Spatio-Temporal Dynamics of a Dieldrin Resistance Gene in Aedes albopictus and Culex quinquefasciatus Populations From Reunion Island

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Abstract

The control of mosquito populations using insecticides is increasingly threatened by the spread of resistance mechanisms. Dieldrin resistance, conferred by point mutations in the *Rdl* gene encoding the γ-aminobutyric acid receptor, has been reported at high prevalence in mosquito populations in response to selective pressures. In this study, we monitored spatio-temporal dynamics of the resistance-conferring Rdl^R allele in *Aedes* (*Stegomyia*) *albopictus* (Skuse, 1895) and *Culex* (*Culex*) *quinquefasciatus* (Say, 1823) populations from Reunion Island. Specimens of both mosquito species were sampled over a 12-month period in three cities and in sites located at lower (<61 m) and higher (between 503 and 564 m) altitudes. Mosquitoes were genotyped using a molecular test detecting the alanine to serine substitution (A302S) in the *Rdl* gene. Overall, the Rdl^R frequencies were higher in *Cx. quinquefasciatus* than *Ae. albopictus*. For both mosquito species, the Rdl^R frequencies were significantly influenced by location and altitude with higher Rdl^R frequencies in the most urbanized areas and at lower altitudes. This study highlights environmental factors that influence the dynamics of insecticide resistance genes, which is critical for the management of insecticide resistance and the implementation of alternative and efficient vector control strategies.

Graphical Abstract
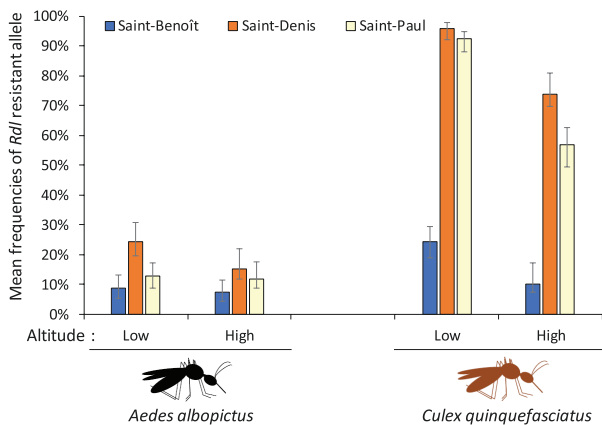
Key words: mosquito, insecticide, resistance, dieldrin, dynamics

In the absence of vaccines or other prophylactic treatments, the use of insecticides to control mosquito populations and disrupt the transmission of mosquito-borne pathogens remains a method of choice. However, such vector control strategies are increasingly threatened by the rapid selection and spread of insecticide resistance mechanisms in mosquito populations (Raymond et al. 1991, Labbe et al. 2005, Ranson and Lissenden 2016, Moyes et al. 2017). Following the widespread usage of insecticides over the last decades, resistance to all classes of synthetic insecticides including organochlorines, organophosphates, carbamates, and pyrethroids has been reported worldwide and among most mosquito species of public health importance (Liu 2015, Labbé et al. 2017). While insecticide resistance impairs insect control, it may also increase the ability of mosquitoes to transmit pathogens (Alout et al. 2013, Atyame et al. 2019), thus stimulating the development of a wealth of alternative, innovative, and environmentally-friendly vector control strategies.

In many insects, resistance to the cyclodiene insecticide dieldrin is conferred by point mutations in its target, the subunit of the γ-aminobutyric acid (GABA) receptor encoded by the *Rdl* gene. The GABA receptor is a major inhibitory receptor in both invertebrates and vertebrates (D'Hulst et al. 2009) that is linked to chlorine-gated channels and consists of five subunits. Each subunit is composed of an extracellular cysteine loop and four transmembrane domains (M1–M4). Early studies in *Drosophila melanogaster* have demonstrated the role of a point mutation in the *Rdl* gene leading to an alanine to serine substitution (A302S) within the M2 transmembrane domain of the receptor (ffrench-Constant et al. 1993). The A302S mutation has been selected in several other insect species and is considered as a converging genetic response to the overuse of cyclodiene insecticides (see ffrench-Constant et al. 2000 for review). In mosquitoes, the A302S substitution has been associated with dieldrin resistance in several species including *Aedes* (*Stegomyia*) *aegypti* (Linnaeus, 1762) (Thompson et al. 1993), *Aedes albopictus* (Tantely et al. 2010, Low et al. 2015, Lebon et al. 2018), *Culex quinquefasciatus* (Tantely et al. 2010), *Culex pipiens* (Taskin et al. 2016), and *Anopheles* (*Cellia*) *arabiensis* (Patton, 1905) (Du et al. 2005). Another substitution in the same position A302G has been also associated with dieldrin resistance in *Anopheles* (*Cellia*) *gambiae* (Giles, 1902), *Anopheles arabiensis* (Du et al. 2005), and *Anopheles* (*Cellia*) *funestus* (Giles, 1900) (Wondji et al. 2011). Dieldrin resistance has been reported at high frequency in *An. funestus* populations in Africa (Wondji et al. 2011) and in *Cx. quinquefasciatus* populations on Reunion Island (Tantely et al. 2010), and attributed to local insecticide pressures. Nowadays, dieldrin and other cyclodiene insecticides have been banned due to their remanence as well as toxicity to nontarget species. Selection of the dieldrin resistant allele *Rdl^R* in mosquito populations may be attributed to other classes of insecticides used in veterinary health (endectocides) and targeting the GABA receptor as antagonists (Ozoe et al. 2013). These cross-reacting insecticides include phenylpyrazoles (e.g. fipronil), ethiprole, pyriprole, and isoxazolines (Ozoe et al. 2013).

