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

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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vectors of the disease in the northern and southern parts of the country, respectively (Wernsdorfer W and Iyengar MO, unpublished data).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]

The first map of *Anopheles* distribution in Tunisia was based on data collected between 1968 and 1974 during the malaria eradication campaign. A literature review by Brunhes and others[7] compiled 12 species in 1999: *An. algeriensis*, *An. cinereus*, *An. claviger*, *An. dthali*, *An. labranchiae*, *An. marteri*, *An. multicolor*, *An. petragnani*, *An. plumbeus*, *An. sergentii*, *An. superpictus*, and *An. ziemanni*. The most recent investigation dating back to the 1990s detected only six species, including those suspected as malaria vectors in Tunisia.[8–10]

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.[11,13,14,17–28] 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.

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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).[29] 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.

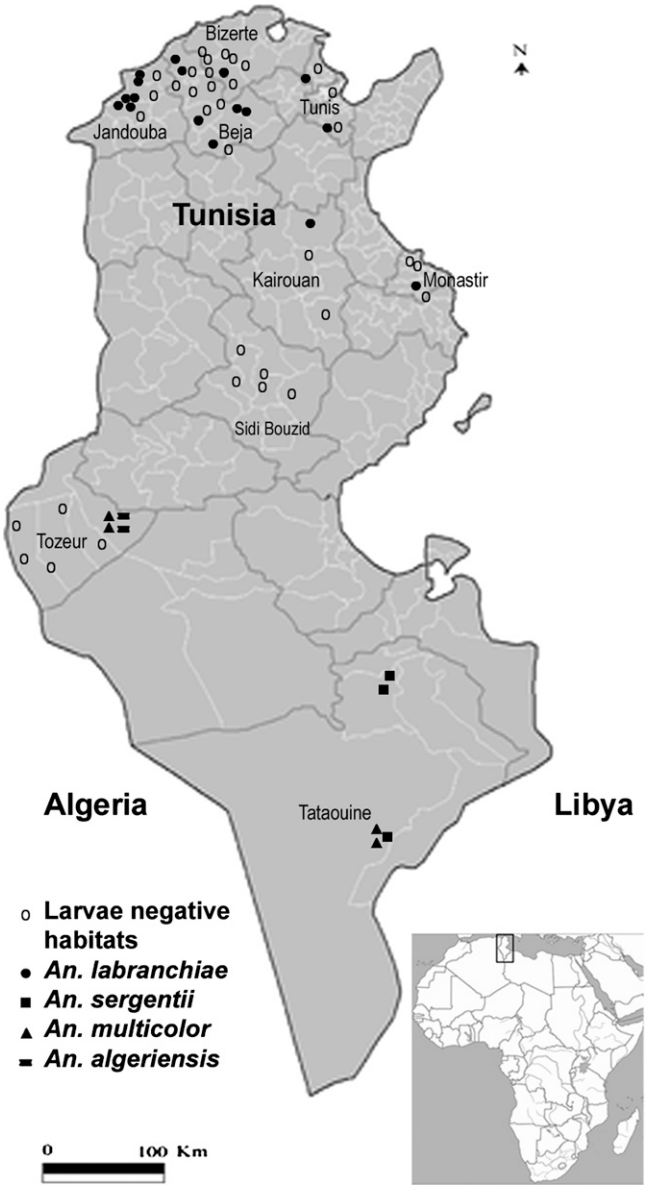

FIGURE 1. Distribution of *Anopheles* mosquitoes in Tunisia.

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.[7] 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.[30]

Genomic DNA was extracted from individual whole-larvae specimens as previously described.[31] Amplification targeted the conserved ITS2 region of the rDNA cluster. The amplification was done with the conditions described previously.[30]

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 χ^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

