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Research Article

Insecticide Resistance in Malaria Vectors in Areas of Seasonal Malaria Transmission in Burkina Faso: Knock-Down Resistance Gene Distribution

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Abstract Resistance to insecticide is the main concern for vectors control based on insecticides. We conducted a two-year monitoring on the resistance to insecticides of *Anopheles gambiae* and *Anopheles funestus* in five localities of Burkina Faso. The susceptibility to insecticides was performed using WHO test. The knock-down time (KDT) and after 24 h mortality was assessed. Mosquitoes were PCR-tested for species, molecular forms and *kdr* gene identification. The specific composition of *Anopheles gambiae* varies according to locality and year of collection. The M form is more represented than the S form and *An. arabiensis*. There is a general increasing of the survival rate to DDT and the frequency of the *kdr* mutation in *Anopheles gambiae*. *An. funestus* was fully susceptible to DDT and pyrethroids in Koubri where it has been collected. These results highlighted the need for monitoring closely the resistance to insecticides in malaria vectors populations.

Keywords malaria vectors; *An. gambiae* s.l.; *An. funestus*; insecticide; *kdr* gene

1 Background

Vector control is a key strategy for malaria control. In the 1990s, large-scale field studies investigating the efficacy of insecticide-treated nets (ITNs) showed that insecticide-treated bednets and curtains were effective in reducing all-cause child mortality [1,3,11,26,32]. These results contributed to the adoption of vector control by ITNs as a cornerstone for malaria control [56]. Apart from reducing child mortality, a wide-scale use of insecticide-treated material reduces malaria transmission and other entomological parameters in many areas worldwide [2, 8,28,31,43]. In Burkina Faso, utilization of insecticide-treated curtains has led to a reduction in the malaria transmission level of more than 90% in protected houses and 50% in unprotected ones [27]. A mass effect of the intervention on the vector's population has been observed, with a marked reduction in vector aggressiveness and sporozoite index in villages situated in the heart of the intervention areas [28]. Much praiseworthy effort has been made over the past ten years in terms of bednet coverage in Africa [34,54,55]. However, the emergence and spreading of malaria vector resistance to current insecticides throughout Africa [5,6,18,19,21,37,38] is a major concern for malaria control programs and scientists, as vector resistance could compromise the efficacy of ITNs [33,42],

Vector resistance to pyrethroids has been reported in West Africa [5,13], East Africa [38,47], Central Africa [22, 35], and China [48]. Many studies conducted in Africa have reported the presence of the *kdr* gene, associated with the "knock-down resistance" mechanism, in *An. gambiae* complex species [15,16,17,18,21,39,46]. The development and spread of malaria vector resistance to insecticides has been attributed to the intensive use of insecticides in agriculture, particularly in cotton cultivation [15,20]. To date, the resistance of *Anopheles* species to the four families of insecticides available for public health use (organochlorines, organophosphates, carbamates, and pyrethroids) is a genuine concern throughout Africa [6,9,37].

We report here the results of two years of monitoring of malaria vector resistance to pyrethroids and DDT in five localities exposed to high insecticide pressure in Burkina Faso. The frequency of the *kdr* gene allele, associated with the "knock-down resistance" mechanism in *An. gambiae*, was assessed. Species and molecular form data were also gathered in order to further characterize the mosquito populations in the study villages.

2 Methodology

2.1 Study site

Burkina Faso is in a tropical climate of Sudanese type in which a long dry season from October to April alternates with a short rainy one from May to September. On the basis of the duration of the rainy season and the total annual rainfall, three climatic zones are determined in the country (Figure 1). Overall, malaria transmission is seasonal, but the duration of the high malaria transmission season varies slightly from the northern to the southern part of the country.

The main malaria vectors are *An. gambiae* s.s., *An. arabiensis*, and to a lesser extent, *An. funestus*. *Plasmodium falciparum* is responsible for more than 95% of malaria infections. According to the national health information system, malaria was the cause of 48% of consultations, 60% of hospitalization, and 47% of deaths in 2010.

