

# Effect of bendiocarb based (Ficam® 80% WP) Indoor Residual Spraying on feeding patterns of malaria vectors in Burkina Faso, West Africa.

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## Research

**Keywords:** Indoor residual spraying, bendiocarb, malaria vectors, mosquito feeding patterns, Burkina Faso

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## Abstract

**Background :** Vector control is a key component in malaria prevention strategies in many malaria endemic countries in Africa. In Burkina Faso, malaria transmission is seasonal in most parts of country, so a single round of spraying should provide effective protection against malaria, provided the insecticide remains effective over the entire malaria transmission season. The primary effects of IRS towards curtailing malaria transmission are : i) to reduce the life span of vector mosquitoes and ii) to reduce the density of the vector mosquitoes.

**Methods :** CDC light trap and pyrethrum spray catches were carried out monthly to determine the change in malaria vector indices in sprayed (Diebouougou) and unsprayed sites (Dano). The females malaria vectors collected by CDC light trap and PSC were used to determine their blood feeding and sporozoites rate using enzyme-linked immunosorbent assay (ELISA) and their biting rate and entomological inoculation rate

**Results:** Three species belonging to the *Anopheles gambiae* complex (*Anopheles gambiae* s.s., *Anopheles coluzzii* and *Anopheles arabiensis*) are present throughout the transmission season, but *An. gambiae* s.s. was the most frequent species of the complex ( $P = 0.0005$ ), comprising 88% of the total collected and the most infected species. Malaria vectors densities were significantly lower in sprayed villages ( $n=4,303$ ) compared with unsprayed villages ( $n=12,569$ ) during post-spraying period ( $P = 0.0012$ ). In addition, mean human biting rate of *An. gambiae* sl and *An. funestus* ss were significantly lower in sprayed areas compared to unsprayed areas ( $P < 0.05$ ). Overall, malaria vector transmission risk was significantly lower in villages which received IRS ( $P = 0.0001$ ) whatever the malaria vectors species (*An. gambiae* sl and *An. funestus* ss).

**Conclusion:** The results showed that in the sprayed area (Diebouougou), vector densities, human biting rates and malaria transmission risks were very lower than unsprayed areas (Dano). The findings also showed a change in vector behavior especially within *An. funestus* which became more zoophagic following IRS. The indoor residual spraying could be recommended as control tool in areas where malaria transmission occurred throughout the year.

**Keywords:** Indoor residual spraying, bendiocarb, malaria vectors, mosquito feeding patterns, Burkina Faso.

## Background

Vector control is a key component in malaria prevention strategies [1]. In Africa, it relies primarily on two effective and complementary tools: long-lasting insecticidal nets (LLINs) and indoor residual spraying (IRS) [2]. Several studies have demonstrated the effectiveness of both tools in reducing the incidence of malaria [3, 4] morbidity and mortality in Africa [5–9]. However, these interventions, especially LLINs, rely on the use of pyrethroid insecticides. Unfortunately, the recent evolution and spread of pyrethroid resistance in West Africa in *Anopheles gambiae* s.s. is a major concern for the sustainability of malaria prevention in Africa [10, 11]. For this reason, the research of alternate solutions using a non-pyrethroid insecticide has become a priority [12]. Indeed, since 2006, the World Health Organization (WHO) encouraged a scale-up of IRS for vector-borne disease control, using one of several classes of insecticide that are suitable for IRS [13]. In 2008, of 108 malaria-endemic burdened countries, 44 reported the use of IRS [14]. The primary effects of IRS towards curtailing malaria transmission are: i) to reduce the life span of vector mosquitoes and ii) to reduce the density of the vector mosquitoes [15]. There are several insecticide formulations currently prequalified by WHO for IRS; namely organophosphates, carbamates, pyrethroids and neonicotinoids. In addition, the effectiveness of IRS depends on many factors, including residual efficacy of IRS formulations, indoor resting behaviour of malaria vectors and local vector susceptibility to the insecticide used for IRS [16]. Residual efficacy of IRS formulations and local vector susceptibility to the insecticide used for IRS were discussed in a separate manuscript about bendiocarb efficacy on walls. The monitoring of behavioral responses of mosquitoes to insecticides is critical to the understanding of how chemicals function in the control of disease transmission [17]. Indeed, in northern Nigeria [18], IRS led to a considerable decrease in the total vector population and reduction in the incidence of malaria among children, the plasmodic index and fever, and an apparent effect on mortality of 1–4-year-old children. In Kenya, IRS with fenitrothion in Kisumu [19] and the use of LLINs in the south coast of Kenya [20], showed a decline in populations of *An. funestus* s.l. and *An. gambiae* s.l. by the IRS, while the high bed net coverage was followed by a much reduced human biting rate and a diminishing role of *An. gambiae* s.s. in malaria transmission.

In Burkina Faso, malaria was the most common cause of outpatient consultations (41.3%), hospitalizations (21.4%) and death (16.4%) in 2018 [21]. According to WHO [22], resistance to at least one insecticide had been identified in 64 malaria-endemic countries, including Burkina Faso [23–27]. Pyrethroid resistance is particularly widespread in Burkina Faso, with high frequency of voltage gated sodium channel mutations reported as long ago as 2006 [28]. However, susceptibility tests performed in 2010–2012 with insecticides belonging to carbamates class (such as 0.1% bendiocarb) have shown a high mortality rate of the local population of malaria vectors and low allelic frequencies of gene  $ace-1^R$  [29] throughout the country including the southwest (Diebouougou). This insecticide molecule was chosen to be applied on walls during pilot study as described in the previous article. Indeed, the preliminary results from this study have shown a short persistence and a vectors resistance to this insecticide. As the international community has now prioritized national and regional elimination with a long-term ultimate goal of malaria eradication [30], the need to understand the biological implications of IRS is paramount. It is important to understand its impact on the behavior of malaria vectors in contact with walls treated with insecticide. In this context, the present study aims to evaluate the effect of a large scale IRS using a non pyrethroid insecticide (bendiocarb) on the behavior of malaria vectors, in the intervention areas (Diebouougou district) compared to untreated areas used (Dano district) as controls.

## Methods

### Study area

Entomological surveys were conducted in the health district of Diebouougou (intervention or sprayed area) and covered four villages (or agglomeration) including Diebouougou center (N10.96741; W 003.24580), Bagane (N10.96397; W003.23422), Loto (N10.96871; W003.23477) and Bapla (N10.87638; W 003.26145). Dano (control or unsprayed area) is situated 42 km from Diebouougou and was utilized as an unsprayed control area. Four villages were sampled,

including Dano sector 1 corresponding to the center of Dano (N11.14288; W 003.05969), Dano sector 2 (N11.13802; W 003.06216), Dano sector 3 (N11.16464; W 003.06374) and Dano sector 4 (N11.14541; W 003.05141) (Fig. 1). Villages chosen in Dieboungou and Dano districts were selected to be representative of the different settings of the areas (peripheral, central, sub-urban, presence of water source, etc) and had the same type of walls such as "banco" (a mixture of mud and water) and cement.

## Implementation of intervention

Indoor Residual Spraying was introduced in 2010 with funding from PMI/USAID, and continued through 2011 in conjunction with the NMCP. During the 2010 spray round, 33,897 structures were sprayed (98.9 percent of the target area), protecting 118,691 persons including nearly 25,000 children under five years of age and more than 2,000 pregnant women. In the 2011 spray campaign, 33,897 structures were sprayed protecting 118,691 people. IRS was implemented in accordance with President's Malaria Initiative Best Management Practices (PMI, 2010) to ensure a high quality of spraying and the safety of the residents, spray operators and the environment. Spray operators were provided with full personal protective equipment including coveralls, gloves, boots and helmet with face visor. Spray operators used either Hudson X-Pert or Goizper IK Vector Control compression sprayers fitted with flat fan nozzles to spray the interior walls and non-metal ceilings of eligible structures in IRS-targeted areas. All spray operators, team leaders and spray supervisors were trained prior to spray operations. Ficom 80%WP (wetable powder) was selected for spraying onto the walls in Dieboungou district. The formulation was applied at 400 mg of bendiocarb active ingredient/m<sup>2</sup> on walls of houses as recommended by the World Health Organization Pesticide Evaluation Scheme (WHOPES) [31, 32]. Spray operation staff informed residents to stay out of the structure for at least two hours after IRS application.

