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Pyrethroid resistance and susceptibility to *Plasmodium falciparum* infection of *Anopheles coluzzii* populations from Yaoundé (Cameroon)

Edmond Kopya, Cyrille Ndo, Billy Tene Fossog, Timoléon Tchuinkam, Parfait Awono-Ambene, Christophe Antonio-Nkondjio

Edmond Kopya
(a) Laboratoire de Recherche sur le Paludisme, Organisation de Coordination pour la lutte Contre les Endémies en Afrique Centrale (OCEAC), P.O. Box 288, Yaoundé, Cameroun.
(b) Faculty of Sciences, University of Yaoundé I, P.O. Box 337, Yaoundé, Cameroon.

Cyrille Ndo
(a) Laboratoire de Recherche sur le Paludisme, Organisation de Coordination pour la lutte Contre les Endémies en Afrique Centrale (OCEAC), P.O. Box 288, Yaoundé, Cameroun.
(b) Vector group, Liverpool School of Tropical Medicine, Pembroke Place, Liverpool L3 5QA, UK
(c) Faculty of Medicine and Pharmaceutical Sciences, University of Douala, Douala Cameroon

Billy Tene Fossog
(a) Laboratoire de Recherche sur le Paludisme, Organisation de Coordination pour la lutte Contre les Endémies en Afrique Centrale (OCEAC), P.O. Box 288, Yaoundé, Cameroun.
(b) Faculty of Sciences, University of Yaoundé I, P.O. Box 337, Yaoundé, Cameroon.

Timoléon Tchuinkam
Malaria Research Unit of the Laboratory of Applied Biology and Ecology (MRU-LABEA), Department of Animal Biology, Faculty of Science of the University of Dschang, P. O. Box 067 Dschang, Cameroon.

Parfait Awono-Ambene
Laboratoire de Recherche sur le Paludisme, Organisation de Coordination pour la lutte Contre les Endémies en Afrique Centrale (OCEAC), P.O. Box 288, Yaoundé, Cameroun.

Christophe Antonio-Nkondjio
(a) Laboratoire de Recherche sur le Paludisme, Organisation de Coordination pour la lutte Contre les Endémies en Afrique Centrale (OCEAC), P.O. Box 288, Yaoundé, Cameroun.
(b) Vector group, Liverpool School of Tropical Medicine, Pembroke Place, Liverpool L3 5QA, UK.

Correspondence
Christophe Antonio-Nkondjio
(a) Laboratoire de Recherche sur le Paludisme, Organisation de Coordination pour la lutte Contre les Endémies en Afrique Centrale (OCEAC), P.O. Box 288, Yaoundé, Cameroun.
(b) Vector group, Liverpool School of Tropical Medicine, Pembroke Place, Liverpool L3 5QA, UK.

Abstract

The current study aims at assessing the influence of pyrethroid resistance on the outcome of *Plasmodium* infection in *Anopheles coluzzii* populations. Mosquito larvae collected in the field were reared in the laboratory, and then separated into two groups. The first group was exposed to permethrin 0.75%, the second acted as control group. Experimental infections were conducted with the two groups with field isolates of *Plasmodium falciparum* by direct membrane feeding assay. Blood fed females were dissected 7 days later to detect oocyst infection. The prevalence of infection between resistant and control was 13.95% and 14.83% respectively. The mean number of oocyst per infected gut in the resistant group was 4.95 whereas it was 3.98 for the control. No significant association between the knock down (*kdr*) allele presence and susceptibility to *Plasmodium* infection was detected. No influence of pyrethroid resistance on the outcome of infection was detected.

Keywords: *Anopheles coluzzii*, Cameroon, Infections, Insecticide resistance, Malaria, *Plasmodium falciparum*.