Reunion Island is a volcanic island of 2,512 km² located in the southwestern part of the Indian Ocean, roughly 700 km East of Madagascar. The island has a population of over 850,000, mainly living along the coastline. Although 12 mosquito species (belonging to *Aedes*, *Anopheles*, *Culex*, and *Orthopodomyia* genera) are currently recognized on the island (Boussès et al. 2013), *Ae. albopictus* and *Cx. quinquefasciatus* are the most abundant species and commonly found in urban, peri-urban, and rural areas, sometimes as high as 1,200 m (Boussès et al. 2013, Delatte et al. 2013). *Aedes albopictus* has been recognized as the main vector responsible for a large chikungunya outbreak that hit Reunion Island in 2005–2006

(Renault et al. 2007) but also as the major vector involved in an ongoing epidemic of dengue that started in 2017 (Santé Publique France 2020). On Reunion Island, *Ae. albopictus* larvae can be found in both artificial (tires or flowerpots) and natural (gullies, rocks or tree holes) breeding sites, while *Cx. quinquefasciatus* larvae are mostly observed in artificial breeding sites with high loads of organic material (Paupy et al. 2001, Delatte et al. 2013). Until 2006, mosquito control programs were implemented using temephos and fenithrothion (organophosphates) against larval and adult stages. Since 2007, temephos was replaced by *Bti* (*Bacillus thuringiensis* var. *israelensis* toxins) for larvae control and deltamethrin (pyrethroids) against adults. A previous investigation of insecticide resistance mechanisms in *Cx. quinquefasciatus* populations from Reunion Island revealed the presence of resistance to organophosphates, pyrethroids, and organochlorines using bioassays (Tantely et al. 2010). Insecticide resistance alleles including *Ester^R*, *ace-1^R*, and *Rdl^R* have also been reported to follow spatial patterns, with higher frequencies of resistant alleles in coastal sites and very low frequencies in one site located in the central highlands (Tantely et al. 2010). In addition, the *Rdl^R* allele was detected for the first time in two samples of *Ae. albopictus* from the island with allelic frequency reaching 30% while even higher *Rdl^R* frequency was recorded in most surveyed *Cx. quinquefasciatus* populations (Tantely et al. 2010).

In the present study, we monitored the dynamics of the *Rdl^R* (A302S) allele in *Ae. albopictus* and *Cx. quinquefasciatus* populations to investigate the spatio-temporal structure of cyclodien/ dieldrin resistance in Reunion Island. To reach this purpose, we sampled and genotyped field populations over time and from sites located at lower and higher altitudes in three cities of Reunion Island. Conclusions presented herein provide original information that will assist in improving vector control programs for better sustainability.