## Mosquito sampling and identification

Following IRS, the Institut de Recherche en Sciences de la Santé (IRSS) monitored the efficacy of IRS on entomological indicators of malaria transmission. Monthly collections were conducted to determine mosquito species composition, biting rates and indoor resting densities. Malaria vector population dynamics were monitored in each of the eight selected villages (four sprayed and four unsprayed controls) using indoor and outdoor CDC light trap collection (CDC LT) and pyrethrum spray catch (PSC). Two months of baseline collections were conducted, before IRS was performed, in June and a week before IRS application in July 2012 in Dano and Dieboungou. The relatively short baseline period of data collection was due to the long dry season in the study area from November until May, during which Anopheles densities and malaria transmission is low. Subsequent monthly entomology surveys were conducted until December 2012.

Indoor CDC LT were installed approximately 1.5 m above the ground next to an occupied (bait) untreated mosquito net (usually in the living room, although many houses have only a single room). Unbaited light traps were also hung outdoors at the same height, approximately 10-20m away from the houses. CDC LT was the preferred trapping method due to concerns about potential disease transmission risks during human landing catch. Moreover, several studies [33, 34, 35] showed a comparability of CDC LT catch size compared with HLC for different Anopheles species. In each village, CDC LT collections were conducted (indoors and outdoors) from 20:00 pm to 06:00 am for a total of four nights per month. Four randomly selected houses were sampled each month, resulting in 16 trap-nights indoors and 16 trap-nights outdoors per month, per site. In addition, indoor resting mosquitoes were collected by pyrethrum spray catches (PSC) in four randomly selected houses in each village. PSC was conducted by laying white sheets on the floor and furniture before spraying a commercial aerosol consisting of 0.64% Pyrethrum EC and 0.75% chlorpyrifos ethyl. PSC was conducted between 06:00 and 09:00 am. The sampling of mosquitoes was carried out in the same bedroom and at the same frequency during the intervention periods.

## Laboratory processing

All anophelines were sorted and assigned to species based on morphological characters using standard identification keys [36]. An. gambiae s.l. females caught in CDC LT were to be dissected to determine the parity rate. Unfortunately, parity rates could not be assessed as females died while in traps and were too dry for dissection. Legs of each female from mosquitoes collected by CDC LT and PSC were tested in PCR for molecular identification of species [37]. The heads and thoraces of host-seeking females were tested by enzyme-linked immunosorbent assay (ELISA) for the detection of Plasmodium falciparum circumsporozoite protein (CSP) using the procedure of Wirtz et al., [38]. The blood meals from freshly fed females collected using PSC were used to assess host preference for blood meal source. A random selection of 30 specimens per month and per district were tested by a direct ELISA [39] using anti-host immunoglobulin G (IgG) conjugated against human, bovine, pig, donkey and sheep blood. All the mosquito samples collected were stored individually in numbered vials with desiccant.

## Data analysis

### Measured parameters

The mean human-biting rate (HBR) was calculated for each species collected by CDC light trap by dividing the total number of captured specimens by the total person-nights for the collection period. The mean indoor resting density was defined as the total number of mosquitoes (per species) collected by PSC divided by the total number of rooms sampled. The circumsporozoite rate was calculated as the proportion of mosquitoes infected with P. falciparum sporozoites. The anthropophilic rate was calculated as the proportion of female malaria vectors with human blood out of the total tested for blood-meal source. The entomological inoculation rate (EIR) was calculated by multiplying the HBR indoor/outdoor and the CSP rate.

All the measured parameters were computed and analysed using the free software GraphPad 5.0 version. Data were compared with the Pearson chi<sup>2</sup> or Fisher exact tests and odds ratio were calculated to determine the impact of IRS in study sites. All calculations were expressed with the statistical significant threshold set at  $P \leq 0.05$ .

## Ethical consideration

Ethical approval for this study was granted by the Ethical Committee of the Ministry of Health in Burkina Faso. The mosquito collectors gave prior informed consent and they were vaccinated against yellow fever. An agreement with health facilities close to sites was also obtained for the free anti-malarial treatment of mosquito collectors who may suffer from malaria.

## Results

### Malaria vector densities and species composition

A total of 26,276 mosquitoes (13,555 anopheline and 12,721 other culicines) were collected from June to December 2012 using both CDC LT (9158 mosquitoes) and PSC collection methods (17,118 mosquitoes). In addition, 9,404 mosquitoes were collected in Dieboungou (sprayed area) between June and December (Table 1) whose 3040 mosquitoes collected in baseline (June-July 2012) and 6,364 mosquitoes during post-spraying period (August-December) compared to unsprayed area with 16,872 collected mosquitoes whose 4303 mosquitoes at baseline and 12,569 mosquitoes in post-spraying period ( $P = 0.0012$ ). A significant difference was observed between the total number collected in sprayed and unsprayed areas. According to species composition, *An. gambiae* s.l. (69.34%) and *An. funestus* s.s. (24.16%) were the most predominant Anopheline species collected in sprayed area (Dieboungou) compared to 45% *An. gambiae* s.l., 19% *An. funestus* s.s., and 36% other culicines (*Culex* sp., *Aedes* sp., *Anopheles* sp etc) (Additional file 1: Figure S1). Their proportion were significantly reduced between sprayed and unsprayed areas ( $P = 0.039$ ). In addition, there was a greater number of culicids collected in sprayed areas compared to unsprayed areas certainly due to impact of IRS. Overall, the total number of collected mosquitoes in sprayed areas (6364 mosquitoes) compared to unsprayed area (12569 mosquitoes) was significant ( $P = 0.001$ ) after spraying period.

Table 1  
Seasonal variation of major vectors densities in sprayed (Dieboungou) and unsprayed (Dano) sites

| Samples                 | CDC indoor collections |      |      |      |     |     |     |      | CDC outdoor collections |     |      |     |     |     |      |      | Pyrethrum indoor collections |      |  |  |
|-------------------------|------------------------|------|------|------|-----|-----|-----|------|-------------------------|-----|------|-----|-----|-----|------|------|------------------------------|------|--|--|
|                         | June                   | July | Aug  | Sept | Oct | Nov | Dec | June | July                    | Aug | Sept | Oct | Nov | Dec | June | July | Aug                          | Sept |  |  |
| Sprayed area            |                        |      |      |      |     |     |     |      |                         |     |      |     |     |     |      |      |                              |      |  |  |
| <i>An. gambiae</i> s.l. | 33                     | 76   | 213  | 106  | 76  | 40  | 22  | 8    | 35                      | 28  | 2    | 3   | 2   | 18  | 234  | 317  | 338                          | 335  |  |  |
| <i>An. funestus</i>     | 0                      | 0    | 27   | 47   | 33  | 28  | 31  | 5    | 15                      | 62  | 9    | 31  | 36  | 41  | 10   | 11   | 8                            | 11   |  |  |
| Other culicids          | 256                    | 220  | 678  | 246  | 309 | 206 | 92  | 15   | 131                     | 433 | 37   | 203 | 183 | 135 | 809  | 865  | 667                          | 149  |  |  |
| Total                   | 289                    | 296  | 918  | 399  | 418 | 274 | 145 | 28   | 181                     | 523 | 48   | 237 | 221 | 194 | 1053 | 1193 | 1013                         | 495  |  |  |
| Unsprayed area          |                        |      |      |      |     |     |     |      |                         |     |      |     |     |     |      |      |                              |      |  |  |
| <i>An. gambiae</i> s.l. | 74                     | 218  | 640  | 244  | 72  | 36  | 14  | 17   | 31                      | 21  | 14   | 2   | 2   | 4   | 469  | 777  | 1926                         | 1160 |  |  |
| <i>An. funestus</i>     | 15                     | 98   | 94   | 109  | 158 | 110 | 50  | 7    | 25                      | 2   | 3    | 3   | 4   | 26  | 118  | 244  | 256                          | 547  |  |  |
| Other culicids          | 150                    | 263  | 413  | 198  | 172 | 159 | 170 | 128  | 77                      | 25  | 20   | 28  | 17  | 74  | 1145 | 447  | 839                          | 844  |  |  |
| Total                   | 239                    | 579  | 1147 | 551  | 402 | 305 | 234 | 152  | 133                     | 48  | 37   | 33  | 23  | 104 | 1732 | 1468 | 3021                         | 2551 |  |  |

