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RESEARCH ARTICLE

A Field Study in Benin to Investigate the Role of Mosquitoes and Other Flying Insects in the Ecology of *Mycobacterium ulcerans*

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Abstract

Background

Buruli ulcer, the third mycobacterial disease after tuberculosis and leprosy, is caused by the environmental mycobacterium *M. ulcerans*. There is at present no clear understanding of the exact mode(s) of transmission of *M. ulcerans*. Populations affected by Buruli ulcer are those living close to humid and swampy zones. The disease is associated with the creation or the extension of swampy areas, such as construction of dams or lakes for the development of agriculture. Currently, it is supposed that insects (water bugs and mosquitoes) are host and vector of *M. ulcerans*. The role of water bugs was clearly demonstrated by several experimental and environmental studies. However, no definitive conclusion can yet be drawn concerning the precise importance of this route of transmission. Concerning the mosquitoes, DNA was detected only in mosquitoes collected in Australia, and their role as host/vector was never studied by experimental approaches. Surprisingly, no specific study was conducted in Africa. In this context, the objective of this study was to investigate the role of mosquitoes (larvae and adults) and other flying insects in ecology of *M. ulcerans*. This study was conducted in a highly endemic area of Benin.

Methodology/Principal Findings

Mosquitoes (adults and larvae) were collected over one year, in Buruli ulcer endemic in Benin. In parallel, to monitor the presence of *M. ulcerans* in environment, aquatic insects were sampled. QPCR was used to detect *M. ulcerans* DNA. DNA of *M. ulcerans* was detected in around 8.7% of aquatic insects but never in mosquitoes (larvae or adults) or in other flying insects.

Competing Interests: The authors have declared that no competing interests exist.

Conclusion/Significance

This study suggested that the mosquitoes don't play a pivotal role in the ecology and transmission of *M. ulcerans* in the studied endemic areas. However, the role of mosquitoes cannot be excluded and, we can reasonably suppose that several routes of transmission of *M. ulcerans* are possible through the world.

Author Summary

Buruli ulcer is a neglected tropical disease due to *M. ulcerans*, an environmental mycobacteria. Modes of transmission to human remain unclear and water bugs and mosquitoes had been incriminated with more or less experimental laboratory evidences and field studies. In this context, we have investigated the presence of *M. ulcerans* DNA in mosquitoes and other flying insect in a highly endemic area of Buruli ulcer in Benin. No trace of the bacteria was found in mosquitoes and other flying insects, while 8,7% of aquatic insects, including water bugs, caught in the same area and in the same period were found positive to *M. ulcerans* DNA. Our results support the hypothesis that mosquitoes don't play a major role in ecology of *M. ulcerans* in our research area and is in favor of a transmission from the aquatic environment.

Introduction

Buruli ulcer, which is caused by *M. ulcerans*, is a neglected tropical disease affecting mostly poor rural communities in West and Central Africa. In 2013, 75% of all new cases of Buruli ulcer worldwide were declared by Ivory Coast, Ghana and Benin. This skin disease, which mostly affects children, causes large ulcerative lesions often leading to permanent disabilities [1,2,3]. The cutaneous lesions are caused by a *M. ulcerans* toxin called mycolactone with cytotoxic, immunomodulatory and analgesic effects [4]. At early stages, Buruli ulcer can be treated with a combination of streptomycin and rifampin for eight weeks; at later stages, antibiotic therapy is associated with extensive surgery [5,6,7,8].

Buruli ulcer occurs mostly in low-lying swampy areas [9,10]. Epidemiological studies have shown that the aquatic environment is the main reservoir of *M. ulcerans*, with many aquatic vertebrates and macro-invertebrates harboring this bacillus. The exact ecological features and mode of transmission of *M. ulcerans* to humans remain to be identified. In recent decades, several studies have suggested that water bugs and mosquitoes may play a role in *M. ulcerans* transmission [11,12,13,14,15,16,17,18,19,20,21,22,23,24,25]. Water bugs have been implicated as potential hosts and vectors of the bacillus in laboratory experiments and field ecology studies in Africa [26,27,28,29,30]. Outside the aquatic environment, adult mosquitoes tested positive for *M. ulcerans* DNA in an area of endemic Buruli ulcer in Australia, leading to the suggestion that these insects might transmit the bacterium to humans [26,28,29,30]. However, this hypothesis was not confirmed by laboratory experiments, and, surprisingly, no study has investigated the possible involvement of mosquitoes in *M. ulcerans* ecology in Africa, the continent with the highest level of endemicity for Buruli ulcer.

