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Mosquitoes sampling strategy for studying West Nile Virus Vectors in Madagascar: Abundance, Distribution and Methods of Catching in High Risk Areas

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ABSTRACT

The West Nile Virus (WNV) is a mosquito-borne virus discovered in 1937, and first described in 1978 in Madagascar. Twenty-six potential mosquito-vector species mainly ornithophilic were described in Madagascar. Investigations on catching methods of mosquitoes vectors of WNV were carried out in two districts located in the Malagasy west coast where high prevalence was detected in 2009 after a serological survey. Five different methods were evaluated during the samplings: CDC light traps and net-trap baited were tested in Mitsinjo district, while human landing catch, CDC light trap, and BioGent (BG) sentinel were used in Masoarivo. One thousand five hundred eleven adult mosquitoes were collected with between 53% and 66% of them captured by CDC light traps in the two districts. Traps baited with chicken (net-trap and BG) captured between 25% and 32% of caught mosquitoes. Culex tritaeniorhynchus, Culex univittatus and Mansonia uniformis were the most abundant species and are also potential vectors of WNV. During the survey, most mosquitoes were collected near a lake or a forest. These 2 ecozones are frequently visited by domestic and wild birds. Results provided a background to conduct a survey in terms of the trapping method choice: CDC light traps and BG traps seem an effective quantitative and qualitative method for a longitudinal study.

Keyword Index: West Nile Virus; Madagascar; Vector; *Culex; Aedes*; *Anopheles*; *Mansonia*; CDC light trap; BG trap; Mosquito sampling.

Background

The West Nile fever is a zoonotic arbovirus infection caused by a virus of the genus Flavivirus affecting several animals including chickens, ducks, geese and equine species. The West Nile Virus (WNV) was first discovered in 1937, December in Uganda [1]. This virus belongs to the genus Flavivirus, family Flaviviridae [2], and is a member of the Japanese encephalitis antigenic complex (Alfuy, Japanese encephalitis, Kokobera, Koutango, Kunjin, Murray Valley encephalitis, St. Louis encephalitis, Stratford, Usutu, and West Nile viruses) transmissible by mosquitoes and many of them can cause febrile, sometimes fatal, illnesses in humans. It is interesting to highlight that these other arboviruses belonging to the same family can also be easily carried by the same mosquito species, which represents a good questioning in term of evolution/adaptation. The WNV can be pathogen for humans, even if most of infected people are asymptotic. The WNV has a worldwide distribution. It can be found in Africa, around the Mediterranean Sea, in Asia, and more recently but largely studied in North America. Since 1994, severe epidemics occurred in human species with some fatal cases. During these last 20 years, epidemics occurred in Algeria in 1994, Romania in 1996, Czech Republic and Tunisia in 1997, Republic Democratic of Congo in 1998, Russia and USA in 1999, Israel in 2000, Canada in 2003 and Greece in 2013 [3].

Mosquitoes, largely bird-feeding species, are the principal vectors of WNV. In North America, WNV is transmitted primarily by *Culex* mosquitoes, but other genera may also be vectors [4]: at least, 59 different mosquito species are considered vectors even if only 10 species are considered to be principal WNV vectors [4], [5], [6]. Except the role of *Culex pipiens* which is the main vector in North America, the role of *Cx. quinquefasciatus, Cx. tarsalis* and *Cx. restuans* species has been particularly highlighted in WNV transmission. In Asia, *Culex quinquefasciatus*, *Cx. tritaeniorhynchus*, and *Cx. vishnui* predominate [7]. Particularly, in India, the species of the *Cx. vishnui* complex are the principal vectors [8]. In Europe, the main vectors are *Cx. pipiens*, *Cx. antennatus*, *Cx. modestus* and *Coquillettidia richiardii* [8]. In Africa and the Middle East, the main vector is *Cx. univittatus*, with important involvement of *Cx. pipiens*, *Cx. poicilipes*, *Cx. neavei*, *Cx. decens* [7]. In Madagascar, 28 species were described as potential vectors of WNV [9]: 11 species belongs to the *Culex* genus, 6 to the *Aedes* genus, 4 to *Anopheles* genus, 2 to *Mimomyia* genus, 1 to *Lutzia* and 1 to the *Aedeomyia* genus, 1 to *Coquilettidia* species, 1 to *Lutzia* species, and 1 to *Mansonia* genus [9]. In Madagascar, WNV was first isolated in 1978, and probably occurred for several years [10]. It was the most abundant arbovirus of the island between 1975 and 1990 [10]. Last serological survey in 1990 revealed a 29.9% seroprevalence of anti-WN antibodies in a non-randomly sample from 5 to 20 years-old people from 12 different regions of Madagascar. It confirmed the high circulation of the virus with a prevalence of antibodies increasing with the age [11]. This study also showed a more important circulation of the virus in the Western Coast of

