C.R.E - COTONOU

Centre de Recherche Entomologique de Cotonou - 06 BP 2604 – Tél / Fax (229) 21 33 08 25
COTONOU (Rép. of Benin) E-mail: akogbetom@yahoo.fr

U.S. PRESIDENT'S MALARIA INITIATIVE ACTION TO REINFORCE MALARIA VECTOR CONTROL IN BENIN

PMI/ABT/IRS OR ACTIVITIES IN BENIN

Entomological Monitoring-Evaluation in the districts of Atacora 6 months after the Indoor Residual Spraying, Benin, West Africa.

Doc/CREC/PMI/Abt/April-November 2012

1. Introduction

Malaria is one of the most critical public-health challenges in Benin. In 2010, malaria accounted for 44.5% of recorded outpatient consultations, 32.2% of all hospital admissions and 36.7% of deaths for children under five. This is why malaria control in Benin and in most African countries is given high priority at both national and international levels. Malaria is not only a major public health problem, but an issue that severely restrains the economic growth of Sub-Sahara Africa (The Abuja Declaration on Roll Back Malaria in Africa 2000). Vector control, good case management, education and communication are the main tools used to fight the disease.

In Benin, Indoor Residual Spraying (IRS) is included in Benin's 2011–2015 national strategic plan for malaria control, and represents one of the main approaches for Benin's malaria prevention strategy. With the support of the Benin Ministry of Health, and the National Malaria Control Program (NMCP), the President's Malaria Initiative (PMI) has led the implementation of IRS to lower malaria transmission in four districts in the Oueme department in southern Benin (from 2008-2010), and all districts within the Atacora department in northern Benin from 2011.

Entomological monitoring is a key component of IRS programming, helping to evaluate the impact of IRS on vector density and, behavior, and identifying species composition in IRS spray areas. Entomological monitoring also assesses the quality of IRS spray operations, and measures the residual life of the insecticide applied, and thereby monitors vector resistance to insecticides.

In 2012, PMI through the Africa Indoor Residual Spray (AIRS) project (implemented by Abt Associates) continues to support entomological surveillance in the Atacora Department, to examine the impact and quality of the 2012 IRS campaign. Recent entomological study results have shown that vector resistance is increasing to carbamates in Benin. The current results from entomological monitoring will help AIRS, PMI and the NMCP determine if carbamates should be used in future IRS campaigns, or if alternative insecticides should be used.

The Entomological Research Center of Cotonou (Centre de Recherche Entomologique de Cotonou) (CREC), an affiliate of the University of Benin, was subcontracted by Abt Associates from April-October, 2012, to complete entomological surveys, vector surveillance, assessment of spraying performance, and provide recommendations for insecticide selection on behalf of the IRS programming supported by the MOH and PMI. Vector resistance surveillance is an important issue. As a matter of fact, vector resistance to many of the insecticides is a growing problem in Benin. In Benin, and particularly in the department of Atacora, it has been reported that the main malaria vector, *Anopheles gambiae*, is resistant to DDT, pyrethroids and a suspected resistance was mentioned for bendiocarb, the current insecticide used for Indoor Residual Spraying. If bendiocarb resistance is verified, the search of alternative effective insecticides becomes increasingly urgent.

CREC's entomological surveillance activities will provide data and information about the following entomological indicators required by PMI and the MOH to evaluate IRS programming:

PMI Primary Indicators:

- Malaria Vector Species identification (species and molecular forms of *Anopheles gambiae s.s*)
- Vector distribution and seasonality (Vector density)
- Mosquito behavior: biting (endophagy or exophagy), vector feeding time and resting (endophily or exophily),
- Vector susceptibility and mechanisms under the resistance
- Quality assurance of IRS (decay of insecticide applied)

PMI secondary Indicators:

- Sporozoite rates, Entomological Inoculate Rate (EIR)
- Parity rates

2. Objectives of the Entomological Surveillance Work to be completed by CREC from April to October, 2012 are:

- Evaluate the effectiveness of various classes of insecticide on the vector susceptibility
- Assess the resting and biting behavior of the vector in IRS –targeted areas
- Evaluate the insecticide decay rates using Wall bioassay (cone test)

- Identify the mosquito species include molecular forms of *Anopheles gambiae s.s*
- Determine the sporozoite rate and the Entomological Inoculation Rate
- Monitor the density of vectors in IRS-targeted areas as compared to one control area
- Assess the entomological profile of malaria in 5 sentinel sites chosen along a transect South-North in Benin (see the second report elaborated and separately sent to Abt).

3. Material and methods

3.1. Study areas

The entomological monitoring in Atacora was carried out in five districts covered by the 2012 IRS Campaign: Natitingou, Tanguieta, Kouande, Materi, and Pehunco, and one control district, Copargo which has similar geography, climate and malaria transmission seasons to Atacora, was selected as a control. All six districts were selected by the NMCP, with guidance from CREC, PMI and AIRS.

Additionally, all entomological monitoring occurs in two villages per district, with one village located in the center of the district, and one village located at the periphery.

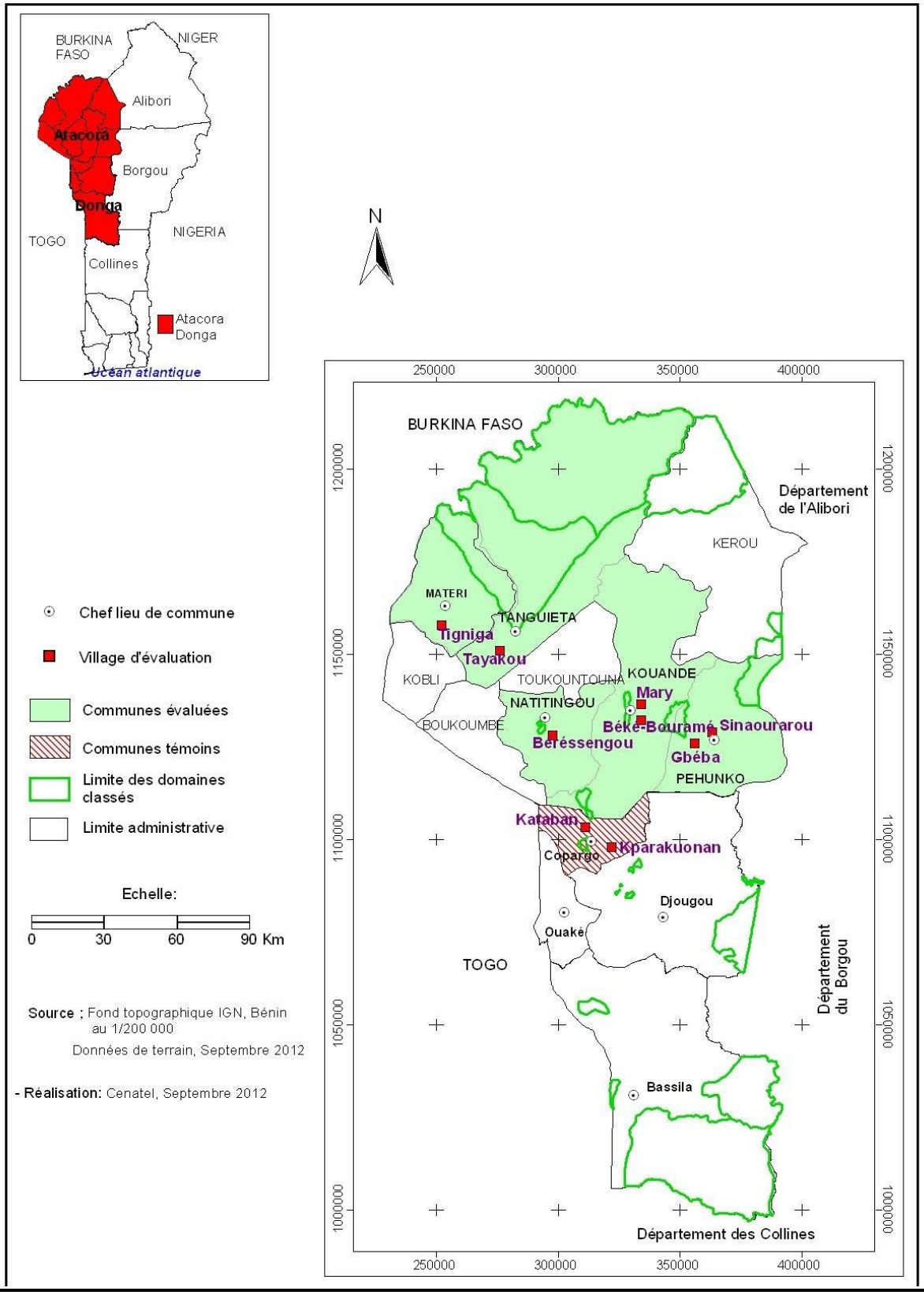


Figure 1: Map of the study area showing localities of mosquito sampling

3.2. Insecticide decay rates using Wall bioassay (cone test)

Insecticide decay evaluation was carried out in 2 districts: Tanguieta and Natitingou. In each district, bioassays were performed in 2 villages (Dondongou and N'dahonta in Tanguieta; Pouya and Dassagaté in Natitingou) and in 4 houses per village. In total, 16 houses were concerned from which 4 control houses

Two strains of mosquito larvae (*Anopheles gambiae* Kisumu, a susceptible colony from insectary and a wild strain of *An. gambiae* reared from larvae and pupae collected) were used for bioassay cone tests. The local wild strain of *An. gambiae* was collected from the 2 districts (Tanguieta and Natitingou).

