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Resting Behaviour of Deltamethrin-Resistant Malaria Vectors, *Anopheles arabiensis* and *Anopheles coluzzii*, from North Cameroon: Upshots from a Two-Level Ordinary Logit Model

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Abstract

The current study was conducted in Garoua, Pitoa, and Mayo-Oulo health districts of north Cameroon, in order to investigate the resting behaviour of deltamethrin-resistant *Anopheles* (*An.*) *gambiae s.l.* populations and build a model of their response to the use of Permanet 2.0 long-lasting insecticidal nets (LLINs). Adult mosquitoes were collected in October and November 2011, using spray catches and window exit traps in 29 clusters with LLINs in use. Sampled *An. gambiae s.l.* were identified down to species and analysed for blood-meal origin, physiological and circumsporozoite protein status. Deltamethrin resistance was assessed using World Health Organization's (WHO's) standard protocol. A two-level ordinary logit model was used to relate the resting behaviour and deltamethrin resistance. Identified species of the *An. gambiae* complex included *An. arabiensis* (90.6%), *An. coluzzii* (7.1%) and *An. gambiae s.s.* (2.3%). They displayed 1.1–4.8% infection rates, 80% indoor-resting density and 56–80% human blood index. Eleven *An. gambiae s.l.* populations over the 15 tested were resistant to deltamethrin (51–89.5% mortality rates). Model results showed a significant dependence of indoor vector density

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on increasing deltamethrin resistance (p-value of <0.01). These behavioural and resistance patterns may lead to increasing malaria transmission in study health districts.

1. Introduction

Malaria still remains a priority problem in the world today. According to the latest estimates, there were about 214 million cases of malaria in 2015 (with an uncertainty range of 205–316 million) and an estimated 438,000 deaths (with an uncertainty range of 236,000–635,000) [1]. People living in the poorest countries are the most exposed to malaria, with 90% of deaths occurring in the African region, mostly among children [1]. The emergence of vector resistance to insecticides is a serious threat in the fight against malaria, since the general use of insecticides in indoor residual spraying (IRS) or in long-lasting insecticidal nets (LLINs) constitutes the only means of mass prevention [2]. In many sub-Saharan African countries, vector resistance to all classes of insecticides has been reported [3–5]. Some studies conducted in south western Chad (close to the North Region of Cameroon), where insecticide-treated nets (ITNs) and LLINs are used in large scale, have revealed deltamethrin resistance in the *Anopheles (An.). gambiae s.l.* populations [3, 6]. Given that the successes in the reduction of malaria burden acquired in the long run may strongly be compromised by vector resistance to insecticides, it is necessary to elaborate strategies for the management of this phenomenon.

In Cameroon, malaria is responsible for 30% of morbidity cases, 36% of outpatient consultation, 67% of childhood mortality and 48% of hospital admissions [7]. Malaria infection is essentially due to *Plasmodium falciparum*, followed by *P. ovale and P. malariae* [8]. Seven anopheline species play major roles in *Plasmodium* parasite transmission, among which are *An arabiensis, An. gambiae* and *An. coluzzii,* three sibling species of the *An. gambiae* complex [9]. However, several studies have reported insecticide resistance in these three mosquito species, threatening the progress towards malaria elimination in the country [10–12]. Field observations have shown that the emergence of insecticide resistance is favoured by the abusive and anarchic use of pesticides in public health and/or in agriculture [11, 13]. The regions that are mostly concerned with resistance are the North, Centre, Littoral, West and North West. Two resistance mechanisms have been noticed, *metabolic-* and *kdr*-based insecticide resistance. *Metabolic*-based resistance is due to altered levels of detoxifying enzymes such as P450s, esterases and glutathione S-transferases, whose expression levels may well be modulated by variation in many genes, making it a quantitative genetic trait [14, 15]. The *kdr*-based insecticide resistance results from a single leucine to phenylalanine or serine amino acid mutation at the voltage gate sodium channel which is the target side of dichlorodiphenyltricholoroethane (DDT) and pyrethroid insecticides [5]. *Metabolic*-based resistance is the major mechanism in the northern tropical regions of Cameroon, whereas multiple insecticide resistance, including *metabolic-* and *kdr*-based resistance mechanisms, is widespread in the southern equatorial regions [16–18].

However, while the major mechanisms of insecticide resistance in malaria vectors are more and more documented, an important research gap remains on detailed understanding of the likely genetic basis of specific behavioural traits, and how surveillance programs should be implemented to best monitor changes in these traits. In other words, how insecticide resistance might interact with vector behavioural response to interventions is very poorly known.

The current study was carried out in three health districts in the North of Cameroon, namely Pitoa, Garoua and Mayo-Oulo, where *metabolic*-based resistance to deltamethrin was reported in *An. gambiae* s.s. and the twin species *An. arabiensis*. These species are the major malaria vectors in the North Region, malaria incidence in these districts (46–62%) is among the highest of the 12 health districts of northern Cameroon region [19, 20]. Deltamethrin LLINs (Permanet 2.0) were distributed there at universal coverage in 2010, for malaria mass prevention. The aim of the study was to assess the resting behaviour of local *An. gambiae s.l.* populations when control interventions are put in place, with respect to their insecticide resistance status.