Madagascar. Climatic factors, particularly temperature, may play a role in this difference (in relation with birds' presence, vector biology, and/or virus survival). In the United States, entomological studies demonstrated that the vectorial capacity of some Culicidae was reduced below 18°C [12].

Although the importance of WNV and its impact on human health remain unknown to the majority of health professionals in Madagascar, the involvement of this arbovirus in the occurrence of cases of encephalitis hospitalized in Antananarivo in 2001 and deaths in 2010 near Mahajanga confirmed that its pathogenicity is not to be neglected and oversight of this infection must be kept regularly. A serological survey showed a high WN prevalence (28.7%) in poultry in September 2009 in the district of Mitsinjo (Figure 1). The second largest lake in Madagascar, Lake Kinkony, is located in this district and represents a natural crossroad where wild terrestrial, aquatic and migratory birds meet. The municipality of Masoarivo was also under our investigation with three lakes under a Peregrin Fund protection plan and where wild migratory birds come during all the year. These two districts were also chosen because outbreaks occurred there and because some villages close to rainforest represents an ideal sample design to estimate the relationship between epizootic cycles and putative sylvatic cycles. Entomological studies were carried out in these places with the aim to provide a good knowledge on the distribution of mosquito's vector species, to compare the mosquito species composition near the water bodies where wild migratory birds came and to increase our sample design for further investigations on the full WNV cycle. The aims of the entomological investigation were to identify the best sampling methods that provide good performance under field condition for the catching of West Nile vector and studying its biology and to identify the best area for a possible longitudinal survey to estimate mosquitoes-bird-human over the seasons.

MATERIALS AND METHODS

Study sites The Mitsinjo district holds the Kinkony Lake which host many wild bird species. Mosquito samples were collected in four villages around this lake: Marofandroboka (16°05S, 45°51W), Amboanjo (16°08S, 45°54W), Morafeno (16°08S, 45°55W) and Mahakary (16°09S, 45°55W) (Figure 1). The site Marofandroboka is distant from the place frequented by wild birds and is located in the forest. The three other villages surrounding the Kinkony Lake seem more favorable areas for the coexistence of wild and domestic birds. Entomological surveys were also conducted in Antsalova area in Masoarivo (19°23S, 44°22E) and Tsakoramby villages (19°02S, 44°25E) and around the Antsamaky lake (19°02S, 44°21E) (Figure 1). The village Masoarivo is far from the lake with wild birds. Only wild birds are present near the lake Antsamaky (*Phoenicopterus* sp, flamingo). Tsakoramby village is an environment where there is a coexistence of domestic and wild birds.