TABLE 1

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

			Chemical characteristics				Physical characteristics								
Governorate	GPS (north/east)	Locality	Temperature (°C)	pH	Dissolved oxygen (mg/L)	Salinity (mg/L)	Habitat type	Altitude (m)	Sunlight exposure	Water turbidity	Substrate	Vegetation	Culicine larvae	Fish	Anopheline species
Biezrte	36°55′/9°22′	Bagrat	32.2	8.8	12.1	506	Suburban	251.7	Sunny	Clear	Muddy	No vegetation	+	–	*An. labranchiae*
Ariana	36°54′/10°03′	Chorfesh	28.6	8.9	11.8	1,480	Rural	54.1	Sunny	Clear	Sandy	No vegetation	+	–	*An. labranchiae*
Ben arous	36°36′/10°09′	Siguel	37.2	8.7	18.5	952.1	Rural	130.7	Sunny	Clear	Sandy	*P. communis*/algae	+	–	*An. labranchiae*
Beja	36°58′/9°00′	Wechtat1	32.8	2.6	14.5	330	Rural	76.9	Sunny	Clear	Sandy	*T. angustifolia*	+	–	*An. labranchiae*
Beja	36°57′/8°59′	Wechtat2	28.1	7.7	25.4	168	Rural	103.7	Sunny	Clear	Rocky	No vegetation	+	–	*An. labranchiae*
Beja	36°30′/9°14′	Bir touta	33.4	8.8	20.2	841	Rural	411.3	Sunny	Clear	Muddy	*P. communis*	+	–	*An. labranchiae*
Beja	36°42′/9°27′	Babouche	31.5	8.4	21	1,751	Rural	261.8	Sunny	Clear	Muddy	Algae	+	+	*An. labranchiae*
Beja	36°41′/9°27′	Deroua	28.5	8.3	10	3,030	Rural	256.2	Sunny	Clear	Muddy	*P. communis*/algae	+	+	*An. labranchiae*
Beja	36°35′/9°11′	Elhamri1	25.6	7.6	14.2	333	Rural	213.76	Sunny	Clear	Muddy	Algae	+	–	*An. labranchiae*
Jandouba	36°55′/8°45′	Kaffaf	27.8	7.3	9.4	235	Rural	65.6	Sunny	Clear	Sandy	No vegetation	+	–	*An. labranchiae*
Jandouba	36°52′/8°43′	Ktayreyia	28.6	7.6	18.3	252	Rural	93.1	Sunny	Clear	Muddy	*P. communis*	+	–	*An. labranchiae*
Jandouba	36°45′/8°32′	Rouii	26.7	7.2	5.3	372	Rural	196.9	Sunny	Clear	Muddy	*T. angustifolia*	+	–	*An. labranchiae*
Jandouba	36°43′/8°35′	Ncham1	33.2	7.1	12.3	392	Rural	672.8	Sunny	Clear	Sandy	Algae	+	–	*An. labranchiae*
Jandouba	36°44′/8°35′	Ncham2	31.6	7.6	10.1	178.1	Rural	646.3	Sunny	Clear	Sandy	No vegetation	+	–	*An. labranchiae*
Jandouba	36°38′/8°39′	Damous	29.5	7.8	11.1	162.5	Rural	337.5	Sunny	Clear	Muddy	No vegetation	+	–	*An. labranchiae*
Kairouan	35°56′/10°01′	Sbikha	28.9	8.9	18.4	1,139	Suburban	136.2	Sunny	Clear	Sandy	Algae	+	–	*An. labranchiae*
Monastir	35°30′/10°46′	Jnaiha	34.5	8.6	15.1	941	Rural	122.1	Shaded	Clear	Muddy	*P. communis*/ *T. angustifolia*	+	–	*An. labranchiae*
Tozeur	33°57′/8°13′	Gite2	24.4	6.5	7.5	9,010	Rural	49.2	Sunny	Clear	Sandy	*P. communis*/ *Juncus* sp.	+	–	*An. algeriensis*
Tozeur	33°58′/8°14′	Gite9	28.6	6.6	10.6	13,660	Rural	57	Shaded	Clear	Sandy	*P. communis*	+	–	*An. multicolor*
Tozeur	33°59′/8°14′	Mejed	36.4	6	2.1	23,600	Suburban	75.3	Sunny	Turbid	Sandy	*Sarcocornia* sp.	+	–	*An. multicolor*
Tozeur	34°00′/8°09′	Chnichina	29.8	6.5	2.7	9,970	Suburban	75.1	Shaded	Clear	Sandy	*P. communis*	+	–	*An. algeriensis*
Tataouine	32°58′/10°22′	Tlalet	21.4	8.4	17.1	6,440	Suburban	282.2	Shaded	Clear	Sandy	*P. communis*/algae	+	+	*An. sergentii*
Tataouine	32°22′/10°23′	Lachouch	24.5	7.7	17.9	36,800	Suburban	315.6	Sunny	Clear	Sandy	No vegetation	+	–	*An. multicolor*/ *An. sergentii*
Tataouine	32°19′/10°23′	Ettboul	24.7	8.5	13.2	4,520	Rural	332.1	Shaded	Clear	Sandy	*P. communis*	+	–	*An. multicolor*
Tataouine	33°03′/10°20′	Ennasr	19.4	8.7	11.3	2,820	Urban	305.9	Sunny	Clear	Sandy	No vegetation	+	–	*An. sergentii*

GPS = global positioning system..