Various treated walls (walls made with plaster cement, plaster and non plaster mud) were tested using the 2 strains. Bioassays were performed with in 24 hrs after spraying for the first time and monthly thereafter for three and four months after IRS. It was done according to the WHO procedures using test cones. Cones were placed on the walls treated with bendiocarb. About 10 females of *An. gambiae* of each strain were introduced per cone and exposed for 30 minutes. For each house, the cones are put at 4 locations: 0.5 m, 1 m, 1.5 m and 2 m. During the tests, the temperature and the relative humidity of exposure and holding periods were registered. The number of mosquitoes knocked down after 30 min and 60 min and dead after 24 hours (holding period), were registered. The number of the houses and GPS coordinates was taken

3.3. Insecticide resistance

A surveillance of the susceptibility of *An. gambiae s.l* to the various classes of insecticides was conducted. The main goal of this surveillance was to evaluate Atacora's local vector susceptibility to various classes of insecticide including bendiocarb, the insecticide currently used in Benin for IRS. There were accelerated production of cotton in Benin since 2 years, particularly in the 4 departments of the north include Atacora. This production of cotton is associated to the use of high quantity of insecticides which might speed up the selection of carbamate-resistance. Pirimiphos methyl was recommended by CREC as an insecticide of choice for IRS following by CREC's experimental huts study in 2011. The limitation was, the data was not supported by representative susceptibility study last year. This year we performed many susceptibility tests using WHO papers impregnated by various insecticides:

bendiocarb (carbamate), pirimiphos methyl and fenitrothion (organophosphate), deltamethrin (pyrethrinoid). Permethrin and DDT were not used due to the very high resistance developed by *An. gambiae* to these two insecticides and mentioned in many papers and reports. This report analyzes data obtained on the susceptibility of *An. gambiae* in Atacora from 2010 to 2012 to appreciate the evolution of bendiocarb resistance.

3.3.1. WHO susceptibility test

a). Mosquito collections.

Mosquitoes were collected during the rainy season (July-August) in districts under IRS in Atacora (Materi, Tanguieta, Kouande, Pehunco, Natitingou) Larvae and pupae were collected using the dipping on breeding sites and then kept in separated labelled bottles related to each locality. Larvae samples were reared up to adult emergence directly on the field for further bioassay tests. It was very difficult to find, in the districts under IRS, productive breeding sites

b). Insecticide susceptibility test

Female mosquitoes aged 2-5 days old were exposed to diagnostic doses of various insecticides for susceptibility tests using insecticide-impregnated papers, as described by the standard WHO testing protocol. The following insecticides were tested: deltamethrin (0.05%), permethrin (0.75%), DDT (4%) and bendiocarb (0.1%) and pirimiphos methyl (0.1%).
For each test, in areas where sufficient specimens of *An. gambiae s.l* were obtained, five test tubes were used: one untreated paper as a control and four treated papers to expose mosquitoes. Control tubes containing filter papers impregnated with silicon oil (insecticide carrier) only, whereas treated papers were impregnated with diagnostic doses of insecticide plus carrier.

Generally, twenty mosquitoes were introduced into each tube, but, in case of insufficiency of *An. gambiae*, the number tested was lower than the number recommended by WHO. Females of *An. gambiae s.l* used in this study were exposed for one hour to insecticide-treated papers and monitored at different time intervals (10, 15, 20, 30, 45, 60 minutes) to record the "knock-down" times. After one hour exposure, mosquitoes were transferred into holding tubes and provided with cotton wools wet with a 10% honey solution. Mortalities were recorded after 24 hours and the susceptibility status of the population was graded according to

the WHO recommended protocol. Dead and survived mosquitoes from this bioassay were separately kept on silicagel at -20ºC for molecular characterization.

3.3.2. Molecular characterization of Anopheles populations using PCR analysis: Species identification, Molecular forms determination, Kdr and Ace^{-1} detection.

In each locality, females of *An. gambiae* samples from the WHO Bioassays were analysed at the molecular level. PCR analysis for species identification was performed to identify various members of *An. gambiae* complex collected in each site. The next set of PCR focused on molecular forms using PCR-RFLP which involved only *An. gambiae s.s.* The PCR forms sub-grouped the *An. gambiae s.s*. into two molecular forms: *An. gambiae s.s. M* and *An. gambiae s.s. S* forms. The last series of PCRs determined the presence of *kdr* mutations in *An. gambiae ss*. populations as described by Martinez-Torres et *al*. The PCR-RFLP diagnostic test was used to detect the presence of G119S mutation (*Ace.1* gene) as described by Weill et al.

In addition to the *kdr* mutation which is the main mechanism of resistance to pyrethroids, this study also addressed *Ace.1* mutation that causes resistance to organophosphates and carbamates.

3.3.3. Metabolic resistance

While insecticide resistance associated with *kdr* is well studied at the physiological, behavioural and population level, much less is known about the enzymes associated with metabolic resistance. In Benin, there is a lack information on the metabolic resistance on *An. gambiae* populations. The present study aimed to provided information on biochemical analysis on mosquitoes populations to detect potential increase in mixed function oxidases (MFO), non-specific esterases (NSE) and glutathione S-transferases (GST) activity.

Larvae of *An. gambiae s.s.* were collected at the two sites and reared at CREC insectary for emergence and testing of adults. Emerging adults female mosquitoes (F_0) aged 6-8 days were fed with guinea pig blood and reared for emergence (F1). Adults 3-5 days were used for biochemical analysis. Sixty adults female F1 aged between 1-3 days from the two sites were used for biochemical based on the methods decribed by Penilla al (1998) to compare the levels of activity of mixed function oxidases (MFO), non-specific esterases (NSE) using α-

naphtyl acetate as a substrate and glutathione S-transferases (GST) in the *An. gambiae s.s.* susceptible Kisumu and the field populations from 2 districts under IRS: Pehunco and Tanguiéta. Individual mosquitoes were homogenized in 200 µl ml distilled water. Each of 10 ml of the homogenate was used for monooxygenase, glutathion S-transferase and protein assay. The other twenty µl ml of homogenate was used for esterases assay.

Glutathione -S-transferase (GST) assay

10 µl of each homogenate was transferred to a microplate well followed by 200 µl of the GSH/CDNB working solution which was prepared by adding 0.060g of glutathione solution(GSH) in 20 ml of Phospahte sodium buffer 0.1M and 0.013gr (in 1 ml of methanol) 1-chloro-2,4-dinitrobenzene (CDNB). The plates were read after 5 mins with the ELISA plate reader at a wave length of 340 nM. GST activity was expressed as: mMoles/ min / mg protein

Monooxygenase (Cytochrome p450) assay

10 µl of homogenate were placed in separate of microtitre plate followed by addition of 80 µl 0.625M potassium phosphate buffer (pH 7.2). Ten mg of 3,3,5'5', Tetramethyl Benzidine(TMBZ) in 5 ml methanol was prepared and a 15 ml of 0.25 M sodium acetate buffer(pH 5.0) was prepared. Two hundred µl of the above TMBZ solution was added in to each well followed by 25 µl of 3% hydrogen peroxide. The plate was read after 2 hours at 630 nm

Esterase assay

20 µl of homogenated were placed in separate wells of microtitre plate. 200 µl of 0.3 mM Alpha/Beta napthyl acetate were added to each well. Leave the plate at room temperature for 1 min and then added 50 µl of fast garnet. After 30 minutes, enzyme activity was determined as an OD value by microplate reader at 450 nm.

Protein assay

The total protein content of individual mosquitoes was determined using the Bio –Rad Protein Assay Kit (Bio -Rad Laboratories) in order to detect the differences in size among individuals that might require correction factors for the enzyme assays

Data analysis

Biochemical assay data (enzymatic activity per mg protein, levels of MFO, NSE, and GST between Kisumu and field populations *An. gambiae s.s.*) were compared using Mann-Whitney non-parametric U-test (Statistica software).

3.3.4. Data interpretation

The resistant status of mosquito samples was determined according to the WHO criteria (WHO, 1998):

97-100% mortality of *An. gambiae s.l* : population considered fully susceptible

80-97% mortality of *An. gambiae s.l* : suspicion of resistance

< 80% mortality of *An. gambiae s.l*: resistance.

Mortality rates were corrected using Abbott formula when control mortality was between 5% and 20%.

Molecular results (PCR *Kdr and Ace-1*) were compared to insecticide susceptibility tests performed with the WHO method to conclude on the *An. gambiae s.l* status of resistance in the districts surveyed.

4.4. Density of vectors, Resting and biting behavior, Mosquito species, sporozoite rate and Entomological Inoculation Rate in IRS areas and control area

4.4.1. Human Landing Catches and Mosquito Collection

In each of the villages selected for mosquito collection, two houses were chosen for human landing catche activities. Human landing catches were used to determine mosquitoes biting time, location of biting (inside and/or outside), and monitor changes in the behavior of mosquitoes after IRS spray occurs.