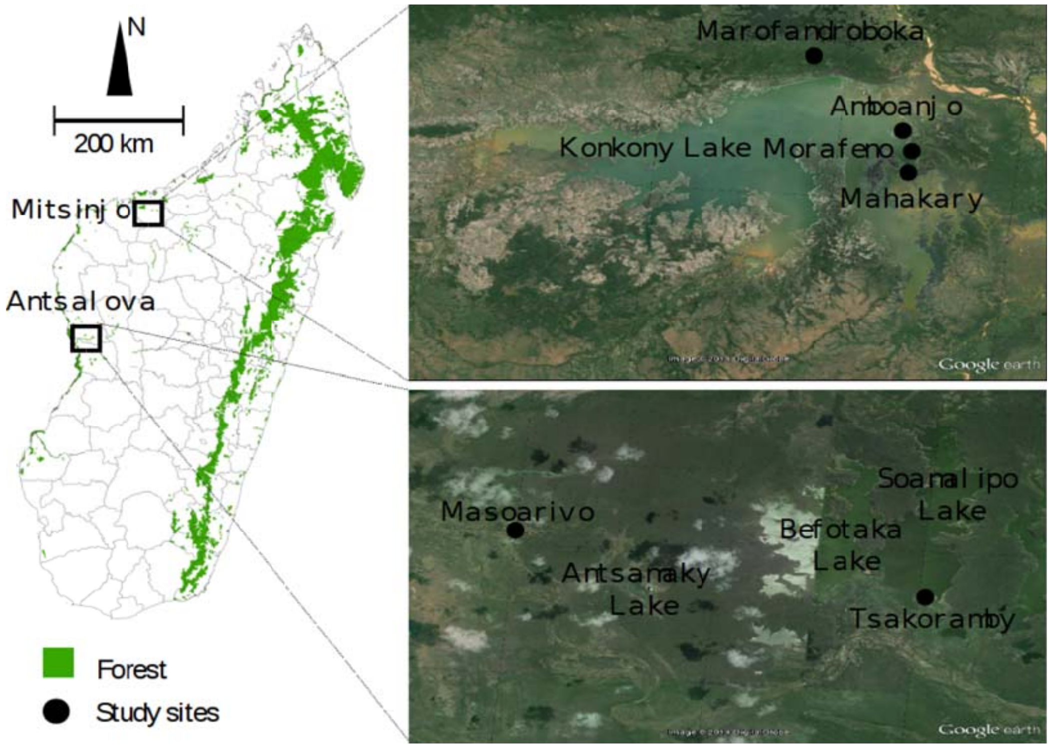

Figure 1. Study sites

Sample design

In Mitsinjo, mosquitoes sampling was performed during the rainy season, on November 2012. Except in the village of Amboanjo where only a night of capture was performed, 2 nights of mosquito catching were done in the three other sites. Ten CDC light traps and 5 net-traps baited with chicken were used for the mosquito sampling in each study site. All light traps were randomly placed in the village to cover various ecotypes (open and close areas, around poultry, in the bushes) (Figure 2).

In Masoarivo, the study was conducted in July 2013, with only a night of capture performed for each study site. Three types of capture were used in parallel: 6 light traps, 3 BG Sentinel and human landing collections. The trapping protocol was performed according to one transect, each line composed of two light traps and a BG sentinel (Figure 2). In the Tsakoramby village, a lake-to-forest transect was performed: the first line of traps was located on Soamalipo Lake, the second line in the village and the third one at the edge of the forest. Around the lake Antsamaky, transect was also a lake-to-forest transect. The first line trap was placed near the lake, the second line in the intermediate zone and the third one in the forest. In the village of Masoarivo, traps were placed randomly in the village to cover various ecotypes such as poultry, open courses, closed course or natural areas at the entrance of the village. As this study focused on the possibility of domestic birds and wild bird transmission, BG sentinel were baited with chicks.

Catching methods

Five different methods were evaluated for collecting mosquitoes from the different habitats (Table 2).

Human landing collections. In Masoarivo, human landing collections were performed for two consecutive nights during the dry season in each selected village. It was done in four different houses with 4 voluntary catchers for each house (2 inside, 2 outside) from 6.00 pm to 6.00 am. According to WHO recommendations [13] human landing catches were made by adult volunteers from the local population. Mosquitoes coming to bite the collectors were detected using a flashlight, collected with glass tubes and placed in the collecting bags.