Materials and Methods

Mosquito Sampling

Aedes albopictus and *Cx. quinquefasciatus* populations were sampled as larvae in three cities of Reunion Island which differ in their population densities; Saint-Benoît (164 inhabitants/km²), Saint-Denis (1,036 inhabitants/km²), and Saint-Paul (433 inhabitants/km²). Samples were collected monthly over a 12-month period from September 2011 to August 2012. This sampling campaign spanned the two climatic seasons recognized on Reunion Island *i.e.* the austral winter from May to October and the austral summer from November to April. In each city, two sample sites were selected: the first one in the coastal area (altitude lower than 61 m) and the second one in an elevated site (altitude between 503 and 564 m). Therefore, mosquitoes were collected from the six following sites: Bourbier-les-rails (Saint-Benoît lower altitude called thereafter Saint-Benoît_LA), Pont-Payet (Saint-Benoît higher altitude or Saint-Benoît_HA), Providence (Saint-Denis_LA), Brûlé (Saint-Denis_HA), Etang (Saint-Paul_LA) and Bois-de-Nèfles (Saint-Paul_HA). The three sites at lower altitude were located in urbanized areas (with predominantly buildings and roads around sampling sites) while sites at higher altitude were located either in urbanized (Saint-Denis_HA), agricultural area (i.e. with predominantly vegetable and sugar cane crops on areas less than 1 ha) (Saint-Paul_HA) or natural area (i.e. without buildings, roads or crops around sampling sites) (Saint-Benoît_HA) (Fig. 1). At each site, eight artificial larval breeding sites each consisting of a 10-L container with tap water were set up and placed 5–10 m apart and larvae of various sizes (2nd to 4th instar larvae) were collected each month. *Ae. albopictus* and *Cx. quinquefasciatus* larvae were collected in the same breeding
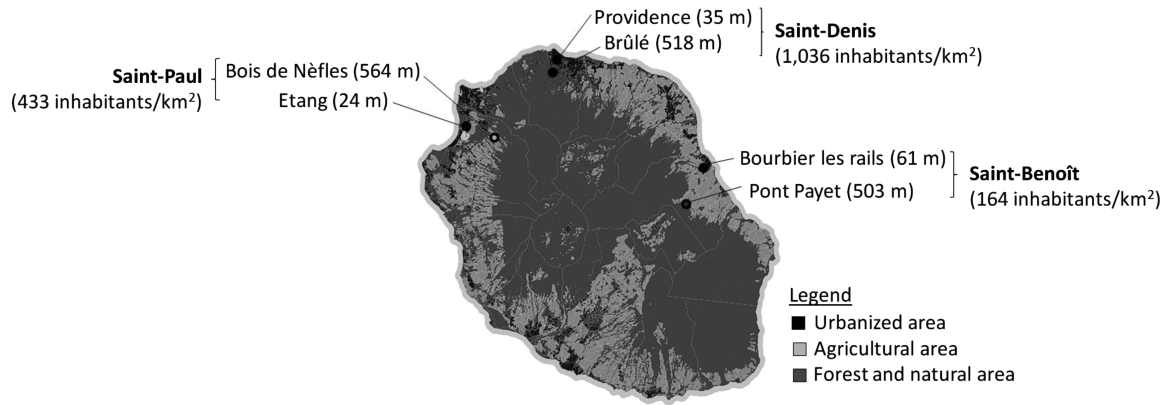
Fig. 1. Map of Reunion Island showing the cities and sites where *Aedes albopictus* and *Culex quinquefasciatus* mosquitoes were collected. This map was modified from the CIRAD AWARE data (https://aware.cirad.fr/layers/geonode:classif_2018_s67_final_code1_com). According to the land use report of Bertrand Ballet of the Ministry of agriculture (https://agreste.agriculture.gouv.fr/agreste-web/download/publication/publie/Dos2103/Dossiers%202021-3_TERUTI.pdf) there was no significant change in the size of agricultural, urbanized, forest and natural areas between 2012 and 2018 on Reunion Island. Therefore, data from 2018 reflects the situation prevailing in 2012 when sampling was carried out. Dark gray corresponds to forest and natural area, light gray to agricultural area and black to urbanized area. In each city, both mosquito species were collected in two sites; the first one at a low altitude (< 61 m) and the other one at a higher altitude (*i.e.* between 503 m and 564 m).

sites and brought to the insect laboratory where they were morphologically identified (Theobald 1910), and eventually stored in 70% ethanol until molecular analyses.

Molecular Typing

Total DNA of single larvae was extracted using a CTAB protocol (Rogers and Bendich 1988). A previously described PCR-RFLP test (Tantely et al. 2010) was used to detect the A302S substitution in the *Rdl* gene in *Ae. albopictus* and *Cx. quinquefasciatus* specimens. Briefly, a 232 bp fragment of the *Rdl* gene was amplified by PCR with mqGABAdir (5′-TGTACGTTCGATGGGTTAT-3′) and mqGABArev (5′-CATGACGAAGCATGTGCCTA-3′) primers. A 30 cycle PCR (each composed of 94°C for 30 s, 52°C for 30 s, and 72°C for 1 min) was performed in the thermocycler using 1 µl (0.5 ng) of a genomic DNA solution in a 25 µl final volume reaction contained 9.5 µl of water, 12.5 µl of MasterMix (Applied Biosystems, Foster City) and 1 µl of each primer (10 µM). PCR products were digested for 3 h at 60°C with 1.5 U of *BstAPI* restriction endonuclease (New England Biolabs, Ipswich), which selectively cleaves the susceptible allele. DNA fragments were run on 2% agarose gel electrophoresis stained with 1X GelRed™ (Biotium Inc.) and visualized under ultraviolet light. On each gel, 3 control PCR-RFLP were included using DNA extracted from lab mosquito lines. Specifically, DNA template of mosquitoes from homozygous susceptible (SS), homozygous resistant (RR), and heterozygous (RS) lines were used.