The study was carried out from 2005 to 2006 in five localities of the country, situated in different geographic and eco-climatic zones (Figure 1). Boromo and Zampa are two rural villages, each located 180 km from Ouagadougou, in the western and eastern parts of the country, respectively. These villages are located in two different climatic zones. Due to the absence of artificial lake, market gardening is not undertaken in these villages, but during the rainy season, rice is cultivated in depressed zones. Both Boromo and Zampa are characterized by intensive cultivation of cotton and the use of insecticide for farming. The annual rainfall is generally higher in these localities (900–1200 mm), and the high malaria transmission season is slightly longer than in the central and northern part of the country. Saaba and Koubri are two suburban areas located 25 km and 30 km away from Ouagadougou, respectively, in a much drier climatic zone. The annual rainfall ranged between 750 mm and 900 mm, and the high malaria transmission is shorter than in Boromo and Zampa. Rice cultivation and market gardening are key activities during the dry season. The presence of dams and favored breeding sites for *Anopheles* are key features of these villages. For instance, in Koubri, the abundance of wells with clean and shady water, due to the presence of two permanent dams, represents appropriate breeding sites for the development of *An. funestus*, in contrast to Saaba where only one semi-permanent dam is present. Irrigated rice cultivation in Koubri offered also another type of breeding site to *Anopheles* species. The fifth locality (Nongremassom) is an urban area located near an artificial lake (dam) in Ouagadougou, in a climatic zone similar to Koubri and Saaba. Rice cultivation in the rainy season and gardening in the dry season are the main farming activities around the lake. This site was chosen because of the microclimate created by the dam and the ecological conditions that are particularly favorable for the development of *An. gambiae* in an urban area. The common characteristic of all these localities is the use of insecticides in agriculture, which is the source of pollution of the larval breeding sites.

2.2 Mosquito collection

For *An. gambiae* s.l., larvae were collected between July and September 2005 and 2006, using the method of "dipping," between 6:00 and 8:00 a.m. Common breeding sites encountered included puddles, channels, brick cellars, ponds, rice paddies, foot prints, and wells. Once collected, larvae were kept in the insectary and fed until reaching the 2- to 5-day-old adult stage. For *An. funestus*, freshly fed females were manually collected indoors from the village of Koubri and directly tested for their susceptibility. The species of this group were morphologically identified, using standard keys of identification of *Anopheles* species [24, 25].

2.3 Susceptibility tests

Susceptibility tests were performed using 2- to 5-day-old unfed adult females of *An. gambiae* s.l. or freshly fed adult females of *An. funestus* s.l. The WHO standard procedure [49] was applied under conditions of 26–27 °C temperature and 82–83% relative humidity. The number of knocked down mosquitoes was recorded at fixed intervals (every 5 to 10 min, depending on knockdown rates) for 60 min. After exposure, the mosquitoes were kept in tubes, provided with glucose-soaked cotton, and held for 24 hours.

All tests on *An. gambiae* s.l. were done with at least 100 females. In the case of *An. funestus*, the number of females tested was sometimes under 100 because of the low density of this species in Koubri, where it was found.

2.4 Mosquito storage, species identification, and detection of the *kdr* mutation

Tested females (alive and dead) were stored at −20 °C until they were analyzed by PCR for presence of the *kdr* mutation and for identification of species and molecular forms. Species typing and molecular characterization of the mosquitoes was done using the protocol of Fanello et al. [23]. The *kdr* allele was identified using the technique of Martinez-Torres et al. [30].

2.5 Data analysis

The data collected were managed using Microsoft Excel, and the analysis was done with XLSTAT (www.xlstat.com/). A logistic regression was performed on the number of knocked down mosquitoes as a function of time. The effect of each insecticide was assessed in terms of 50% and 95% knockdown times (KdT50 and KdT95) and confidence limits were calculated. Student's *t*-test was used to examine whether there was a significant difference between KdT95 values observed in 2005 and 2006 in each location.