During the post IRS study period, indoor resting densities of malaria vectors were significantly lower in sprayed villages ( $n = 1,798$ ) compared with unsprayed villages ( $n = 8,607$ )  $P = 0.0051$  (Table 1). There was also a significant difference for total Anopheline catch by indoor CDC LT, with 1,527 in the untreated area compared with 623 in the IRS area ( $P = 0.0069$ ). When broken down to species, *An. funestus* indoor resting (PSC) and host-seeking (CDC LT) densities (CDC LT: sprayed =  $n = 166$  vs unsprayed  $n = 521$  with  $P = 0.004$ ; PSC: sprayed  $n = 99$  vs unsprayed  $n = 2,136$ ;  $P = 0.0079$ ) and *An. gambiae* s.l. indoor resting densities (PSC: sprayed  $n = 1,076$  vs unsprayed  $n = 4,944$ ;  $P = 0.0005$ ) were significantly lower in sprayed sites compared with control villages (Table 1).

### Monthly biting and resting behaviour of *Anopheles gambiae* s.l. and *An. funestus* s.s. following IRS

#### Baseline data

In Dano (unsprayed), in June 2012, the *An. gambiae* s.l. indoor human biting rate was estimated at 4.6 bites per person per night by CDC LT collection and 13.6 bites/person/night in July 2012 (Fig. 3A). However, in Dieboungou the *An. gambiae* s.l. human biting rates by indoor CDC LT were found to be lower at 2 and 5 b/p/n indoors in June and July respectively. The *An. funestus* indoor human biting rate was less than 1 b/p/n (Fig. 4A). A similar trend was recorded for indoor resting densities, with Dano having approximately double the catch size of Dieboungou (Fig. 5A&B). The highest resting densities by indoor PSC

collection with a mean value in July reaching 49 *An. gambiae* s.l. per house per night in Dano (Fig. 5A). The catch size was generally low in outdoor CDC collections in both sites (Fig. 3B & 4B).

## Post-spraying data

A summary of mean biting rates is presented in Fig. 3 for *An. gambiae* s.l. and Fig. 4 for *An. funestus* ss. In addition, the number of mosquitoes collected by month and by site is summarized in Table 2&3.

Table 2

Monthly *An. gambiae* s.l. sporozoite rate and entomological inoculation rate from Dano unsprayed area and Diebouyou (sprayed area) from June to December, 2012.

|  | June | July  | August | September | October | November | December | 2012 Total    |
|--|------|-------|--------|-----------|---------|----------|----------|---------------|
| Dano (unsprayed area)  |      |       |        |           |         |          |          |               |
| Total <i>An. gambiae</i> s.l. (CDC-LT) collected                                     | 91   | 249   | 661    | 258       | 74      | 38       | 18       | 1389          |
| CDC trap-nights  | 32   | 32    | 32     | 32        | 32      | 32       | 32       | 224           |
| (indoors + outdoors)   |      |       |        |           |         |          |          |               |
| HBR per night  | 2.84 | 7.78  | 20.66  | 8.06      | 2.31    | 1.19     | 0.56     | 6.2           |
| Total <i>An. gambiae</i> s.l. tested by CSP  | 78   | 45    | 44     | 14        | 21      | 27       | 13       | 242           |
| Sporozoites rate   | 0    | 6.7   | 13.6   | 14.2      | 09.5    | 11       | 30.8     | 7             |
| EIR p/night  | 0    | 0.521 | 2.809  | 1.145     | 0.219   | 0.131    | 0.173    | 0.714 (mean)  |
| EIR p/month*   | 0    | 15.64 | 84.28  | 34.35     | 6.591   | 3.92     | 5.19     | 21.42 (mean)  |
| Dano 5-month EIR post-IRS August-December 2012 = 134 infectious bites per person     |      |       |        |           |         |          |          |               |
| Diebouyou (sprayed area)   |      |       |        |           |         |          |          |               |
| Total <i>An. gambiae</i> s.l. (CDC-LT) collected                                     | 41   | 111   | 241    | 108       | 79      | 42       | 40       | 662           |
| CDC trap-nights  | 32   | 32    | 32     | 32        | 32      | 32       | 32       | 224           |
| (indoors + outdoors)   |      |       |        |           |         |          |          |               |
| HBR per night  | 1.28 | 3.47  | 7.53   | 3.38      | 2.47    | 1.31     | 1.25     | 2.95          |
| Total <i>An. gambiae</i> s.l. tested by CSP  | 42   | 49    | 56     | 25        | 114     | 62       | 30       | 378           |
| Sporozoites rate   | 0    | 6.1   | 5      | 8         | 14      | 3.2      | 0        | 0.052         |
| EIR p/night  | 0    | 0.212 | 0.377  | 0.27      | 0.346   | 0.042    | 0        | 0.1778 (mean) |
| EIR p/month*   | 0    | 6.35  | 11.29  | 8.1       | 10.36   | 1.26     | 0        | 5.34 (mean)   |
| Diebouyou 5-month EIR post-IRS August-December 2012 = 31 infectious bites per person |      |       |        |           |         |          |          |               |

Table 3

Monthly *An. funestus* ss sporozoite rate and entomological inoculation rate from Dano (unsprayed area) and Diebougou (sprayed area) from June to December, 2012.