1. Introduction

Challenges deriving from the rapid evolution of pyrethroid resistance on malaria vectors vectorial capacity still remain not fully understood [1, 2]. Two main mechanisms conferred pyrethroid resistance: target site insensitivity and metabolic resistance. Target site insensitivity or knockdown resistance (*kdr* resistance), is induced by two mutations the first lead to a leucine to phenylalanine substitution (L1014F) West Africa *kdr* allele and the second, lead to a leucine to serine substitution (L1014S) East Africa *kdr* allele [3, 4]. Metabolic resistance is conferred by overexpression of enzymes belonging to three classes, esterases, glutathione S transferase and cytochrome P450 monooxygenases. Although pyrethroid resistance increases the survival capacity and thus the life span of mosquitoes in treated environments, its impact on malaria transmission or *Anopheles* mosquito susceptibility to *Plasmodium* remains unclear. Several factors including the level of mosquito anthropophily, biting rate, mosquito life span and the duration of parasite development in the mosquito influence transmission from mosquito to human and the intensity of malaria transmission [5]. Before being transmitted by the mosquito, *Plasmodium* gametocytes ingested during a blood meal undergoes a complete cycle in the mosquito stomach called the sporogonic cycle which last 12 to 14 days according to the temperature. At the end of this cycle, sporozoites are released in the hemolymph from where, they migrate through to the salivary glands and are inoculated during subsequent blood meals [6]. Several parameters including mosquito immune system and other exogenous factors (antibodies, phagocytosis, temperature) influence the success of this cycle [6]. There are increasing evidences supporting the influence of insecticide resistance genes on *Plasmodium* sporogonic cycle [7]. Gene expression analysis during the sporogonic cycle showed cytochrome P450 genes differentially expressed at different phases of parasite infection and in different tissues [7]. The detoxification enzyme *CYP6M2* and several other detoxifications genes such as *CYP6AG1, CYP6M4, CYP6M1, CYP4G17, CYP9J5* and *CYP12F3* were reported to be strongly up regulated in the midgut and the fat body shortly after ingestion of an infected blood meal or during *Plasmodium* infection [7, 8]. Experimental infections assays using three resistant *An. gambiae* strains with field isolates of *Plasmodium falciparum* showed that mosquitoes carrying the *kdr* resistant alleles were more infected than susceptible [9]. Because pyrethroid resistance has increased dramatically over the past decade across Africa, it becomes

urgent to assess the genetic and biological implications of this evolution on mosquito vectorial capacity and malaria transmission.

In Cameroon, the evolution of pyrethroid resistance has been particularly important in major cities where mosquitoes of the *An. gambiae* complex happen to be the main vectors [10-12]. In the city of Yaoundé, a cross sectional survey reported an annual prevalence of 35% of *Plasmodium falciparum* asexual parasites carriers, 4.4% of gametocyte carriers in the general population and an entomological inoculation rate of 33 infected bites/man/year [13]. Experimental infections studies analysing the vectorial competency of the main vectors in the area *An. coluzzii* and *An. gambiae* showed infections to be more prevalent in *An. coluzzii* however no difference in the infection intensity was detected between the two species [14]. Studies conducted so far in the city of Yaoundé reported a good adaptation of both *An. coluzzii* and *An. gambiae* to polluted habitats in urban and periurban settings [15, 16]. It is still unclear whether the current adaptation of mosquitoes to the urban environment combined to the rapid evolution of pyrethroid resistance is affecting malaria transmission dynamic. In the present study, we assessed if the high prevalence of pyrethroid resistance in *An. coluzzii* has influenced its susceptibility to *Plasmodium falciparum*.

Materials and Methods
Study sites
The study took place in Yaoundé (3° 51'N 11° 30'E) the capital city of Cameroon. The city is situated within the Congo-Guinean phytogeographic zone characterized by a typical equatorial climate with two rainy seasons extending from March to June and from September to November. The annual average rainfall in Yaoundé is 1,700 mm. The city is situated 800 m above sea level and is surrounded by many hills.