Mortality after 24 hours was calculated and the proportions of dead mosquitoes were compared using a chi-squared test. The *kdr* allele frequencies were compared between

Table 1: Mortality rate, knock-down time, and resistance of malaria vectors to insecticides in the study area.

Insecticide	Localities	% of mortality (total tested)		KDT50 (95%CI)		KDT95 (95%CI)		Situation of resistance	
		2005	2006	2005	2006	2005	2006	2005	2006
DDT 4%	Nongremassoum*	**93.14**	**96.12**	**30.52**	**15.76**	**66.77**	**72.36**	**PsR**	**PsR**
		102	103	(29.01–32.11)	(14.32–17.20)	(60.70–75.08)	(61.50–88.63)		
	Saaba	**99.01**	**92.52**	**41.34**	**34.84**	**67.69**	**69.5**	**S**	**PsR**
		101	107	(39.76–43.04)	(33.28–36.50)	(63.00–71.11)	(63.67–77.43)		
	Koubri**	**96.25**	**100**	**27.96**	**29.48**	**67.25**	**60.5**	**PsR**	**S**
		80	106	(26.26–29.76)	(28.12–30.91)	(59.86–77.87)	(55.55–67.11)		
	Boromo	**94.28**	**88.24**	**31.91**	**37.36**	**73.45**	**79.91**	**PsR**	**PsR**
		105	102	(29.92–33.89)	(35.51–39.42)	(65.87–84.49)	(71.92–91.18)		
	Zampa	**89.56**	**56**	**32.14**	**47.52**	**72.27**	**105.69**	**PsR**	**PrR**
		115	100	(30.85–33.51)	(44.74–50.97)	(66.54–79.69)	(91.61–127.92)		
Permethrine 0.75%	Nongremassoum		**100**	**/**	**13.33**	**/**	**25.63**		**S**
			106	/	(12.55–14.07)	/	(23.75–28.12)		
	Saaba	**93.22**	**98.1**	**11.46**	**12.07**	**28.19**	**38.35**	**PsR**	**S**
		118	105	(10.65–12.25)	(11.00–13.11)	(25.85–31.21)	(34.44–43.60)		
	Koubri**		**100**		**7.48**		**12.84**		**S**
			28		(6.45–8.48)		(11.06–16.20)		
	Boromo	**100**	**98.13**	**12.42**	**5.16**	**22.39**	**34.93**	**S**	**S**
		100	107	(11.67–13.13)	(3.99–6.27)	(20.78–24.58)	(29.48–43.50)		
	Zampa	**100**	**100**	**17.33**	**12.94**	**31.91**	**27.51**	**S**	**S**
		101	102	(16.46–18.18)	(12.07–13.76)	(29.66–34.90)	(25.33–30.40)		
Deltamethrine 0.05%	Nongremassoum		**100**		**4.9**		**9.28**		**S**
			105		(4.37–5.34)		(8.26–11.10)		
	Saaba	**100**		**8.43**		**18**		**S**	
		112		(7.81–9.04)		(16.46–20.08)			
	Boromo	**100**	**98.98**	**11.7**	**5.82**	**24.67**	**13.02**	**S**	**S**
		209	98	(11.14–12.25)	(5.20–6.40)	(23.26–26.37)	(11.60–15.17)		
	Zampa	**99.04**	**100**	**12.35**	**7.32**	**27.31**	**14.83**	**S**	**S**
		104	100	(11.51–13.17)	(6.73–7.89)	(25.09–30.22)	(13.44–16.78)		
Lambdacyhalothrine 0.05%	Boromo	**98.9**		**14.64**		**26.59**		**S**	
		91		(13.81–15.44)		(24.63–29.22)			

*No test was performed in 2005 at Nongreassoum. Data in the column 2005 are those of a first test performed in 2006.
**At Koubri, sensitivity tests were performed only on *An. funestus*. As in Nongremassoum, no test was performed in 2005 at Koubri.

sites and between *An. gambiae* subpopulations using a chi-squared test. The level of significance was set at $P \leq .05$.