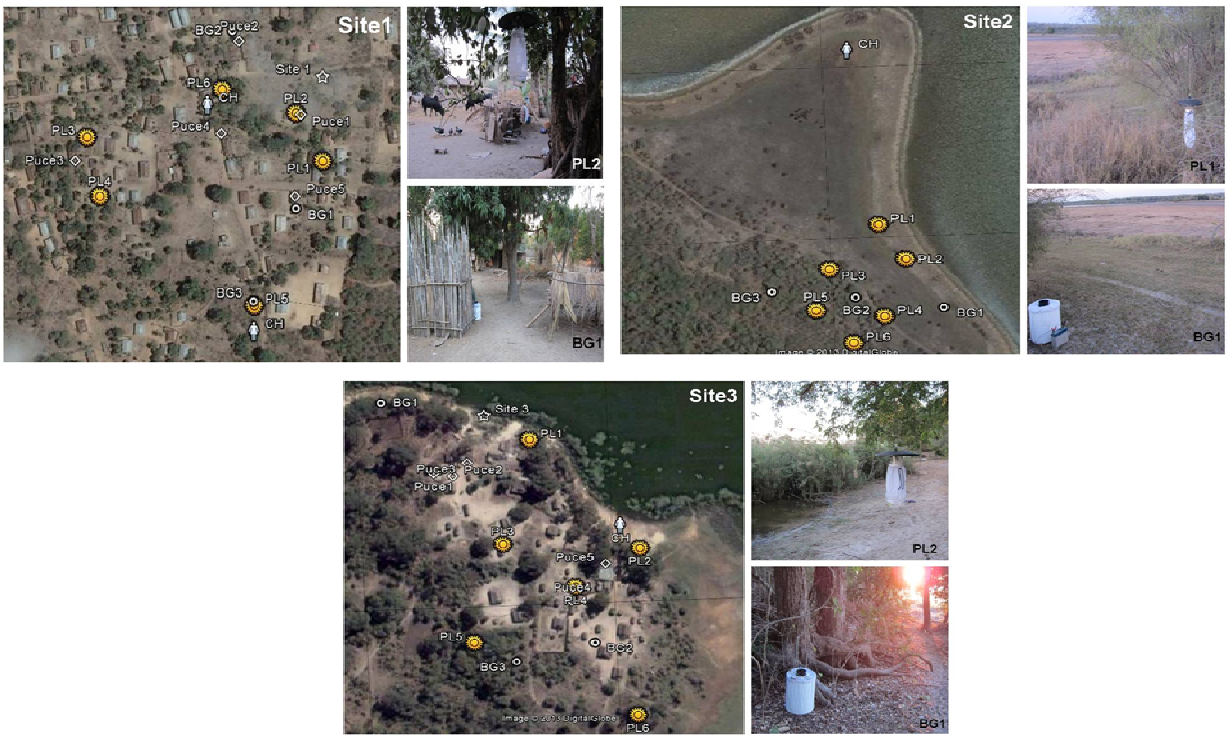

Figure 2: Different ecotypes and catching methods
BG : BioGent Sentinel, PL : Light trap, CH : Human Landing Catch

CDC light traps. This trap is a system that incorporates a mini-light source attracting mosquitoes which are drawn in through the top of the trap and forced downward by the fan into the collection bag-net. Live-trapped females can be counted and tested for mosquito-borne arboviruses. Light-traps were turn on with 6V battery. As in all Mitsinjo study sites, light-traps used in Masoarivo sites were set on before sunset (around 6.00 pm) and off after sunrise (after 6.15 am). In the morning, contents of each bag-net were sorted on a chill table.

BG sentinel trap. The BG traps were used in Masoarivo site. The BG-Sentinel mosquito trap (Biogents, Regensburg, Germany) is essentially a collapsible, white fabric container with white gauze covering its opening. In the middle of the gauze cover, air is sucked into the trap through a black catch pipe by an electrical fan, drawing approaching mosquitoes into a catch bag. Young chicken were used as baits in BG-sentinel traps, powered by 12V battery (PS-1270 F1, Power Sonic, UK). Traps were placed at 5.00 pm (before the sunset) and removed at 6.15 am (after the sunrise).

Net-trap baited. Net-traps were used in Mitsinjo study sites. This trap consisted of a wood frame covered with an untreated mosquito net, which was raised slightly above the ground to allow mosquitoes to enter the trap at its base. One another untreated mosquito-net was then placed under the first one with 5 chickens used as baits. During the night, mosquitoes are attracted by chickens, flying upwards and placed on top of the external net-trap. Mosquitoes were collected with aspirators in the morning before the sunrise.

Data analysis

Chi square tests, Student t tests were realized with R statistical software.

RESULTS

One thousand five hundred and eleven mosquito adults

Back-Pack collection. Aspirations were done outdoors during the days in Mitsinjo. Poultry and bushes were systematically aspirated with the back-pack aspirator using progressive down- and upward movements with a speed approximating 1 meter per second.