|   | June | July      | August | September | October | November | December | 2012 Total   |
|---|------|-----------|--------|-----------|---------|----------|----------|--------------|
| Dano (unsprayed area)   |      |           |        |           |         |          |          |              |
| Total <i>An. gambiae</i> s.l. (HLC) collected                                       | 22   | 123       | 96     | 112       | 161     | 114      | 76       | 704          |
| CDC trap-nights<br>(indoors + outdoors)   | 32   | 32        | 32     | 32        | 32      | 32       | 32       | 224          |
| HBR per night   | 0.69 | 3.84      | 3      | 3.5       | 5.03    | 3.56     | 2.38     | 3.14         |
| Total <i>An. gambiae</i> s.l. tested by CSP   | 18   | 78        | 77     | 24        | 58      | 23       | 26       | 304          |
| Sporozoites rate (%)  | 0    | 1.3       | 1.3    | 4.2       | 5.2     | 0        | 0        | 1.7          |
| EIR p/night   | 0    | 0.05      | 0.039  | 0.147     | 0.262   | 0        | 0        | 0.071 (mean) |
| EIR p/month*  | 0    | 1.5       | 1.17   | 4.41      | 7.85    | 0        | 0        | 2.13 (mean)  |
| Dano 5-month EIR post-IRS August-December 2012 = 13 infectious bites per person     |      |           |        |           |         |          |          |              |
| Diebougou (sprayed area)  |      |           |        |           |         |          |          |              |
| Total <i>An. gambiae</i> s.l. (HLC) collected                                       | 5    | 15        | 92     | 56        | 64      | 64       | 72       | 368          |
| CDC trap-nights<br>(indoors + outdoors)   | 32   | 32        | 32     | 32        | 32      | 32       | 32       | 224          |
| HBR per night   | 0.16 | 0.47      | 2.88   | 1.75      | 2       | 2        | 2.25     | 1.64         |
| Total <i>An. gambiae</i> s.l. tested by CSP   | 8    | 11        | 50     | 44        | 43      | 37       | 16       | 209          |
| Sporozoites rate (%)  | 0    | 9.1       | 0      | 0         | 2.3     | 2.7      | 0        | 2            |
| EIR p/night   | 0    | 0.043     | 0      | 0         | 0.046   | 0.054    | 0        | 0.02 (mean)  |
| EIR p/month*  | 0    | 1.2796875 | 0      | 0         | 1.38    | 1.62     | 0        | 0.61 (mean)  |
| Diebougou 5-month EIR post-IRS August-December 2012 = 3 infectious bites per person |      |           |        |           |         |          |          |              |

In the period post-IRS (August to December) the mean indoor biting rate per person per night (bpn) was significantly different for *An. gambiae* s.l. females between the two areas, with the highest mean biting rate observed in the unsprayed sites (mean = 6.55 bites per person per night from August to December) compared to sprayed sites (mean = 3.18 bites per person per night). A significant difference was observed ( $P = 0.015$ ).

The peak from indoor biting density of *An. gambiae* sl occurred in August with about 40 bites/person/night in Dano (unsprayed) and decreased progressively to December, when it was less than 5 b/p/n at the end of the rainy season (Fig. 3A). The same pattern was observed in the intervention area but with less than 15 *An. gambiae* sl bites per person per night. The human biting rate and mean number of *An. gambiae* sl per house from indoor collections (CDC LT and PSC) in sprayed sites was half a time lower compared to unsprayed sites (Odds ratio<sub>(CDC LT)</sub> = 0.51, 95% CI= [0.34–0.67] with  $P = 0.001$  and Odds ratio<sub>(PSC)</sub> = 0.30, 95% CI= [0.21–0.43]. Outdoor biting rates were particularly low in both sites, with a mean of < 3 bites per person per night (Fig. 3B). But, the exposure to mosquito bites outdoors was slightly, but more increased in Diebougou (sprayed area) after treatment compared to Dano, the unsprayed area but the difference was not significant ( $P > 0.05$ ).

Similar results were observed in *An. gambiae* s.l. and *An. funestus* ss biting rates (Fig. 4) in PSC collection (Fig. 5B) with a mean biting rate of 2.00 bpn in Diebougou compared with 4.00 in Dano during the post-spraying period August-December (Odds ratio<sub>(CDC LT indoors)</sub> = 0.28, 95% CI= [0.11–0.35] with  $P = 0.035$  (Table 3).

## Plasmodium falciparum circumsporozoite and entomological inoculation rates (EIR) in *An. gambiae* sl and *An. funestus* ss

The results of CSP-ELISA assays and entomological inoculation rate of *An. gambiae* s.l. and *An. funestus* ss are presented in Table 2 and 3 respectively. Overall, 2051 *An. gambiae* sl and 1072 *An. funestus* ss specimens were screened for the circumsporozoite protein from June to December 2012 in the two areas. The sporozoites and entomological inoculation rate (EIR) were calculated by grouping indoor and outdoor collections of *An. gambiae* s.l. and *An. funestus* ss due to low number sampled in indoor and outdoor collection (Additional file 2: Table S1; Additional file 3: Table S2). So, during the post-IRS period (August-December), the mean CSP rate differed between unsprayed and sprayed areas for CDC LT method. The average sporozoites rates were significantly different (more than 2-fold) between the unsprayed areas (average sporozoite rate = 15.82%; 95% CI: [8.94–23.49]) and the sprayed areas (average sporozoites rate = 6.05%; 95% CI: [3.509–12.59]) ( $t = 2.475$ ;  $df = 9$  with  $P = 0.022$ ) (Table 2). The highest sporozoites rates were observed in Dano in August (average sporozoite rate = 13.6%; 95% CI: [9.68–17.33]) and September reaching an average of 14.2%. The similar trends were also observed in *An. funestus* ss

sporozoites rate (Table 3) but in lowest proportions (average sporozoite rate = 2.14%; with 95% CI: [0.25–3.15] in unsprayed areas and average sporozoites rate = 1% with 95% CI: [0.33–1.8] in sprayed area) with a significant difference ( $P = 0.035$ ).

The major contributor to the EIR, both in the control and intervention areas, was *An. gambiae* s.l. (70%). The indoor EIR reached 134 infective bites/person during the five-month post-IRS in the unsprayed area Dano. IRS appears to have reduced the EIR four-fold in the sprayed area (31 infective bites /person) after spraying with  $P = 0.0001$ . *An. funestus* ss contributed also to the transmission in the two areas, with the similar results (EIR reduced 4-fold in sprayed area) compared to *An. gambiae* sl after spraying (mean EIR in Dano = 13 bi/p/n vs mean EIR in Diebougou = 3 bi/p/n with  $P = 0.003$ ).

## **An. gambiae sl and An. funestus ss blood meal sources**

The results presented in Figs. 8 and 9 include data from indoor and outdoor CDC collections and indoor PSC collections, for *An. gambiae* sl and *Anopheles funestus* ss from the two areas. Irrespective of the sampling month, the proportion of *An. gambiae* sl blood-fed on human was highest, reaching more than 80% of the total of 335 females analysed, both in the sprayed and unsprayed areas. No female was recorded blood-fed only on animals. For *An. funestus* ss the feeding patterns were quite different, especially in the sprayed area (Diebougou) where females showed a large range of hosts. Out of 132 females analysed for their blood-fed origin, about 20–40% of *An. funestus* ss females had taken a mixed blood meal (human, bovine, and goat). A potential effect of the IRS on *An. funestus* ss is that the proportion of human blood meals decreased being replaced by animal and mixed blood meals.

## **Discussion**

The study showed that two species *An. gambiae* s.l. and *An. funestus* ss are predominant vectors of malaria transmission in study areas [28] collected by CDC light trap and pyrethrum spray catches. The results also indicated that the *An. gambiae* sl entomological inoculation rate was 4-fold lower in sprayed area compared to the unsprayed area, after the implementation of the IRS primarily due to a lower indoor biting rate and a significant decrease of malaria vectors sporozoite rates. However, the indoor resting density of vectors declined in the sprayed houses following IRS, but increased slightly in October, probably due to the relatively short residual duration of bendiocarb indicated in a separate manuscript. This drastic drop could be also due to the lethal effect of bendiocarb on the anophelines resistant to pyrethroids [40]. The biting rates observed outdoors were slightly higher in intervention areas compared to control areas and may be an early sign of biting behavior change. In conclusion IRS did not reduce the endophily behaviour from that of the baseline but had significantly reduced the density of mosquitoes resting indoors in sprayed area compared to the unsprayed area. In addition, EIR might have been impacted by the observed outdoor biting behaviour, and the reduced residual efficacy of the insecticide after September. The overall indoor biting rate of *An. funestus* ss was twofold greater in the unsprayed area. Indeed, the susceptibility status, taxonomy, and the role of *An. funestus* in malaria transmission was well documented in this area at west (Lena) and southwestern Burkina Faso (Soumouso) [44].