Adult mosquitoes were collected for 2 consecutive nights, every month, using human landing catches with one collector (human acting as a landing catch) placed indoor, and another collector placed outdoor in each of the houses used for mosquito collection. All Anopheles mosquitoes caught during the night collections were identified to species. All vector species that were collected except gravid and half-gravid mosquitoes were transported to a small field laboratory for dissection using a microscope, to determine the parous rates. The heads/thoraxes of the vector species were analyzed by ELISA method to look for CSP antigens. Abdomens of females of the vector species were used for PCR analyses, to identify sibling species and molecular forms.

4.4.2. Window Exit Trap Catches and Indoor Pyrethrum Spray Catches

The Window Exit Trap Catches and the Indoor Pyrethrum Spray Catches were completed to estimate the total density of mosquito species in the treated houses and the proportion of female mosquitoes exiting from the houses.

In the selected villages in the 5 study districts, four bedrooms from separate houses (not from houses that were used for human landing catches) were selected for window exit trap catches and indoor pyrethrum spray catches. This activity took place over two nights, with mosquito collection occurring in the morning. This activity occured twice per month.

The Window Exit Trap activity measures exophily induced by the presence of insecticide on treated walls. For this activity exit traps were positioned over the windows of each study bedroom for two nights per month. All mosquitoes escaping the study bedrooms via the windows were thereby trapped. Mosquito collections were completed the following morning, using a mouth aspirator. Mosquitoes that were still alive during the collection were transferred into plastic cups supplied with 10% honey solution, with mortality rate recorded after 24 hours holding period.

After window exit trap collections, Indoor Pyrethrum Spray Catches were conducted. The study bedrooms were sprayed with Pyrethrum (mixed with water) and a white canvas placed on the floor. After 10 minutes, all fallen mosquitoes were collected from the floor and placed in petri dishes, to measure the number of mosquitoes in the room, and develop data for endophily behavior.

5. Results

5.1. Insecticide decay rates using wall bioassay (cone test)

The first cone bioassay test aimed to assess the quality of spraying was done in selected houses 24 hours after spraying. Subsequent tests were done on a monthly basis to determine the persistence of the sprayed insecticide on the wall. For the bioassay cone tests, 2 strains of *An. gambiae* were used: the susceptible strain (Kisumu) and the local wild strain collected from Tanguieta and Natitingou. We have used 16 houses in the 4 villages for bioassay tests, 12 treated houses and 4 control houses. The number of mosquitoes knocked down after 30 min and 60 min and dead after 24 hours was registered (table I, figure 1 a and b below) in all

houses where the tests have been done. For the control, the percentage of dead mosquitoes at the end of the test was less than 5% for all the testes

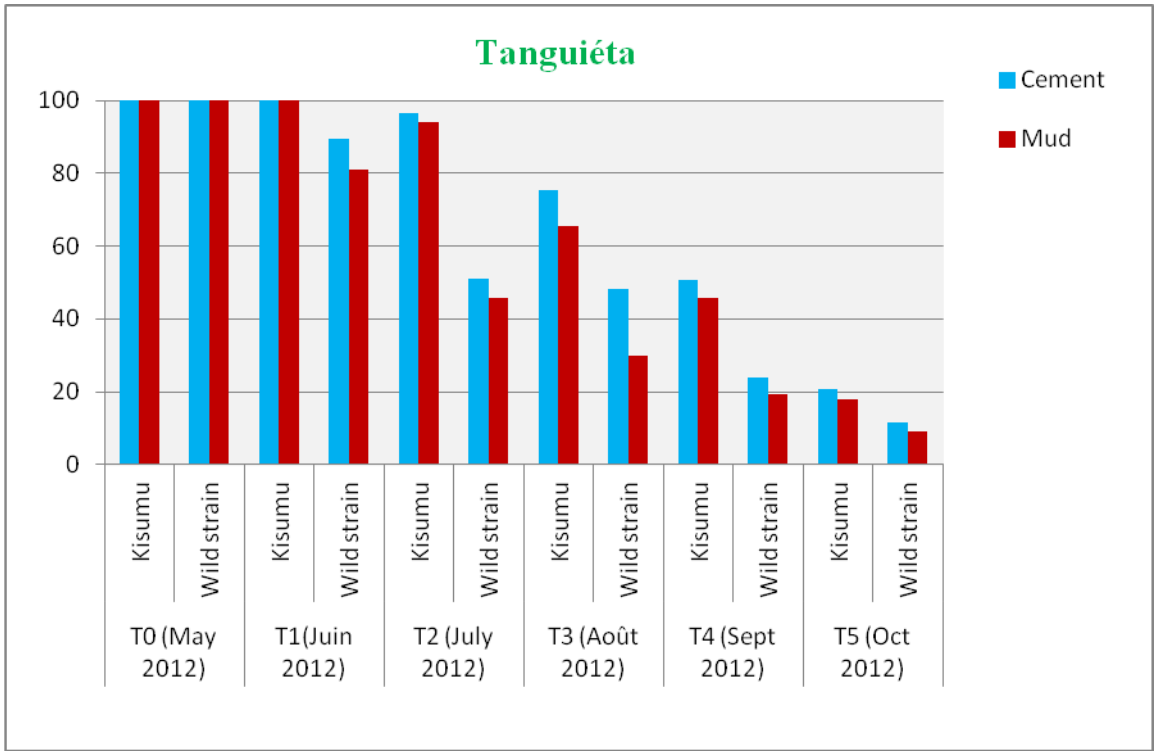
Figure 1 a (Tanguiéta) and b (Natitingou) shows the variability of bio-availability of bendiocarb on the walls after IRS. For the wild strain of *An. gambiae* collected from the field, 2 months after IRS, the mortality of mosquitoes exposed to cement walls has decreased from 100% at T0 to 84.75% in Tanguieta and 53.6% in Natitingou. Three months after IRS, only the half of mosquitoes exposed to treated walls were killed after the holding time. These low mortalities might be due to the resistant status of the local populations of *An. gambiae* s.l to bendiocarb. Vector mortality of the exposure mosquitoes was higher for susceptible colonies as compared to the wild mosquitoes. For example, two months after spraying exposure test mortality rates ranged between 94.2% to 96.55% in Tanguieta and 81.92% to 90.24% Natitingou. The mortality rates were higher on the cement walls as compared to the mud. Three months after IRS, the mortality rates are very low: 72.17-75.54% for Kisumu and 29.75-48.15% for the wild population on the cement walls (table I and fig-1). Five months after IRS, less than 20% of Kisumu *An. gambiae* and less than 10% of the wild populations from Tanguieta and Natitingou were killed after exposure to treated walls.

In conclusion, bendiocarb was effective on the wild and laboratory strains of *An. gambiae s.l* for 2 months in Atacora in terms of lethal effect. Three months after IRS, less than 80% of exposed both susceptible and wild mosquitoes died after 24hrs holding time.

Table I: Mortality rates of Kisumu, the susceptible strain of *An. gambiae,* and the wild *An. gambiae,* after 30 minutes exposure to bendiocarb in WHO cone test 5 months after IRS

		T0 (May 2012)		**T1 (Juin 2012)**		**T2 (July 2012)**		**T3 (Août 2012)**		**T4 (Sept 2012)**		**T5 (Oct 2012)**	
		Kisumu	Wild strain	Kisumu	Wild strain	Kisumu	Wild strain	Kisumu	Wild strain	Kisumu	Wild strain	Kisumu	Wild strain
Tanguiéta	Cement	100	100	100	89,47	96,6	51,16	75,54	48,15	50,74	23,91	20,81	11,5
	Mud	100	100	100	81,03	94,2	45,73	65,35	29,75	45,6	19,2	17,99	8,96
Natitingou	Cement	100	100	100	84,75	90,2	53,6	57,6	40,31	41,41	31,11	19,86	12,4
	Mud	100	100	97,5	66,39	81,9	49,5	56,71	39,13	37,78	27,47	15,44	8,6

a)



b)

