators including vertebrates predators in different types of habitats. Additionally, we examined whether time duration after collection influenced the sensitivity of PCR to detect predators.

## Materials and Methods

***Anopheles* Abundance and Predator Collection.** Our study was conducted in six wetlands (Wetland A: 1 × 1 m, B: 15 × 20 m, C: 5 × 5 m, D: 2 × 2 m, E: 2 × 1 m, F: 3 × 3 m) near Lake Victoria (<ca. 500 m) in Mbita, Western Kenya (0°26'S, 34°12'E), on December 2, 3, and 7, 2009. This period was within the short rainy season. Water depth in wetlands ranged from 0.03–0.3 m. The environment of the study site has been described previously (Minakawa et al. 2008).

To estimate the density of *Anopheles* larvae, samples of 30 dips from the water surface were examined in each wetland using a standard dipper (diameter of dip at mouth: 11.5 cm, height: 5.5 cm, volume: 350 ml). To collect mosquito predators, two field assistants swept each wetland using a 3-mm mesh D-frame dipnet (0.28 m wide) for about 30 min. The collected specimens were immediately transferred to 99% ethanol in the field. Two hours after the first fixation, specimens were transferred to fresh 99% ethanol and were identified to genus, family, and order in the laboratory.

Dominant Dytiscidae (*Laccophilus* spp.) and Corixidae (*Micronecta* spp.) collected from wetland D were divided into two groups to examine differences in positive reaction rates at different times after collection (immediately after or 24 h after collection). The predators of the former group were transferred within 1 min to 99% ethanol in the field.

The latter group was kept in a plastic container without food for 1 day (300 mm diameter; 400 mm height) filled with dechlorinated tap water to a depth of 50 mm before fixation in 99% ethanol.

**PCR of Prey Found in the Midgut of Predators.** PCR analysis was performed to confirm whether predators (Anuran larva, Odonata nymphs, Hemiptera, and Coleoptera) feed on *Anopheles* larvae. Sample *An. gambiae* s.l. larvae and predators were analyzed with the rDNA-PCR method (Scott et al. 1993). *An. gambiae* s.l. larvae and predators were placed on clean paper for 30 min to remove ethanol before DNA extraction (see Ohba et al. in press). For the PCR analysis, we used the whole body of predatory insects <5 mm in body length, the abdomen of predatory insects  $\geq$ 5 mm in body length, and the stomach of anuran larvae (tadpoles). DNA was extracted using REDExtract-N-Amp Tissue PCR Kit (Sigma, St. Louis, MO, USA). The extraction solution (20  $\mu$ l) and tissue preparation solution (5  $\mu$ l) were mixed, and each individual sample was homogenized in a 1.5-ml tube and incubated at room temperature for 10 min followed by incubation at 95°C for 3 min. Neutralization solution (20  $\mu$ l) was added to the sample and mixed by vortexing. The resultant mixture was used directly for the PCR. Multiplex PCR was conducted using 1 primer for *An. gambiae* s.l. (*An. arabiensis*, *An. gambiae*, and *An. merus*). PCRs were performed in a total reaction mix volume of 5.0  $\mu$ l that contained 0.5

µl of template DNA, 1.0 µl of REDExtract-N-Amp ReadyMix (TaKaRa Bio Inc., Shiga, Japan), and 1 pmol of each primer. Primer sequences were as described by Scott et al. (1993): UNG: GTGTGCCCCCTTCCTCGATGT, GA: CTGGTTTGGTCGGCACGTTT, ME: TGACCAACCCACTCCCTTGA, AR: AAGTGCCTTCTCCATCCTA. The PCR reaction mixture was heated to 95°C for 1 min and then subjected to 35 cycles of PCR amplification: 94°C for 25 s, 50°C for 25 s, and 72°C for 30 s, followed by 72°C for 5 min. The amplified DNA was loaded onto 2% agarose gel with a 100-bp ladder loading marker (Bio-Rad, Richmond, CA), stained with ethidium bromide solution (Wako Inc., Tokyo, Japan), and visualized on an ultraviolet (UV) transilluminator (TF-20C; Vilber Lourmat, Marne-la-Vallée, France).