Statistical Analyses

We analyzed the dynamics of Rdl^R allele frequency over time in the six selected populations of *Ae. albopictus* and *Cx. quinquefasciatus* using R software 3.4.0 (R Core Team 2017). Resistant allele frequencies were plotted over time using "ggplot2" package (Wickham et al. 2016) and presented with a loess smoother line and the associated 95% confidence interval. The Hardy–Weinberg equilibrium test was performed to examine the departure of homozygous susceptible (SS), homozygous resistant (RR), and heterozygous (RS) from panmixia in each collection site. We tested the influence of three explanatory variables, namely the "city" where each population was collected (a three-level categorical variable: Saint-Benoit, Saint-Denis, and Saint-Paul), the "altitude" (a two-level categorical variable: high

and low) of each breeding site as well as the sampling "month". We performed two independent analyses of the resistant allele frequency (response variable) for each mosquito species, using the generalized linear mixed-effect models (package "glmmTMB", Brooks et al. 2017) with a binomial error structure. Both maximal models included the variables 'city' and "altitude" and their interaction, as fixed effects. As we used the same artificial traps over the sampling period, the "month" variable was used as a random variable to account for pseudo-replication between two consecutive collections and was compared with a generalized linear model with no random effect based on the lower AIC (Akaike's Information Criterion). Significance of variables and selection of the minimal model was assessed using the "ANOVA" procedure within the package "car" (Fox and Weisberg 2011), which performs a type III error test for the fixed effects of the linear mixed-effects models. Mean frequencies of the Rdl^R allele were computed and posthoc tests (package "emmeans", Lenth et al. 2019) were carried out to assess differences between estimates and Bonferroni corrections were applied for multiple comparisons. As the random variable "month" had a significant influence on allelic frequency, we also tested whether there was a significant trend over time. To this aim, we used a nonparametric smoother for the variable "month" in a generalized additive mixed-model (package "mgcv", Wood 2011).

Results

A total of 2855 mosquitoes ($N = 1,446$ and $N = 1,409$ for *Ae. albopictus* and *Cx. quinquefasciatus*, respectively) representing 142 time points over one year ($N = 72$ and $N = 70$ collections for *Ae. albopictus* and *Cx. quinquefasciatus*, respectively) were genotyped to estimate allelic and genotypic frequencies (Table 1).

Ae. albopictus Displayed Lower *Rdl*R Frequencies Than *Cx. quinquefasciatus*

Overall, the Rdl^R allelic frequency was lower in *Ae. albopictus* (ranging from 7.5% to 24.5%) than in *Cx. quinquefasciatus* (ranging from 10.1% to 96%). The occurrence of homozygous resistant specimens (RR) was very low in *Ae. albopictus* in all cities while in *Cx. quinquefasciatus* RR was low only in Saint-Benoit

and higher in other cities (Table 1). The Hardy–Weinberg equilibrium test performed to analyze the Rdl^R genotypic frequency in each collection did not show a significant difference between the expected and the observed values for each collection (all $P > 0.11$), suggesting that all sampled populations were at genetic equilibrium. We then examined the effects of city, altitude, and sampling month on the frequency of the Rdl^R allele for each of the two mosquito species.

For both species, the Rdl^R frequency was significantly influenced by the *city* (χ^2 = 57.25, $P < 0.001$ and χ^2 = 263.02, $P < 0.001$ for *Ae. albopictus* and *Cx. quinquefasciatus*, respectively; Table 2), *altitude* (χ^2 = 8.06, $P = 0.0045$ and χ^2 = 29.6, $P < 0.001$ for *Ae. albopictus* and *Cx. quinquefasciatus*, respectively; Table 2) and the *month* of sampling (χ^2 = 14.91, $P = <0.001$ and χ^2 = 8.91, $P = 0.003$ for *Ae. albopictus* and *Cx. quinquefasciatus*, respectively; Table 2). In *Cx. quinquefasciatus* populations, the *city* by *altitude* interaction influenced the frequency of the Rdl^R allele (χ^2 = 19.66, $P < 0.001$; Table 2) revealing that frequency variation associated with altitude was distinct among cities. Higher frequencies of Rdl^R were observed in *Cx. quinquefasciatus* populations as compared to *Ae. albopictus* in all samples except in Saint-Benoît_LA in February and Saint-Benoît_HA in September, January, May, July, and August (Fig. 2). For example, in the Saint-Denis_LA site, the Rdl^R frequencies for *Cx. quinquefasciatus* varied slightly between 78.1% (95% confidence interval [95 CI]: 59.6–90.1%) and 100% (95 CI: 90.8–100%, Fig. 2) with almost all specimens being homozygous for the Rdl^R allele (208 RR out of 225 specimens) while very few heterozygous (17 RS specimens out of 225) and no susceptible (SS) individuals were detected (Table 1). In contrast, the frequencies of Rdl^R in *Ae. albopictus* from the same site ranged from 9.1% (95 CI: 2.9–22.6%) to 44.7% (95 CI: 29.0–61.5%, Fig. 2) with most specimens being susceptible (136 SS out of 241) or heterozygous (92 RS out of 241) while a minority of mosquitoes were homozygous resistant (13 RR out of 241). A similar pattern was observed at the Saint-Paul_LA site (Fig. 2; Table 1).