2.6 Ethical approval

Ethical approval to conduct the study was obtained from the Ministry of Health, Burkina Faso. Meetings were held with the community in the localities before the start of the study. Signed informed consent was obtained from the head of each locality, before larvae and adult mosquito collection.

3 Results

Over the course of the two-year survey, 2,283 adult females of *An. gambiae* s.l. and 214 adult females of *An. funestus* s.l. were tested for susceptibility to pyrethroid and DDT.

3.1 Mortality rate and knock-down time

Mortality rates after 1 hour of exposure to DDT 4%, permethrin 0.75%, deltamethrin 0.05%, and lambdacyhalothrin 0.05% are presented in Table 1, along with 50% and 95% knockdown times, and the resistance status of *An. gambiae* s.l. and *An. funestus* s.l. obtained with the different insecticides.

3.1.1 Pyrethroid-related mortality rates

Mortality rates of *An. gambiae* s.l. and *An. funestus* s.l. to permethrin, deltamethrin, and lambdacyhalothrin varied from 98.10% to 100% in all of the localities during the two years of survey, except in Saaba, where a mortality rate of 93.22% was obtained in 2005 with permethrin for *An. gambiae* s.l. (Table 1). According to WHO [49] criteria, these results indicate that vectors are susceptible to pyrethroids, except permethrin in Saaba in 2005 where resistance is possible, but requires confirmation. No evidence of significant difference was observed between years when comparing *An. gambiae* s.l. mortality rates for exposure to permethrin and deltamethrin. These data indicate that these vectors are still susceptible to these compounds.

3.1.2 Pyrethroid knock-down times

The times after which 50% of females were knocked down (KdT50) after exposure to each of the three insecticides (permethrin 0.75%, deltamethrin 0.05%, and lambdacyhalothrin 0.05%) decreased in 2006 or remained similar to those of 2005 in all the localities (Table 1). In contrast, the times after which 95% of females were knocked down (KdT95) under the influence of pyrethroid in 2006 increased in Saaba ($t = -4.07$; $P = .0001$) and Boromo ($t = -4.02$; $P = .0001$) for permethrin and decreased in 2006 in Zampa ($t = 2.37$; $P = .019$). The KdT95 for deltamethrin declined in Boromo ($t = 9.60$; $P < .0001$) and Zampa ($t = 8.30$; $P < .0001$) in 2006.

3.1.3 DDT-related mortality rates

DDT tests showed that *An. funestus* was susceptible in Koubri, with mortality rates of 96.25% and 100% in 2006 (Table 1); no evidence of a significant difference was found between the two tests conducted in 2006 with DDT in this village: Chi-2 = 4.0402, $P = .078$. In contrast, *An. gambiae* s.l. was resistant to DDT in all the study localities (Table 1), with mortality rates varying from 56% to 96.12% during the two years of study, except in Saaba, where a 99.01% mortality rate was observed in 2005. For *An. gambiae* exposed to DDT, a remarkable decrease in mortality rate was observed in 2006, with statistically significant differences between years, in Zampa (Chi-2 = 18.827, $P < .001$) and Saaba (Chi-2 = 5.281, $P = .022$). In Saaba, the mosquito population switched from being susceptible to DDT in 2005 to showing some resistance in 2006.

3.1.4 DDT knockdown times

In 2006, DDT KdT50 for *An. gambiae* s.l. declined in Saaba, whereas this species showed some resistance to DDT, regarding the result of the mortality rate of the same year. In Boromo and Zampa, an increase in DDT KdT50 was observed (Table 1). In Nongremassoum, a decrease in DDT KdT50 was observed in the second test conducted in 2006 compared with the first test in the same year. The DDT KdT95 increased in Zampa in 2006 ($t = -4.06$; $P = .001$). However, in the other localities, no evidence of a significant change was observed between 2005 and 2006 (95% CI); $P = 0.45$, 0.64, and 0.34, respectively, in Nongremassoum, Saaba, and Boromo.