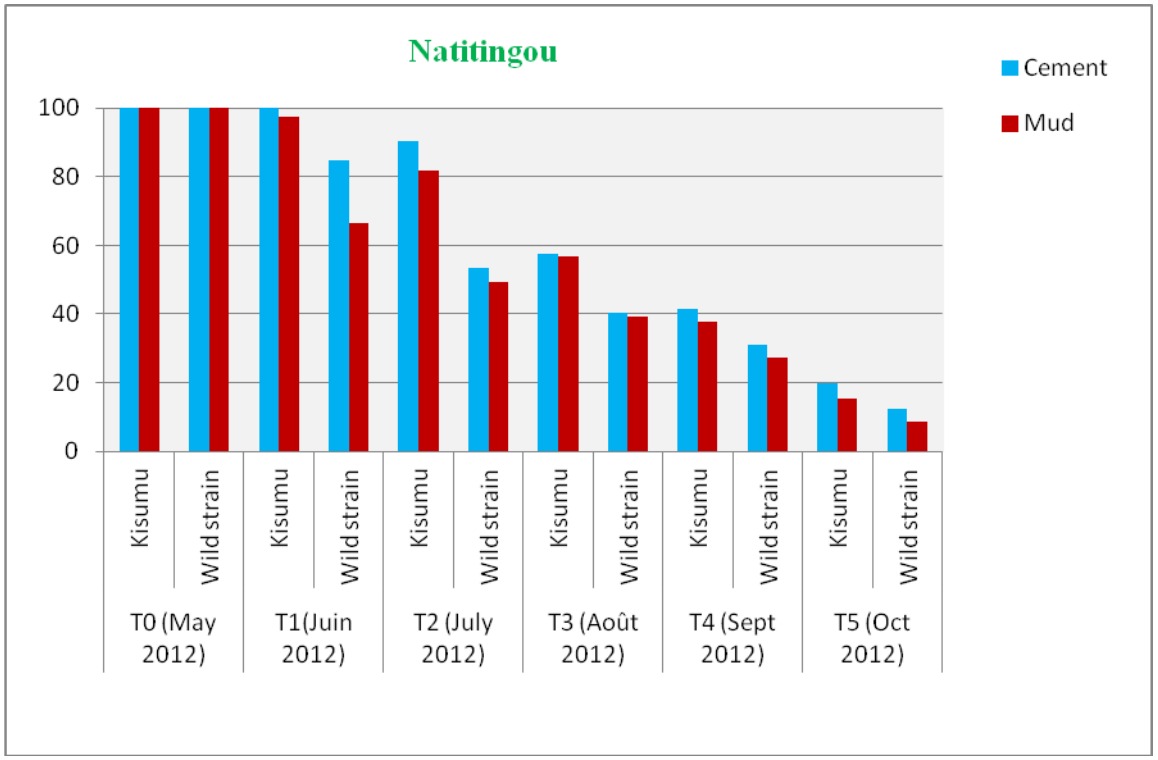


Figure 1: Mortality rates of Kisumu, the susceptible strain, and the wild *An. gambiae* females from Tanguiéta (a) and Natitingou (b) after 30 minutes exposure to bendiocarb in WHO bioassay cone test 5 months after IRS.

5. 2. Dynamics of vector resistance in Atacora from 2010 to 2012

5.2.1. Results of susceptibility tests using WHO bioassay tubes (Tables II and diagrams D1 to D6)

During the study period, larvae of *An. gambiae* were collected at 2 periods (July/August and October) and reared to have 2-5 days adult mosquitoes to perform WHO bioassay tube tests. More than 2000 females of *An. gambiae* were used for susceptibility tests. Mosquitoes were collected in 6 districts: Tanguiéta, Natitingou, Matéri, Kouané and Pehunco in IRS districts and in Copargo the control district. The susceptibility of *An. gambiae* was evaluated to 3 classes of insecticides in 2012: Pyrethrinoids (deltamethrin), carbamates (bendiocarb and propoxur) and organophosphates (pyrimiphos methyl and fenitrothion).

The table II below and diagrams D1 to D6 shows the mortality rates of *Anopheles gambiae s.l* collected in July and August 2012 (during the rainy season) and in October (at the end of the rainy season) after their exposure to insecticide impregnated papers. The data of 2012 were compared with those of 2010 (before the IRS campaign). The purpose of this comparison was to assess the dynamics of resistance to insecticides, especially to carbamates and organophosphates. It was also an opportunity, for the first time, to compare the susceptibility of *An. gambiae s.l* to bendiocarb and pirimiphos methyl.

- ✓ **High resistance to DDT and deltamethrin in Atacora**

DDT was tested only in 2010 just before the IRS campaign (Table II). The results showed a high resistance of *An. gambiae* s.l to DDT. Besides, the mortality rate was only 13% in Tanguiéta and 18% in Toucountouna. Similar results were obtained with permethrin. As a matter of fact, since year 2000, a high resistance to this insecticide was registered. Today, many articles have shown that *An. gambiae* has developed a high resistance to permethrin everywhere in Benin. Based on these results, inclusion of these two insecticides in the subsequent testing was found to be less important.

Regarding deltamethrin, 828 *An. gambiae s.l* were tested in 2010 in nine districts of Atacora department. The results showed very low mortality rates ranging from 27% to 51% (Table II). The 2010 test mortality rates were 31% in Natitingou and Tanguiéta slightly lower than 2012 data, which is 57.14% and 42.11% respectively in July/September and 45.83% and 49.25% respectively in Tanguiéta and Kouandé in October. The data demonstrate that there is a sign of reversal of the vector to susceptibility status to deltamethrin after the introduction of carbamates though it very slow.

- ✓ **Full resistance to bendiocarb and suspected resistance to propoxur in Atacora**

Before IRS campaign in 2010, *An. gambiae* s.l was relatively susceptible to bendiocarb (Table II). As a matter of fact, the susceptibility of 765 *An. gambiae* s.l to this product was tested and the result obtained showed high susceptibility in the nine districts of Atacora; the result showed mortality rates ranging between 95% and 98%. Two years later in 2012, the situation has changed. Surprisingly, *An. gambiae s.l* has developed a high level of resistance to bendiocarb. The mortality rates recorded now were low: 63.41% in Tanguiéta, 61.9% in Natitingou, 78.57% in Kouandé and 58.61% in Matéri. However, it is important to point out that the quick spread of vector resistance to bendiocarb may not be mainly linked to the use of this insecticide for IRS campaigns, rather to the high use of agricultural insecticides against cotton pests in recent years. Regarding propoxur, another insecticide of the same class (carbamate), mortality rates registered in Tanguiéta (88.13%) and Kouandé (90%) are relatively low showing a suspected resistance of *An. gambiae* to this insecticide according to WHO 1998 classification. Using the new classification (WHO, 2011), these 2 rates would be ranged as resistance indicator. However, the resistance level of *An. gambiae* observed in 2012 in Atacora is lower the bendiocarb one.

- ✓ **High susceptibility to pirimiphos methyl and suspected resistance to fenitrothion**

On the other hand, unlike bendiocarb, *An. gambiae s.l* was very susceptible to pirimiphos methyl: 100% mortality rate in Natitingou, Kouandé and Matéri and 99.09% in Tanguiéta in July/October and the same full susceptibility in October (100% mortality in all districts). For fenitrothion (another organophosphate), we noted a decrease in the susceptibility (suspected resistance) of *An. gambaie s.l* in Tanguiéta in July/August, but the sample size was very small to make deduction. Additional tests performed in October and data registered confirm the suspected resistance status of this mosquito (83.67% mortality in Tanguiéta, 87.80 in Kouandé and 94.79 in Matéri.

Figures 2b below shows the frequency of the dead time following the exposure of *An. gambiae* to insecticides. This frequency is globally slow for all insecticides. Neither of insecticides tested have provided 100% mortality during the 60 minutes of exposure time showing they are not characterized by knock-down action. In parallel. Only pirimiphos methyl and fenitrothion have induced 80% mortality after the 120 minutes following the exposure to impregnated papers.

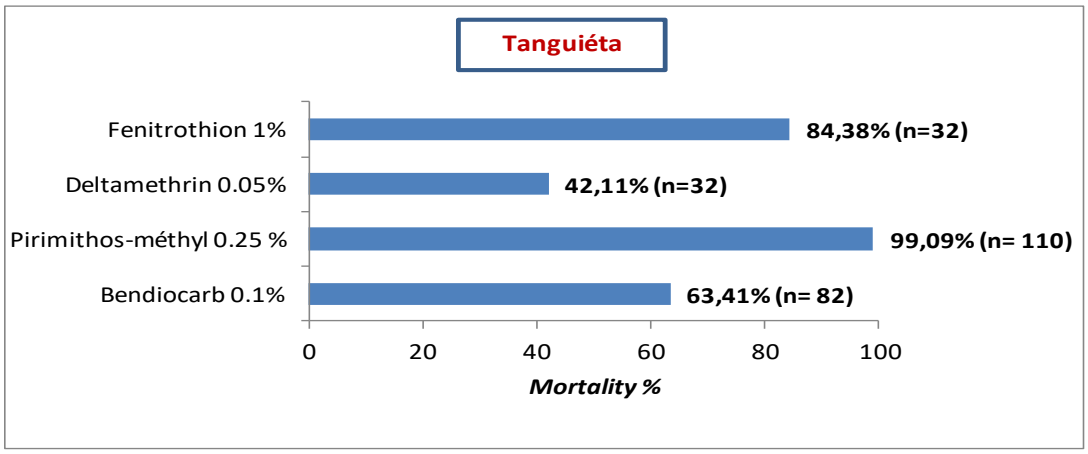
Table II: Susceptiblity status of *An. gambiae* population collected in Atacora from 2010 to 2012.