The use of vector control tools and behaviors of the host would be the main factors that modify the behavior of human blood feeding observed on *An. gambiae* s.l.. Indeed, recent studies [41, 42] showed that the long-term indoor application of residual insecticides contributes towards an increased tendency for outdoor feeding among malaria vector populations. The treatment had a great positive impact within *An. funestus* decreasing the human host-seeking in interventions sites compared to control sites. It is probably due to behavior change that this species is opting to go outside to seek a bloodmeal. It is important to highlight the exophagic host seeking activities and the exophilic behaviour that resulted in the search for blood in animals and mixed meals analyzed which more developed by *An. funestus* in the intervention area than in the control area where this vector remains mainly endophagic and endophilic. Indeed, after treatment there is less of *An. funestus* collected in intervention area compared to control area. Moreover, the exophagic host seeking recorded in intervention area was more pronounced than that obtained with *An. gambiae* s.l. Gillies and De Meillon [36] stated "...*Funestus* shows a closer adaptation to human dwellings than any other African anopheline. In many areas it spends the greater part of its adult life in houses, which has made it one of the most vulnerable of species to attack with residual insecticides". This statement is clearly valid in this case. Such a clear response to indoor insecticides makes the emergence of insecticide resistance in this species all the more likely [43]. Moreover, the results of impact of IRS on malaria transmission by *An. gambiae* s.l. indicated that the transmission was lower compared to the control area, after the implementation of the IRS, where the biting rate of *Anopheles* dropped drastically. This drastic drop is due to the lethal effect of bendiocarb on the anophelines resistant to pyrethroids [40] even though such efficacy did not last more than three months (discussed in a separate manuscript). The biting rates observed in outdoors were higher in intervention areas compared to control areas assuming a less pronounced behavior change of vectors.

In conclusion IRS did not reduce the endophily behavior from that of the baseline though it had significantly reduced the density of mosquitoes resting indoors compared to the control area. In addition, the findings have also shown *An. gambiae* s.l. were particularly anthropophilic in the two areas with few cases of mixed blood meals and no pure animal blood meals identified. This feeding pattern was the inverse for *An. funestus* in the intervention area where more females showed a large plasticity of the host range (zoo-anthropophilic). The results have shown that *An. gambiae* s.s. S-form was the major malaria vector species biting in the southwestern region in Burkina Faso. This corroborates previous reports [28–29] of the anopheline distribution in Burkina Faso, which explained the abundance of the S molecular form by the ecological characteristics of this region.

## **Conclusion**

The pilot study of IRS with bendiocarb appeared to have a significant impact on malaria transmission in the sprayed areas, as measured by EIR. Indeed, the results illustrated that IRS was strong enough to reduce mosquito abundance, sporozoite rate, and EIR in pyrethroids resistance areas. However, the baseline period indicated intrinsic differences in biting rates between Dano and Diebougou before IRS.

The findings also showed a change in vector behavior, with *An. funestus* becoming more zoophagic after IRS. Furthermore, the residual efficacy of IRS did not last more than three months. In areas of high transmission, other insecticides with a longer life span covering the malaria transmission season need to be

explored, in combination with LLINs.

## Declarations

### Availability of data and materials

All relevant data and material are available upon request.

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### Authors' contributions

KRD designed the study and drafted the manuscript. ASH, DDS and SPS participated in the field study, the sample collection, the laboratory and data analysis and the drafting of the manuscript; DDS and ASH participated in the data analysis; SP and DDS participated in the laboratory analysis. ASH and RKD participated in the study design. MN, SP, GAO and AD corrected the first draft of the manuscript and participated to data analysis. All authors read and approved the final version. KDR is guarantor of the paper.

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### Ethics approval and consent to participate

Ethical approval for this study was granted by the Ethical Committee of the Ministry of Health in Burkina Faso. The mosquito collectors gave prior informed consent and they were vaccinated against yellow fever. They were also subjected to regular medical check-ups with preventive treatments of malaria.

### Consent for publication

Not applicable.

### Competing interests

The authors declare that they have no competing interests.

## Supporting Information

**Additional file 1 : Figure S1.** A) overall density of mosquitoes per species and B) proportion of species composition from June to December 2012 in unsprayed and sprayed

**Additional file 2: Table S1.** Infection rate for *P. falciparum* calculated by circumsporozoite protein (CSP) ELISA from the head and thoraxes of *An. funestus*

**Additional file 3: Table S2.** Infection rate for *P. falciparum* calculated by circumsporozoite protein (CSP) ELISA from the head and thoraxes of *An. gambiae* sl

## References

1. World Health Organization. *Global technical strategy for malaria 2016-2030*. World Health Organization, 2015.
2. Okumu FO, Moore SJ. Combining indoor residual spraying and insecticide-treated nets for malaria control in Africa: a review of possible outcomes and an outline of suggestions for the future. *Malar J.* 2011; 10:208.
3. Lengeler C. Insecticide-treated bed nets and curtains for preventing malaria. *Cochrane Database Syst Rev.* 2004;2:CD000363.
4. Pluess B, Tanser FC, Lengeler C, Sharp BL. Indoor residual spraying for preventing malaria. *Cochrane Database Syst Rev.* 2010;4:CD006657.