Identification

Mosquito identification was performed by mean of a binocular microscope and according to Grjebine (1966) [14] and Fontenille (1989, Unpublished paper) morphological keys. Identifications were carried out in the field right after the collection. All the data were consigned with respect to hour, locality, genus, species, sexes, feeding status and method of collection. have been collected during this study in two different sites with different catching methods (Table 1). Among the 5 genus and 30 species, a total of 1,085 individuals representing 73.3% of the 1,511 mosquitoes belonged to 11 potential WNV vector species.

Table 1. Mosquito species and number of mosquitoes caught in Antsalova and Mitsinjo

	Antsalova			Mitsinjo				Total	
Species	Antsamaka	Masoarivo	Tsakoramby	Amboanjo	Mahakary	Marofandroboka	Morafeno	N	%
Aedeomyia madagascarica	1		3	4	4		4	16	1,08
Aedes aegypti						8		8	0,54
Aedes albocephalus						108	1	109	7,36
Aedes albodorsalis						3		3	0,20
Aedes durbanensis						8		8	0,54
Aedes fowleri				1		4	3	8	0,54
Aedes moucheti	18							18	1,22
Anopheles coustani		2	2		2			6	0,41
Anopheles funestus			59		9			68	4,59
Anopheles fuscicolor	1		102					103	6,95
Anopheles gambiae s.l.	1			1	14	1	1	18	1,22
Anopheles grassei				4	7			11	0,74
Anopheles maculipalpis		1						1	0,07
Anopheles pauliani	1	3	5		1		1	11	0,74
Anopheles pharoensis	4				89		5	98	6,62
Anopheles sp	1		3					4	0,27
Anopheles squamosus					1			1	0,07
Culex annulioris					1			1	0,07
Culex antennatus	1		97	1	6		4	109	7,36
Culex bitaeniorhynchus				9		2	3	14	0,95
Culex decens	2							2	0,14
Culex pipiens					10			10	0,68
Culex poicilipes	5		263					268	18,10
Culex sp	2		2					4	0,27
Culex tritaeniorhynchus	19	1		4	18	30	10	82	5,54
Culex univittatus			1	12	37	1	6	57	3,85
Ficalbia circumtestacea	9							9	0,61
Mansonia africana			1					1	0,07
Mansonia sp	1		1					2	0,14
Mansonia uniformis	131	1	275		5	8	2	422	28,49
NA sp	1							1	0,07
Uranotaenia alboabdominalis			1					1	0,07
Uranotaenia balfouri			4					4	0,27
Uranotaenia sp	3							3	0,20
Total	201	8	819	36	204	173	40	1481	100,00
Number of species	13-17	5	12-15	8	14	10	11		

Mitsinjo District

Four hundred eighty eight adult mosquitoes (485 females and 3 males) from 5 genus and 20 species were collected during 7 nights in the Mitsinjo districts. Ten different species were collected in Marofandroboka (the furthest place from the lake) while 11 species were collected in Amboanjo and in Morafeno, and 14 species in Mahakary. Only 4 species were present in the 4 villages: *An. gambiae s.l.*, *Cx. tritaeniorhynchus*, *Cx. univittatus*, and *Ma. uniformis*;

the 3 last ones being potential vectors of WNV while *An. gambiae s.l.* is the main malaria vector in Madagascar. *Culex annulioris*, *Cx. pipiens*, *Anopheles funestus* and *An. squamosus* were only found in Mahakary, and *Aedes albocephalus* and Ae. *durbanensis* only in Marofandroboka. In Marofandroboka, 89% of the collected mosquitoes were potential vectors: *Ae. albocephalus* represented 62% of the individuals, *Cx. triteaniorhynchus* 17%, *Ma. uniformis* and *Ae. aegypti* (5% each) and *Cx. univittatus* with one specimen. In Amboanjo, 6/11 species are potentially involved in the WNV transmission: *Cx. univittatus* (55%), *Cx. tritaeniorhynchus* (9%), *Cx. antennatus* (1%), *An. coustani* (1%), *Ae. albocephalus* (1%) and *Ma. uniformis* (1%). In Morafeno, 5 species (*Cx. tritaeniorhynchus* with 25%, *Cx. univittatus* with 15%, *Cx. antennatus* with 10%, *Ma. uniformis* with 5% and *Ae. albocephalus* with 3%) were potential vectors. In Mahakary, 38% of the total individuals of the 14 collected species were considered as potential vectors (*Cx. univittatus* 18%, *Cx. tritaeniorhynchus* 9%, *Cx. pipiens* 5%, *Cx. antennatus* 3%, *Ma. uniformis* 2% and *An. coustani* 1%).