2. Main body

2.1. Experiments

2.1.1. Study area

The field survey was carried out in the North Region of Cameroon from October 2011 to November 2011 in 27 villages in three health districts: Garoua, the regional capital city (9° 30N; 13°40E), Pitoa (9°21N; 13°31E), situated 15 km from Garoua, and Mayo-Oulo (9°46N; 13° 44E), 140 km from Garoua. This is a cotton-cultivated area where there is an extensive use of LLINs for human protection with approximately 87% of households having at least one net and 69% of households having at least one net per two people [21]. The North Region of Cameroon makes up 66,090 km^2 of the Northern half of the Republic of Cameroon with a population density of 13 people/km^2. Neighbouring territories include the Far North Region to the north, the Adamawa Region to the south, Nigeria to the west, Chad to the east and Central African Republic to the south-east. The districts and their study clusters are given in **Figure 1**.

2.1.2. Mosquito collection

Mosquitoes were collected during a cross-sectional survey in 29 clusters including 12 clusters in the Garoua health district, 10 clusters in the Pitoa health district and seven clusters in the Mayo-Oulo health district (**Table 1**). Two separate data sets were collected: one on mosquito's resting behaviour while another was collected on insecticide resistance. For the resting behaviour data, two techniques of adult mosquito collection were used: window exit traps (WETs) and indoor spray catches (ISCs).

WET were placed over the windows of bedrooms to collect mosquitoes that attempt to escape during the night [22]. Ten rooms per cluster were used to collect mosquitoes during two consecutive nights. WETs were placed at 6.00 pm over the windows of each bedroom till 6.00
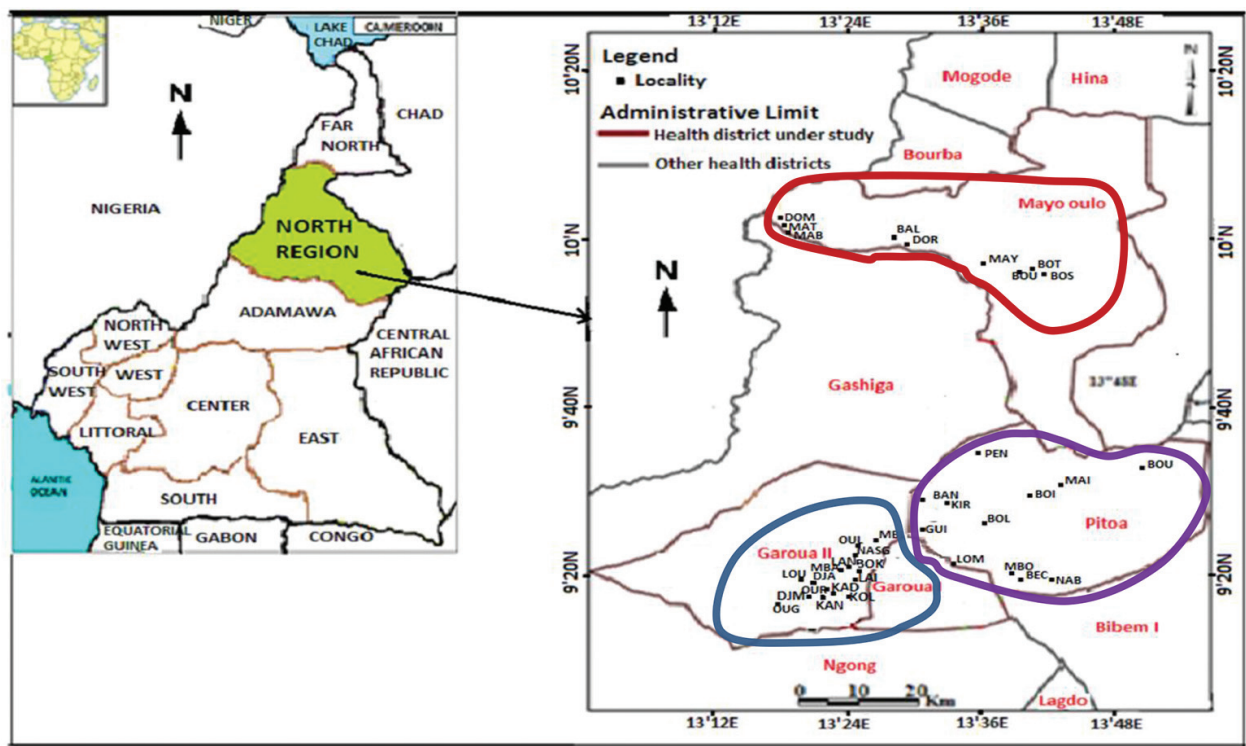
Figure 1. Map of Cameroon showing the study region, health districts and study clusters. The red chart encloses cluster of the Mayo-Oulo Health District (MAY: Mayo-Oulo; BOU: Bouyoum; BOT: Batoum; BOS: Bossoum; DOR: Dourbaye; MAB: Maboni; BAL: Balla). The purple chart encloses cluster of the Pitoa Health District (MAL: Mayo Lebri; BEC: Be Centre; BOI: Boula-Ibbi; BOL: Boulgou; BOUS: Boussa GUI: Guizigaré; NASP: Nassarao; MBO: Mbolom; PEN: Pena KIR; Kirombo). The blue chart encloses cluster of the Garoua Health Districts (DJA: Djamboutou I; DJM: Djamboutou II; KAD: Kanadi II; KAN: Kanadi I; LAN: Laïndé II; LOU: Loundérou; MBA: Mboum-aviation; MBI: Mbilga; BOK: Bocki; NASG: Nassarao; OUL: Oulo Lawane; OUG: Ouro-Garga).

am. Mosquitoes were then collected inside the trap in the morning, between 6 and 8 am using a mouth aspirator.