Larval collections in Yaoundé were carried out in five districts situated in the city centre: Mokolo, Messa, Olezoa, Ahala and Combattant. Studies conducted in Yaoundé indicated that 75 - 85% of mosquitoes in these districts were susceptible to permethrin (11).

The present study was conducted under the ethical clearance N° 216/CNE/SE/09 delivered by the Cameroon National Ethics Committee Ref N° IORG0006538-IRB00007847-FWA00016054.

Mosquito collection
Mosquitoes were collected in breeding habitats and reared until the adult stage then were separated in two batches. The first batch constituted of mosquitoes aged between 2 - 3 days was exposed for one hour to 0.75% permethrin impregnated papers. Insecticide susceptibility tests were performed according to WHO procedures between 25 and 27 °C [17]. After exposure, mosquitoes were kept in a room at 25 °C with 70 - 75% humidity and fed with a 10% glucose solution and the number of dead mosquitoes was recorded 24 hours post-exposure. Tests using untreated papers were systematically run as controls. Mosquitoes still alive after 24 hours observation period were considered as resistant and starved for 24 hours before being infected. The second batch considered as control was exposed to untreated papers and then starved for 24 hours before blood feeding with an infected blood meal.

Mosquito identification
Anopheline larvae were collected and reared until the adult stage before being identified morphologically using the Gillies and Coetzee keys [18]. Mosquitoes belonging to the *Anopheles gambiae* complex were subjected to PCR assays designed for species and molecular forms identifications [19]. Genomic DNA used for molecular analysis was extracted from the legs of adult mosquitoes according to Cornel & Collins [20] protocols. To screen for the presence of the *kdr* alleles (1014F and 1014S), DNA extracted from individuals exposed to insecticide was tested using the TaqMan assay [21].

Experimental infections
Mosquitoes were placed in cups of at least 25 individuals covered with netting. An equivalent number of mosquitoes of the two mosquitoes batches (resistant and control) were used for experimental infections. At least five replicates were conducted. Starved females aged 3 to 6 days were allowed to feed for 35 minutes on a membrane feeder with blood from a gametocyte carrier. Before feeding, the gametocyte carrier serum was replaced by a non-immune AB serum to avoid human transmission blocking factors. At the end of the feeding, unfed females were removed from cups. Blood fed females were kept in the insectary with daily access to a 10% glucose solution. Mosquitoes were dissected 7 days post infection in sterile phosphate buffered saline solution under a binocular microscope. Experimental infections were conducted during two consecutive infection sessions. The first session from April to May 2012 (during the small rainy season) was conducted using nine gametocyte carriers, the second infection session from September to October 2012 (during the long rainy season) used 6 gametocyte carriers.

Statistical analysis
The data generated from infection experiments including the number of mosquitoes tested, the number of mosquitoes found infected, the status of mosquitoes resistant and control, the period of infection and the density of gametocytes per µl of blood were registered in an excel database. The data was grouped into control and resistant before being analyzed. The correlation between gametocyte density and oocyst infection prevalence or oocyst number was assessed using regression analyses. Comparison between means was assessed using ANOVA or Kruskals Wallis test in case of inequality of sample variances. Percentages were compared using the chi square test. The difference was recorded as significant for $P<0.05$. All analysis were performed using the software MedCalc V13.3.3.

Results
Selection of permethrin resistant specimens and experimental infections
Adult susceptibility tests were conducted to select mosquitoes displaying resistance to permethrin. Of a total of 2367 mosquitoes aged 2 to 3 days old exposed to 0.75% permethrin, 1083 were recorded as dead this giving rise to a mortality rate of 45.76%. Survivors were used for experimental infections. A total of 3157 mosquitoes including survivors to permethrin and unexposed females (control) were used for experimental infections. Gametocyte carriers used for the study had a gametocyte density ranging from 7 to 285 gametocyte/µl. Only

912 females *Anopheles coluzzii* successfully fed on *Plasmodium falciparum* gametocyte carriers blood. There was no significant difference between the feeding rates of mosquitoes considered as resistant and the control (P >0.05). Mosquitoes used for the study were genotyped and were all *An. coluzzii*. The infection rate varied from 13.95% for resistant and 14.83% for control and was not significantly different (P = 0.93). The mean number of oocysts per infected gut in resistant mosquitoes was 4.95 with a range of 1-28 and in the control group was 3.98 with a range of 1-39 (Table 1). The difference was not significant (F = 1.8; P = 0, 14).