The objective of this study was to investigate the presence of *M. ulcerans* DNA in flying insects, including mosquitoes, in an area of Buruli ulcer endemicity in Benin. We monitored, in parallel, the levels of *M. ulcerans* in the aquatic environment, as a marker of the presence of the bacterium in the study area.

Materials and Methods

Study area

The study was carried out in the Oueme administrative area in South-East Benin, where Buruli ulcer has been endemic for several decades [31,32,33,34,35,36]. Sampling was carried out in three districts crossed by the Oueme River (Bonou, Adjohoun and Dangbo). The districts were selected for study because they are accessible throughout the year (including the rainy season) and because data were available for relevant epidemiological studies. Flying insects were sampled at four sites and aquatic sampling was carried out at nine sites (Fig 1).

The Oueme River originates in the Taneka hills in the Atacora Mountains and flows into the Atlantic Ocean close to Cotonou. The study area is characterized by a subequatorial climate with two rainy seasons. The first rainy season extends from April to July and the second extends from October to November. Mean annual precipitation is 1122 mm, and temperatures range from 22°C to 26°C. There are two main types of soil: alluvial soils, which are fertile but liable to flooding, and sandy soils, which are less fertile but suitable for growing coconut, palm,

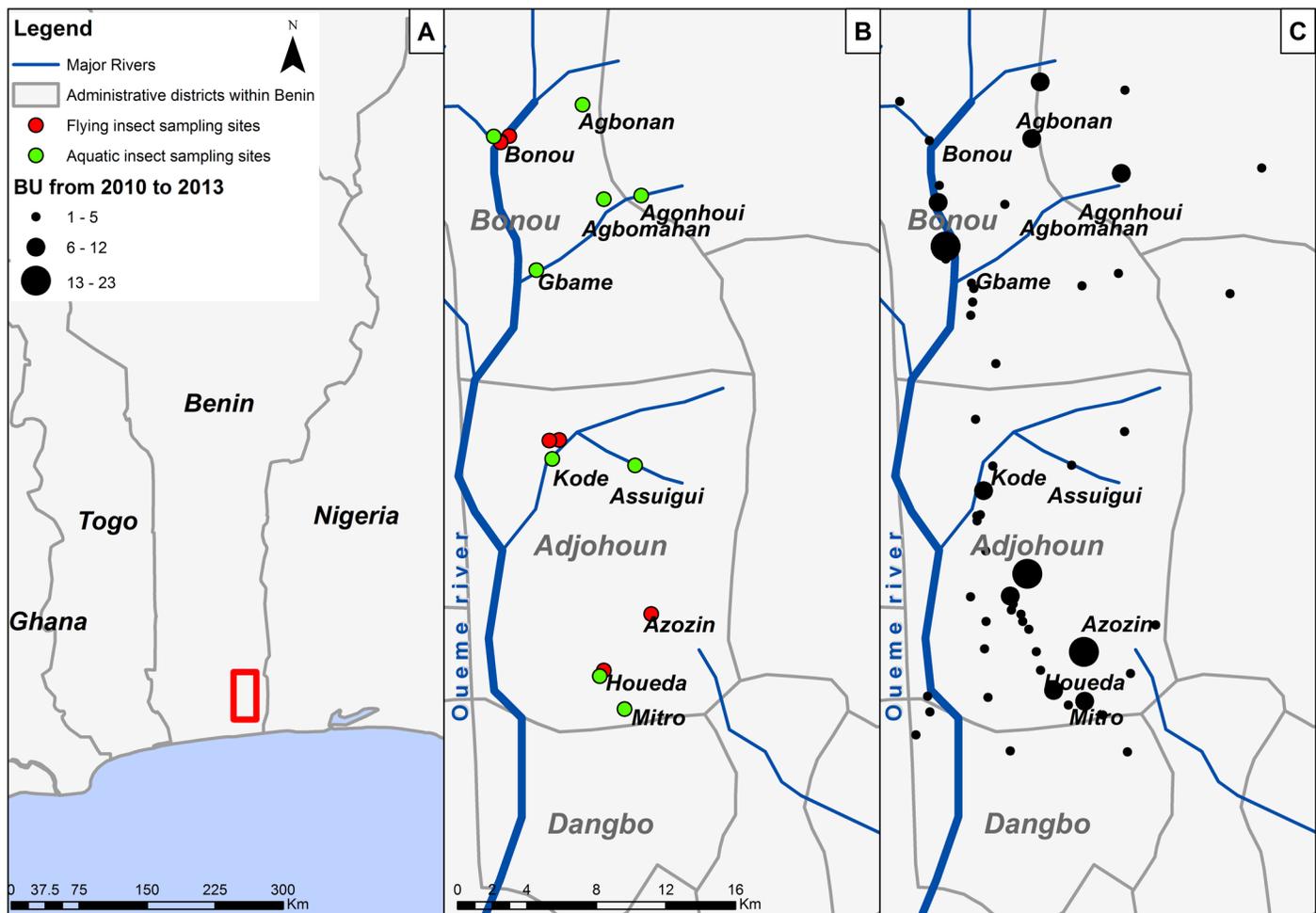


Fig 1. Location of the sites used for environmental sampling in the area of Buruli ulcer endemicity in the Oueme Valley in Benin. A. Area of Buruli ulcer endemicity in the Oueme Valley in South-East Benin (red rectangle). B. Map indicating the sites at which flying insects and larvae were collected (red points) and aquatic sampling was carried out (green points). C. Map showing the locations along the Oueme River of Buruli ulcer patients, from 2010 to 2013 (cumulative cases in black points).

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and other tropical trees. Most of the population in this area is engaged in farming (rice, maize, cassava, cowpeas, market garden crops, etc.), fishing and trade. The natural vegetation consists of grassy savannah and swampy mangrove forest.

Flying insect sampling