ISCs were done in the morning, from 6 to 8 pm, to sample mosquitoes resting inside rooms used for WETs. After spreading white sheets to cover the entire floor space and objects, rooms were sprayed with commercial aerosols containing pyrethroids. Mosquitoes fall on to the white sheets 10 min after spraying and were carefully picked up.

Female anophelines collected from WET and ISCs were morphologically identified using identification keys [23, 24]; they were separated according to their physiological status (unfed, freshly fed, half gravid and gravid) and individually preserved in tubes with desiccant for subsequent laboratory analyses.

For insecticide resistance testing, samples of anopheline larvae and pupae were collected from the maximum number of breeding sites. In each cluster, all open water bodies were inspected. Samples of immature anophelines were collected from active breeding sites by the dipping method [22]; they were pooled per cluster and brought to a local insectary, then reared in spring water. Larvae were fed on Tetramin Fish Food (Tetra Werke Company, Inc., Blacksburg, VA, USA) and pupae were kept in a cage to emerge into adults. Emerging adults were

Health district	Population of district	Selected health areas	Distance to headquarters (km)	Human population	Clusters
		Pitoa	0	32,327	GUI, BOL
		Banaye	13	6296	PEN, KIR
Pitoa	108,611	Be Centre	22	4341	BEC, MBO, NASP
		Bouba Ibib	40	15,262	BOI, MAL, BOUS
Total				**58,226**	
		Kolleré	0	27,692	KAD, KAN
		Nassarao	12	7224	NASG, BOK, MBI
Garoua	316,957	Laïndé	10	56,569	LAN, OUL, MBA
		Djamboutou	7	40,122	DJA, DJM, OUG, LOU
Total				**165,212**	
		Mayo-Oulo	0	28,203	MAY, BOT, BOU
Mayo-Oulo	91,501	Dourbaye	10	16,106	DOR, BAL, MAB
		Doumo	42	8502	BOS
Total				**52,811**	

BAL: Balla; BEC: Be Centre; BOI: Boula-Ibbi; BOK: Bocki; BOL: Boulgou; BOS: Bossoum; BOUS: Boussa; BOU: Bouyoum; BOT: Batoum; DOR: Dourbaye; GUI: Guizigaré; DJA: Djamboutou I; DJM: Djamboutou II; KAD: Kanadi II; KAN: Kanadi I; KIR: Kirombo; LAN: Laïndé II; LOU: Loundérou; MAB: Maboni; MAY: Mayo-Oulo; MBA: Mboum-aviation; MBO: Mbolom; MAL: Mayo Lebri; MBI: Mbilga; NASG: Nassarao; OUL: Oulo Lawane; NASP: Nassarao; OUG: Ouro-Garga; PEN: Pena.

Table 1. Health districts and study clusters in the north Cameroon region.

maintained on 10% sugar solution. Adult anophelines were then morphologically identified as belonging to the *An. gambiae* complex by means of reference keys [23, 24] and used for susceptibility tests.

2.1.3. Insecticide susceptibility test

Insecticide susceptibility tests were performed on mosquitoes aged 2–4 days, emerged from field-collected larvae and pupae. *An. gambiae s.l.* females were exposed for 1 h to 0.05% deltamethrin-impregnated papers using WHO susceptibility test kits and standard protocol for adult mosquitoes [25] under ambient room temperature (25–28°C) and 70–80% relative humidity. Test kits including deltamethrin-impregnated papers, test tubes and accessories were supplied by the Vector Control and Research Unit, University Sains Malaysia (Penang, Malaysia). Filter paper sheets (12 × 15 cm, Whatman N°1) were impregnated with the discriminating dosage of deltamethrin (0.05%) (pyrethroid insecticide), mixed with acetone and silicon oil. Other batches of filter paper sheets were impregnated with acetone + silicon solution for use as control. Acetone acted as the solvent and silicon oil as a carrier. The purchased impregnated papers were stored at 4°C until the date of the test.

Each full set of bioassays was performed with five batches of 20–25 non-blood fed, 2–4-day-old females: four batches were exposed to insecticide-impregnated filter papers and one batch was exposed to untreated filter paper and served as a control. The number of mosquitoes knocked down was recorded at 5-min interval during the 1-h long exposure to deltamethrin-impregnated papers. After exposure to insecticide-impregnated papers or control papers, mosquitoes were transferred to holding tubes and provided with cotton pads soaked with 10% sugar solution. The mortality rates were determined 24 h post exposure.

The resistance status in the mosquito populations was determined according to WHO's criteria [25]:

▪ A mortality rate in the range of 98–100% indicates susceptibility;

▪ A mortality rate between 90 and 97% suggests possible resistance to be confirmed;

▪ A mortality rate less than 90% indicates resistance.

The above criteria are recommended on the grounds that a greater than 2% survival at the diagnostic concentration is considered unlikely to be due to chance alone, and provided that all the test conditions are met.

Tests with mortality of the controls greater than 20% were taken all over again.

2.1.4. Laboratory analysis

The head and thorax of field-collected female *An. gambiae s.l.* were examined for *P. falciparum* circumsporozoite protein (CSP) by enzyme-linked immunosorbent assay (ELISA) [26, 27]. Sporozoite rates were determined as the number of anophelines positive for circumsporozoite protein ÷ the total number of specimens tested by ELISA CSP.

Blood-meal sources were identified by ELISA [28]. The technique identifies human, bovine, ovine (sheep and goat), equine (horse and donkey), pig or chicken hosts. Human blood index (HBI) was determined for each species, that is, the proportion of female anophelines that were found with human blood in their stomachs thereby giving an indication of anthropophilic rate. The HBI was determined as the number of human feeds ÷ (number of human feeds + number of animal feeds).