**Statistical Analysis.** To examine the effect of time duration after collection, a logistic regression analysis was applied to the data of the positive reactions in the two dominant predators, Dytiscidae (*Laccophilus* spp.) and Corixidae (*Micronecta* spp.). The response variable was binary (positive or negative reaction) for the mosquitoes ingested by predators. The time duration after collection (immediately or 24 h after collection) were used as explanatory variables. *Micronecta* spp. was the dominant predator in two wetlands that had different densities of *Anopheles* larvae (1.10 in wetland B and 0.13 in wetland C per dip; Table 1), which might have influenced the encounter rate and ease of

capture by predators. Moreover, one habitat was covered with taller vegetation. We expected that the differences in habitat condition influenced the positive rates of the two populations of *Micronecta* spp. and that the PCR method could detect such differences. A logistic regression analysis was applied to the data from habitat B and C. The reaction (positive or negative) was used as the response variable, and wetland (B or C) was included as the explanatory variable. The significance level for all tests was set at 0.05. All statistical tests were conducted using JMP software (JMP version 8.0, SAS Institute, 2008).

## Results

Among the 164 *An. gambiae* s.l. larvae, the PCR analysis revealed that 162 were *An. arabiensis* (98.8%) and 2 were *An. gambiae* (1.2%). Therefore, our analysis focused on *An. arabiensis* prey. Over three study days, 330 aquatic predators were collected from six wetlands and killed (Table 1). The positive detection rate of *An. arabiensis* DNA in whole predators was 54.2% (179 out of 330), which indicates that predators had recently ingested this mosquito. The predator community and the positive rates of PCR were different among the six wetlands. For instance, amphibian larvae (tadpoles) were limited to wetland A, Odonata nymphs were collected from wetland C, E, and F, and Hemiptera



and Coleoptera were collected from B, C, and D. Odonata had the highest positive rate (70.2%), followed by Hemiptera (62.8%), Anura (41.7%), and Coleoptera (18%) (Table 1). Among the Odonata, Libellulidae (*Pantala* spp.) had a higher positive rate than Zygoptera. Corixidae (*Micronecta* spp. A, B, and nymphs) and Mesoveliidae were found to have positive rates <50%, whereas Belostomatidae (*Dyplonychus* spp.) had the lowest positive rate of all the Hemipteran predators. The semi-aquatic Hemiptera, Mesoveliidae, was found to have a higher positive rate (8 out of 9). The diving beetle, *Laccophilus* spp. (<5 mm in body length, classified into small-bodied species; Ohba and Takagi 2010) was the dominant coleopteran predator in this study.

The results of the positive rates immediately after collection were markedly different from those 24 h after collection in two dominant predator species (Logistic regression analysis: predator species:  $df = 1$ , likelihood ratio  $\chi^2 = 8.99$ ,  $P = 0.003$ ; time after collection:  $df = 1$ , likelihood ratio  $\chi^2 = 24.57$ ,  $P < 0.001$ ; Fig. 1). In both predator species, positive reactions were detected in individuals killed immediately after collection, but no positive reactions were found in those killed 24 h after collection (no. positive per no. tested, Dytiscidae: immediately after collection = 8 out of 20, 24 h after collection = 0 out of 20; Corixidae: immediately after collection = 6 out of 6, 24 h after collection = 0 out of 3).

The logistic regression analysis showed that *Micronecta* living in wetland B had higher positive rates of *An. arabiensis* in the midgut than those in wetland C (no. positive per no. tested, 20 out of 23 in wetland B, 28 out of 64 in wetland C;  $df=1$ , likelihood ratio  $\chi^2 = 14.14$ ,  $P < 0.001$ ; Fig. 2).

