

## Variation in Pyrethroid Resistance Phenotypes in *Anopheles darlingi* in an Area with Residual Malaria Transmission: Warning of Suspected Resistance in French Guiana

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**Abstract.** *Anopheles darlingi* is the main vector of malaria in South America. In French Guiana, malaria transmission occurs inland and along the rivers with a regular reemergence in the lower Oyapock area. Control against malaria vectors includes indoor residual spraying of deltamethrin and the distribution of long-lasting impregnated bednets. In this context, the level of resistance to pyrethroids was monitored for 4 years using CDC bottle tests in *An. darlingi* populations. A loss of susceptibility to pyrethroids was recorded with 30-minute knock-down measured as low as 81%. However, no pyrethroid molecular resistance was found by sequencing a 170 base pair fragment of the S6 segment of domain II of the voltage-gated sodium channel gene. Fluctuation of resistance phenotypes may be influenced by the reintroduction of susceptible alleles from sylvatic populations or by other mechanisms of metabolic resistance.

*Anopheles darlingi* is the main vector of malaria in South America. For decades, this species has been targeted by vector control program using indoor residual spraying (IRS) and bednet distribution. *Anopheles darlingi* has the ability to adapt its behavior across its distribution.<sup>1–3</sup> Known as a sylvatic mosquito, its density drastically increases in the presence of human settlements. However, little evidence of insecticide resistance exists in this species.<sup>4,5</sup>

In French Guiana, a French overseas territory, *An. darlingi* is found across the territory and remains strongly associated with malaria outbreaks that occur along the rivers and inland close to goldmine settlements.<sup>6–9</sup> A drastic decrease of malaria incidence has been observed the last 20 years, but the area of Saint Georges de l'Oyapock (STG), at the border with Amapa state (Brazil), remains impacted.<sup>10</sup> In riverine areas with high malaria transmission, deltamethrin-based control has been implemented. It is noteworthy that only molecules from the pyrethroid family are authorized for killing of adult mosquitoes under French regulation. In the context of malaria control, chemical methods to reduce vector populations must be carefully monitored.

Two populations of *An. darlingi* from French Guiana were studied. The first one was located at La Césarée (LC) farm (5°0' 54.59' N; 52°32' 2.35' O), a malaria-free location located 45-minute drive west from Cayenne, the capital town. This equine and bovine farm is surrounded by humid savannah. No insecticidal treatment is performed in this area; the population at LC was considered a reference for this study. The second site was situated in Blondin (BL) hamlet (3°52' 31.2' N; 51°53' 12.9' O), accessible by 5-minute boat ride from STG and located on the Brazilian border.

Mosquito collections were performed in 2014, 2015, 2016, and 2018 using either supervised human landing collection (HLC) or, because of a malaria outbreak in 2018 in BL, mosquito magnet (MM) trap was used (Table 1). Local volunteer residents were trained on HLC and informed of the associated risks. Malaria prophylaxis was proposed and information on the medication was provided. Collectors who participated in the HLC activities gave their free, express, and informed consent. Mosquito magnet collections were performed by retrieving the nets every 30 minutes to 1 hour and storing the samples in cups to prevent mosquito deterioration. At the reference LC site, female mosquitoes were stored in cups<sup>11</sup> and transported by car to our Cayenne main laboratory within 2 hours, where test conditions were controlled at 28°C and 90% relative humidity. Those collected in BL were stored in cups of approximately 25 mosquitoes and transported to our field laboratory in STG within 4 hours of collection. This laboratory room maintained field temperature and humidity (approx. 28°C and above 80% humidity) throughout the day. Samples collected by either method from two field populations were subject to CDC bottle tests.<sup>12</sup> Groups of female mosquitoes were exposed to one of the four insecticides for each time point and site with a minimum of four replicates each. Control bottles coated with solvent contained 10–25 field-collected female mosquitoes for each time point and site (Table 1). Insecticide resistance was evaluated at the diagnostic dosages recommended by the CDC for deltamethrin (12.5 µg/bottle), permethrin (21.5 µg/bottle), and alpha-cypermethrin (12.5 µg/bottle). In addition, malathion (50 µg/bottle) was tested for BL collections in 2014 and 2015 (Table 1). For the three pyrethroids (deltamethrin, permethrin, and alpha-cypermethrin), the number of knocked-down mosquitoes, both dead and unable to fly, was recorded every 5 minutes in the pyrethroid-impregnated bottles for a minimum of 30 minutes (diagnostic time, 30' KD) or until 100% of the mosquitoes were knocked down (Table 1). The number of dead mosquitoes in the malathion-impregnated