Genomic DNA was extracted from each selected *An. gambiae s.l.* specimen as described by Collins et al. [29] and each individual was identified to the species level using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) [30]. This method allows simultaneous identification of species of the *An. gambiae* complex.

2.2. Statistical analysis

2.2.1. Descriptive analysis

Pareto charts were used for univariate analysis of the qualitative variables *species, district, locality, phy. status* (physiological status), *csp* (circumsporozoite protein status) and *behaviour* (resting behaviour). Box plots were used to see the distribution of the susceptibility variable

(*loc. suscep*) vis-à-vis the modalities of the qualitative variables and results obtained were tested using the Kruskal-Wallis rank sum test.

2.2.2. Missing data

The *k*-nearest neighbour method of classification with random assignment was used to impute missing observations in the data set, so the missing value in a variable on an individual was imputed by one of its *k*-nearest neighbours picked at random. As a result of this randomness in imputations, constructed models were implemented each on 10 imputed data sets and parameter estimates were obtained for each model by taking the average over the respective 10 imputed data sets estimates [31].

2.2.3. Model specification

A two-level ordinary logit model was used to establish a relationship between *An. gambiae* species caught indoors and at exit traps and the status of deltamethrin resistance in *An. gambiae s.l.* from the same clusters [31]. This model was used because mosquitoes, which were considered as level-1 units, were clustered within localities, considered as level-2 units and measurements were taken at level 1 and 2 units. The model was interested in the effect of resistance on the odds of the probability that a given species *k* in locality *j* will be found indoors against the probability that it will be found at the level of the exit trap. The model therefore has the form: where

$$
\log\left[\frac{\Pr(\text{behavior}_{jk}=1|x_{jk},z_j,\mu_j)}{1-\text{Prbehavior}_{jk}=1|x_{jk},z_j,\mu_j)}\right] = \gamma + \beta_{\text{res}}\text{loc.suscep}_j + \beta_{A.gamM}1_{\text{species}_{jk}=A.gamM}+
$$
$$
\beta_{A.gamS}1_{\text{species}_{jk}=A.gamS} + \beta_P 1_{csp_{jk}=P} + \beta_{\text{Gravid}}1_{phy.status_{jk}=\text{Gravid}} + \beta_{H.grav}1_{phy.status_{jk}=H.grav}
$$
$$
+ \beta_{\text{Unfed}}1_{phy.status_{jk}=\text{Unfed}} + \text{loc.ran.effect}_j, \tag{1}
$$

- x_{jk} is a realization of the vector of level-1 covariates (*species*, *csp* and *physiological status*) for a mosquito *k* in locality *j*,

- z_j is a realization of the level-2 covariate (*locality susceptibility*). It is the percentage of deltamethrin susceptibility of locality *j*,

- μ_j = loc.ran.effect$_j$ is the locality *j*'s random effect in the model assumed to follow a normal distribution with mean 0 and variance σ^2,

- γ, β_{res}, $\beta_{A.gam\,M}$, $\beta_{A.gam\,S}$, β_p, β_{Gravid}, $\beta_{H.grav}$, β_{Unfed} and σ^2 are the model parameters to be estimated and

- $1_{X_{jk}=x} = \begin{cases} 1 & \text{if} X_{jk} = x \\ 0 & \text{otherwise} \end{cases}$

2.2.4. Estimation of parameters

The maximum marginal likelihood method was used to estimate the parameters of the model. Solutions to the likelihood equations were approximated using Fisher's method of scoring

with numerical approximations of integrals obtained using the Gauss- Hermite quadrature (GHQ) method [31]. Analyses were done in EXCEL and in *R*, using the *R*-function *sabre* in the *R*-package *sabreR* [31–33].

2.3. Results

2.3.1. Vector densities, infection rates and blood-meal origins

A total of 1135 *Anopheline* mosquitoes were collected from October 2011 to November 2011 in 29 clusters across the three study health districts (Garoua, Pitoa and Mayo-Oulo). On the bases of morphological identification, 10 anopheline species were identified in collected mosquito samples, including:

- -three species of the *An. gambiae s.l.* complex representing more than half of the total population of *anopheles* vectors (609/1135= 53.7%),

- -*An. funestus* group (408/1135 = 35.9%),

- -*An. rufipes* (82/1135 = 7.2%),

- -*An. pharoensis, An. nili, An. coustani, An. maculipalpis* and *An. paludis* representing each less than 5% of the total analysed samples.

Among the samples belonging to the *An. gambiae* complex, 299/374 (80%) specimens were successfully identified down to species using PCR-RFLP. *An. arabiensis* was the predominant species (271/299 = 90.6%), followed by *An. coluzzii* (21/299 = 7.1%) and *An. gambiae* s.s. (7/299 = 2.3%) (**Table 2**).