Mosquitoes From Lower Altitude Sites are More Resistant Than Those From Elevated Sites

When comparing the Rdl^R frequencies between sites with low and high altitudes for each city, a significant difference was observed in both mosquito species (Table 2). For *Cx. quinquefasciatus*, Rdl^R frequency was higher in low altitude sites compared to the corresponding high-altitude sites ($P < 0.001$, for all pairwise comparisons). For *Ae. albopictus*, Rdl^R frequency was also higher in low altitude sites but the difference was only significant in Saint-Denis ($P = 0.0011$) but not in Saint-Paul and Saint-Benoît ($P = 0.64$ and $P = 0.54$, respectively).

Pairwise comparison for *Ae. albopictus* between sites with low altitude (see Fig. 2) revealed significant differences between Saint-Benoît_LA and Saint-Denis_LA ($P < 0.001$) and between Saint-Denis_LA and Saint-Paul_LA ($P < 0.001$) and no significant difference was observed between Saint-Benoît_LA and Saint-Paul_LA ($P = 0.1$). For *Cx. quinquefasciatus*, a significant difference was observed between Saint-Benoît_LA and Saint-Denis_LA ($P < 0.001$) and between Saint-Benoît_LA and Saint-Paul_LA ($P < 0.001$) while no significant difference was observed between Saint-Denis_LA and Saint-Paul_LA ($P = 0.051$). The analysis between high altitude sites (see Fig. 2), revealed a significant difference between the three sites for *Cx. quinquefasciatus* ($P < 0.001$ for all pairwise comparisons). For *Ae. albopictus*, Rdl^R frequency was significantly higher in Saint-Denis compared to Saint-Benoît only ($P < 0.001$) but no significant difference was observed between Saint-Benoît_HA and Saint-Paul_HA ($P = 0.069$), and between Saint-Denis_HA and Saint-Paul_HA ($P = 0.253$).

Finally, there was a significant difference in Rdl^R frequency between months in *Ae. albopictus* ($P < 0.001$) and in *Cx. quinquefasciatus* ($P = 0.003$). We thus tested whether this variation followed a trend during the 12-month period but no significant trend of Rdl^R allelic frequency over the year was noted ($P = 0.4969$ and $P = 0.1710$ for *Ae. albopictus* and *Cx. quinquefasciatus*, respectively) suggesting that the Rdl^R allele was not associated with cyclical change on Reunion Island over the 12-month period.

Discussion

Here, we examined spatio-temporal dynamics of the resistance-conferring Rdl^R allele in field populations of *Ae. albopictus* and *Cx. quinquefasciatus* from Reunion Island. As previously highlighted using *Ae. albopictus* and *Cx. quinquefasciatus* mosquitoes from

Table 1. Estimated RdlR allelic frequencies and genotypes in *Aedes albopictus* and *Culex quinquefasciatus* over one year in the sampling sites

City	Altitude	N	SS	RS	RR	Rdl^R frequency	N	SS	RS	RR	Rdl^R frequency
			Aedes albopictus					*Culex quinquefasciatus*			
			Number per genotype					Number per genotype			
Saint-Benoit	Low	236	197	37	2	0.087 (0.054; 0.13)	256	137	113	6	0.244 (0.188; 0.296)
	High	246	210	35	1	0.075 (0.045; 0.115)	184	148	35	1	0.101 (0.074; 0.171)
Saint-Denis	Low	241	136	92	13	0.245 (0.197; 0.309)	225	0	17	208	0.96 (0.923; 0.98)
	High	216	155	56	5	0.153 (0.117; 0.22)	248	33	63	152	0.74 (0.699; 0.809)
Saint-Paul	Low	253	191	59	3	0.128 (0.086; 0.171)	270	0	40	230	0.926 (0.882; 0.95)
	High	254	199	50	5	0.118 (0.089; 0.175)	226	42	111	73	0.569 (0.495; 0.627)

In each city, mosquitoes were collected in a site at lower altitude (low) and in a site at higher altitude (high). The numbers between brackets indicate 95% confidence intervals calculated from binomial distribution. SS, homozygous susceptible specimens; RS, heterozygous specimens; RR, homozygous resistant specimens.