5. Alonso PL, Lindsay SW, Armstrong JR, Conteh M, Hill AG, David PH, et al. The effect of insecticide-treated bed nets on mortality of Gambian children. *Lancet*. 1991;337:1499–02.
6. Alonso PL, Lindsay SW, Armstrong Schellenberg JR, Keita K, Gomez P, Shenton FC, et al. A malaria control trial using insecticide-treated bed nets and targeted chemoprophylaxis in a rural area of The Gambia, West Africa.
7. The impact of the interventions on mortality and morbidity from malaria. *Trans R Soc Trop Med Hyg*. 1993;2:37–44.
8. D'Alessandro U, Olaleye BO, McGuire W, Langerock P, Bennett S, Aikins MK, et al. Mortality and morbidity from malaria in Gambian children after introduction of an impregnated bednet programme. *Lancet*. 1995;345:479–83.
9. Binka FN, Kubaje A, Adjuik M, Williams LA, Lengeler C, Maude GH, et al. Impact of permethrin impregnated bednets on child mortality in Kassena-Nankana district, Ghana: a randomized controlled trial. *Trop Med Int Health*. 1996;1:147–54.
10. Reimer LJ, Tripet F, Slotman M, Spielman A, Fondjo E, Lanzaro GC: An unusual distribution of the kdr gene among populations of *Anopheles gambiae* on the island of Bioko, Equatorial Guinea. *Insect Mol Biol* 2005, 14:683–688.
11. Protopopoff N, Verhaeghen K, Van Bortel W, Roelants P, Marcotty T, Baza D, D'Alessandro U, Coosemans M: A high increase in kdr in *Anopheles gambiae* is associated with an intensive vector control intervention in Burundi highlands. *Trop Med Int Health* 2008, 13:1479–1487.
12. Zaim M, Guillet P: Alternative insecticides: an urgent need. *Trends Parasitol* 2002, 18:161–163.
13. Kolaczinski, K., Kolaczinski, J., Kilian, A., & Meek, S. Extension of indoor residual spraying for malaria control into high transmission settings in Africa. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, 2007 : 101(9), 852-853.
14. World Health Organization World Malaria Report 2009. Geneva : World Health Organization (2009);
15. World Health Organization. Global Malaria Programme : Indoor Residual Spraying—Use of indoor residual spraying for scaling up global malaria control and elimination: WHO Position Statement. *Geneva*, 2006.
16. Ranson H, Abdallah H, Badolo A, Guelbeogo WM, Keraf-Hinzoumbe C, Yangalbe-Kalnone E, et al. Insecticide resistance in *Anopheles gambiae*: data from the first year of a multi-country study highlight the extent of the problem. *Malar J*. 2009 : 8:299.
17. Sungvornyothin S, Chareonviriyaphap T, Prabaripai A, Trirakhupt V, Ratanatham S, Bangs MJ: Effects of nutritional and physiological status on behavioral avoidance of *Anopheles minimus* (Diptera: Culicidae) to DDT, deltamethrin and lambda-cyhalothrin. *J Vector Ecol* 2001, 26:202–215.
18. Molineaux, L., Gramiccia, G., & World Health Organization. Le projet Garki: recherches sur l'épidémiologie du paludisme et la lutte antipaludique dans la savane soudanienne de l'Afrique occidentale, 1980.
19. Payne D, Grab B, Fontaine RE, Hempel JH: Impact of control measures on malaria transmission and general mortality. *Bull World Health Organ* 1976, 54:369–77.
20. Mutuku FM, King CH, Mungai P, Mbogo C, Mwangangi J, Muchiri EM, Walker ED, Kitron U: Impact of insecticide-treated bed nets on malaria transmission indices on the south coast of Kenya. *Malaria J* 2011, 10:356.
21. Institut national de la statistique et de la démographie. Annuaire statistique 2018. Ministère de l'économie, des finances et du développement. 2018. [http://www.insd.bf/n/content/pub\\_periodiques/annuaires\\_stat/Annuaire\\_stat\\_nationaux\\_BF/Annuaire\\_Statistique\\_National\\_2018.pdf](http://www.insd.bf/n/content/pub_periodiques/annuaires_stat/Annuaire_stat_nationaux_BF/Annuaire_Statistique_National_2018.pdf)
22. WHO. World Malaria Report, 2012. Geneva: World Health Organization.
23. Diabaté A., Baldet T., Chandre F., Akogbeto M., Darriet F., Brengues C., Guiguemdé T.R., Guillet P., Hemingway J., Hougard J.M. The role of agricultural use of insecticides in resistance to pyrethroids in *Anopheles gambiae* s.l. in Burkina Faso. *Am J Trop Med Hyg*. 2002: 67; 617-622.
24. Diabaté A., Baldet T., Chandre F., Dabiré K.R., Kengne P, Simard F., Guiguemdé T.R., Guillet P, Hemingway J., Hougard J.M. Kdr mutation, genetic marker to assess events of introgression between the molecular M and S forms of *An. Gambiae* (Diptera: Culicidae) in the tropical savannah area of West Africa. *J. Med. Entomol.*2003 :40(2) ; 195-198.
25. Diabate A., Brengues C., Baldet T., Dabiré K.R., Hougard J.M., Akogbeto M., Kengne P, Simard F., Guillet P, Hemingway J., Chandre F. The spread of the Leu-Phe kdr mutation through *Anopheles gambiae* complex in Burkina Faso : genetic introgression and de novo phenomena. *Trop Med Int Health*. 2004 :9; 1267-1273.
26. Dabiré K.R., Diabaté A., Djogbenou L., Ouari A., N'Guessan R., Ouédraogo J.B., Hougard J-M., Chandre F., Baldet T.. Dynamics of multiple insecticide resistance in the malaria vector *Anopheles gambiae* in a rice growing area in South-Western Burkina Faso. *Malar J* 2008 :7 ; 188.
27. Dabiré K.R., Diabaté A., Namountougou M., Toe K.H., Ouari A., Kengne P, Bass C., Baldet T. Distribution of pyrethroid and DDT resistance and the L1014F kdr mutation in *Anopheles gambiae* s.l. from Burkina Faso (West Africa). *Trans R Soc Trop Med Hyg*, 2009 : 103 ; 1113-1120.
28. Dabiré, K. R., Diabaté, A., Namountougou, M., Djogbenou, L., Wondji, C., Chandre, F., ... & Baldet, T. Trends in insecticide resistance in natural populations of malaria vectors in Burkina Faso, West Africa : 10 years' surveys. In *Insecticides-Pest Engineering :2012*. InTech
29. Namountougou, M., Simard, F., Baldet, T., Diabaté, A., Ouédraogo, J. B., Martin, T., & Dabiré, R. K. Multiple insecticide resistance in *Anopheles gambiae* sl populations from Burkina Faso, West Africa. *PLoS One* 2012 : 7(11) ; e48412.
30. Feachem RGA, Phillips AA, Hwang J, Cotter C, Wielgosz B, Greenwood BM, Sabot O, Rodriguez MH, Abeyasinghe RR, Ghebreyesus TA, Snow RW: Shrinking the malaria map: progress and prospects. *Lancet* 2010, 376 :1566–1578.
31. WHO, 2015. WHO recommended insecticides for indoor residual spraying against malaria vectors. [http://www.who.int/whopes/Insecticides\\_IRS\\_2\\_March\\_2015.pdf?ua=1](http://www.who.int/whopes/Insecticides_IRS_2_March_2015.pdf?ua=1). Accessed 04/16/2017.
32. WHO, 2013. Report of the Sixteenth WHOPES working group meeting. Geneva: [who.int/iris/bitstream/10665/90976/1/9789241506333\\_eng.pdf](http://www.who.int/iris/bitstream/10665/90976/1/9789241506333_eng.pdf). Accessed 09/09/2018.

33. Lines, J. D., Curtis, C. F., Wilkes, T. J., & Njunwa, K. J. Monitoring human-biting mosquitoes (Diptera: Culicidae) in Tanzania with light-traps hung beside mosquito nets. *Bulletin of entomological research*. 1991: *81*(1), 77-84.
34. Costantini, C., Sagnon, N. F., Sanogo, E., Merzagora, L., & Coluzzi, M. Relationship to human biting collections and influence of light and bednet in CDC light-trap catches of West African malaria vectors. *Bulletin of Entomological Research*. 1998 : *88*(5) ; 503-511.
35. Kilama, M., Smith, D. L., Hutchinson, R., Kigozi, R., Yeka, A., Lavoy, G., ... & Greenhouse, B. Estimating the annual entomological inoculation rate for *Plasmodium falciparum* transmitted by *Anopheles gambiae* s1 using three sampling methods in three sites in Uganda. *Malaria J*. 2014 : *13*(1) ; 111.
36. Gillies MT, De Meillon B: *The Anophelinae of Africa south of the Sahara*. Johannesburg: Publication of the South African Institute for Medical Research; 1968:54.
37. Scott JA, Brogdon WG, Collins FH. Identification of single specimens of *Anopheles gambiae* complex by polymerase chain reaction. *AmJTrop Med Hyg*. 1993 :49 ;520-529.
38. Wirtz, R. A., Burkot, T. R., Graves, P. M., & Andre, R. G. Field evaluation of enzyme-linked immunosorbent assays for *Plasmodium falciparum* and *Plasmodium vivax* sporozoites in mosquitoes (Diptera: Culicidae) from Papua New Guinea. *Journal of medical entomology*. 1987 : *24*(4) ; 433-437.
39. Beier, J. C., Perkins, P. V., Wirtz, R. A., Koros, J., Diggs, D., Gargan, T. P., & Koech, D. K. Bloodmeal identification by direct enzyme-linked immunosorbent assay (ELISA), tested on *Anopheles* (Diptera: *Culicidae*) in Kenya. *Journal of medical entomology*. 1988 : *25*(1) ; 9-16.
40. Akogbéto, M. C., Padonou, G. G., Gbénou, D., Irish, S., & Yadouleton, A. Bendiocarb, a potential alternative against pyrethroid resistant *Anopheles gambiae* in Benin, West Africa. *Malaria J*. 2010 : *9*(1) ; 204.
41. Reddy, M. R., Overgaard, H. J., Abaga, S., Reddy, V. P., Caccone, A., Kiszewski, A. E., & Slotman, M. A. Outdoor host seeking behaviour of *Anopheles gambiae* mosquitoes following initiation of malaria vector control on Bioko Island, Equatorial Guinea. *Malaria J*. 2011 : *10*(1) ; 184.
42. Russell, T. L., Govella, N. J., Azizi, S., Drakeley, C. J., Kachur, S. P., & Killeen, G. F. Increased proportions of outdoor feeding among residual malaria vector populations following increased use of insecticide-treated nets in rural Tanzania. *Malaria J*. 2011 : *10*(1) ; 80.
43. Casimiro, S., Coleman, M., Mohloai, P., Hemingway, J., & Sharp, B. Insecticide resistance in *Anopheles funestus* (Diptera: *culicidae*) from Mozambique. *Journal of medical entomology* 2006 : *43*(2) ; 267-275.
44. Dabire, K. R., Baldet, T., Diabaté, A., Dia, I., Costantini, C., Cohuet, A., ... & Fontenille, D. (2007). *Anopheles funestus* (Diptera: Culicidae) in a humid savannah area of western Burkina Faso: bionomics, insecticide resistance status, and role in malaria transmission. *Journal of medical entomology*, *44*(6), 990-997.