## Discussion

As previously confirmed using serological methods (Service 1973, 1977), our study found that the PCR method could detect the presence of *An. gambiae* s.l. in the guts of predators. There were no positive reactions obtained from Dytiscidae and Corixidae predators 24 h after collection, which suggests that their stomach contents were completely digested. Hence, the PCR method was only useful when predators had undigested *An. gambiae* in their midgut (<24 h). Schielke et al. (2007) found that molecular assay can successfully detect larval DNA immediately after ingestion in Hemiptera and Odonata DNA extracts, but prey DNA detection varied over time and for different predators, ranging from 4 to 24 h after ingestion. In the present study, the positive rate in Odonata was higher than that in Hemiptera (Table 1). This variation in DNA prey detection appears to be related to the predator digestion type (external versus internal), which in turn affects the amount and quality of prey DNA (Schielke et al. 2007).

Schielke et al. (2007) obtained a positive result 1 h after ingestion in Lestidae (Odonata) but did not detect mosquito DNA in Notonectidae (Hemiptera). The authors propose that this may be due to Hemiptera feeding behavior; they inject digestive enzymes into their prey, ingest the liquefied material, and discard the rest of the body. This reduces the amount and quality of larval DNA in Hemiptera compared with Odonata, which ingest the entire larva.

Although only *Micronecta* spp. was investigated, the positive rates of *An. arabiensis* in the midgut of the population in wetland B were higher than those in wetland C (Fig. 1). However, the number of *An. gambiae* s.l. in wetland C was lower than that in wetland B, while the vegetation density in wetland C was higher than that in wetland B. Therefore, the difference in positive rates between the two populations of *Micronecta* spp. may be due to different environmental complexity in the habitats and encounter rates of *An. gambiae* s.l. The role of environmental complexity in the outcome of aquatic predation requires further study. Regardless, our results suggest that the PCR method can be used for comparing predators from different types of habitats.

Much attention has been given to identifying various predators of mosquitoes (for a review, see Mogi 2007; Quiroz-Martinez and Rodriguez-Castro 2007). Jenkins (1964) compiled a list of 220 species of invertebrate predators of mosquitoes; however, only a

few of these are potential candidates for biological control. Of these species, aquatic Hemiptera (Notonectidae, Belostomatidae, Nepidae, and Naucoridae), which inhabit rice fields and wetlands, are ecologically important mosquito predators (Mogi 2007, Quiroz-Martinez and Rodriguez-Castro 2007). In the present study, more than 60% of aquatic Hemiptera showed a positive detection rate for *An. arabiensis* DNA (Table 1). Although it has been suggested that Belostomatidae are important mosquito predators (Saha et al. 2007, 2010), this family was found to have the lowest positive rates of the Hemiptera included in our study. A previous serological study (Service 1977) also showed that the positive rate in Belostomatidae (genus *Sphaerodema*) was lower than for other Hemipteran species. Assuming that the digestion rates of prey are similar among aquatic Hemipterans, the Belostomatidae might not be important predators of *An. gambiae* s.l. On the other hand, the positive rate of the semi-aquatic Mesoveliidae in our study was >90% (Table 1). Because Mesoveliidae inhabit the water surface (e.g., Chen et al. 2005), they directly attack *An. gambiae* s.l. larvae just under the water surface.

The positive rates of Dytiscidae were considerably lower compared with the other taxa (Table 1). Beetles captured in the present study were <9 mm in body length and were classified as a small-bodied species. According to Ohba and Takagi (2010), predation rates are higher in medium-bodied species (9–20 mm in body length) than in small- (<9

mm) and large-bodied (>20 mm) species. In a preliminary field survey in May 2009, the density of medium-bodied predatory diving beetles (Coleoptera, Dytiscidae) in wetland habitats was higher during the high rainy season than during the low rainy season (O.S., unpublished data). Given these preliminary findings, it would be interesting to collect Dytiscidae during the rainy season to determine their positive rates and examine their role as predators of mosquito larvae.