A total of 6/696 anopheline mosquitoes tested by ELISA CSP showed the presence of the circumsporozoite protein, corresponding to 0.86% global infection rate. These specimens belonged to three species: *An. coluzzii, An. arabiensis* and *An. funestus*. The infection rates per

Species	Point of collection	Physiological status (or gonotrophic state) (%)				
		Unfed	Freshly fed	Half gravid	Gravid	Total (%)
An. arabiensis	Indoors	55 (70.5)	99 (84.6)	18 (62.1)	45 (95.7)	217 (80.2)
	Exit traps	23 (29.5)	18 (15.4)	11 (37.9)	2 (4.3)	54 (19.8)
An. coluzzii	Indoors	3 (75.0)	15 (88.2)	0	0	18 (85.7)
	Exit traps	1 (25.0)	2 (11.8)	0	0	3 (14.3)
An. gambiae	Indoors	1 (50.0)	3 (60.0)	0	0	4 (57.1)
	Exit traps	1 (50.0)	2 (40.0)	0	0	3 (42.9)
Total	Indoors	59 (92.2)	117 (84.2)	18 (62.8)	45 (95.7)	239 (79.9)
	Exit traps	25 (7.8)	22 (15.8)	11 (37.9)	2 (4.3)	60 (20.2)

Table 2. Residual and exit trap faunas in relation to species of the *Anopheles gambiae* complex and physiological status. %: proportion of mosquitoes collected indoors versus exit traps.

species were 4.8% (1/21), 1.1% (3/271) and 0.5% (2/408) for *An. coluzzii, An. arabiensis* and *An. funestus,* respectively.

Four hundred and ninety-seven (497) anophelines were tested for blood-meal origins, among which 229 meals were successfully determined. They included 55.5% (127/229) meals from human, 31.4% (72/229) from cattle and 4.8% (7/229) from sheep and pig (**Table 2**). There were 8.3% (21/229) mixed blood meals from human + pig, human + cattle, human + cattle + sheep, cattle + sheep, cattle + pig and cattle + sheep + pig. The three species of the *An. gambiae* complex (*An. arabiensis, An. coluzzii* and *An. gambiae s.s.*) displayed high human blood indexes (HBI), ranging from 0.56 to 0.8, suggesting high anthropophagic tendencies compared with *An. funestus* (HBI = 0.42, $p < 0.005$) (**Table 3**).

Species	H	C	S	P	H/C	H/P	H/C/S	C/S	C/P	C/S/P	Total	HBI
An. arabiensis	94	39	2	2	1	5	1	2	0	0	146	0.69
An. coluzzii	7	3	1	3	2	0	0	0	0	0	16	0.56
An. gambiae	4	1	0	0	0	0	0	0	0	0	5	0.80
An. funestus	22	29	1	2	0	3	1	1	1	2	62	0.42
Total	127	72	4	7	3	8	2	3	1	2	229	0.62
(%)	55.5	31.4	1.7	3.1	1.3	3.5	0.9	1.3	0.4	0.9	100	

H: human; C: cattle; S: sheep; P: pig; H/P: human + pig; H/C: human + cattle; H/C/S: human + cattle + sheep, C/S: cattle + sheep, C/P: cattle + pig; C/S/P: cattle + sheep + pig.

Table 3. Origin of blood meals of the four major malaria vectors in the study health districts.

Figure 2. Mortality rates of *Anopheles gambiae s.l.* populations 24 h post exposure to 0.05% deltamethrin-impregnated papers. The red line at 90% mortality rate indicates the threshold below which a mosquito population is classified as resistant to the insecticide used for susceptibility test [25]. BAL: Balla; BEC: Be Centre; BOK: Bocki; BOS: Bossoum; BOUS: Boussa; DOR: Dourbaye; GUI: Guizigaré; DJM: Djamboutou II; KIR: Kirombo; MAY: Mayo-Oulo; MBA: Mboum-aviation; MBO: Mbolom; MAL: Mayo Lebri; OUG: Ouro-Garga.

2.3.2. Status of deltamethrin resistance in An. gambiae s.l. populations

One thousand two hundred and thirteen (1213) females of the *An. gambiae s.l.* were tested for their susceptibility to deltamethrin. They were from 15 field populations, including six from the Pitoa health district, four from the Mayo-Oulo health district and five from the Garoua health district. Recorded mortality rates are presented in **Figure 2**. According to WHO's criteria, 11 populations among the 15 tested were found resistant to deltamethrin with mortality rates ranging from 51 to 89.5%. Resistance was suspected in three populations, that is, those from Kirambo and Boussa in the Pitoa health district and Mayo-Oulo in the Mayo-Oulo health district, with 92–94% mortality rate. Only the Ouro-Garga population was found susceptible to deltamethrin. These data showed that deltamethrin resistance was widespread in the three study health districts.

2.3.3. Resting behaviour and physiological statuses of the species of the An. gambiae complex

Among the three identified sibling species of the *An. gambiae* complex, high proportions of *An. arabiensis* (217/271 = 80%) and *An. coluzzii* (18/21 = 86%) samples were caught indoors compared with exit traps collection (20 and 14%, respectively), showing their high endophilic behaviour in the study areas (**Table 2**). These proportions were not significantly different between the two species (*p*-value = 0.25). However, similar rates of *An. gambiae* s.s. specimens were caught indoors versus in exit traps, although the sample size was very small.

Considering the physiological status of collected mosquitoes, all the four gonotrophic states (unfed, freshly fed, half gravid and gravid) were found in *An. arabiensis* samples collected indoors or in exit traps, with freshly fed and unfed mosquitoes being predominant. Meanwhile, only unfed and freshly fed gonotrophic states were found in analysed *An. coluzzii* and *An. gambiae s.s.* samples, raising the question about the behavioural patterns (endophilic/exiting) of half gravid and gravid mosquitoes of these two species.

The overall indoor-resting rate for the three species of the *An. gambiae* complex was 80% (239/299), increasing from half gravid (18/29 = 62%), to freshly fed (117/239 = 84.2%), unfed (59/84 = 92%) and gravid mosquitoes (45/47 = 96%) (**Table 2**). Conversely, the highest rate of exiting mosquitoes was recorded among the half gravid samples (11/29 = 40%) and the lowest among the gravid samples (2/47 = 4%).