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TABLE 1  
Percentages of knocked-down mosquitoes at 30 minutes (30' KD) and the SD among replicates in CDC bottle test for *Anopheles darlingi* populations collected in Blondin (BL) and La Césariée (LC)

Insecticide 2015	Site 39	Year HLC	Week* 95	CM 4	N 100	n 0	30' KD 30'	SD	Minutes for 100%	
Deltamethrin (12.5 µg/bottle)	BL	2014	39	HLC	81	4	85	11	45'	
			41	HLC	88	4	93	4	> 40'	
			42	HLC	67	4	100	0	30'	
			39	HLC	116	5	100	0	30'	
			38	HLC	110	4	92	2	45'	
			39	MM	315	12	100	0	20'	
			38	HLC	81	4	100	0	30'	
			33	MM	170	8	100	0	20'	
			35	MM	131	4	100	0	15'	
			40	HLC	117	6	98	3	> 30'	
Alpha-cypermethrin (12.5 µg/bottle)	BL	2016	38	HLC	131	4	81	3	65'	
			39	MM	109	4	100	0	25'	
			2018	36	HLC	215	9	100	0	25'
				36	MM	91	4	100	0	15'
				37	MM	105	4	100	0	30'
			38	MM	114	4	100	0	15'	
Permethrin (21.5 µg/bottle)	BL	2014	39	HLC	82	4	93	5	90'	
			41	HLC	72	4	85	11	> 30'	
			42	HLC	89	4	100	0	25'	
			39	HLC	150	7	100	0	30'	
			38	HLC	106	4	92	4	40'	
			42	HLC	158	4	93	2	45'	
			39	MM	316	12	100	0	25'	
			2015	37	HLC	153	6	100	0	25'
				37	HLC	136	6	100	0	30'
				33	MM	185	8	100	0	20'
35	MM	95		4	100	0	20'			
2018	42	HLC	85	4	100	0	30'			
	39	HLC	95	4	100	0	30'			
	LC	2015	37	HLC	153	6	100	0	25'	
		2016	37	HLC	136	6	100	0	30'	
LC	2018	33	MM	185	8	100	0	20'		
		35	MM	95	4	100	0	20'		
		42	HLC	85	4	100	0	30'		
		39	HLC	95	4	100	0	30'		

Test results are shown for each insecticidal molecule, year, and week (Wk). In addition, the method of collections (CM), that is, human landing catch (HLC) and mosquito magnet trap (MM), the number of mosquitoes tested (N) and replicates (n) are mentioned. Dark gray: resistant populations with 30' KD < 90%; light gray: 98% < 30' KD < 99% possible resistance; white: susceptible 30' KD > 98%. All of the collection weeks for BL village correspond to the dry season during which most of the malaria transmission occurs<sup>13</sup>; in LC there is no malaria transmission.

bottles was recorded at 30 minutes (Table 1). Morphological species identification was performed after the test according to keys specific to the region.

Raw data were corrected by Abbott's formula when needed.<sup>13</sup> KT50 values, the theoretical time to knock down/kill 50% of mosquitoes exposed to pyrethroids, was calculated based on 30' KD with a minimum of four time points producing from 0% to 100% of KD at 30 minutes of exposure. The probit regression of the knock-down response over time was performed in BioRssays (Montpellier, France)<sup>14</sup> in the R environment, which produces P value for the logistic regression significance. In addition, ratios of KT50 usually calculated with the KT values divided by the same values for reference strain (population from LC) were calculated for each insecticide by dividing the obtained values by the minimal KT in the panel.

All mosquitoes were identified as *An. darlingi*. All populations with a 30' KD above 98% were diagnosed as susceptible. Tests performed with malathion in BL mosquito collections revealed susceptibility to that molecule with 100% of mosquitoes dead at 30 minutes in 2014 and 2015. As expected, the LC population was susceptible to all pyrethroids at all collection times (Table 1). However, we observed that pyrethroid 30' KD values varied and some were below the 90% threshold for resistance in BL (Table 1). In 2014 and 2016, when samples were collected and tested for multiple time points in the same season, susceptibility varied from one collection to the next. In 2015 and 2018, susceptibility was recorded for only 1 week of collection samples.