During the Mitsinjo experiment, the individuals caught with the light traps represented 62% of mosquitoes (N= 322 individuals) following by the net trap baited (32,8%). Four mosquitoes individuals were caught with the Back-Pack method (0,8%). No mosquito larvae were found in potential breeding sites of the four sites in Mitsinjo.

Masoarivo site

On thousand twenty three mosquitoes were caught during the three nights in Masoarivo: 542 mosquitoes (53%) with the light traps and 256 (25%) with BG sentinel traps. Thirteen mosquito species including 8 species of potential WNV vectors were collected with light traps, and 7 species (3 potential WNV vectors) with BG traps. In parallel, we captured 225 mosquitoes on human bait. In Masoarivo, 3 mosquito adults were captured during 1h30 (3 species), 105 during 1h near the Antsamaka Lake (5 species) and 117 during 1h30 in Tsakoramby (5 species) (Table 2).

Qualitatively and quantitatively, the results were different in the two districts. Quantitatively with these traps, six mosquitoes were captured in Masoarivo, 91 in Antsamaka around the lake, and 702 in Tsakoramby. Qualitatively, three species were captured in Masoarivo, 10 in Antsamaka around the lake, and 8 in Tsakoramby.

Transect and vector species distribution

The mosquitoes' abundance and composition species varied according to the three transects. Mosquitoes' biodiversity and density were high in areas which seem to be the most frequented by vertebrate host such as humans and domestic birds in the Tsakoramby village (transect 2) and wild birds (transect 1) near the Antsamaky Lake. The two types of trappings (BG and light traps) were complementary with the capture of different species in different proportions. BG traps proved to be *Mansonia uniformis* very specific (a well-known WNV vector) and BG results were comparable to human bait results. In addition, species composition varied spatially, with more individuals caught from the lake and from the forest, and less in the intermediate ecosystem. In Tsakoramby and around the Antsamaky Lake, 2 transects were done with the traps but no statistical difference existed between the number of mosquitoes, neither between the considered species. And, finally, the proportion of potential vectors was statistically the same despite the different zones and traps types.

DISCUSSION

The results were unexpected. Indeed, 71.08% of the trapped mosquitoes were potential WNV vectors. Even if we designed this study to choose the best methods to follow up mosquitoes in our transversal studies, we cannot imagine such a high percentage of vectors.

Table 2. Number of mosquitoes caught with different trapping methods in Mitsinjo and Antsalova

Trapping	District: Antsalova	District: Mitsinjo	Total
BG sentinel	256		256
Back-Pack Aspirator		3	3
Human Landing Catch	225		225
Double Nets		129	129
Light traps	547	321	868
Total	1028	453	1481

Vector species

The main WNV vectors involved were ornithophilic mosquitoes (Mathiot et al. 1983), but we choose these sites in order to obtain ornithophilic mosquitoes. Indeed, the birds, especially the wild migratory birds, play a central role in the epidemiology of this zoonosis as the primary host and reservoir [10]. Classically, the main vectors involved in the cycle are ornithophilic mosquitoes of the genus *Culex*. Surprisingly, our study reports that other three genus such as *Aedes* (*Ae. aegypti*, *Ae. albocephalus*) *Anopheles* (*An. coustani*, *An. pauliani*) and *Mansonia* (*Ma. africana*, *Ma. uniformis*) which possessed an anthropophlic behavior were already found naturally infected with WNV [15], [16], [17], [18], [19], [20]. These species include *Ae. aegypti*, *Ae. albopictus*, *Ae. albocephalus*, *Cx. pipiens*, *Cx. antennatus*, *Cx.*

tritaeniorhynchus, Cx. univittatus, Cx. poicilipes and *Mansonia uniformis*.