2.3.4. Effect of deltamethrin resistance on the resting behaviour

The two level logit regression analysis given in **Table 4** showed a statistically significant dependence of resting behaviour on deltamethrin resistance. Fitting the mean parameter estimates in the model, we obtained the following:

$$log\left[\frac{\text{Pr}(behavior_{jk}=1|x_{jk},z_j,\mu_j)}{1-\text{Pr}(behavior_{jk}=1|x_{jk},z_j,\mu_j)}\right] = -14.4626 + 0.1398 loc.suscep_j - 2.36991_{species_{jk}=A.gamM}$$
$$-2.0974\_1_{phy.status_{jk}=Gravid} + 1.29431_{phy.status_{jk}=Unfed} + loc.ran.effect_j \quad (2)$$

This model showed that the estimated coefficient of the locality resistance variable (*loc.suscep*)

Variable	Mean estimate of parameter	Variance of imputation method	Standard error estimate	Signal-to-noise ratio	95% C.I. Approximation	Approximated P-value
Threshold intercept	−14.463	4.662	2.265	6.385	(−18.902, −0.0232)	0.000
Resistance loc. suscept	0.140	0.000701	0.0271	5.158	(0.0866, 0.193)	0.000
Species cies (*An. Arabiensis* = ref) A. coluzzii A. gambiae s.s.	−2.370 −0.301	0.199 0.711	0.470 0.885	5.0471 0.340	(−3.290, −1.450) (−2.0357, 1.434)	0.000 0.734
csp (Abs = ref) Presence	−16.865	0.265	355.814	0.0474	(−14.259, 680.531)	0.962
Phy.Status (Freshly fed = ref) Gravid Half Gravid Unfed	−2.0974 −0.678 1.294	0.0615 0.185 0.0855	0.264 0.451 0.307	7.936 1.502 4.210	(−2.615, −1.580) (−1.563, 0.207) (0.691, 1.897)	0.000 0.133 0.000
Variance of locality random effect (σ^2)	3.673	0.0864	0.311	11.803	(3.0627, 4.283)	0.000

Table 4. Mean of estimates of parameters in the two-level logit regression model and their corresponding precisions.

	p-Value					
	Behaviour	Locality	District	Species	Physiological status	csp
Susceptibility to deltamethrin	0.0041	2.2e–16	2.2e–16	0.18	4.1e–06	Observations in same group

Table 5. Kruskal-Wallis rank sum tests of independence between the deltamethrin susceptibility variable and the qualitative variables.

has a positive sign, indicating that the species were more likely to be endophilic (stay indoors) with decreasing values of mortality rates to deltamethrin susceptibility test (increasing deltamethrin resistance). The result was significant at the 5% level (*p*-value = 2.502e–07). This result was confirmed by the Kruskal-Wallis rank sum test (*p*-value = 0.0041) (**Table 5**).

Furthermore, there was varying effect of physiological status on the resting behaviour. **Figure 3** shows the relationships between resting behaviour and deltamethrin resistance for the *An. arabiensis* (A) and *An. coluzzii* species (B) with different physiological statuses.

With specimens of the *An. arabiensis* species, the probability that an unfed specimen was endophilic decreased from 1.00 to 0.31, as the mortality to susceptibility test increased from 50 to 100% **(Figure 3A)**. The total difference in probability was 1.00–0.31 = 0.69. Thus, when the mortality rate was less than 80%, the probability that the unfed *An. arabiensis* was endophilic was greater than 0.88 and when mortality was greater than 98%, the probability was less than 0.38. The effect gradually decreased on freshly fed (total difference in probability was 1.00 −
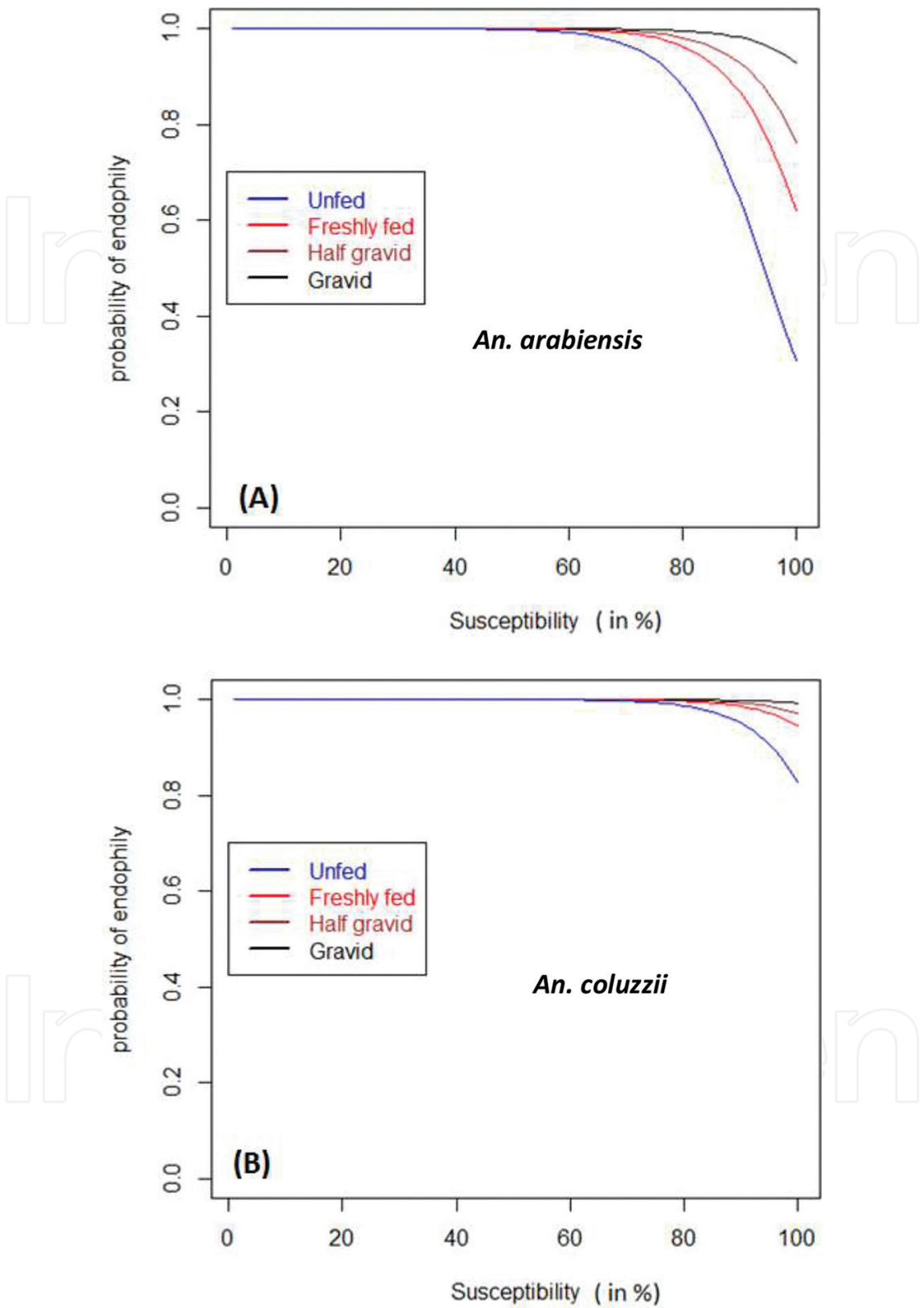
Figure 3. Endophily curves for *An. arabiensis* (A) and *An. coluzzii* (B) in relation to physiological statuses and mortality rates to susceptibility tests.

