Larval Habitats Characterization and Species Composition of Anopheles Mosquitoes in Tunisia, with Particular Attention to Anopheles maculipennis Complex

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Laboratoire de Parasitologie Médicale, Biotechnologies et Biomolécules, Institut Pasteur de Tunis, Tunis, Tunisia; Laboratoire de Parasitologie-Mycologie, Institut Pasteur de Tunis, Tunis, Tunisia; Maladies Infectieuses et Vecteurs: Écologie, Génétique, Évolution et Contrôle (MIVEGEC; UMR IRD 224, CNRS 5290, UMI, UM2), Centre IRD France-Sud, Montpellier, France; Direction de l’Hygiène du Milieu et de la Protection De l’Environnement, Ministère De La Santé Publique, Tunis, Tunisia; Observatoire National des Maladies Nouvelles et Emergentes, Tunis, Tunisia

Abstract. In Tunisia, malaria transmission has been interrupted since 1980. However, the growing number of imported cases and the persistence of putative vectors stress the need for additional studies to assess the risk of malaria resurgence in the country. In this context, our aim was to update entomological data concerning Anopheles mosquitoes in Tunisia. From May to October of 2012, mosquito larval specimens were captured in 60 breeding sites throughout the country and identified at the species level using morphological keys. Environmental parameters of the larval habitats were recorded. Specimens belonging to the An. maculipennis complex were further identified to sibling species by the ribosomal deoxyribonucleic acid (rDNA)-internal transcribed spacer 2 (ITS2) polymerase chain reaction (PCR) technique. In total, 647 Anopheles larvae were collected from 25 habitats. Four species, including An. labranchiae, An. multicolor, An. sergentii, and An. algeriensis, were morphologically identified. rDNA-ITS2 PCR confirmed that An. labranchiae is the sole member of the An. maculipennis complex in Tunisia. An. labranchiae was collected throughout northern and central Tunisia, and it was highly associated with rural habitat, clear water, and sunlight areas. Larvae of An. multicolor and An. sergentii existed separately or together and were collected in southern Tunisia in similar types of breeding places.

INTRODUCTION

Until its elimination in 1980, malaria was endemic in Tunisia, with an annual mean incidence of 10,000 cases.1 Anopheles (An.) labranchiae Falleroni, 1926 and An. (Cellia) sergentii Theobald, 1907 were reported as the main incriminated vectors of the disease in the northern and southern parts of the country, respectively (Wernsdorfer W and Iyengar MO, unpublished data). Since 1903 and mainly after the World War II, numerous control campaigns combining environmental interventions, vector control and screening, and treatment of infected people have led to successful interruption of autochthonous malaria transmission.2,3 Currently, only imported cases (mainly from sub-Saharan African countries and caused by Plasmodium falciparum) and some post-transfusion cases are observed.4,5 However, the increase of the annual incidence of imported cases of malaria associated with the persistence of Anopheles mosquitoes highlights the risk of a resumption of the disease transmission in Tunisia.1,3,5,6


Despite the public health importance of An. labranchiae, An. sergentii, and An. multicolor, their larval biology has not, to our knowledge, been explored. A good understanding of larval habitat diversity and selection can provide relevant information about areas that are at higher risk of malaria transmission and could improve vector control implementation through targeted strategies.

Most of the important malaria vectors are members of species complexes or species groups, which are often difficult to distinguish morphologically from one another. Members of the Maculipennis complex have different ecologies, biological attributes, and vectorial capacities, and hence, correct species identification is necessary for a better understanding of their potential roles in malaria transmission in areas where they are known to occur. Species that are endemic to Europe, Asia, and North Africa and considered to belong to this group are An. labranchiae, An. atroparvus, An. messeae, An. sacharovi, An. maculipennis, and An. melanoon. According to most studies, with the exception of An. sacharovi, these species are impossible to distinguish morphologically at the adult as well as larval stages,11–14 even if some reports suggest possible diagnostic characters for some members of the complex at the larval stage.15,16 Recent studies based on the analysis of DNA sequences have leveraged part of the problem by providing a straight-forward and reliable polymerase chain reaction (PCR)-based molecular identification tool that allows species discrimination within the Maculipennis group in many countries, including Italy, Romania, Iran, England, Algeria, Morocco, and Greece.13,14,17–28 These studies have retained An. labranchiae as the only member of the Maculipennis complex found in Morocco and Algeria,22 whereas species composition within the Maculipennis complex is still pending for Tunisia.

This study aimed to update available data on the endemic Anopheles species present in Tunisia and their geographical distribution and determine their larval habitat preference.