This study focused on the adult stage of mosquitoes and other flying insects and the immature stages of mosquitoes. Insects were collected during four surveys in June, July, November, and December 2013, at four sites in the Bonou Centre, Kode, Gbada and Houeda areas (Fig 1). The collection periods correspond to the start, middle and end of the rainy season and the dry season, respectively. Flying insects were collected with Centers for Disease Control (CDC) light traps. A CDC light trap consists of a 150 mA incandescent light bulb and a fan, powered by 6 V batteries. At each survey, once consent had been received from the heads of household, insects were trapped from two selected houses in each village, over a period of two days. Traps were placed both indoors and outdoors at each house, from 6:00 pm to 6:00 am, corresponding to the period from dusk to dawn. The indoor traps were suspended from the ceiling, about 2m above the ground. The outdoor traps were hung on trees at about the same height. The insects collected were identified in the field in two steps. In the first step, mosquitoes were separated from the other insects. All mosquitoes were identified to species level under stereoscopic microscopes, according to morphological criteria in dichotomous keys [37,38,39]. They were counted and stored, in pooled groups of up to 15 individuals of the same species, in 70% ethanol for transport to the laboratory. In the second step, the remaining flying insects were identified to order level on the basis of their morphology under a stereoscopic microscope, with the appropriate keys [40,41]. They were stored in 70% ethanol, in pooled groups of up to 15 individuals from the same order, and were transported to the laboratory for PCR analysis (Fig 2).

Sampling of mosquito larvae

During each survey, mosquito larvae were collected throughout the selected area by dipping with a 350 ml ladle. Samples were collected from various temporary and permanent bodies of water constituting potential habitats for the development of populations of mosquito larvae. All larvae were transported in clean water, in plastic containers, to the field laboratory. Larvae were identified to genus level with appropriate morphological keys [37,38,39]. The larvae of each genus were then separated into two groups. The larvae of the first group were preserved in 70% ethanol, in pools of 20 individuals for each genus. The larvae of the second group were reared to emergence. The resulting adults were then stored in 70% ethanol, in pools of up to 15 individuals. Exuviae were also preserved in 70% ethanol, in pools of 20, for laboratory analysis (Fig 2).