It is interesting to note that among the five species caught in Masoarivo, 4 species are described as WNV vectors. In terms of individuals, this results in 6 out of the 9 individuals which can be vectors. Also this is the first study reporting the presence of *Ae. albodorsalis, Ae. moucheti*, *Aedeomyia madagascarica*, *Anopheles grassei*, *Culex annulioris*, *Ficalbia circumtestacea* in the western domain. Four of these species used to live only in forested area of the eastern domain [16], [9]. *Aedes moucheti* is only present with certainty in Nosy Be [21] and *Culex annulioris* occurs only in the forested area of the central and eastern domain [22], [20]. The majority of these species are not involved in disease transmission. *Culex annulioris* is the only species involved in Sindbis virus [23] and Middelburg virus transmission
(http://www.pasteur.fr/recherche/banques/CRORA /).

Trapping

In order to evaluate the distribution and the relative density of a mosquito vector, the choice of the trapping method is important [24]. For WNV vectors, the most efficient and reproductive traps were represented by the light traps and the BG sentinel traps. Indeed those two traps can catch mosquitoes in high number and they seem complementary because the vectors collected with these 2 traps did not belong to the same species.

Until now BG traps failed in catching mosquito species in Madagascar. Generally, this method has been used with artificial BG-Lure ® in Central Highlands without providing interest results [9]. This highlights the importance of the lure depending on the study and the aim of the study.

The backpack trap, the larvae collection and the human bait did not appear reproducible enough to be used during a repeated transversal study. Concerning the two first traps (backpack and larval collection), the number of mosquitoes we were able to catch was not sufficient enough. Indeed, with only 6 mosquitoes (representing 0.8 %) for backpack and 0 mosquitoes for larvae, it could not be representative of the ecological reality in the wetlands ecotypes. But the use of backpack can be adapted during epidemics in order to catch engorged mosquitoes from outdoor resting places, and particularly [25] pit traps, dug in and around both villages [20]. Despite the important number caught with the human baits, this trap could not be selected. First, in the wetland ecotype in the West of Madagascar, the sanitary problem is important (FVR, WNV, Malaria…). Plus, the high density of WNV vectors and other disease vectors is a warning against this method. More, we were able to catch less species in term of biodiversity, with often one very prominent species. Depending on the technician skills, or the involvement of the inhabitants, this method is not reproducible enough. At least, this is not necessary to estimate the human risk when the majority of the transmission we are looking for, is a bird-to-bird transmission.

Spatiality and Contacts

The importance of the spatial variation in term of biodiversity of mosquito species is important to be understood in order to study an ecotype. For example, in Masoarivo, we tested ecological gradients in two different environments: around a lake either in a natural protected park, or in a village. An important spatial variation was observed in less than 100 meters. This result demonstrated the importance to choose the right places where to put the traps in order to take in consideration the overall diversity and to increase the probability of trapping mosquitoes to obtain a more representative snapshot. More than the study of WNV vectors, the importance of the spatial distribution is not enough discussed generally and not taken into consideration when scientists come to their conclusions.

Our study focused on the role of mosquitoes vector in WNV transmission in the cohabitation area where stay wild and domestic birds (Tsakoramby, Mahakary, Morafeno and Amboanjo villages), and non-cohabitation area (Marofandroboka, Masoarivo and Antsamaky Lake). The next step is a longitudinal study including virological detection and isolation of WNV in the potential vectors but also in the other species in order to determine whether they are able to transmit the WNV and play a role in the onset of the epidemics or WNV maintenance. *Ae. albopictus, Ae. aegypti* , *Cx. tritaeniorhynchus*, *Cx. univittatus* have been described to be able to transmit vertically WNV [26], [27].

CONCLUSION

From this preliminary field study, the net trap is an efficient and simple way of collecting WN vector species. BG sentinel is only used as alternative methods if it is associated with small vertebrate host (Lemur or poultry). CDC light trap can be applied more intensively for exploring vector composition in this longitudinal study. Detection and isolation of WN virus in pool of mosquitoes, the components of vectors capacity led to consider these species as good vectors of WNV in Madagascar and further studies on vector competence are required.

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