Reunion Island, the A302S mutation confers strong resistance to dieldrin in larval bioassays (Tantely et al. 2010, Lebon et al. 2018), therefore *Rdl^R* allelic frequency provides a relevant proxy of dieldrin resistance in mosquitoes' natural populations. Mosquitoes were collected monthly at high and low altitude sampling sites in three cities: Saint-Benoît, Saint-Denis, and Saint-Paul. The presence of *Rdl^R* was detected in almost all samples for both *Ae. albopictus* and *Cx. quinquefasciatus*. As dieldrin has been banned since 1994, this widespread presence of *Rdl^R* in both mosquito species throughout the island likely results from selective pressures exerted by other pesticides targeting GABA receptor (Buckingham et al. 2005).

Table 2. Statistical analyses of the frequency of the resistant *Rdl*R allele in *Aedes albopictus* and *Culex quinquefasciatus* populations from Reunion Island

Source	*Aedes albopictus*			*Culex quinquefasciatus*		
	χ²	d.f.	P-value	χ²	d.f.	P-value
City	57.25	2	<0.001	263.02	2	<0.001
Altitude	8.06	1	0.0045	29.6	1	<0.001
City × altitude	-	-	-	19.66	2	<0.001
Month	14.91	1	<0.001	8.91	1	0.003

In these analyses, the influence of city (three-level categorical variable: Saint-Benoit, Saint-Denis, and Saint-Paul), altitude (two-level categorical variable: low and high) and month (as a random variable) were tested. *d.f.* is the degree of freedom and χ² is the Chi-square value. Significance of variables was obtained after downward selection based on Akaike's Information Criterion (AIC).

Cross-reacting pesticides such as fipronil are widely used for domestic purposes to control termites and ants on the island. In addition, agricultural and veterinary use of pesticides, endectocides, and other chemicals are also known driving forces in the selection of insecticide resistance in mosquito field populations (Diabate et al. 2002, Nkya et al. 2013, Ozoe et al. 2013, Reid and McKenzie 2016) and the study of these pesticide-use practices warrants further investigation.