Table 1: Results of the experimental infections using resistant and unexposed *An. coluzzii* females seven days after blood feeding on *Plasmodium falciparum* gametocyte carriers blood.

Status	N blood fed	N diss	N positive (oocysts)	% of positive (CI 95%)	Mean oocystes/gut (CI 95%)
Resistant	204	129	18	13.95% (8.3 - 22.05)	4.95 (3.4 - 6.5)
Control	708	344	51	14.83% (11 - 19.5)	3.98 (2.44 - 5.52)
Total	912	473	69	14,59% (11.3 - 18.5)	4.28 (3.17 - 5.39)

N: number; diss: dissected; CI 95% confidence interval, Control: mosquitoes unexposed to permethrin

Variation of oocyst infections according to the gametocytes density

Regression analysis was conducted to assess the correlation between gametocyte densities and the prevalence of infection at the oocyst stage in both the resistant and the control group. A positive and significant correlation was recorded when the prevalence of infection at the oocyst stage was plotted against Log of gametocyte density in the control group ($R^2 = 0.45$; F = 17.37; P = 0.0004) whereas a positive but not significant correlation was recorded in the resistant group ($R^2 = 0.32$; F = 4.74; P = 0.054) (Figure 1). Regression analysis assessing the correlation between the number of oocyst against Log of gametocyte density showed a positive and significant correlation in the control group ($R^2 = 0.25$; F = 15.69; P = 0.0003) whereas a positive but not significant correlation was recorded in the resistant group ($R^2 = 0.19$ F = 4.7; P = 0.04). As a general picture, it appeared that mosquito displaying high oocyst counts were those who fed on blood with high gametocyte densities (Figure 2).
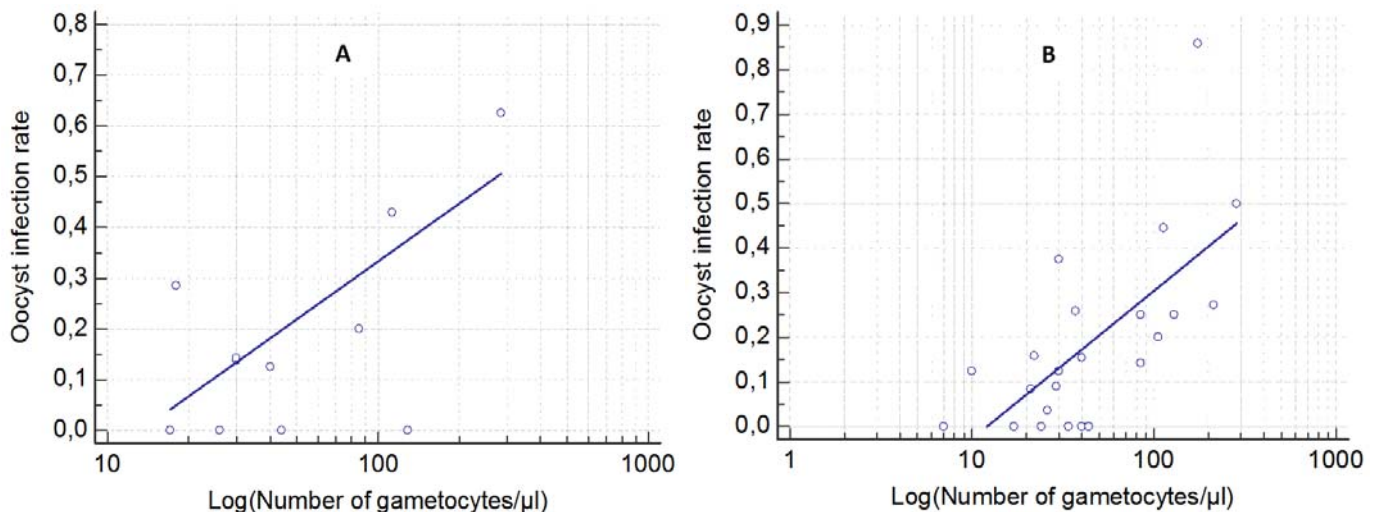