0.62 = 0.38) and half gravid specimens (total difference in probability was 1.00 − 0.76 = 0.24). The effect of deltamethrin resistance on the resting behaviour of gravid *An. arabiensis* was not significant.

With specimens of the *An. coluzzii* species, the effect of deltamethrin resistance on the resting behaviour was not significant ($p > 0.8$) in general as shown in **Figure 3(B)**. Nevertheless, the probability of endophily in unfed *An. coluzzii* was slightly decreased between 80 and 100% mortality rates.

2.4. Discussion

The current study revealed high indoor densities and an obvious infectivity of deltamethrin-resistant *An. arabiensis* and *An. coluzzii* populations, leading to contact with humans and ongoing malaria transmission in the study districts no matter high coverage of LLINs.

Most important malaria vectors such as *An. gambiae*, *An. arabiensis* and *An. funestus* [34] prefer to feed in the middle of the night when most humans are typically asleep, immobile and vulnerable to attack. Feeding indoors at night is, therefore, the behaviour that is targeted by the use of LLINs to protect sleeping humans. Indoor feeding is then often followed by resting within the same sheltered domestic structure for one or two nights while the blood meal is digested and eggs are developed. Applications of insecticides to houses by IRS, to kill mosquitoes resting on inner surfaces of the walls and roof after they have fed upon the human occupants, are therefore a highly effective strategy for controlling populations of vectors that rest indoors as a matter of preference. Overall, the community-wide mass effect of LLINs and IRS can have a dramatic impact on the population size of stereotypical vectors that depend heavily upon feeding on humans and resting inside houses [35]. However, there are a number of possible impacts that insecticide use indoors could have on mosquito behaviour including changes in biting phenology, the rate of endophagy and endophily. Therefore, understanding that the limits of IRS, LLINs or any other vector-control strategy are primarily defined by the behavioural traits of mosquitoes [36–38] is fundamental. Patterns of malaria vector behaviours have been examined in quite a good number of studies where vectors undergo modifications on their behaviour to facilitate avoidance or circumvention of insecticides [39]. However, better understanding the interactions between insecticide resistance phenotypes in field malaria vector populations, the resistance mechanisms and subsequent behavioural patterns in the presence of LLINs at household level is essential.

The phenotype of deltamethrin resistance in *An. arabiensis* and *An. coluzzii* recorded in this study was consistent with previous studies conducted in the north Cameroon Region [11, 19, 40]. Furthermore, these data provided evidence of widespread deltamethrin resistance in species of the *An. gambiae* complex from this region. In *An. arabiensis* field populations from Pitoa, a set of constitutively overexpressed antioxidant genes (superoxide dismutases *SOD2* and *SOD3*, the glutathione S-transferase *GSTS1* and the thioredoxin-dependent peroxidase *TPX4*) and a single P450 (*CYP4G16*) were previously associated with increased tolerance to deltamethrin [15]. Indeed, metabolic-based resistance was likely the main DDT and pyrethroid resistance mechanisms in *An. gambiae s.l.* in this area, since the *kdr L1014F* allele, which is

recessive, was found at low frequency and heterozygous state in August 2011 just before the current survey [40].