The frequencies of *Rdl^R* significantly differed between the two mosquito species with consistently lower *Rdl^R* frequencies in *Ae. albopictus* ranging from 7.5% to 24.5% for each city over the 12-month period as compared to *Cx. quinquefasciatus*, with *Rdl^R* frequencies reaching 96% over the same period (Table 1). Most analyzed *Ae. albopictus* specimens were susceptible, while *Cx. quinquefasciatus* mosquitoes from sites such as Saint-Denis_LA and Saint-Paul_LA had *Rdl^R* frequencies that were close to fixation (Fig. 2), with a majority of homozygous resistant mosquitoes. In a previous study using mosquitoes sampled in 2008, Tantely et al. (2010) observed similar results with low *Rdl^R* frequencies in *Ae. albopictus* (30% and 10% in Saint-Denis and Ravine à Jacques, respectively), while *Rdl^R* was almost fixed in most *Cx. quinquefasciatus* samples from the northwestern coast of the island. Although the study by Tantely et al. (2010) relied on a single time point, data gathered in the present study further supports a long-term persistence of this dieldrin resistance with a similar *Rdl^R* allelic frequency in mosquito populations on Reunion Island. This suggests that the selective pressures acting on *Rdl^R* are weak or discontinuous and that fitness cost associated with *Rdl^R* is low. The difference in *Rdl^R* frequencies between both mosquito species could be explained by their ecology. Indeed, *Cx. quinquefasciatus* larvae breed in artificial breeding
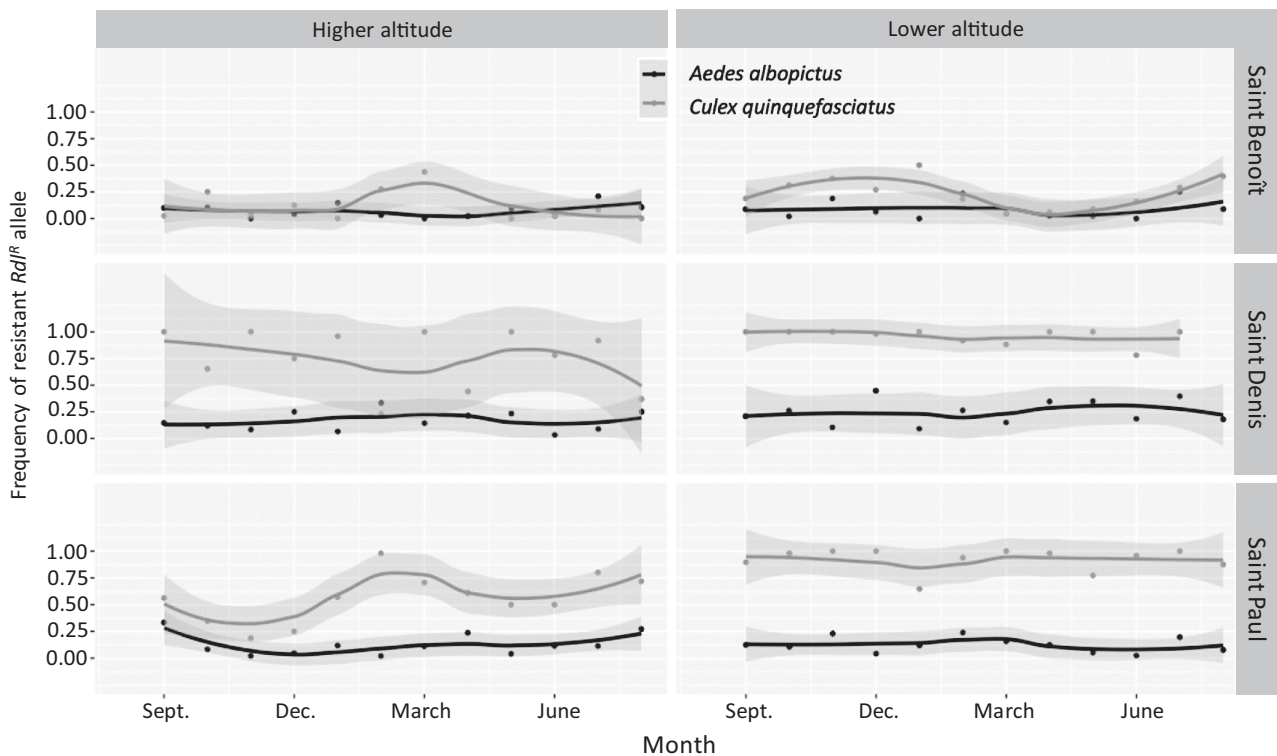
Fig. 2. Dynamics of the resistant *Rdl*R (A302S) allele frequencies in field populations of *Aedes albopictus* and *Culex quinquefasciatus*. Mosquitoes of both species were sampled monthly as larvae in breeding sites set up in three cities (Saint-Benoît, Saint-Denis and Saint-Paul) of Reunion Island. In each city, two sites were selected: the first one at a low altitude (< 61 m) and the second one at a high altitude (between 503 and 564 m). Dots represent the resistant allele frequencies, and the line represent the less smoother line with the associated 95% confidence indicated in grey.

habitats with polluted water (i.e. manhole sewers, sites created from leakage of sewage canal, wastewater blocked in pipes and under bridges, etc…) often located in watersheds and therefore heavily exposed to xenobiotics. By contrast, *Ae. albopictus* larvae are adapted to smaller either artificial (tires or flower pots) or natural (gully, rock, or tree holes) breeding sites (Paupy et al. 2001, Delatte et al. 2013) with smaller water volumes, which may be under lower selective pressure.

The dynamics of *Rdl^R* also displayed an altitudinal pattern, with higher *Rdl^R* allele frequencies in the coastal than in the elevated areas. This altitudinal pattern of insecticide-resistance is consistent with the previous study in Reunion Island where frequencies of insecticide resistant alleles in *Cx. quinquefascitus* sampled in sites along the coast were higher compared to mosquitoes from a single mountainous site located at Plaine des Palmistes (altitude of 948 m), in the center of the island (Tantely et al. 2010). This pattern appears robust as it is found in both mosquito species, and we propose three nonexclusive hypotheses to account for this trend. Firstly, the observed pattern may be related to soil erosion and leaching by rain. Indeed, chemicals potentially present in elevated sites would be frequently leached by runoff water and accumulate on the coast thus increasing the selection pressure in larvae breeding sites located at low altitude. Secondly, because the abundance of mosquitoes generally decreases with altitude due to climatic conditions (e.g. lower temperatures) (Asigau et al. 2017), mosquito control measures are less stringent in elevated sites than in the coast where vector control from the public and the private sector is continuous over the year in response to higher abundance of mosquitoes. However, it should be noted that since 2007, mosquito control programs in Reunion Island have been carried out using *Bti* (*Bacillus thuringiensis* var. *israelensis* toxins) against larvae and deltamethrin (pyrethroids) against adults, none of these insecticides targeting the GABA receptor. It should be pointed that insecticides used for agriculture pest control (e.g. pyrethroids, avermetins, nicotinoids) may as well exert significant pressure. Thirdly, selection by pesticides and other chemicals resulting from domestic purposes may explain the contrasted pattern of *Rdl^R* distribution. Indeed, according to the land use data (Fig. 1), all sites at lower altitude are located in urbanized and more densely populated areas, while sites at higher altitude are located either in urbanized (Saint-Denis_HA), agricultural (Saint-Paul_HA) or in natural (Saint-Benoît_HA) areas (Fig. 1). Interestingly, among elevated sites, *Rdl^R* frequency was higher in the urbanized and most densely populated site of Saint-Denis_HA than in Saint-Paul_HA and Saint-Benoît_HA, located in an agricultural and a natural area, respectively.