Fig 1: Correlation between oocyst infection rate and Log of gametocyte density in An. coluzzii resistant group (A) and control group (B).
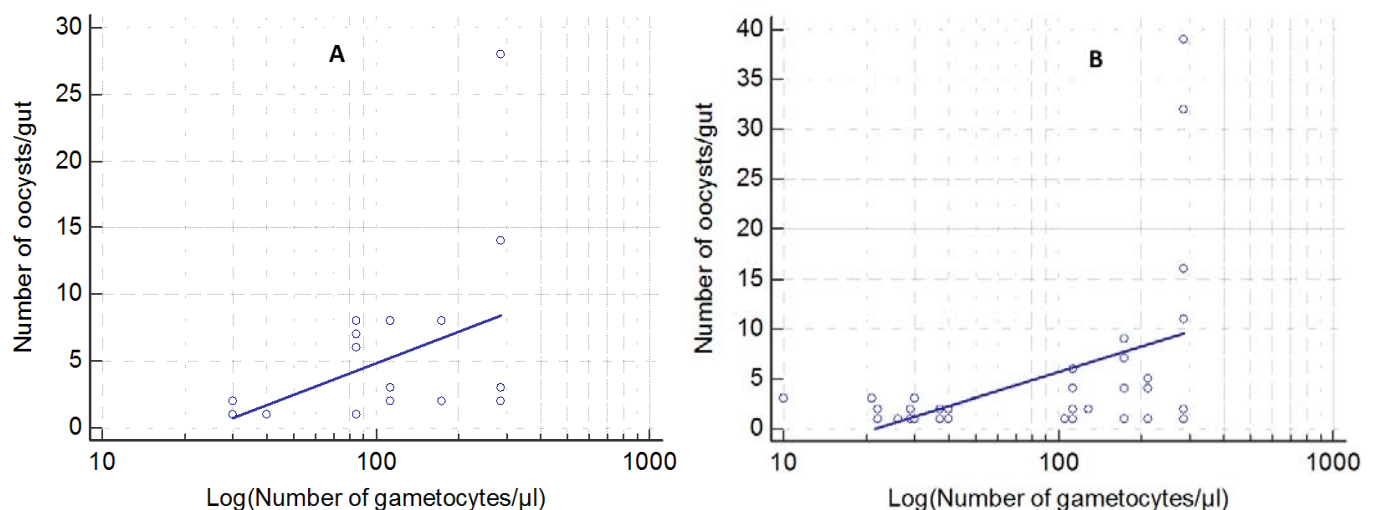

Fig 2: Correlation between oocyst number and Log of gametocyte density in An. coluzzii resistant group (A) and control group (B).