The most abundant species of the *An. gambiae* complex encountered in the three study health districts was the *An. arabiensis*, while *An. coluzzii* and *An. gambiae s.s.* occurred in small ranges. The predominance of *An. arabiensis* in the analysed samples was consistent with the distribution of species of the *An. gambiae* complex in north Cameroon [11, 15, 19]. Since data collection for the current study was done between October and November 2011, the observed distribution of sibling species of *An. gambiae* complex may vary periodically [41]. Significant variations in the relative frequencies of *An. arabiensis* and *An. gambiae* were previously recorded over time and between localities in the north Cameroon. More interestingly, both species were resistant to DDT and deltamethrin, and the resistance steadily increased throughout the rainy season [11]. In other studies, *An. arabiensis* populations have shown some variations in biting or resting behaviour at different locations, with some of these variations being explained by seasons or historical use of insecticides [42].

Substantial variations have also been recorded in the current study. Most of the indoor-collected samples were freshly fed or unfed, suggesting that the vectors might tend to increase the chance of feeding on humans by remaining indoors, regardless of the presence of the LLINs. This behavioural pattern could therefore lead to increasing malaria risk in north Cameroon, since the sporozoite rates in mosquito samples were 4.8 and 1.1% for *An. coluzzii* and *An. arabiensis,* respectively, suggesting regular malaria transmission in households with LLINs. Furthermore, the human blood index was high (56–80%) for the three sibling species, demonstrating their anthropophilic tendencies. Further variations were linked to mosquito physiological status. While all the four gonotrophic states were found in analysed *An. arabiensis* samples (indoors or exiting), only unfed and freshly fed gonotrophic states were found in *An. coluzzii* and *An. gambiae s.s.* samples, suggesting outdoor-resting habit (exophily) for half gravid and gravid mosquitoes of these two species, although the small size of the collected and analysed samples ($N = 21$ for *An. coluzzii* and $N = 7$ for *An. gambiae*) precludes any strong conclusion in these cases. Around 40% of half gravid *An. arabiensis* samples were found in exit traps and almost the same proportion of specimens of this species displayed animal blood meals (cattle, pigs and sheep), sometimes mixed with human blood. This variability of resting behaviour of *An. arabiensis* and to a lesser extent *An. coluzzii* was confirmed by the two-level logit regression analysis in relation with deltamethrin resistance. The decrease of insecticide resistance tended to induce exophily in unfed specimens, while the probability for gravid *An. arabiensis* to remain indoors no matter their susceptibility status was high. Although endophily of gravid mosquitoes was consistent with blood digestion and egg development processes, it could also be enhanced in the specimens displaying resistance to deltamethrin.

Data reported in this study put forward a high plasticity of feeding and resting behaviour in the three species of the *An. gambiae* complex from the study areas. Accordingly, resistant malaria vectors, which have developed the capacity of blood feeding or resting in houses in the presence of LLINs, render LLINs useless if they are not in good state (torn, holed or not large enough). The physical integrity of LLINs in the specific context of vector resistance to

pyrethroid insecticides is essential for the sustainability of malaria mass prevention. These findings concord with those reported in Benin, where households in the areas with resistant mosquitoes showed high rates of blood feeding, while freshly insecticide-treated nets provided no protection once holed. By contrast, sleeping under a holed LLIN in the location where susceptible mosquitoes were common decreased the odds of being bitten by 66% and the majority of mosquitoes were killed by the treatment [43].

When the LLIN is intact, a side effect of physiological resistance would be the reduction in vector behavioural responsiveness to the insecticide [44–46]. Pyrethroid-resistant mosquitoes showed reduced irritability to insecticides, which allow them to rest on the surface for longer periods than susceptible mosquitoes, thus increasing the dose of insecticide received and likely the subsequent mortality rates [46]. In most cases, the effect of physiological resistance is unquantified and dependent on the mechanism of resistance [45]. The *Kdr* mutation is considered a relatively weak form of resistance compared to metabolic-based resistance, and it is usually only when the *kdr* occurs along with metabolic-based resistance that control fails [47, 48]. Furthermore, the current study has demonstrated that control failure may also result from a cumulative effect of insecticide resistance mechanisms and the profile of physiological status of targeted vector population.

With untreated nets, such effects of insecticide resistance and behavioural traits may not occur, and personal protection may be afforded provided the nets are tucked in, maintained in good condition and sufficiently large so that the sleepers do not make contact with the net [49]. Subsequently, this protection may result in a reduction of malaria morbidity compared with areas without nets at all. However, the obtained level of protection remains lower than that conferred by the LLINs which remain one of the most cost-effective tools for malaria mass prevention, even in some areas where vectors have developed pyrethroid resistance [46, 50, 51].

According to WHO, when insecticide resistance is confirmed, pre-emptive actions must be taken to manage this resistance and to ensure that the effectiveness of insecticides used for malaria vector control is preserved [25]. Trials of the combination of LLINs and IRS with different classes of insecticides (carbamates or organophosphates) as a resistance management strategy are necessary in north Cameroon, in order to guide malaria vector-control strategies in this region.

3. Conclusions

The data generated in the current study provide further evidence for cumulative effects of deltamethrin resistance and physiological status of *An. arabiensis* and to lesser extent *An. coluzzii* populations on the patterns of their behavioural responses to LLINs in north Cameroon. It is unique in drawing a model based on analysis of insecticide resistance, indoors/exiting mosquito densities and physiological statuses data. The high densities of vectors inside houses and regular contact with human may lead to increasing malaria transmission in study health districts regardless of the coverage of LLNs. These findings are valuable for the development of resistance management strategies, and effective tools for malaria control and elimination in the study areas.

Acknowledgements

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