To further investigate the fluctuation of KD response with pyrethroids, KT50 and associated ratio (RKT) were obtained.

First, they confirmed the trends observed for the end-point measures at 30 minutes. La Césariée population had the lowest KT values overall, whereas KT from BL-2016-38 were the highest recorded in BL for all insecticides tested. The lowest values at LC were used to calculate RKT50 for each insecticide (Table 2). The ratio calculated with those values highlighted that the population of BL-2014-38 exposed to deltamethrin and that of BL-2016-38 exposed to any pyrethroids had a resistance ratio (RKT50) above 2, demonstrating incipient resistance, as it takes twice as much time to knock down mosquitoes in BL than in LC. Second, we observed that the slope of regression was lower in BL than in LC populations, suggesting that even if the data do not support full resistance, knock down takes longer in BL than in LC.

To understand the mechanisms underlying the suspected resistance, the voltage-gated sodium channels (*vgsc*) gene was amplified and sequenced from individualized surviving (N = 16) and dead (N = 32) mosquitoes exposed to permethrin from BL in 2014. Both strands of 170 base pairs fragment of the *vgsc* was sequenced as described in Orjuela (2019).<sup>15</sup> The codons 1010, 1013, and 1014 at the S6 segment of domain II is used to identify point mutations, which have been associated with insecticide resistance in different species of *Anopheles* malaria vectors. No mutation on the *vgsc* fragment was detected. The sequences obtained showed 100% identity with amino acid sequences already published<sup>15</sup> (GenBank accession number MN053065). The observations described here, made over multiple years, raise the concern of insecticide resistance development in *An. darlingi* population in places where the use of

TABLE 2  
Results of significant regressions performed per pyrethroid molecule, site, year, and week

Insecticide	Site	Year	Week	N	n	KT50 (CI95)	RKT50 (CI95)	Slope (SE)	Intercept (SE)
Deltamethrin	BL	2014	39	81	11	11.3 (8.56–13.79)	2.09 (0.98–4.47)	2.64 (0.25)	–2.81 (0.38)
			42	67	6	5.95 (4.57–7.24)	1.07 (0.41–2.77)	2.77 (0.28)	–2.14 (0.33)
		2015	39	116	6	7.64 (5.84–9.07)	1.38 (0.59–3.22)	3.49 (0.44)	–3.08 (0.52)
			2016	38	110	10	13.64 (12.59–14.64)	2.46 (1.39–4.36)	4.56 (0.29)
	LC	2018	39	315	5	6.9 (6.55–7.25)	1.24 (0.69–2.24)	5.43 (0.24)	–4.55 (0.23)
			2018	33	170	5	5.53 (4.96–6.04)	–	4.97 (0.46)
		2018	35	131	4	5.93 (5.53–6.33)	1.07 (0.5–2.27)	6.45 (0.56)	–4.99 (0.47)
			37	105	7	8.43 (7.72–9.12)	1.81 (0.94–3.49)	4.41 (0.3)	–4.08 (0.32)
Alpha-cypermethrin	BL	2015	40	117	6	7.43 (5.54–9.01)	1.59 (0.73–3.48)	3.02 (0.38)	–2.63 (0.46)
			2016	38	131	14	20.34 (19.42–21.24)	4.37 (2.51–7.58)	4.88 (0.2)
		2018	39	109	6	7.02 (6.41–7.6)	1.5 (0.74–3.05)	4.75 (0.35)	–4.02 (0.35)
			2018	36	215	6	4.65 (4.18–5.09)	–	3.87 (0.25)
	LC	2018	36	91	4	7.08 (6.54–7.61)	1.52 (0.7–3.28)	6.37 (0.55)	–5.42 (0.5)
			37	105	7	8.43 (7.72–9.12)	1.81 (0.94–3.49)	4.41 (0.3)	–4.08 (0.32)
		2018	38	114	4	6.53 (5.82–7.2)	1.4 (0.67–2.91)	5.14 (0.49)	–4.19 (0.46)
			2018	39	109	6	7.82 (5.83–9.77)	1.18 (0.32–4.29)	2.6 (0.23)
Permethrin	BL	2014	39	82	9	7.82 (5.83–9.77)	1.18 (0.32–4.29)	2.6 (0.23)	–2.32 (0.33)
			41	72	6	10.44 (7.94–12.45)	1.58 (0.5–4.98)	2.64 (0.38)	–2.69 (0.48)
		2015	39	150	6	9.78 (8.19–11.05)	1.48 (0.47–4.61)	3.63 (0.41)	–3.6 (0.5)
			2016	38	106	9	16.92 (16.06–17.76)	2.56 (0.86–7.58)	5.89 (0.34)
	LC	2018	39	316	6	9.22 (8.74–9.69)	1.39 (0.47–4.11)	5.04 (0.24)	–4.86 (0.26)
			2015	37	153	5	6.59 (5.12–7.71)	–	4.51 (0.6)
		2016	37	136	7	8.72 (8.12–9.3)	1.32 (0.43–4.02)	5.09 (0.31)	–4.78 (0.33)
			2018	33	185	5	6.78 (6.41–7.16)	1.02 (0.33–3.18)	6.1 (0.38)
2018	35	95	5	7.84 (7.02–8.62)	1.18 (0.37–3.74)	4.5 (0.37)	–4.02 (0.38)		