Aquatic sampling

Samples were collected from the principal sources of water for domestic washing, bathing, fishing and recreation. The sampling sites were located in nine villages in the three districts: Bonou Centre, Agbonan, Agbomahan, Agonhoui, Gbame, Kode, Assigui, Houeda, and Mitro (Fig 1). Aquatic sampling was carried out with the same methods at each site, at least twice, between January 2013 and December 2013. Invertebrates and fish were captured with a square net (32 x 32 cm and 1 mm in mesh size), from the surface down to a depth of 0.2 to 1 m, over a distance of 1 m. A sample was considered to correspond to all the insects collected in 10 such sweeps with the net. All insects were preserved in 70% ethanol for laboratory identification. For the detection of *M. ulcerans* DNA, the insects were sorted into pooled groups, each including no more than 20 specimens from the same family. For each body of water, we collected plant

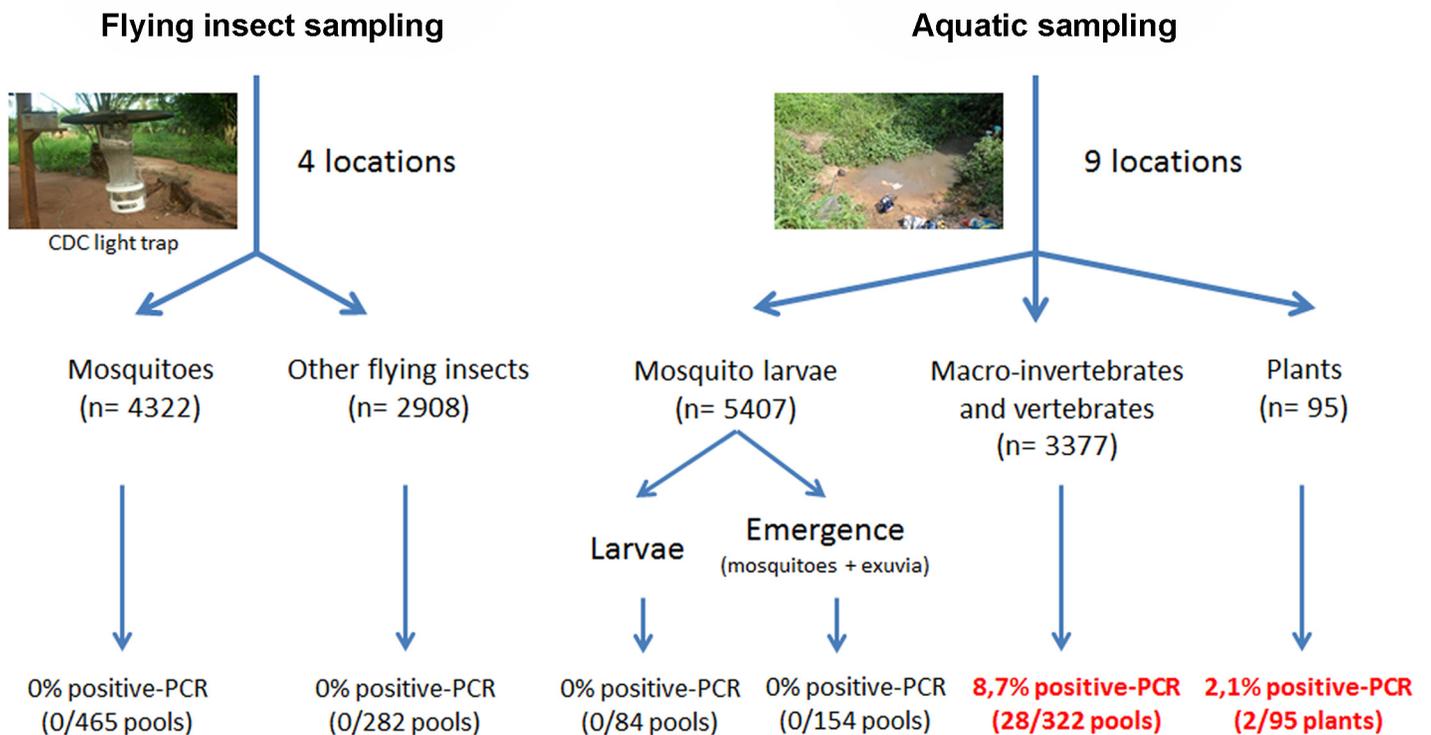


Fig 2. Schematic summary of the environmental sampling and detection of *M. ulcerans* by PCR.

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samples from the predominant and the second most frequent types of living plant. Each of these plant samples consisted of one to five plant leaves, stems or roots, depending on the size of the plant sample. They were placed directly in a clean 100 ml bottle containing 70% ethanol (Fig 2).