Districts	DDT		Deltamethin						Bendiocarb						Pirimithos methyl				Fenitrothion			
	October 2010		October 2010		July 2012		October 2012		October 2010		July 2012		October 2012		July 2012		October 2012		July 2012		October 2012	
	n	%M	n	%M	n	%M	n	%M	n	%M	n	%M	n	%M	n	%M	n	%M	n	%M	n	%M
Tanguiéta	39	13	93	31	32	42,1	72	45,83	95	95	82	63,4	106	63,2	110	99,09	90	100	32	84,38	49	83,67
Natitingou	65	12	90	31	21	57,1	_	_	92	97	84	61,9	_	_	58	100	_	_	_	_	_	_
Kouandé	63	14	68	37	_	_	67	49,25	42	98	74	78,6	90	78,89	61	100	81	100	_	_	82	87,80
Cobly	63	14	68	37	_	_	_	_	42	98	_	_	_	_	_	_	_	_	_	_	_	_
Matéri	65	12	124	27	_	_	_	_	94	98	29	58,6	73	58,9	31	100	72	100	_	_	96	94,79
Pehunco	54	17	73	31	_	_	74	67,57	83	95	_	_	101	79,21	_	_	89	98,88	_	_	89	89,89
Boukoumbé	70	11	67	31	_	_	_	_	56	95	_	_	_	_	_	_	_	_	_	_	_	_
Toukountounan	65	12	94	31	_	_	_	_	88	95	_	_	_	_	_	_	_	_	_	_	_	_
Kérou	68	12	72	51	_	_	_	_	89	96	_	_	_	_	_	_	_	_	_	_	_	_

Figure 2a. Diagram showing data obtained after WHO susceptibility tests in Atacora in July and October 2012