Resistance appears to increase with urbanization as *Rdl^R* frequencies are highest in the most urbanized and densely populated cities of the island, i.e. in Saint-Denis (1,036 inhabitants/km^2), then in Saint-Paul (433 inhabitants/km^2) and in Saint-Benoît (164 inhabitants/km^2). A number of investigations have supported the role of urbanization in shaping the distribution of insecticide resistance in mosquitoes (Jones et al. 2012, Li et al. 2018, Satoto et al. 2019, Tabbabi et al. 2019). A survey in *Ae. albopictus* populations from Guangzhou (China) showed increased insecticide resistance in adults and larvae from urban areas than suburban and rural environments, a trend that was correlated to an increased use of insecticides in urban areas (Li et al. 2018). Similarly, Tabbabi et al. (2019) described increased resistance to organophosphate insecticides in *Culex* (*Culex*) *pipiens* (Linnaeus, 1758) populations from most urbanized areas as compared to populations from natural areas in Tunisia. Hence, in urban areas, mosquito larvae are likely more exposed to pollutants thus increasing the selective pressure towards insecticide resistance

mechanisms (see Nkya et al. 2013 for review). It has been shown that *An. gambiae* larvae collected in petroleum polluted breeding sites were significantly more tolerant to the insecticide permethrin compared to mosquitoes sampled in nonpolluted areas (Djouaka et al. 2007). Similar results were observed in *Ae. albopictus* and *Ae. aegypti* larvae with an increased tolerance to various insecticides after exposure to environmental xenobiotics (Poupardin et al. 2008, Suwanchaichinda and Brattsten 2002). This increased tolerance to insecticides has been shown to be related to the induction of detoxification enzymes (Suwanchaichinda and Brattsten 2002, Poupardin et al. 2008, Riaz et al. 2013), although cross-resistance with other mechanisms should not be excluded. Overall, this suggests a correlation between urbanization and the presence of chemicals targeting the GABA receptor in larvae breeding sites on Reunion Island. This assumption could be examined in future investigations by comparing physio-chemical properties of water collected in larvae breeding sites sampled from cities with different levels of urbanization.

Conclusion

In the present study, we investigated the spatio-temporal dynamics of the dieldrin resistant *Rdl^R* allele in *Ae. albopictus* and *Cx. quinquefasciatus* populations from Reunion Island. We found a significant difference in *Rdl^R* frequency between sampled cities and between low and high altitudes. The reported patterns may result from selective pressures of various origins and targeting the GABA receptor and/or absence of a fitness cost associated with *Rdl^R*. Our results suggest a correlation between urbanization and insecticide resistance in mosquito field populations that needs to be confirmed. The consistency of this pattern between 2008 and 2012 requires a comprehensive understanding of the driving forces at play in the environment. Particular attention must therefore be given to the spatial and temporal evolution of insecticide resistance genes and to the underlying environmental drivers. The dynamics of *Rdl^R* in mosquito vectors of Reunion Island stressed sustained surveillance of resistance to improve its management.

Author Contributions

CL: Investigation, Methodology, Writing—original draft; HA: Formal analysis, Writing—original draft, Writing—reviewing and editing; SZ: Investigation, Writing—original draft; JSD: Conceptualization; Resources—Provision of study materials, Writing—original draft; MW: Conceptualization, Writing—original draft, Writing—reviewing and editing; PT: Conceptualization, Resources—Provision of reagents and materials, Funding acquisition, Writing—original draft, Writing—reviewing and editing; CA: Investigation, Methodology, Supervision, Validation, Visualization, Writing—original draft, Writing—reviewing and editing.

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