Extraction and purification of DNA

Pooled insect bodies were ground and homogenized in 50 mM NaOH. Tissue homogenates were heated at 95°C for 20 min. The samples were neutralized with 100 mM Tris-HCl, pH 8.0. DNA was extracted from the homogenized insect tissues with the QIAquick PCR purification kit (Qiagen), according to the manufacturer's recommendations. Negative extraction and purification controls were included in each series of manipulations. The homogenizers were decontaminated by incubation overnight in 1 M NaOH, to eliminate any traces of DNA. For each aquatic plant sample, the material was cut into small pieces with a scalpel and then ground in 50 mM NaOH. The extract was heated and neutralized and the DNA was purified with the Mobio purification kit, according to the manufacturer's recommendations.

Quantitative PCR

Oligonucleotide primer and TaqMan probe sequences were used for detection of the IS2404 sequence and the ketoreductase B (KR) domain of the mycolactone polyketide synthase (*mls*) gene from the plasmid pMUM001 [13,42,43]. PCR mixtures contained 5 µl of template DNA, 0.3 µM of each primer, 0.25 µM probe, and Brilliant QPCR Master Mix (Agilent Technologies) in a total volume of 25 µl. Amplification and detection were performed with a Thermocycler StepOne (Applied Biosystems), using the following program: heating at 95°C for 10 min,

followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. DNA extracts were tested at least in duplicates, and negative controls were included in each assay. Quantitative readout assays were set up, based on an external standard curve generated with five tenfold serial dilutions of *M. ulcerans* (strain 1G897) DNA. Samples were considered positive only if both the IS2404 sequence and the gene sequence encoding the ketoreductase B domain (KR) were detected, with threshold cycle (Ct) values strictly < 35 cycles. An inhibition control was performed as previously described [44] and 10% negative controls (water alone) were included in each assay.

Data analysis

Mosquito abundance was compared between sites and between seasons in nonparametric Kruskal–Wallis tests.

Results

Diversity of flying insect orders collected

We collected 7230 flying insects from nine orders: Coleoptera, Diptera, Heteroptera, Homoptera, Hymenoptera, Lepidoptera, Neuroptera, Odonate, Tricoptera. At all sites, Diptera was by far the most frequent order of flying insects caught, accounting for 84% of all insects trapped. Heteroptera was the least abundant order at each site and was not detected at Gbada and Houeda (Table 1).

Diversity of mosquito species collected

The 6047 dipteran specimens collected during the four surveys included 4322 mosquitoes from 10 species. *Mansonia africana* (50%), *Culex nebulosus* (27%), and *Culex quinquefasciatus* (22%) were the most abundant species, accounting for 98% of all the mosquitoes trapped. The four least represented species were *Anopheles pharoensis*, *Aedes vittatus*, *Culex decens*, and *Culex fatigans*, with no more than four individuals each (S1 Table).

Spatio-seasonal variation of the total mosquitoes and flying insects collected

No significant differences in the abundance of the mosquitoes and other flying insects caught were found between sites ($p > 0.05$). Flying insects were significantly more abundant ($p < 0.05$) in the wet season than in the dry season, whereas no significant difference in mosquito densities was observed within seasons ($p > 0.05$; S2 Table).

Table 1. Orders of flying insects collected during the surveys.

	Gbada	Bonou	Houeda	Kode	Total
Coleoptera	37	31	16	59	143
Diptera	1136	1033	1822	2056	6047
Heteroptera	0	1	0	1	2
Homoptera	29	5	10	6	50
Hymenoptera	11	71	2	39	123
Lepidoptera	85	41	138	98	362
Neuroptera	54	53	28	67	202
Odonates	2	2	2	2	8
Tricoptera	78	86	31	98	293
Total	1432	1323	2049	2426	7230

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Collection of larvae

During the surveys, we collected a total of 5407 mosquito larvae. These larvae were identified as *Culex* spp., *Anopheles* spp. and *Aedes* spp. In total, 3146 mosquito larvae belonged to the genera *Culex* and *Anopheles*. *Culex* spp. were the most abundant, accounting for 66.35% of the mosquito larvae collected. For the adults emerging in the laboratory following the rearing of larvae collected in the field, 2261 individuals belonging to the genera *Culex*, *Anopheles* and *Aedes* were identified. *Culex* was the most abundant genus, accounting for 79.08% of the sample (S3 Table).

Diversity of aquatic sampling

During the survey, we collected 3377 aquatic vertebrates and macro-invertebrates from various bodies of water in the Oueme administrative area (Table 2). Insecta accounted for 72% of the animals collected, with a majority of Hemiptera. The bodies of water studied were of various natures (flooded land, river and swamp) and were scattered around the Oueme, making it possible to sample diverse types of specimens from different ecological niches. In total, 95 plants were collected from the various bodies of water. They were identified as belonging to the Poaceae, Lemnaceae, Nymphaeaceae, Araceae and Potamogetonaceae families.