For *An. funestus*, no evidence of a significant change was observed between KdT95 values obtained during the two tests conducted in 2006 in Koubri ($P = .18$); the KdT50 values were also similar (Table 1).

It is important to note that *An. gambiae* s.l., which was susceptible to DDT and resistant to permethrin in Saaba in 2005, became resistant to DDT and susceptible to permethrin in the same locality in 2006 (Table 1).

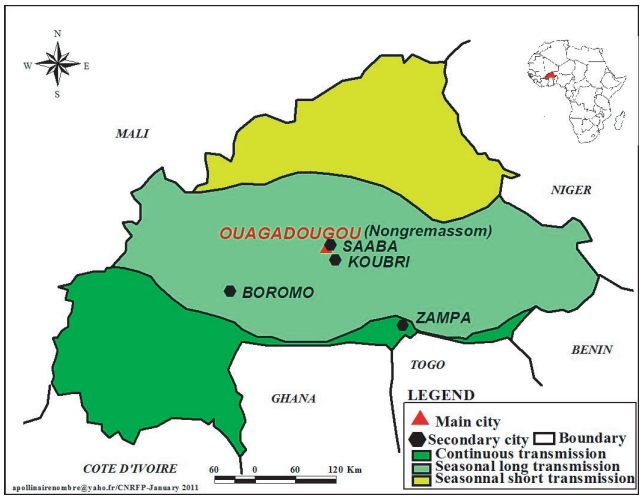

Figure 1: Geographical situation of the study site localities.

3.2 Species and molecular forms of *An. gambiae* complex mosquitoes

All *An. gambiae* s.l. females used for susceptibility tests during the two-year study period were subsequently genotyped for determination of species and molecular forms. A total of 1,151 females in 2005 and 1,132 in 2006 were analyzed. *An. arabiensis* and the two molecular forms, M and S, of *An. gambiae* s.s. were present in the study site. With respect to *An. gambiae* s.s., the molecular form M was more frequent in all study locations, with proportions ranging from 46% to 59% in 2005 and 46% to 88% in 2006. *An. arabiensis* and the S molecular form of *An. gambiae* s.s. were poorly represented: 8% to 37% in 2005 and 1% to 32% in 2006 for *An. arabiensis* and 17% to 32% in 2005 and 11% to 29% in 2006 for the S molecular form of *An. gambiae* s.s. Figures 2 and 3 show the distribution of *An. gambiae* family members in 2005 and 2006 in the study site.

3.3 Kdr gene distribution in *An. gambiae* s.l.

The distribution of the *kdr* gene in the study site during the two years is summarized in Table 2.

The *kdr* gene was generally found in *An. gambiae* s.l. in 2005 and 2006. However, a detailed analysis indicated that, in *An. gambiae* s.s., the *kdr* gene was absent in the M molecular form in Boromo in 2006 and in the S molecular form in 2005 in Saaba. Regarding *An. arabiensis*, the *kdr* gene was only observed in Saaba and Nongremassoum, in 2005 and 2006, respectively.

In general, we noted an increase in the frequency of the *kdr* gene in 2006 compared with 2005 for *An. gambiae* s.l. The differences were statistically significant in Zampa (Chi-2 = 63.93; $P < .001$) and Saaba (Chi-2 = 11.14; $P < .001$).

The highest frequency of the *kdr* gene in *An. gambiae* s.l. was found at Zampa.

Table 2: Kdr gene distribution in the study areas in 2005 and 2006.