The present study showed that the PCR method is useful for detecting a variety of predators for malaria vectors in natural aquatic habitats. Future studies can apply the PCR method to terrestrial predators such as spiders, assassin bugs, and shorefly (Service 1971, 1973, 1977, Minakawa et al. 2007, Futami et al. 2008) to determine whether these organisms prey upon adults in the *An. gambiae* complex.

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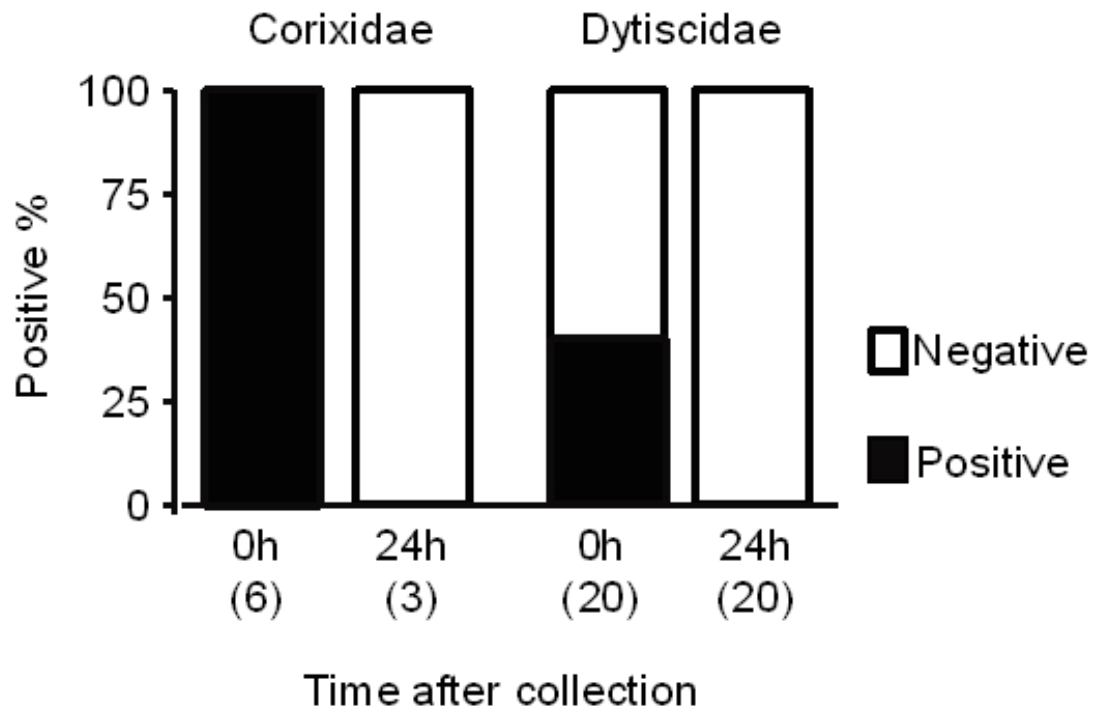
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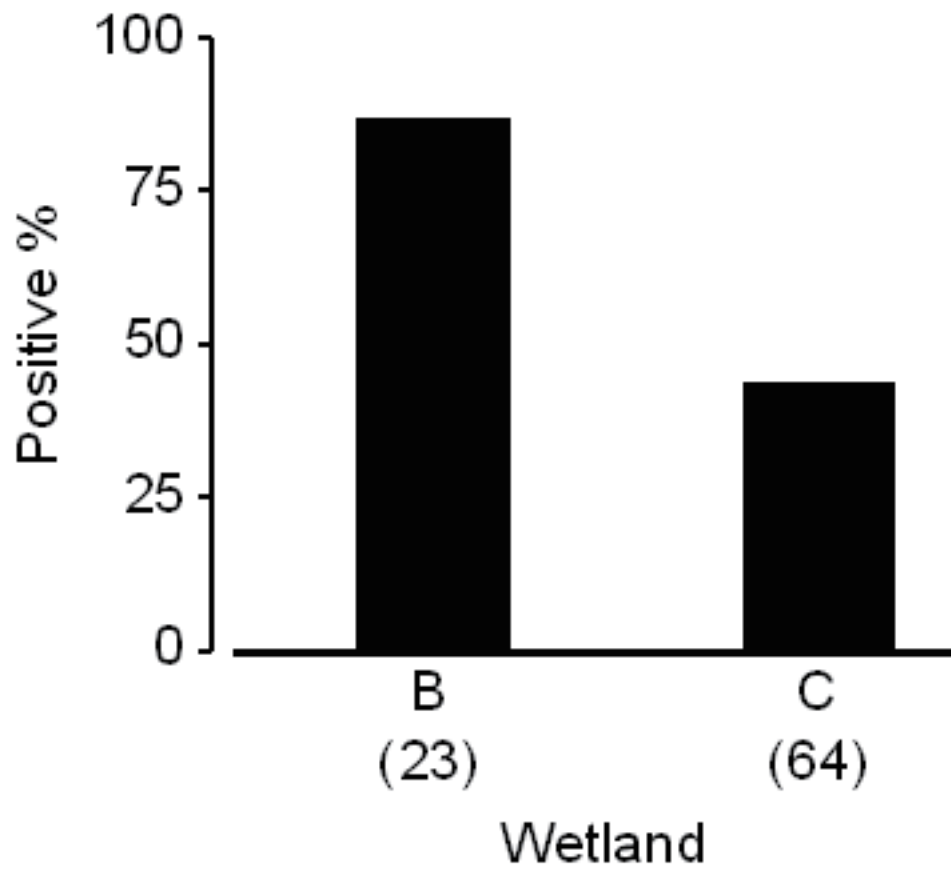
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**Figure legends**