Detection of *M. ulcerans* DNA in environmental samples

We tested flying insects, larvae, aquatic vertebrates and invertebrates, and plants collected in 2013 from various sites in Oueme for the presence of *M. ulcerans* DNA. We found that 942 pools of flying insects (corresponding to the 7230 captured flying insects and the 5407 collected larvae) tested negative for *M. ulcerans* DNA by PCR. Positive PCR results were obtained for 8.7% (28/322) of aquatic animal sample pools from the various bodies of water. No positive specimens were obtained at two sites, and 5.5 to 25% of the sample pools at the other seven sites tested positive (Table 2 and S4 Table). Decapoda was the invertebrate family with the highest level of mycobacterial contamination (26%). We performed 295 PCR analyses on the 95 plants collected. These analyses were carried out on leaves, stems and roots, and three samples tested positive for *M. ulcerans* DNA by PCR: a leaf pool and a stem pool from the same

Table 2. Presence of *M. ulcerans* in aquatics vertebrates and macro-invertebrates collected in different areas of Oueme.

		Mitro	Houeda	Agonhoui	Agbomahan	Agbonan	Assigui	Kode	Gbame	Bonou	Total	
Vertebrates	Fish	17 (0/5)	1 (0/1)	54 (0/6)	-	2 (0/2)	88 (1/5)	3 (0/1)	3 (0/2)	-	169 (1/22)	
	Anura	6 (0/4)	12 (1/4)	19 (0/4)	14 (0/2)	79 (0/3)	18 (1/3)	2 (0/1)	2 (0/1)	12 (0/1)	164 (2/24)	
Invertebrates	Insecta	Odonata	36 (0/5)	5 (0/3)	17 (1/4)	-	44 (0/4)	10 (0/4)	2 (0/3)	6 (0/2)	22 (0/1)	142 (1/26)
		Hemiptera	676 (2/36)	216 (1/23)	22 (2/12)	6 (0/3)	269 (1/17)	59 (3/13)	111 (1/15)	122 (0/15)	109 (0/7)	1590 (10/141)
		Coleoptera	288 (2/17)	118 (2/8)	1 (0/1)	-	6 (0/2)	32 (0/5)	75 (1/9)	53 (0/7)	27 (1/4)	600 (6/53)
		Diptera	-	-	-	-	100 (1/2)	-	-	1 (0/1)	1 (0/1)	102 (1/4)
		Mollusca	100 (0/4)	16 (0/2)	148 (0/6)	-	-	1 (0/1)	3 (0/1)	-	-	268 (0/14)
		Crustacea	Decapoda	5 (0/1)	-	93 (2/9)	59 (0/5)	-	150 (5/11)	1 (0/1)	-	308 (7/27)
	Araneae		7 (0/1)	3 (0/2)	18 (0/4)	-	1 (0/1)	2 (0/1)	3 (0/1)	1 (0/1)	-	35 (0/11)
	Total	1135 (4/73)	371 (4/43)	372 (5/46)	79 (0/10)	501 (2/31)	360 (10/40)	200 (2/32)	189 (0/29)	171 (1/14)	3378 (28/322)	

The figures correspond to the abundance and the figures in brackets correspond to the number of positive qPCR pools (number of positive pools/total pools). qPCR targeting the KR and IS2404 sequences was used to detect *M. ulcerans*. Only sample pools testing positive for both sequences were considered positive.

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plant from a water body in Kode and a leaf pool from Mitro (S4 Table). Both plants concerned belonged to the Poaceae plant family.