## Figures

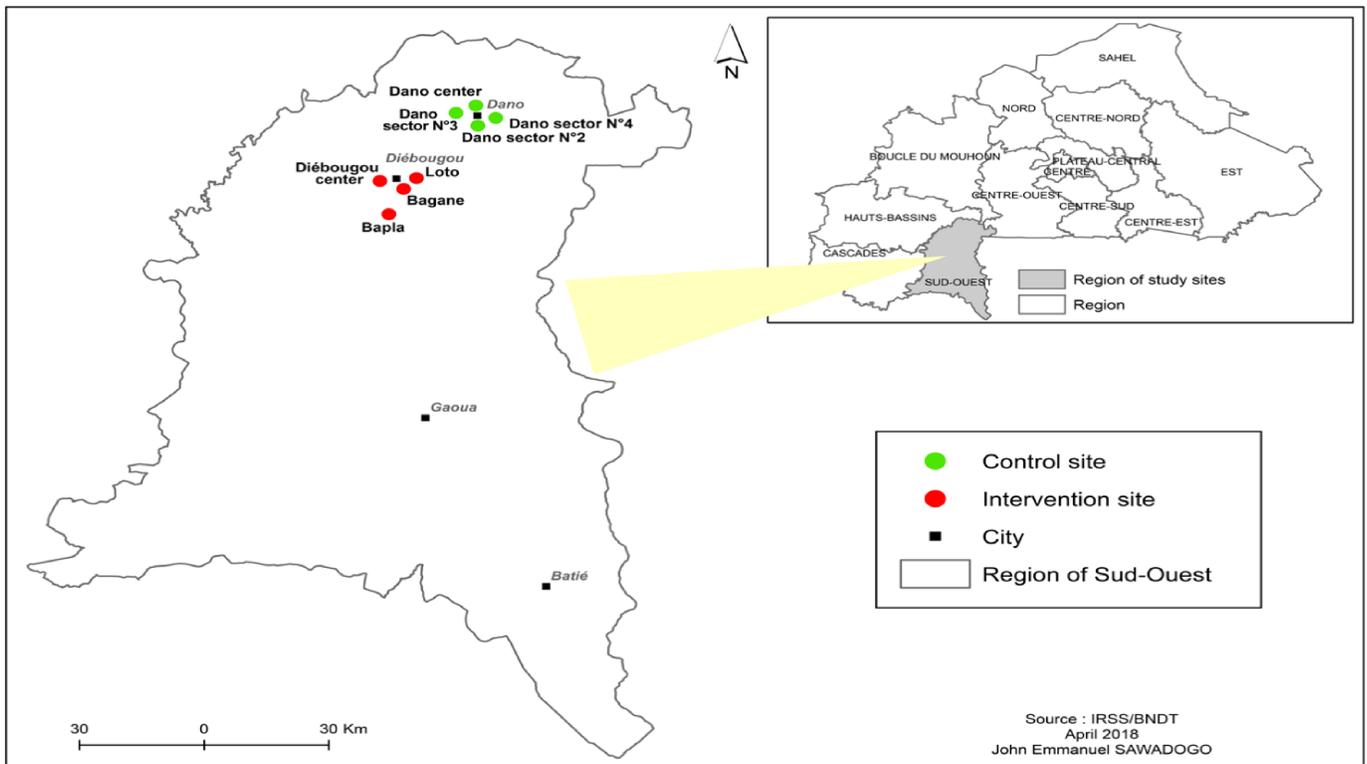


Figure 1

Location of study sites

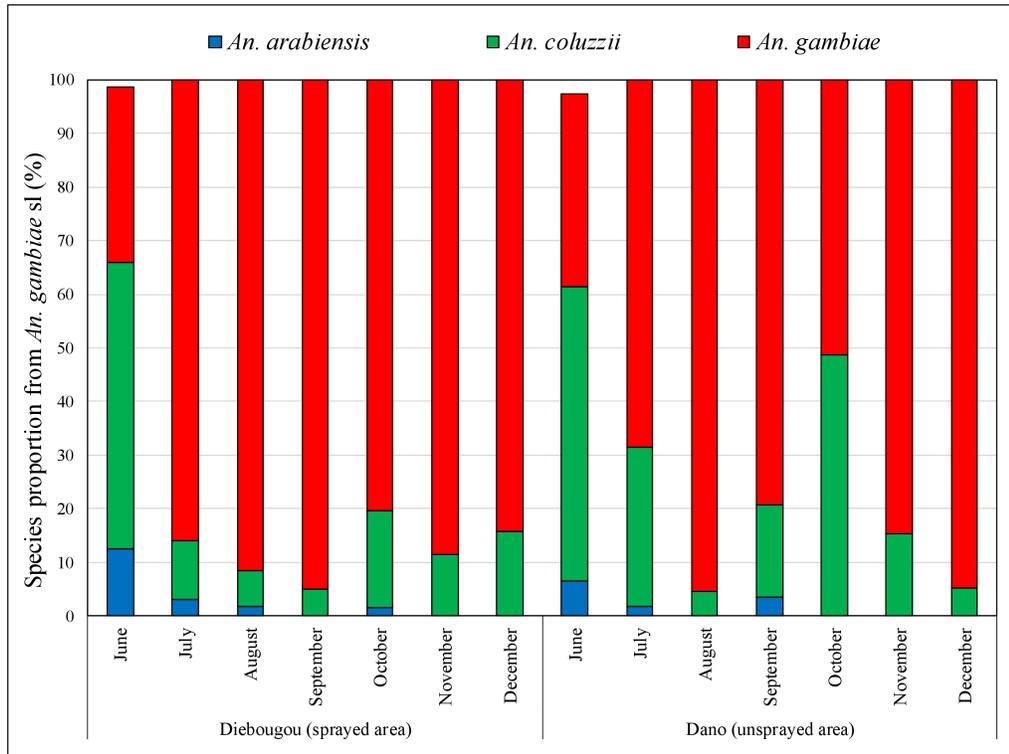


Figure 2

Species composition within the *An. gambiae* complex collected by CDC light-traps in sprayed (Diebougou) and unsprayed areas (Dano).