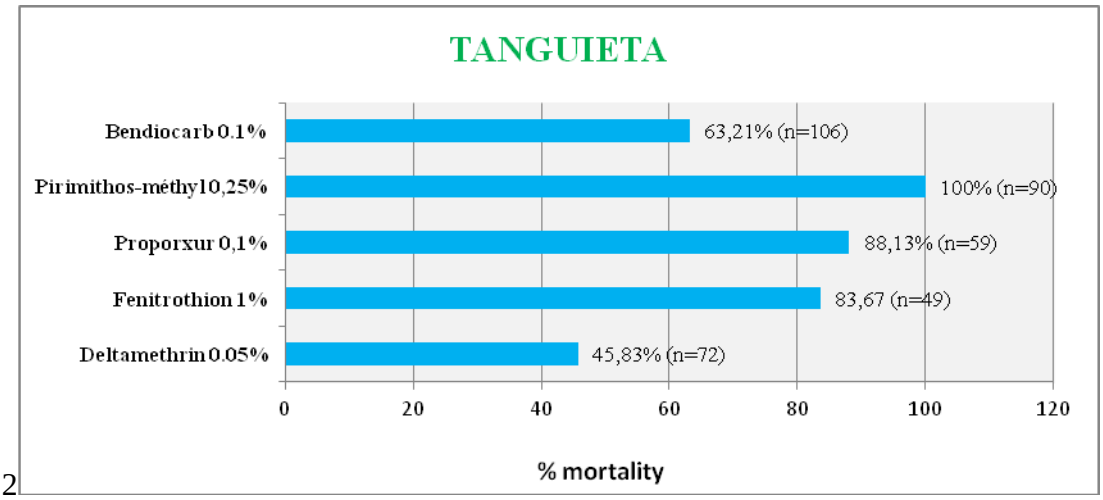
D 1- Tanguiéta

- July



2012

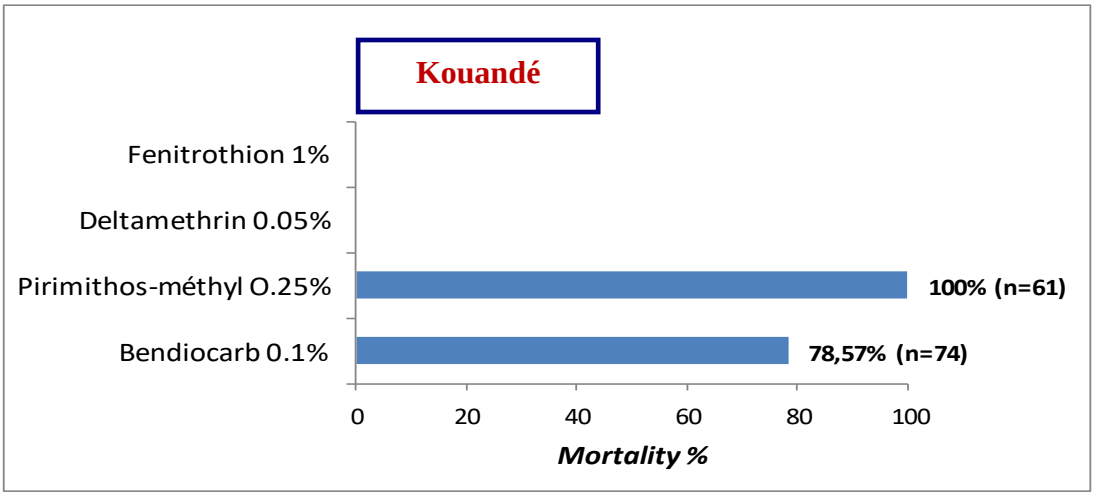
- October



2012

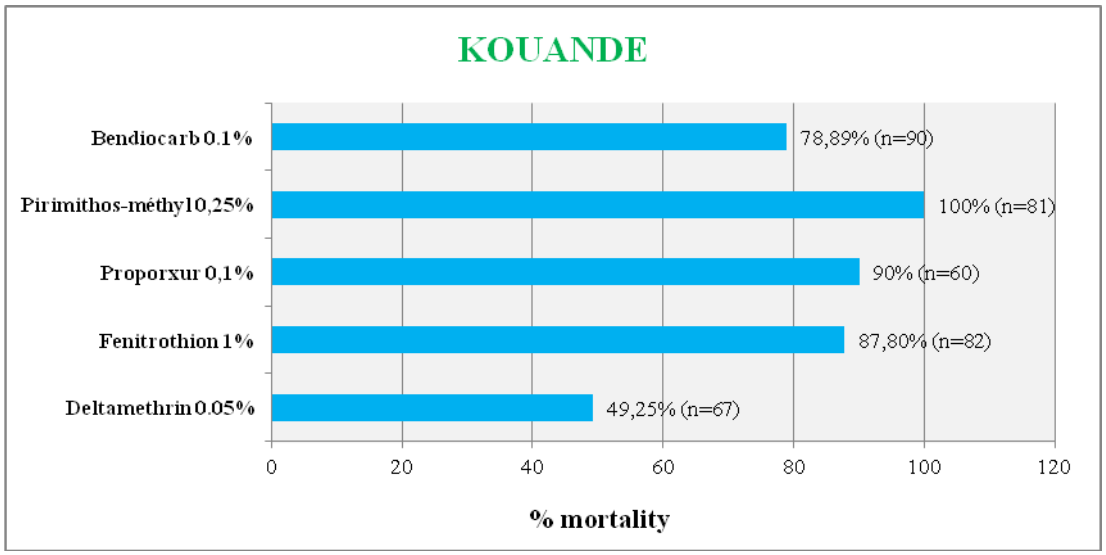
D 2- Kouandé

- July



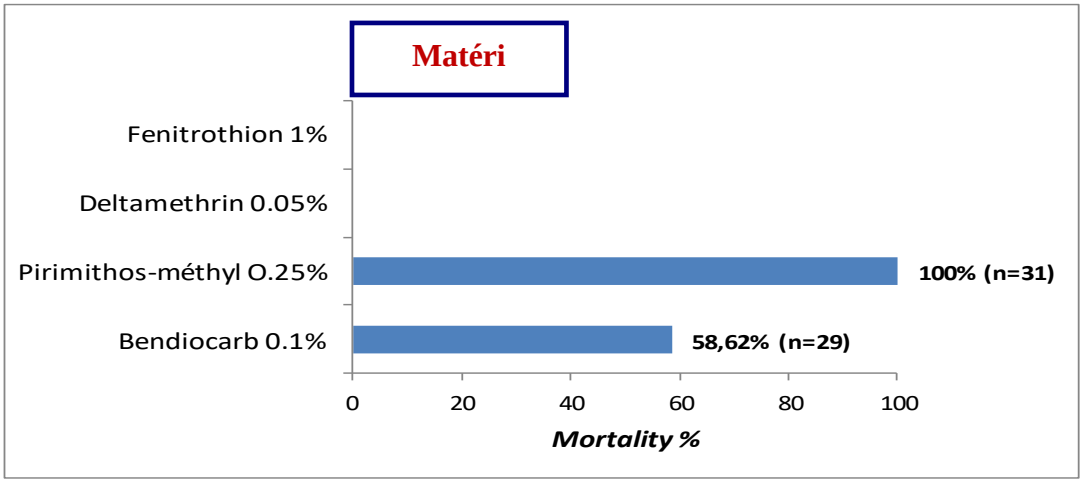
2012

- October 2012



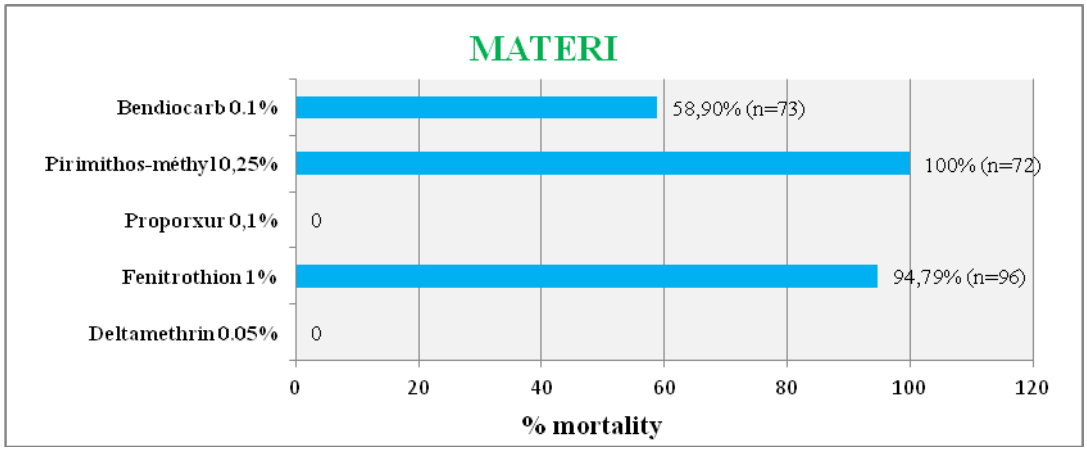
D 3- Matéri

- July



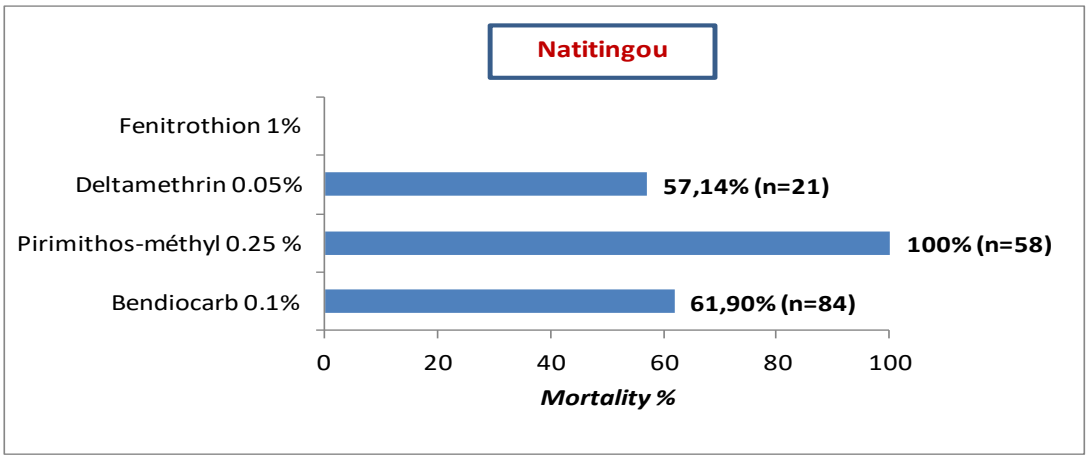
2012

- October 2012



D 4- Natitingou

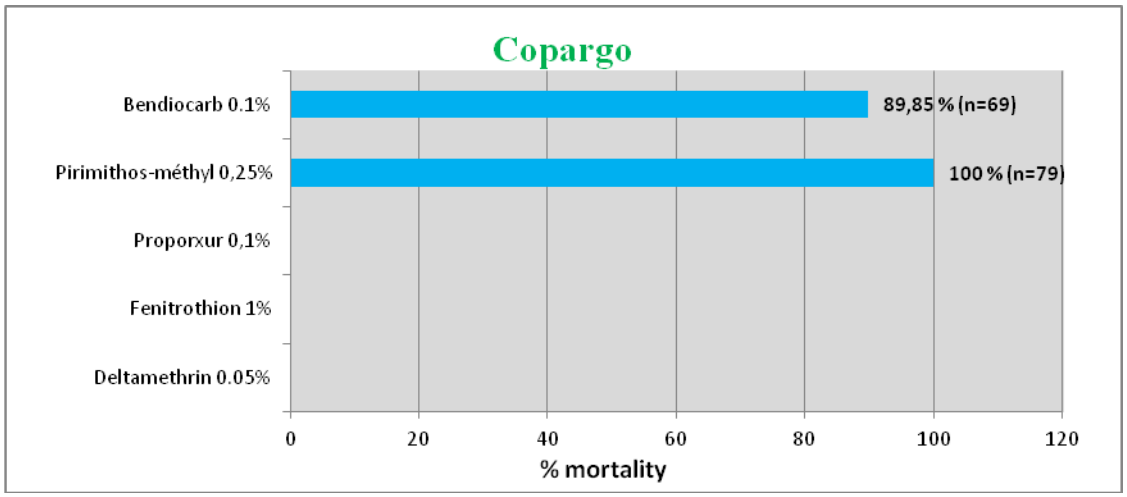
- July



2012

D 5- Copargo

- October 2012



D6- Pehunco

October 2012

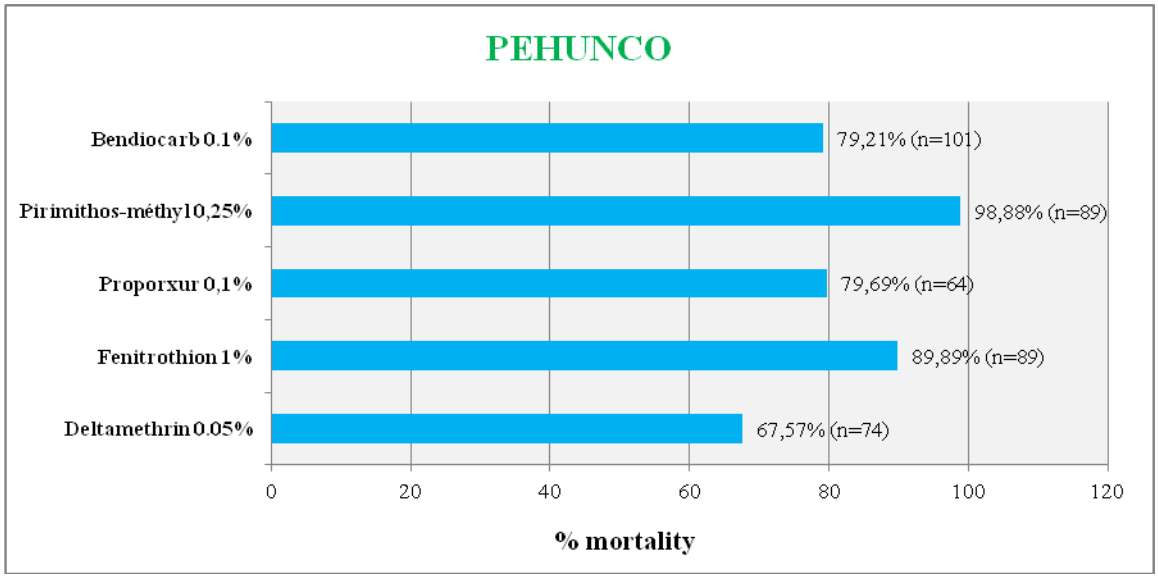
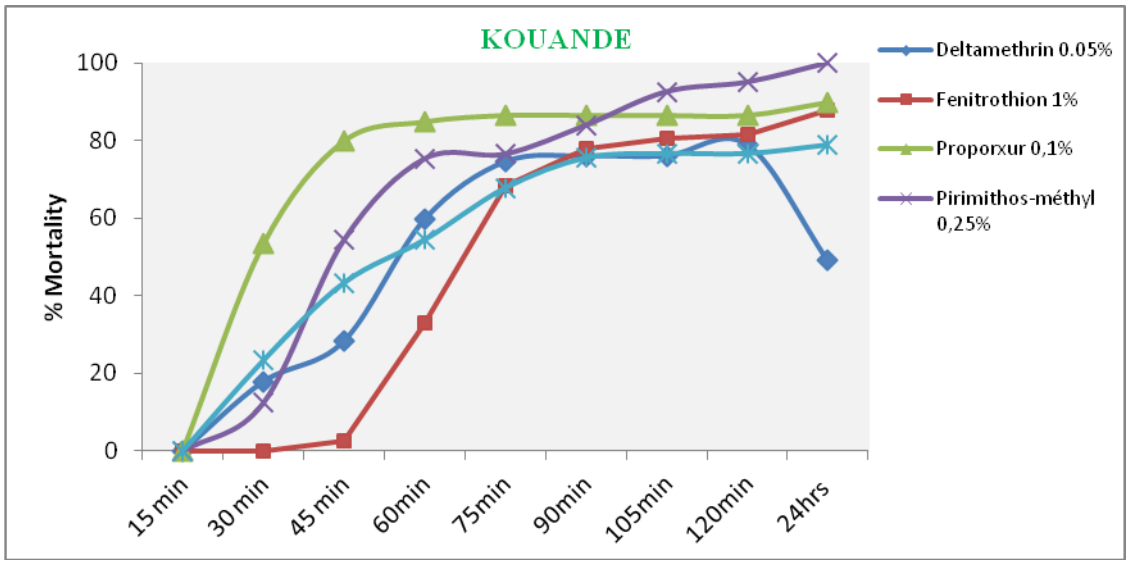
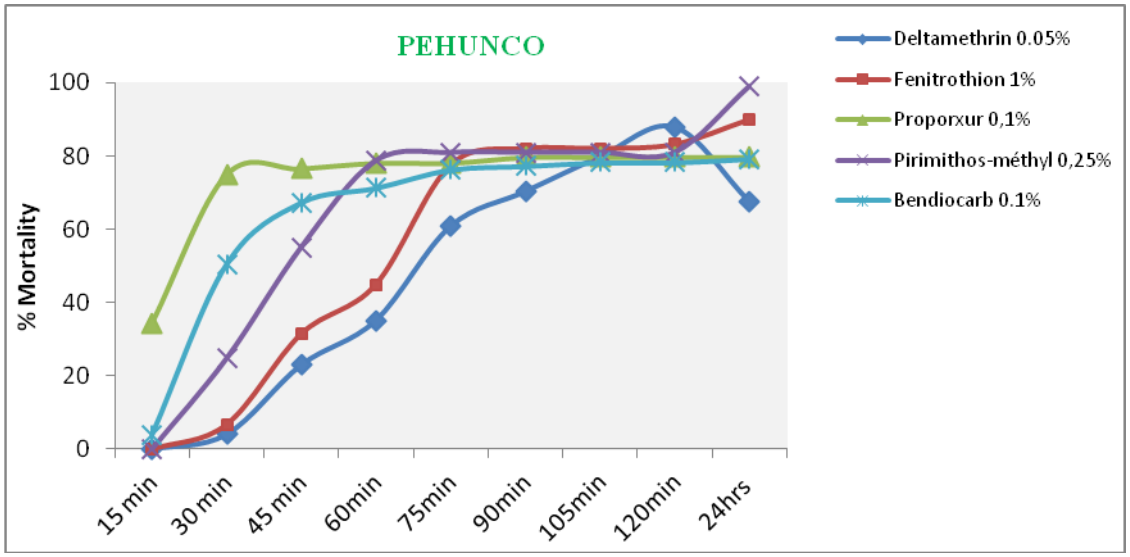
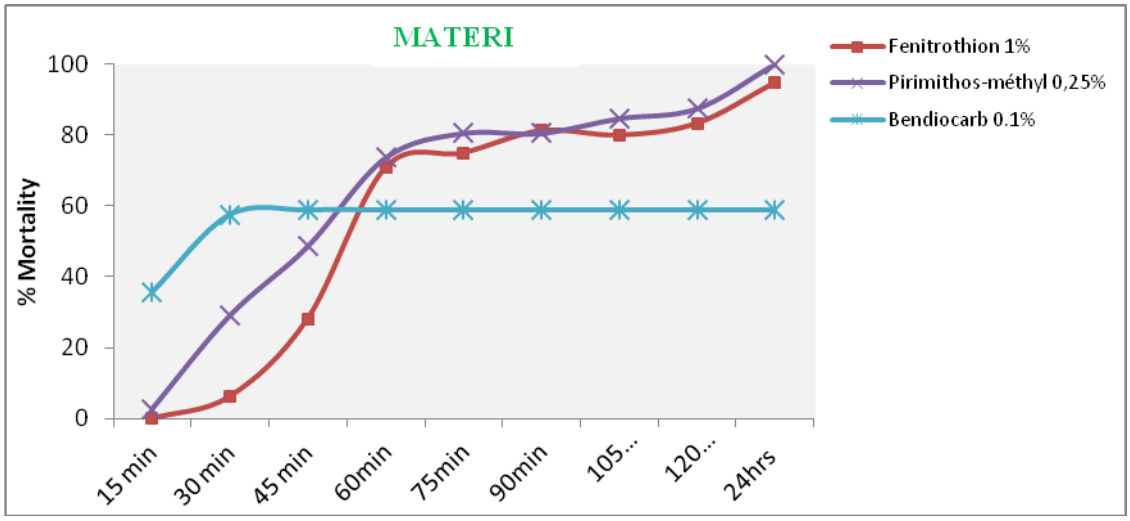