* Address correspondence to Karim Aoun, Laboratory of Medical Parasitology, Biotechnologies, and Biomolécules (LR 11 IPT 06) and Department of Parasitology and Mycology, Pasteur Institute of Tunis, Tunisia, 13 PI Pasteur, BP 74, 1002 Tunis, Tunisia. E-mail: karim.aoun@pasteur.rns.tn

doi:10.4269/ajtmh.14-0513
MATERIALS AND METHODS

Larval sampling and habitat characterization. In total, 60 localities in nine governorates from northern, central, and southern Tunisia were visited during the 2012 summer season (May to October) (Figure 1 and Table 1). The selection of the region of interest was based on bibliographic research and data provided by the Regional Directories of Public Health. Anopheline mosquito larvae were collected from each larval development site using the standard dipping technique (350-mL dipper). Arbitrarily, sites with over 200 larvae after 15 dips were considered as hosting high larval densities. Sites with less than 100 larvae were considered as low-density habitats, and sites with no anopheline larvae after 15 dips were recorded as negative. The Anopheles larvae were separated from the culicine larvae and classified as early- (I and II) or late-instar (III and IV) stage. The late instars were preserved in 70% ethanol and transported to the laboratory for morphological identification.

Environmental variables, including chemical and physical characteristics of larval development sites, were recorded during larval collections. Chemical characteristics, including dissolved oxygen, salinity, and pH, were measured using a digital multimeter (CP1000/Wagtech WTD, Palintest Ltd, Gateshead, UK). Water temperature at the time of collection was also recorded. Physical characteristics of the mosquito larval habitats included altitude, habitat type (i.e., rural, suburban, or urban), sunlight exposure (i.e., sunny versus shaded), water turbidity (clear versus turbid), and substrate (i.e., muddy, sandy, or rocky). Presence/absence of vegetation (Phragmites communis, Typha angustifolia, Juncus sp., Sarcocornia sp., and algae), predators (Gambusia affinis), and competitors (e.g., culicinae larvae) was noted.

Anopheles larvae identification. Third- and fourth-instar larvae were morphologically identified in the field using the standardized key for the mosquitoes of Mediterranean Africa. Twenty larvae of the Maculipennis complex were then randomly selected from positive larval collections sites to be further identified into sibling species by the ribosomal deoxyribonucleic acid (rDNA)—internal transcribed spacer 2 (ITS2) PCR technique.

Genomic DNA was extracted from individual whole-larvae specimens as previously described. Amplification targeted the conserved ITS2 region of the rDNA cluster. The amplification was done with the conditions described previously.

Statistical analysis. The associations of the environmental variables with the occurrence of Anopheles mosquito larvae were tested using SPSS software (IBM SPSS statistics 20). A descriptive analysis of the data was carried out. The quantitative variables were described by means ± SEMs, and for the categorical variables, the percentages were calculated. The $\chi^2$ or Fisher's exact test was used to compare qualitative variables (percentages), whereas t test or analysis of variance (ANOVA) and the corresponding non-parametric tests (Mann–Whitney and Kruskal–Wallis tests, respectively) were used to compare the quantitative variables (means).

RESULTS

Morphological identification of anopheline mosquito larvae. In total, 25 of 60 water collections prospected revealed the presence of anopheline mosquito larvae (41.7%) (Figure 1). They produced a total of 647 anopheline mosquito larvae. According to morphological identification, four species belonging to two subgenera were recorded, including members of the Maculipennis complex: most probably An. (An.) labranchiae ($N = 252$; 38.9%), An. (Ce.) multicolor ($N = 233$; 36%), An. (Ce.) sergentii ($N = 150$; 23.2%), and An. (An.) algeriensis ($N = 12$; 1.9%).

Molecular identification. The rDNA-PCR technique performed on 20 specimens collected from 11 larval habitats amplified a single 374-base pair (bp) -long fragment, which was expected from An. labranchiae, therefore confirming morphological identification and suggesting the presence of a single member of the Maculipennis complex in Tunisia.

Habitat characterization of anopheline mosquito larvae. Six hundred forty-seven larvae of Anopheles, including An. labranchiae, An. multicolor, An. sergentii, and An. algeriensis, were collected from 25 habitats located in northern, central, and southern Tunisia (Figure 1); 4 of 10 studied variables were significantly associated with species distribution: water...
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<th>Locality</th>
<th>Temperature (°C)</th>
<th>pH</th>
<th>Dissolved oxygen (mg/L)</th>
<th>Salinity (mg/L)</th>
<th>Habitat type</th>
<th>Altitude (m)</th>
<th>Sunlight exposure</th>
<th>Water turbidity</th>
<th>Substrate</th>
<th>Vegetation</th>
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<td>-</td>
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**Table 1**

Larval habitats characterization and composition species of *Anopheles* mosquitoes in Tunisia

GPS = global positioning system.
temperature \((P = 0.031)\), salinity \((P = 0.001)\), bottom surface \((P = 0.022)\), and habitat type \((P = 0.017)\) (Tables 2 and 3). These characteristics were found to be the key factors that are associated with species occurrence.