**Fig. 1.** Comparison of positive rates of *An. arabiensis* in different time after collection of Corixidae and Dytiscidae. Numbers in parentheses show sample size.



**Fig. 2.** Comparison of positive rates of *An. arabiensis* in *Micronecta* spp. inhabiting in each wetland. Numbers in parentheses show sample size.

Table 1. continued.

Site	E			F			Total		
	No. <i>An. gambiae</i> larvae per dip	0.23		0.13					
Predators	No. tested	No. positive	Positive %	No. tested	No. positive	Positive %	No. tested	No. positive	Positive %
Amphibia									
Unidentified tadpole	-	-	-	-	-	-	24	10	41.7
Total	-	-	-	-	-	-	24	10	41.7
Odonata									
Libellulidae ( <i>Pantala</i> spp.)	20	14	70.0	29	22	75.9	49	36	73.5
Zygoptera	-	-	-	-	-	-	8	4	50.0
Total	-	-	-	-	-	-	57	40	70.2
Hemiptera									
Corixidae ( <i>Micronecta</i> spp. A)	-	-	-	-	-	-	48	47	97.9
Corixidae ( <i>Micronecta</i> spp. B)	-	-	-	-	-	-	39	22	56.4
Corixidae ( <i>Micronecta</i> nymphs)	-	-	-	-	-	-	48	26	54.2
Belostomatidae ( <i>Diplonychus</i> spp.)	-	-	-	-	-	-	25	6	24.0
Notonectidae	-	-	-	-	-	-	14	5	35.7
Pleidae	-	-	-	-	-	-	3	2	66.7
Mesoveliidae	-	-	-	-	-	-	11	10	90.9
Total	-	-	-	-	-	-	188	118	62.8
Coleoptera									
Dytiscidae ( <i>Laccophilus</i> spp.)	-	-	-	-	-	-	54	10	18.5
Dytiscidae (species A)	-	-	-	-	-	-	1	0	0
Dytiscidae (species B)	-	-	-	-	-	-	1	0	0
Larva of Dytiscidae (unidentified)	-	-	-	-	-	-	1	1	100.0
Larva of Hydrophilidae (unidentified)	-	-	-	-	-	-	4	0	0
Total	-	-	-	-	-	-	61	11	18.0