TABLE 2
Statistical analysis results: distribution of species (percentage) according to qualitative environmental conditions

	Sunlight situation		Fauna		Water transparency		Bottom surface		Habitat type	
	Shaded ($N = 5$)	Sunlight ($N = 20$)	Absence ($N = 22$)	Presence ($N = 3$)	Clear ($N = 24$)	Turbid ($N = 1$)	Muddy/rocky ($N = 9$)	Sandy ($N = 16$)	Rural ($N = 18$)	Suburban/urban ($N = 7$)
An. labranchiae (%)	33.3	78.9	68.2	66.7	70.8	0	100	50	83.3	16.7
Others (%)	66.7	21.1	31.8	33.3	29.2	100	0	50	28.6	71.4
P value*	0.059		NS		NS		0.022		0.017	

NS = not significant.
*Fisher's exact test.

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 *An. labranchiae* (30.5°C ± 3.09°C) than others species (26.15°C ± 5.35°C). Likewise, analysis of the association between chemical characteristics of habitats and species revealed that salinity mean was significantly higher among *An. labranchiae* (Tables 2 and 3).

An. labranchiae is significantly less frequent in sandy bottom surface (50%) than other types of bottom surface (rocky and muddy; 100%; $P = 0.022$), 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 *Gambusia* fish only coexisted in three habitats where the predators were recently introduced (Table 1).

The 252 *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 *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).

An. multicolor and *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). *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 *Anopheles* were found in this study, despite the sampling effort and the appropriate season of the captures corresponding to the *Anopheles* activity period in Tunisia: *An. labranchiae*, *An. multicolor*, *An. sergentii*, and *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 *An. cinereus* and *An. claviger*.[8,9] *An. labranchiae* was the predominant species in northern Tunisia, whereas *An. sergentii* and *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. *An. labranchiae* was the only widely distributed species throughout northern and central Tunisia in subhumid and semiarid climate,

TABLE 3
Statistical analysis results: comparison of quantitative environmental conditions by species

Code species	Altitude (m)	Salinity (mg/L)	Dissolved oxygen (mg/L)	pH	Water temperature (°C)
An. labranchiae					
Mean	237.0924	20,160.747	14.5776	7.7494	30.5118
N	17	17	17	17	17
SD	187.55885	79,847.2568	5.12223	1.46602	3.09493
Median	196.9400	506.000	14.2000	7.7800	29.5000
Others					
Mean	186.5488	13,352.500	9.9013	7.3588	26.1500
N	8	8	8	8	8
SD	131.84250	11,476.1800	5.45234	1.08320	5.35057
Median	178.7600	9,490.000	10.9300	7.1000	24.6000
Total					
Mean	220.9184	17,982.108	13.0812	7.6244	29.1160
N	25	25	25	25	25
SD	170.59059	65,569.1193	5.57845	1.34522	4.36441
Median	196.9400	1,139.000	12.3300	7.7000	28.6000
Test statistics					
Mann–Whitney *U*	57.000	9.000	39.000	45.000	31.000
Asymptomatic significance (two-tailed)	0.522	0.001	0.091	0.180	0.031

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,[32,33] 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.[33] 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.[34] 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.[10,35] 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,[22,36–40] Algeria,[22,41,42] and Tunisia.[10,35] 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.[43–48] Laboratory studies performed with *An. labranchiae* revealed that this species can transmit *P. ovale*,[45] whereas populations collected in Italy were refractory to African strains of *P. falciparum*.[44,45] Nevertheless, recent research with populations from Corsica (France) and Principina (Grosseto, Italy) have indicated that the *P. falciparum* cycle can be successfully completed in *An. labranchiae*.[49,50] Moreover, *An. labranchiae* has also been involved in autochthonous transmission of *P. vivax* in Corsica, Greece, and Italy.[46–48] *P. vivax* malaria has been reported from different regions of Tunisia, and the number of imported cases is on the rise,[51] 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.[52] 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.[53] In Egypt, *An. multicolor* and *An. sergentii* have been found infected with *P. vivax* and *P. falciparum* in natural conditions.[54] *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,[9] catholic feeding preferences, and exophilic behavior, the species does not presently and did not historically pose a risk.[55–57]

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.

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