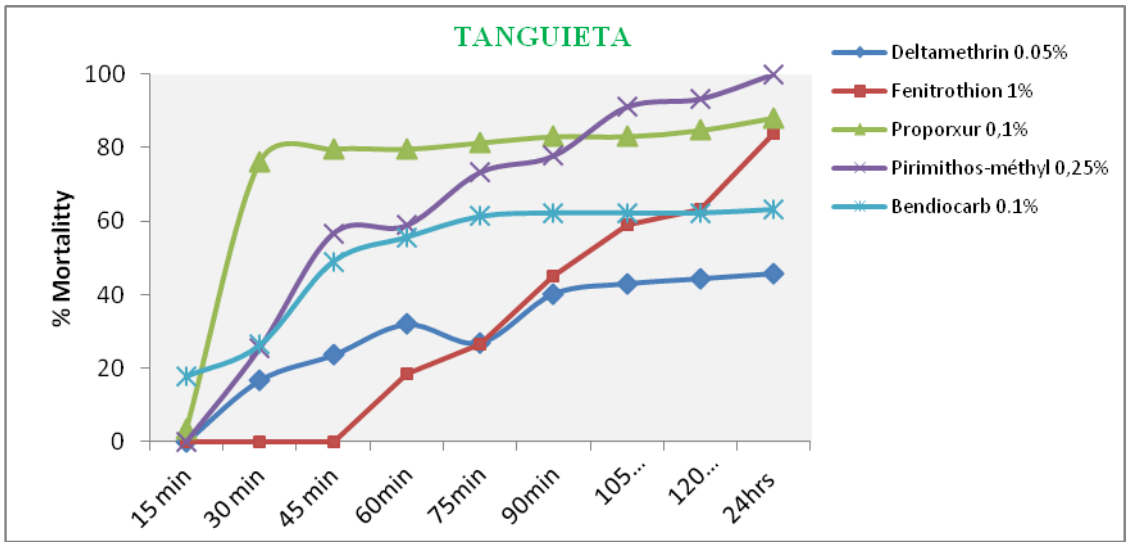
Figure 2a. Diagram showing data obtained after WHO susceptibility tests in Atacora in July and October 2012

2. b. Evolution of *An. gambiae* dead time during the 60 minutes of exposure to insecticides and 60 minutes of holding time.






MATERI
% Mortality
100
80
60
40
20
0
15 min
30 min
45 min
60min
75min
90min
105...
120...
24hrs
Fenitrothion 1%
Pirimithos-méthyl 0,25%
Bendiocarb 0.1%


TANGUIETA
% Mortalitty
100
80
60
40
20
0
15 min
30 min
45 min
60min
75min
90min
105...
120...
24hrs
Deltamethrin 0.05%
Fenitrothion 1%
Proporxur 0,1%
Pirimithos-méthyl 0,25%
Bendiocarb 0.1%

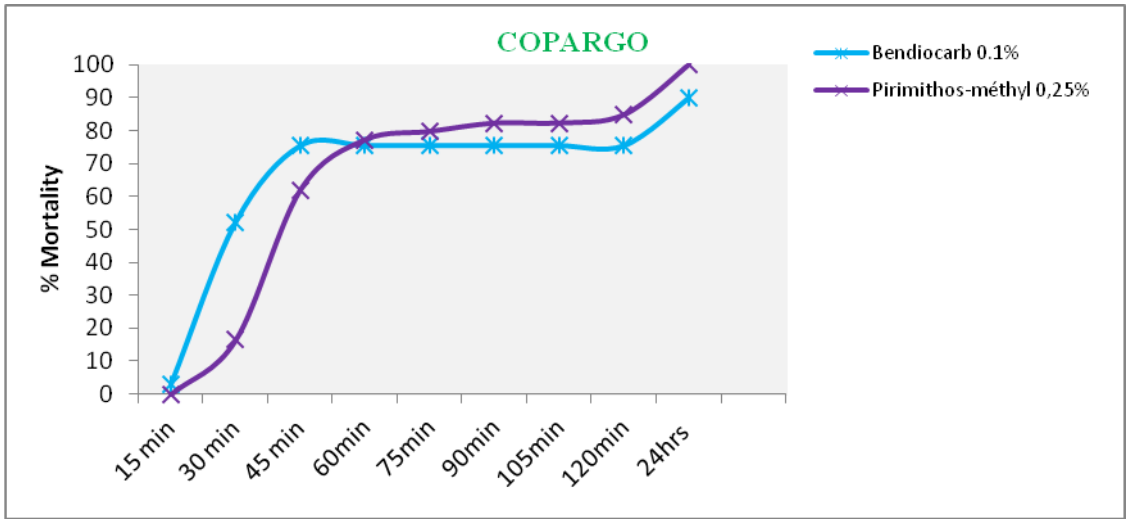


Figure 2b: Evolution of *An. gambiae* dead time during the 60 minutes of exposure to insecticides and 60 minutes of holding time.