Variation of oocyst infection according to the *kdr* genotypes

All dissected mosquitoes including infected specimens and uninfected mosquitoes were genotyped to assess the prevalence of the *kdr* allele. This included 110 specimens genotyped as homozygotes (RR), 48 as heterozygotes (RS) and 13 as susceptible (SS). Over 98% of mosquitoes detected with *kdr* had the West Africa *kdr* allele (1014F). The prevalence of mosquitoes carrying the *kdr* allele and becoming infected varied greatly between groups (Figure 3). No significant association between *kdr* allele presence and susceptibility to *Plasmodium* infection was detected (P = 0.10). Equally, no significant association was recorded when comparing the prevalence of oocyst infection and *kdr* genotypes (P > 10) (Table 2).
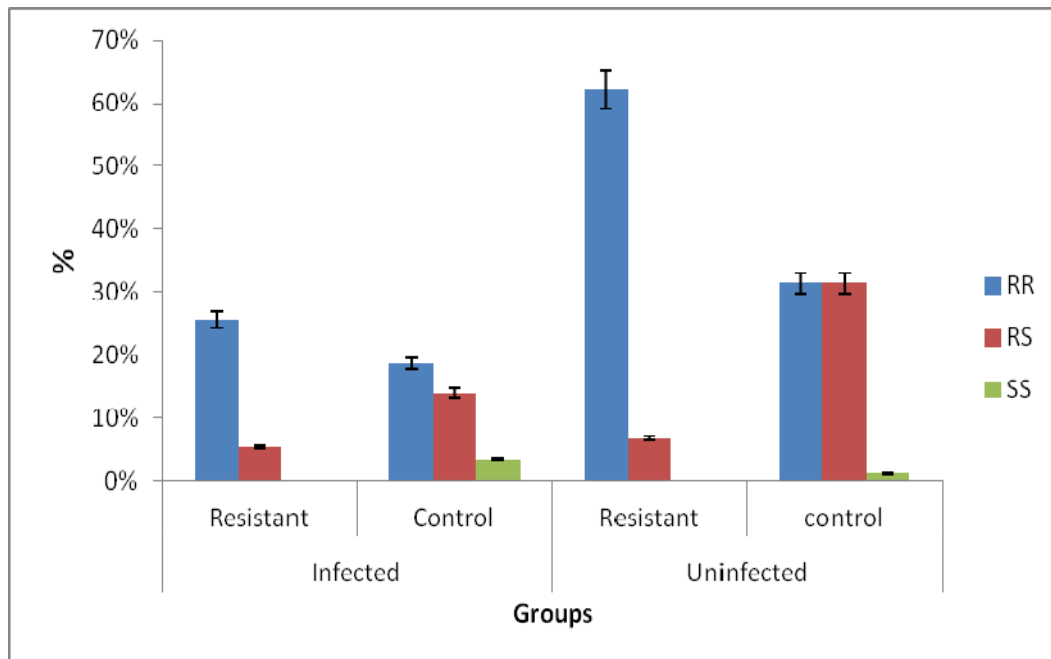

Fig 3: Diagram presenting the prevalence of mosquitoes carrying the kdr allele, infected and uninfected in both the control and exposed group (bars with standard errors) (RR: homozygote for kdr, RS: heterozygote for kdr, SS: susceptible).

Table 2: Comparison of the prevalence of *Plasmodium falciparum* oocyst infection between different kdr genotypes.

	kdr genotypes		
	RR vs RS	**RR vs SS**	**RS vs SS**
OR (95% CI)	0.96 (0.46 - 1.98)	0.16 (0.02 - 1.59)	0.117 (0.02 - 1.73)
P	0.91	0.11	0.13

OR, odds ratio, RR: homozygote, RS: heterozygote, SS: susceptible, 95% CI: 95% confidence interval.