Zone	Localities	*An. gambiae* s.l.		*An. arabiensis*		M form		S form	
		2005	2006	2005	2006	2005	2006	2005	2006
Urban	Nongremassoum		**0.027** (415)		**0.008** (61)		**0.049** (287)		**0.011** (42)
Peri-urban	Saaba	**0.009** (332)	**0.056** (209)	**0.017** (89)	**0.000** (2)	**0.008** (141)	**0.057** (176)	**0.000** (48)	**0.005** (22)
Rural	Boromo	**0.029** (500)	**0.033** (306)	**0.000** (40)	**0.000** (92)	**0.029** (284)	**0.000** (131)	**0.042** (155)	**0.013** (61)
	Zampa	**0.030** (319)	**0.268** (202)	**0.000** (111)	**0.000** (2)	**0.050** (140)	**0.159** (139)	**0.048** (53)	**0.115** (57)

The allelic frequency is in bold and the number of mosquitoes tested in brackets.

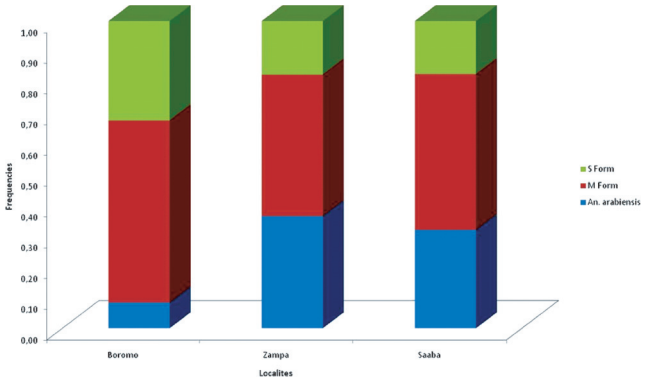

Figure 2: Distribution of species and molecular forms of *An. gambiae* s.l. in the study area in 2005.

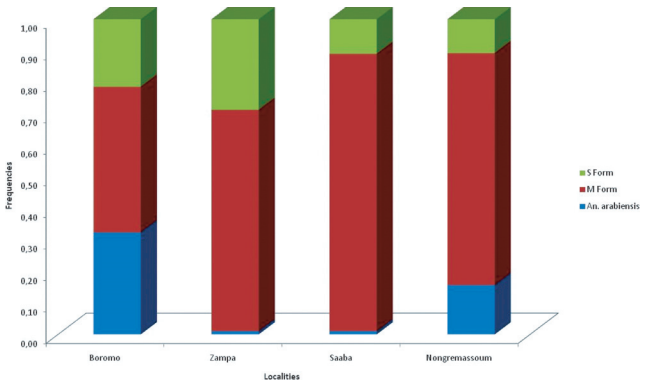

Figure 3: Distribution of species and molecular forms of *An. gambiae* s.l. in the study area in 2006.

4 Discussion

Our results regarding the *An. gambiae* s.l. population's mortality rates after exposure to DDT indicate possible and/or probable resistance in the study site.

The increase in KdT50 in Boromo and Zampa in 2006 indicates a decrease in vector susceptibility to DDT. Moreover, the presence and increase in frequency of the *kdr* gene in some locations during the second year of the study are suggestive of the presence of resistance. Finally, given that the mortality rate is under 95% [49], and considering the fact that each test with *An. gambiae* s.l. was done with a sample size of up to 100 females, we strongly suspect the presence of vector resistance. It is also worth mentioning that vector resistance to DDT was observed in all four localities, which included rural (Boromo and Zampa), suburban (Saaba), and urban (Nongremassoum) areas.

Formerly a major component of the global campaign to eradicate malaria, the use of DDT has been compromised, not only because of resistance, but also because of its environmental effects [4, 29].

Our results showed that *An. funestus* s.l. is still susceptible to DDT in Koubri. However, it would be advisable to perform tests with mosquito samples from various localities of the country to get a more representative picture of *An. funestus* susceptibility to DDT in the country.