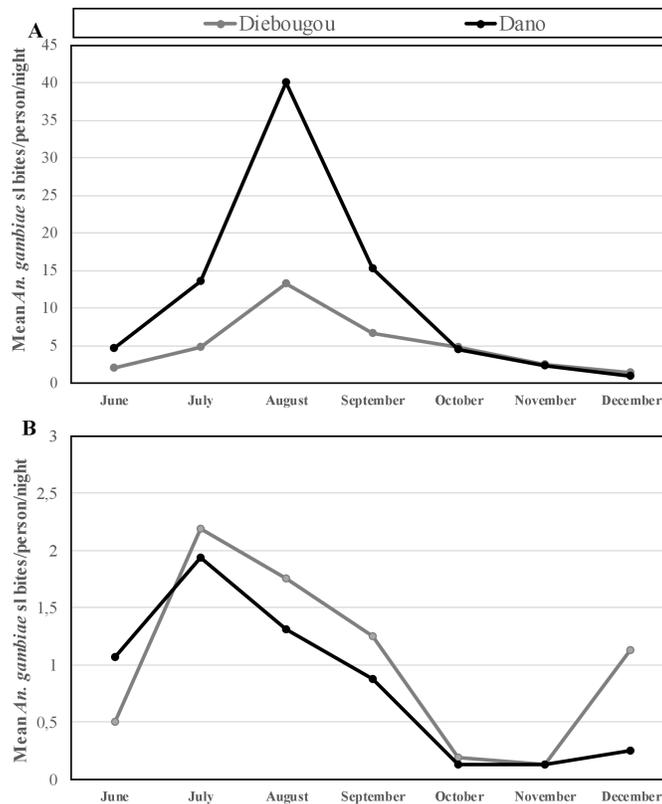
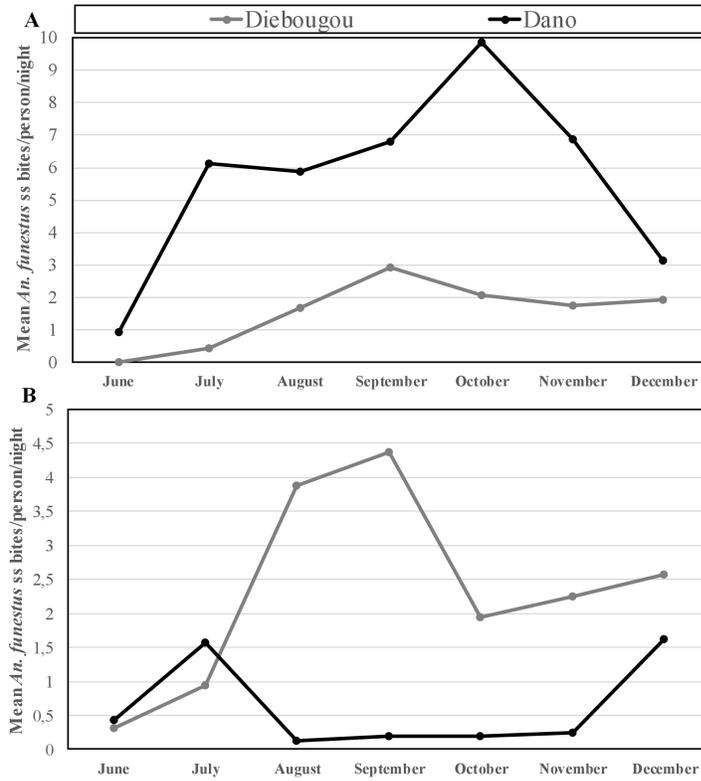
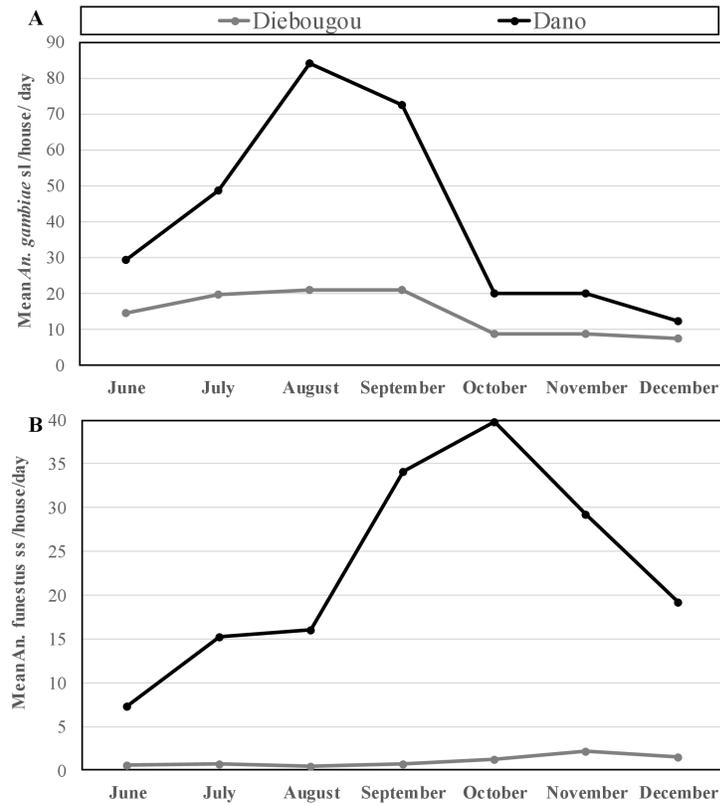


Figure 3

Mean *An. gambiae* sl bites per person per night collected by CDC light-traps in sprayed (Diebouougou) and unsprayed areas (Dano) in A) Indoor collection and B) Outdoor collections before and after spraying

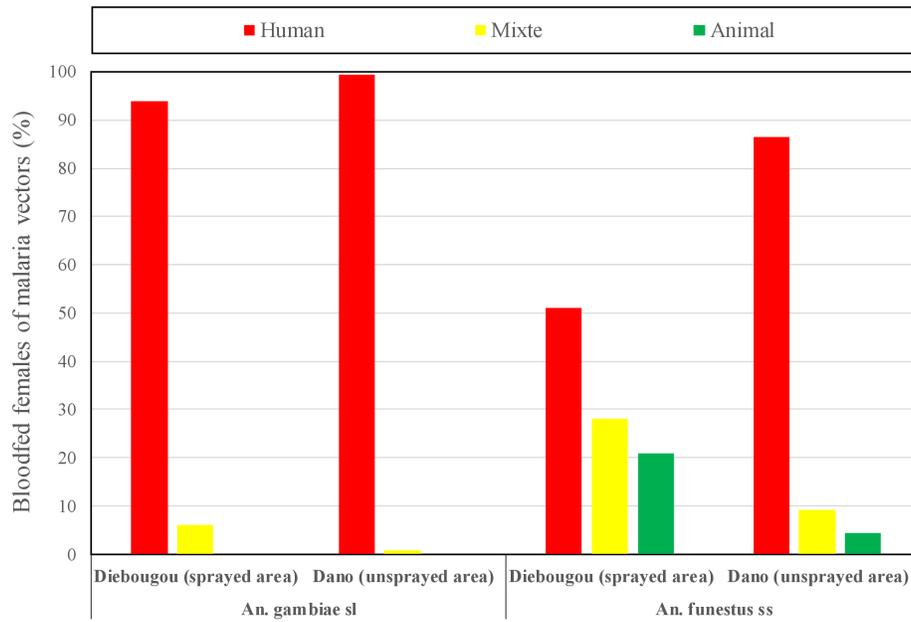


**Figure 4**  
Mean *An. funestus* ss bites per person per night collected CDC light-traps in sprayed (Diebouougou) and unsprayed areas (Dano) in A) Indoor collection and B) Outdoor collections before and after spraying.



**Figure 5**

Mean number of mosquitoes /houses from indoor PSC collection in sprayed (Diebougou) and unsprayed areas (Dano) in A) *An. gambiae* sl and B) *An. funestus* ss before and after spraying



**Figure 6**

Proportion of *An. gambiae* sl and *An. funestus* ss blood-fed on humans, animals or mixed from sprayed area (Diebougou) and unsprayed area (Dano).

## Supplementary Files

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