Discussion

The ecological characteristics and mode of transmission of *M. ulcerans* are not entirely understood, and several fundamental questions remain unanswered. One key concern relates to the routes by which *M. ulcerans* crosses the human skin barrier. There are currently two main hypotheses: (i) direct contact between an existing wound and water containing *M. ulcerans*; (ii) the inoculation of *M. ulcerans* into the skin [2,45]. Comparisons with the modes of transmission of other environmental mycobacteria in immunocompetent humans (e.g. *M. fortuitum*, *M. chelonae*, *M. xenopy*) and recent studies of *M. ulcerans* [46] have suggested that direct inoculation into the skin is the most likely mode of transmission. In this context, the two most likely scenarios for the inoculation with the bacterium are either inoculation by an active vector harboring *M. ulcerans*, as described for various microorganisms, including parasites (e.g. *Leishmania* sp. or *Plasmodium* sp.), arboviruses (e.g. the Dengue and Chikungunya viruses), and bacteria (e.g. *Yersinia pestis* and *Borrelia* sp.), or inoculation by a mechanical vector, such as aquatic plant thorns or sharp leaves, biting or sucking insects (bacilli present on the outside of the insects) [13,15,16,17,18,19,20,21,25,26,27,28,29,30,47,48,49,50,51,52]. *M. ulcerans* ecology is highly complex. It is therefore possible for these scenarios to co-exist, and their importance or significance is dependent on a number of different criteria (e.g. human behavior, including access to drinking water, rural or urban life and work, fauna and flora biodiversity, presence of permissive species, season).

Several experimental studies have explored the role of aquatic hemipterans as passive or active vectors of *M. ulcerans*. These approaches were supported by various environmental and epidemiological studies conducted in Africa. However, the importance (unique, major, or marginal) of this transmission route has yet to be established and other transmission routes should therefore be explored. For instance, it has been suggested that mosquitoes act as vectors of *M. ulcerans* in Australia, but, surprisingly, this possibility has never been explored in Africa. In this context, the aim of our study was to assess the role of mosquitoes in *M. ulcerans* ecology. We carried out an extensive field study in an endemic area in Benin, involving temporal and spatial monitoring of the presence of *M. ulcerans* in mosquitoes and other flying insects, used as a control for the distribution of *M. ulcerans* in aquatic flora and fauna.

M. ulcerans DNA was detected in various aquatic macro-invertebrates and vertebrates, and some aquatic plants. The global rate of detection was about 9%, consistent with the findings of other environmental studies [26,27,28,29,30]. *M. ulcerans* DNA was not detected in any of the flying insects collected in CDC light traps inside and around houses over the same period (including mosquito families in which *M. ulcerans* DNA was detected in Australia). As only one type of sampling method was used to collect flying insects (CDC light traps), it is possible that this introduced a bias in terms of species diversity. Nevertheless, in a recent study performed in the same area with three other types of sampling method for mosquito collection, the three most abundant mosquito species were the same as in our study, and eight of the 14 species identified were common to our study [53].

Our results suggest that mosquitoes and non-aquatic flying insects are not involved in the ecology and dissemination of *M. ulcerans* in an area of South-East Benin in which Buruli ulcer is highly endemic, and confirm that the aquatic environment is the main environmental reservoir of the bacillus. However, a role for mosquitoes in other areas, including Australia, cannot be definitively excluded.

The ecology and mode of transmission of micro-organisms may differ between geographic locations, with biological diversity affecting bacterial adaptation and human activities. This concept could be applied to *M. leprae*, a mycobacterium that also causes a dermatosis. Indeed, a recent study has suggested that the ecological features, reservoirs and transmission routes of *M. leprae* may differ between continents. It has been shown that, in North America, wild armadillos harbor the same strain of *M. leprae* as leprosy patients. Leprosy may thus be a zoonosis in this region [54]. This situation cannot be transposed to other continents in which leprosy is highly endemic such as Africa and Asia, where there are no armadillos and no other mammal is known to harbor the bacillus. A similar situation may apply to *M. ulcerans*. In Australia, mammals such as possums have been shown to be hosts of *M. ulcerans* and may play a key role in its dissemination, together with mosquitoes. However, there are no possums in Africa, and *M. ulcerans* has never been detected in the tissues of any mammal other than humans in Africa.

Based on the results of various studies performed in recent decades and aiming to decipher the ecological characteristics of *M. ulcerans*, it seems likely that *M. ulcerans* can be transmitted via several routes, potentially differing between locations in different parts of the world.

Supporting Information

S1 Table. Mosquito species collected during the surveys.

(DOCX)

S2 Table. Total mosquitoes and other insects collected per site and per season.

(DOCX)

S3 Table. Mosquito larvae and emerged adults sampled during the surveys.

(DOCX)

S4 Table. Ct values for qPCR-positive samples.

(DOCX)

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Author Contributions

Conceived and designed the experiments: EM AD JFG. Performed the experiments: EM AD KC JB BZ. Analyzed the data: EM SE JFG AD KC BZ. Contributed reagents/materials/analysis tools: EM JB. Wrote the paper: EM AD BZ.

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