The increasing KdT50 for DDT in Boromo and Zampa supports the low mortality rates observed in these localities. In Saaba, the decline in DDT KdT50 together with the low mortality rate is suggestive of the presence of other mechanisms of resistance. In the other sites, the level of resistance appears to be stable.

Malaria vectors still remain susceptible to permethrin, but in a context of resistance to DDT, the phenomenon of cross-resistance to permethrin cannot be ruled out. The case of Saaba where mosquitoes were resistant, only to permethrin in 2005 and only to DDT in 2006, suggests that other mechanisms other than kdr are involved in resistance to DDT or pyrethroids. Mosquitoes collected in the study villages were also found to be susceptible to deltamethrin and lambdacyhalothrin.

With regard to pyrethroids, the low KdT50 obtained in 2006 as compared with 2005 indicates an increase in vector susceptibility. The increase in permethrin KdT95 in Saaba and Boromo in 2006 compared with 2005 suggests development of resistance to this insecticide in the future, as knockdown time is an early indicator of the development of resistance.

In Burkina Faso, the main malaria vector is *An. gambiae* s.l. and, to a lesser extent, *An. funestus* s.l. [12, 40, 41].

The *Anopheles gambiae* complex members included in this study were *An. arabiensis* and the M and S molecular forms of *An. gambiae* s.s.

Our results on the distribution of species and molecular forms of the *An. gambiae* complex showed a predominance of the M molecular form of *An. gambiae* s.s. in the study site. This corroborates with results of previous studies

in Burkina Faso [14,27,36]. The *An. gambiae* M form is associated with the dry season and irrigation in arid areas; however, this M form of *An. gambiae* s.s. is characterized by a remarkable ecological flexibility which enables it to adapt well in different environments [7,44,45]. *An. funestus*, which generally contributes to malaria transmission during the dry season [12,40,41], was present in relatively small numbers in the study site in 2005 and 2006. This may be because shady permanent and semi-permanent deep waters, favorite breeding sites for this vector, were rare in the study villages.

As has been reported in previous studies, our study confirmed the presence of the *kdr* gene in Burkina Faso [10, 15,18] and its presence in the M molecular form of *An. gambiae* s.s. [17], sometimes at higher frequencies than in the S molecular form [39]. Our findings on the presence of the *kdr* gene in *An. arabiensis* are consistent with those from previous studies [10,16,18].

The increase in *kdr* gene frequency in 2006 within *An. gambiae* s.l. may suggest an increase in resistance. However, a two-year follow-up does not seem long enough to come to any definitive conclusions regarding such an increase. The observation of such increased frequency could simply be explained by random variations. In the study villages, insecticides are widely used, in an unregulated manner, for agricultural purposes (unpublished data). Could such behavior explain the quick changes in *kdr* frequencies?

5 Conclusion

DDT-resistant *An. gambiae* s.l. was present in all locations of the study, whereas *An. funestus* remained sensitive to DDT. However, the low number of tests performed on *An. funestus* and the fact that only mosquitoes from one locality were tested call for caution in the interpretation of these results. Overall, malaria vectors are still susceptible to pyrethroids in the localities involved in the study.

The increase in frequency of the *kdr* gene in the second year of the study, and the question of whether this is a consequence of random variation or insecticide pressure, is a serious concern. This is particularly true in the context of the re-introduction of indoor residual spraying (IRS) with DDT [50,51,53] towards which malaria vectors were found to be resistant in our study villages. Regarding IRS and considering the WHO position statement on treated mosquito nets [52], it becomes urgent to implement a strategy to address insecticide resistance management, including an effective monitoring strategy to guide decision making on malaria control policy.

Authors' contributions E. Ilboudo-Sanogo, N. Sagnon, and S. B. Sirima conceived and designed the study. E. Ilboudo-Sanogo and A. Badolo performed and supervised susceptibility tests. A. Badolo supervised field larva collection. N. Sagnon and W. M. Guelbeogo performed PCRs. E. Ilboudo-Sanogo, A. Badolo, and S. B. Sirima wrote the article.

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