The mean water temperature was significantly higher among \textit{An. labranchiae} \((30.5^\circ\text{C} \pm 3.09^\circ\text{C})\) than others species \((26.15^\circ\text{C} \pm 5.35^\circ\text{C})\). Likewise, analysis of the association between chemical characteristics of habitats and species revealed that salinity mean was significantly higher among \textit{An. labranchiae} (Tables 2 and 3).

\textit{An. labranchiae} is significantly less frequent in sandy bottom surface \((50\%)\) than other types of bottom surface \((33.3\%)\), significantly more common in rural \((83.3\%)\) than urban and suburban \((28.6\%); P = 0.017)\) areas, and more frequent in sunlight areas \((78.9\%)\) versus shaded one \((33.3\%)\), with a tendency to significance \((P = 0.059)\).

In this study, the other measured parameters, such as dissolved oxygen \((P = 0.091)\), altitude \((P = 0.522)\), pH \((P = 0.18)\), fauna \((P = 1)\), and water transparency \((P = 0.32)\), differed between species but were not significantly associated with species distribution (Tables 2 and 3).

Anopheline larvae and \textit{Gambusia} fish only coexisted in three habitats where the predators were recently introduced (Table 1).

The 252 \textit{An. labranchiae} larvae were collected in 17 habitats located in northern and central Tunisia. It was the only species encountered at these sites (Figure 1), and it always occurred at low density in the breeding sites. The most common habitats for \textit{An. labranchiae} larvae in Tunisia were rural \((88.2\%)\) with clear water \((100\%)\), no larvivorous fishes \((88.2\%)\), and sunny areas \((94.1\%)\) (Table 1).

\textit{An. multiolor} and \textit{An. sergentii}, the suspected vector species in central and southern Tunisia during the endemic period, were found separately in 83.3% of the positive breeding places of both species, where they frequently occurred at low density. They were collected together in only 16.7% of the positive breeding places of both species, corresponding often to high-density larval habitats (Figure 1 and Table 1). The most common habitats for both species are characterized by clear water \((83.3\%)\), no larvivorous fishes \((83.3\%)\), and sand substrate \((100\%)\) (Table 1). \textit{An. algeriensis}, reported as a non-vector species during the endemic period in Tunisia, was collected at low density from only 8% of larval habitats.

### DISCUSSION

Only four species of \textit{Anopheles} were found in this study, despite the sampling effort and the appropriate season of the captures corresponding to the \textit{Anopheles} activity period in Tunisia: \textit{An. labranchiae}, \textit{An. multicolor}, \textit{An. sergentii}, and \textit{An. algeriensis}. Between 1968 and 1974 (i.e., during the malaria eradication campaign), 12 species had been reported.\(^7\)

None of these species are specific to Tunisia. More recent investigations found six species, including \textit{An. cinereus} and \textit{An. claviger}.\(^8,9\) \textit{An. labranchiae} was the predominant species in northern Tunisia, whereas \textit{An. sergentii} and \textit{An. multicolor} were prevalent in southern Tunisia. These results are similar to those of previous studies.\(^8,9\)

As reported by Krida and others,\(^10\) anopheline larvae were found in rural, suburban, and urban habitats. \textit{An. labranchiae} was the only widely distributed species throughout northern and central Tunisia in subhumid and semiarid climate.
respectively (Figure 1). Our results showed that habitats sustaining the development of *An. multicolor*, *An. sergentii*, and *An. algeriensis* were not significantly different in relation to the environmental variables measured (Tables 2 and 3). The three species were captured in localities with arid climate located in southern Tunisia. Only water temperature, salinity, habitat type, and bottom surface were associated with species distribution. As reported in other studies, the existence and abundance of *Anopheles* immature stages were not correlated with water temperature, dissolved oxygen, pH, and bottom surface. However, a significant role of temperature and light exposure on *Anopheles* distribution was supported by Christophe and others. Surprisingly, exceptional tolerance to low pH of *An. labranchiae* larvae was observed (pH 2.6). However, no study showed comparative results for *Anopheles* mosquitoes, and additional investigations are required on the larvae biology of this species.