BL = Blondin; LC = La Césarée. Knocked-down time 50 (KT50), ratio KT (RKT50) with confidence interval at 95% (CI95), and parameters of regression (slope and intercept with their standard errors) are represented. The number of mosquitoes used in each test is given (N). The number of time points per regression is also mentioned (n).

pyrethroids is widespread.<sup>16</sup> The population living in STG along the Oyapock River suffers from recurrent malaria transmission (between 30% and 50% *Plasmodium vivax* malaria prevalence depending on the year<sup>16</sup>). In recent years, IRS was only conducted together with spatial spraying to control outbreaks, while the distribution of LLINs occurred in 2021 with deltamethrin-impregnated nets and in 2016 with alpha-cypermethrin-impregnated nets. In BL, LLIN usage reached 95.7%.<sup>16</sup> Deltamethrin is also used for IRS and occasionally for *Aedes* control. Communities use household insecticides containing pyrethroids for several domestic pest control purposes.

In 2014, BL was the epicenter of malaria transmission in STG. After several years without any large outbreaks, a drastic resurgence appeared in Trois Palétuviers in 2017. This hamlet is situated downstream of STG on the Oyapock river. At the same time, the French Guiana population suffered from chikungunya and Zika outbreaks against which pyrethroid-based control was performed. None of these insecticidal applications are implemented in LC. Despite this frequent and regular use of insecticides, *An. darlingi*-resistant populations are rare across its distribution, unlike *Aedes aegypti*, which have shown a drastic increase of resistance.<sup>17</sup> In fact, reliable efficacy of chemical control methods against *An. darlingi* is observed, in contrast to *Ae. aegypti* populations that became resistant. The hypothesis formulated at that time was that populations of *An. darlingi* remained susceptible because of the continuous introduction of naive mosquitoes with susceptible alleles from forested areas to urbanized ones. Population genetic studies tend to support this hypothesis.<sup>18</sup> *Anopheles darlingi* resistance development seems to be slowed down or nullified by this dilution effect. This factor may partially explain the variation in pyrethroid susceptibility in our monitoring data.

Resistance to pyrethroids is a crucial issue in French Guiana. As a European territory, the use of mosquito adulticides

is restricted to pyrethroids. The resulting monotherapy creates pressure on mosquito populations to develop resistance.

Insecticide resistance surveillance should be sustained in areas that experience malaria transmission with regular monitoring of resistance and associated molecular markers to detect resistance early and develop an appropriate management plan. In our small sample set mutations in the voltage-gated sodium channel, gene were not seen, but resistance to pyrethroids can be mediated by other mechanisms, as previously studied in Colombia.<sup>15</sup> The *An. darlingi* populations from Colombia exhibit metabolic resistance involving oxidases and esterases.<sup>19</sup> Further investigation on mechanisms of metabolic resistance is required to maintain an effective toolbox of molecules for vector control and reduce malaria transmission in the region.

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