Discussion

One of the main questions arising from the increasing prevalence of insecticide resistance in malaria vectors is to know if it does affect the outcome of *Plasmodium* infection in mosquito. During the present study, mosquito characterized phenotypically as resistant to permethrin were compared to control to assess their vectorial competency. No significant difference of the infection prevalence at the oocyst stage between resistant and control was recorded. These findings were different from recent studies suggesting pyrethroid resistant mosquitoes more efficient in parasite transmission than susceptible mosquitoes [9, 22]. The low power of assessing any difference of our study could come from the fact that the general population (including both susceptible and resistant) was used as control. Although bioassay analysis suggested a similar prevalence of permethrin resistant and susceptible individuals in the population, the use of the general population as control could have reduced the power of the analysis because of the small difference that could exist between resistant and susceptible individuals in the field. Thus the use of the general population as control could also have reduced risks of overestimating the real contribution of pyrethroid resistant mosquitoes to malaria transmission. It is rather possible that transmission intensity might be high in resistant compare to control due to the high average number of oocysts recorded per gut in this group. Yet the following still deserve further investigations since infections at the sporozoite stage were not assessed. The prevalence of oocyst infections in each group was low and could likely be the result of the low infectivity of gametocytes carriers blood. Indeed several factors including gametocyte maturity, the mosquito immune system, temperature, digestive enzymes or the presence of monoclonal *Plasmodium falciparum* isolates were reported to influence the outcome of infection in natural mosquito populations [23, 24]. Recent studies in the city of Yaoundé, indicated that the abundance of Enterobacteriaceae in the midgut can also increase mosquito susceptibility to *Plasmodium falciparum* infection [25]. The following underlying the important number of factors that could contribute in shaping the vectorial competency and vectorial capacity of local vector populations.

Correlation analysis showed on their part that, the outcome of infections from different feeding assay varied according to the density of gametocytes in the blood meal. It also appeared from the study that a considerable number of mosquitoes became infected after ingesting blood meals containing very low gametocyte densities. The following could likely be the

result of gametocyte aggregation as reported earlier [26] or an evolution of mosquito vectorial competency. Studies assessing the vectorial competency of *An. gambiae* and *An. coluzzii* in Yaoundé reported different infection prevalence between the two species supporting a high vectorial competency for *An. coluzzii* compare to *An. gambiae* [14]. In the city of Yaoundé, *An. coluzzii* is mainly distributed in the city centre while *An. gambiae* is abundant in rural and periurban areas [15, 16]. But it is still unknown whether the adaptation of *An. coluzzii* to urban centre could have important influence on the intensity of malaria transmission. The number of oocysts per infected gut also appeared positively correlated to gametocytes density in the blood meal. The following is in agreement with previous studies showing a strong linear and positive relationship between gametocytes or ookinetes densities and oocyst density [23].

Although the prevalence of oocyst infections was high in mosquitoes displaying the *kdr* allele, no significant difference was detected between mosquitoes carrying the *kdr* allele in the resistant and the control groups. Recent investigations by Alout *et al.* [9] using two *An. gambiae* strains (one resistant the other susceptible) sharing a common genetic background suggested the presence of *kdr* alleles influencing the outcome of *Plasmodium* infection. In their study Alout *et al.* [9] detected a high and significant parasite prevalence at both the oocyst and sporozoite stages in resistant compare to control. Similar findings were recorded in Senegal by Ndiath *et al.* [22] using F3 progeny of *An. gambiae* and *An. coluzzii*. It is still unknown under which mechanism the presence of the *kdr* mutation influence malaria vector vectorial competency. Gene expression analysis of pyrethroid resistant *An. gambiae* mosquitoes suggested the upregulation in these mosquitoes of antimicrobial genes involved in the anti-*Plasmodium* immune response which might potentially influence the response to malaria infection [27]. Several detoxification genes including CYP6M2, CYP6M3, CYP6AG1, CYP9J5, CYP49A1, CYP12F4, CYP6Z1 were also reported differentially expressed during the midgut invasion by *Plasmodium* [28]. Studies conducted by Felix *et al.* [8] showed that the number of cytochrome P450 genes upregulated decrease significantly between day 1 after blood meal and day 11 post blood meal. With the fast evolution of pyrethroid resistance, understanding how this is affecting mosquitoes vectorial capacity is becoming urgent in order to improve the fight against pyrethroid resistant malaria vectors.

Conclusion

Our study objective was to assess the existence of any relationship between pyrethroid resistance and the outcome of infection in *An. coluzzii*. Although the study did not provide clear evidences of any influence of pyrethroid resistance on the outcome of infection, the rapid evolution of pyrethroid resistance in malaria vector populations requests further attention.

Acknowledgements

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