The species belonging to Maculipennis complex are difficult to identify because of the morphological overlap that exists within the groups. The molecular identification of species revealed the presence of a single member of the Maculipennis complex in Tunisia, namely *An. labranchiae*. The proportion of *An. labranchiae* (identified according to morphological characters) might have been correctly reported in previous entomological surveys in the area. The distribution of *An. labranchiae* is somewhat unusual, in that it is believed to be the only African member of the Maculipennis complex. It is highly abundant and widespread in the Maghreb countries: Morocco, Algeria, and Tunisia. It is assumed that *An. labranchiae* was the principal malaria vector in a large part of the country, particularly in the northern governorates. However, data are quite confusing because of the scanty and old infectivity tests conducted. Laboratory studies performed with *An. labranchiae* revealed that this species can transmit *P. ovale*, whereas populations collected in Italy were refractory to African strains of *P. falciparum*. Nevertheless, current research with populations from Corsica (France) and Principina (Grosseto, Italy) have indicated that the *P. falciparum* cycle can be successfully completed in *An. labranchiae*. Moreover, *An. labranchiae* has also been involved in autochthonous transmission of *P. vivax* in Corsica, Greece, and Italy. *P. vivax* malaria has been reported from different regions of Tunisia, and the number of imported cases is on the rise, highlighting a risk for the re-emergence of local foci in Tunisia. Furthermore, *An. labranchiae* has been involved in the epidemic transmission of *P. falciparum*, *P. malariae*, and *P. vivax* during recent epidemics in Morocco. It will be necessary to assess the vector competence of local *An. labranchiae* populations from Tunisia for sub-Saharan strains of African malaria parasites to more accurately assess the risk for re-emergence of malaria transmission in the highly populated northern parts of the country.

*An. sergentii* has been incriminated in malaria transmission in the southern part of Tunisia (Wernsdorfer W and Iyengar MO). The role of *An. multicolor* where it exists with *An. sergentii* or alone in the oases remains unknown. *An. multicolor* has not been incriminated in nature, but it is suspected to be a vector on epidemiological grounds, because it has been found alone in some oases where malaria is transmitted. In Egypt, *An. multicolor* and *An. sergentii* have been found infected with *P. vivax* and *P. falciparum* in natural conditions. *An. algeriensis* is only considered a potential or secondary malaria vector in endemic regions without proof of natural transmission. Because of its scarcity in Tunisia, catholic feeding preferences, and exophilic behavior, the species does not presently and did not historically pose a risk.

The presence of putative malaria vector species together with high numbers of imported malaria cases each year in Tunisia highlight a risk for re-emergence of autochthonous transmission in the country. Additional investigations are required on the ecology, bionomics, and vector competence of local *Anopheles* populations to implement tailored vector surveillance and control programs and prevent re-emergence of the disease.

Received August 12, 2014. Accepted for publication November 4, 2014.

Published online January 5, 2015.

Acknowledgments: We are grateful to the Regional Directors of Public Health that participated in the study. A special thanks to Dr. S. Fassi, Dr. A. Houerbi, Dr. L. Sakhr, Dr. T. Barhoumi, Dr. A. Hedfi, Dr. M. Raouane, Mrs. S. Kilani, H. Hajlaoui, A. Mribai, H. Delli, H. Aloui, A. Rezeigni, N. Mejri, S. Ben Bdira, N. Shili, E. Trilla, A. Khilfi, H. Gherbi, and S. Traoui for their help. We also thank Dr. Thierry Baldet for his advice and Dr. I. Ben Abda, Dr. E. Siala, and Dr. M. Ben Abid for their assistance.

Financial support: This study was supported by the Tunisian Ministry of High Education and Research in the frame of the Research Laboratory LR 11-IPT-06.

Authors’ addresses: Ahmed Tabbabi, Adel Rhim, Aida Bouratbine, and Karim Aoun, Laboratoire de Parasitologie Médicale, Biotechnologies et Biomolécules and Laboratoire de Parasitologie-Mycologie, Institut Pasteur de Tunis, Tunis, Tunisia, E-mails: tabbabiahmed@gmail.com, adel.rhaim@pasteur.rns.tn, aida.bouratbine@pasteur.rns.tn, and karim.aoun@pasteur.rns.tn. Philippe Bousse`s, Cécile Brengues, Didier Fontenille, and Frédéric Simard, Maladies Infectieuses et Vecteurs: Écologie, Génétique, Évolution et Contrôle (MIVEGEC; UMR IRD 224, CNRS 5290, UMI, UMC), Centre IRD France-Sud, Montpellier, France, E-mails: philippe.bousses@ird.fr, cecile.brengues@ird.fr, didier.fontenille@ird.fr, and frederic.simard@ird.fr. Jabeur Daaboub, Direction de l’Hygiène du Milieu et de la Protection de l’Environnement, Ministère de la santé publique, Tunis, Tunisia, E-mail: jadaaboub@yahoo.fr. Nissaf Ben-Alaya-Bouafif, Observatoire National des Maladies Nouvelles et Emergentes, Tunis, Tunisia, E-mail: nissaf.bouafif@rns.tn.

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