5.2.2. Results of molecular analysis

5.2.2.1. Kdr and Ace-1 mutations

The frequency of Kdr mutation that confers resistance to pyrethroids and DDT is high in all districts of Atacora (Table IIIa). Table IIIa shows the variation of the frequency of Kdr gene allele from April (one month before IRS) to August (4 months after IRS). Generally, the frequency is high everywhere and doesn't increase. Ace 1 mutation that confers resistance to carbamates and organophosphates is particularly high in July 2012 in Tanguiéta and Kouandé (Table IIIb,c) within *An. gambiae s.l* population. The frequencies registered in Tanguieta (0.38) and Kouande (0.36) were particularly high. It is the first time we registered such a high frequency in Benin. On other hand, 3 specimens of *An. gambiae s.l* among 65 were found with homozygote genotype of Ace-1 allele. The number of mosquitoes tested for Ace-1 identification in some districts like Tanguieta and Kouande are very low.

5.2.2.2. Dynamics of the frequency of Ace-1 gene allele in Atacora from 2010 to 2012

The table IIIb shows the frequency of Ace-1 gene, responsible of vector resistance to carbamates. The development of the frequency of this gene was followed from 2010 to 2012 in order to study how it progresses over time; this was to make a comparison between the molecular resistance due to this gene and the phenotypic resistance displayed by susceptibility tests. In fact, the evolution of the frequency of Ace-1 gene was followed at different moments: in September and October 2010 (rainy season) (Table IV), in December 2011 (end of rainy

season) (Table V), in January, February and April 2012 (dry season) (Tables VI and VII) and finally in July 2012 (rainy season) (Table IIIb and Table IIIc). For the molecular analyses, two types of mosquito samples were used. The mosquitoes analyzed during the rainy season were adults obtained from larvae reared in insectary whereas those analyzed during the dry season were adults collected in Atacora by landing catch at night using aboriginal volunteers. The analysis of the various results from 2010 to 2012 altogether doesn't show a significant increase in the frequency of Ace-1 mutation. Yet, the frequencies observed in July 2012 (0.36 in Kouandé and 0.38 in Tanguiéta) seem high. Moreover, the Tables IIIb, c and Tables IV-VII below shows that Ace-1 frequencies were higher during the rainy season (September and October 2010, December 2011 and July 2012) than during the dry season (January, February and April 2012). The difference in the frequency of Ace-1 mutation observed during these two periods could be justified. In fact, this probably is because rainy season is the season when the use of agricultural insecticides is required.

Table III.a: Variation of *kdr* mutation

	April-2012					May-2012					June-2012					July-2012					August-2012					April-August 2012				
	n	RR	RS	SS	F(kdr)	n	RR	RS	SS	F(kdr)	n	RR	RS	SS	F(kdr)	n	RR	RS	SS	F(kdr)	n	RR	RS	SS	F(kdr)	n	RR	RS	SS	F(kdr)
Pehunco	13	8	4	1	**0,77**	143	99	44	0	**0,85**	9	6	3	0	**0,83**	48	39	9	0	**0,91**	10	4	6	0	**0,7**	223	156	66	1	**0,85**
Tanguiéta	5	4	1	0	**0,90**	16	10	6	0	**0,81**	16	12	2	2	**0,81**	9	4	5	0	**0,72**	10	2	8	0	**0,6**	56	32	22	2	**0,80**
Matéri	5	2	3	0	**0,70**	33	19	13	1	**0,77**	13	10	3	0	**0,88**	45	28	17	0	**0,81**	22	15	7	0	**0,84**	118	74	43	1	**0,80**
Natitingou	8	6	2	0	**0,88**	73	44	28	1	**0,79**	10	7	3	0	**0,85**	11	9	2	0	**0,91**	17	11	6	0	**0,82**	119	77	41	1	**0,82**
Kouandé	4	2	2	0	**0,75**	58	38	18	2	**0,81**	42	28	13	1	**0,82**	7	2	5	0	**0,64**	18	9	8	1	**0,72**	129	79	46	4	**0,80**
Copargo	51	28	23	0	**0,77**	173	95	75	3	**0,77**	78	63	15	0	**0,90**	50	34	15	1	**0,83**	46	32	14	0	**0,85**	398	252	142	4	**0,81**

Table III.b: Variation of *Ace.1* mutation

	April-2012					May-2012					June-2012					July-2012					August-2012					April-August 2012				
	N	RR	RS	SS	F(Ace.1)	N	RR	RS	SS	F(Ace.1)	n	RR	RS	SS	F(Ace.1)	n	RR	RS	SS	F(Ace.1)	n	RR	RS	SS	F(Ace.1)	n	RR	RS	SS	F(Ace.1)
Pehunco	13	0	0	13	**0**	143	0	6	137	**0,02**	9	0	0	9	**0**	48	0	5	43	**0,05**	10	0	2	8	**0,10**	223	0	13	210	**0,03**
Tanguiéta	5	0	0	5	**0**	16	0	0	16	**0**	16	0	0	16	**0**	8	1	4	3	**0,38**	10	0	3	7	**0,15**	55	1	7	47	**0,08**
Matéri	5	0	0	5	**0**	33	0	0	33	**0**	13	0	1	12	**0,04**	45	0	2	43	**0,02**	22	0	5	17	**0,11**	118	0	8	110	**0,03**
Natitingou	8	0	0	8	**0**	73	0	0	73	**0**	10	0	0	10	**0**	11	0	0	11	**0,00**	17	0	0	17	**0,00**	119	0	0	119	**0**
Kouandé	4	0	0	4	**0**	58	0	4	54	**0,03**	42	0	2	40	**0,02**	7	1	3	3	**0,36**	18	1	4	13	**0,17**	129	2	13	114	**0,07**
Copargo	51	0	0	51	**0**	173	0	0	173	**0**	78	0	6	72	**0,04**	50	1	7	42	**0,09**	46	0	5	41	**0,05**	398	1	18	379	**0,03**

Table III c. Species identification, molecular forms and frequency of the *kdr, Ace.1* alleles and génotypes in *An.gambiae s.l* from Atacora in July 2012.

		Species		Molecular forms			*kdr mutation*				*Ace-1 mutation*			
	N tested	Aa	Ag	M	S	MS	RR	RS	SS	F(*kdr*)	RR	RS	SS	F(*Ace-1*)
Pehunco	48	0	48	2	45	1	39	9	0	0,91	0	5	43	0,05
Tanguiéta	8	0	8	0	8	0	4	5	0	0,72	1	4	3	0,38
Matéri	45	0	45	0	44	1	28	17	0	0,81	0	2	43	0,02
Natitingou	11	0	11	0	9	2	9	2	0	0,91	0	0	11	0,00
Kouandé	7	0	7	1	6	0	2	5	0	0,64	1	3	3	0,36
Copargo	50	0	50	1	47	2	34	15	1	0,83	1	7	42	0,09

5.2.2.3. Implication of *Ace.1* mutation in bendiocarb resistance.

Dead and alive *An. gambiae* after susceptibility tests to bendiocarb were separated and analyzed for Ace-1 mutation by PCR. Any heterozygous RR mosquito was found neither within alive mosquitoes, nor within dead mosquitoes. Two genotypes were found: The RS genotype, particularly within alive mosquitoes and the SS genotype (figure 3). By other hand, a not neglected number of SS genotype (>20%) was found within dead mosquitoes that probably means existence of other mechanisms of bendiocarb resistance.

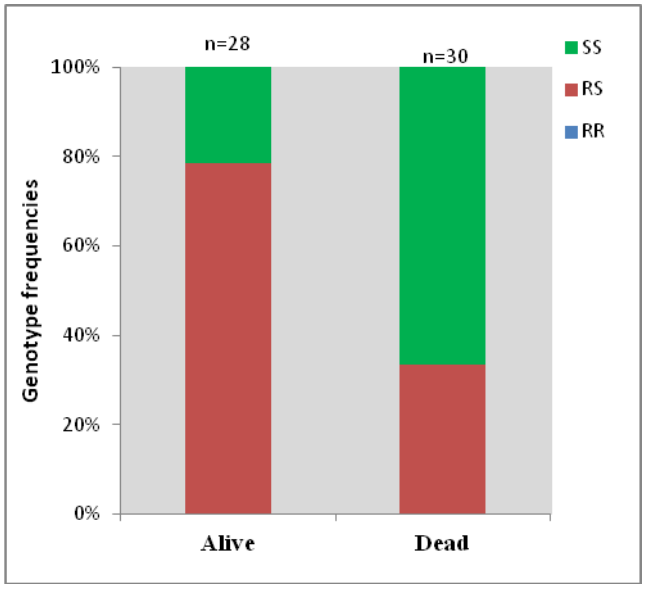


Figure: 3 Ace.1 genotype frequencies found in alive (A) and dead (D) *Anopheles gambiae* from WHO susceptibility tests to Bendiocarb.

5.2.3. Metabolic resistance

A significant increase in the level of both GSTs and cytochrome monooxygenases was observed in the Pehunco and Tanguieta populations compared to the susceptible Kisumu strain. For GSTs, Fig 1 shows an increase in the mean quantity of CNDB converted compared to the Kisumu strain ($P<0.05$).

For Cytochrome monooxygenases, an increase level in the amount of cytochrome C equivalents/mg of protein was detected in the Pehunco and Tanguieta populations when compared to the Kisumu strain ($P<0.05$) (Fig2)

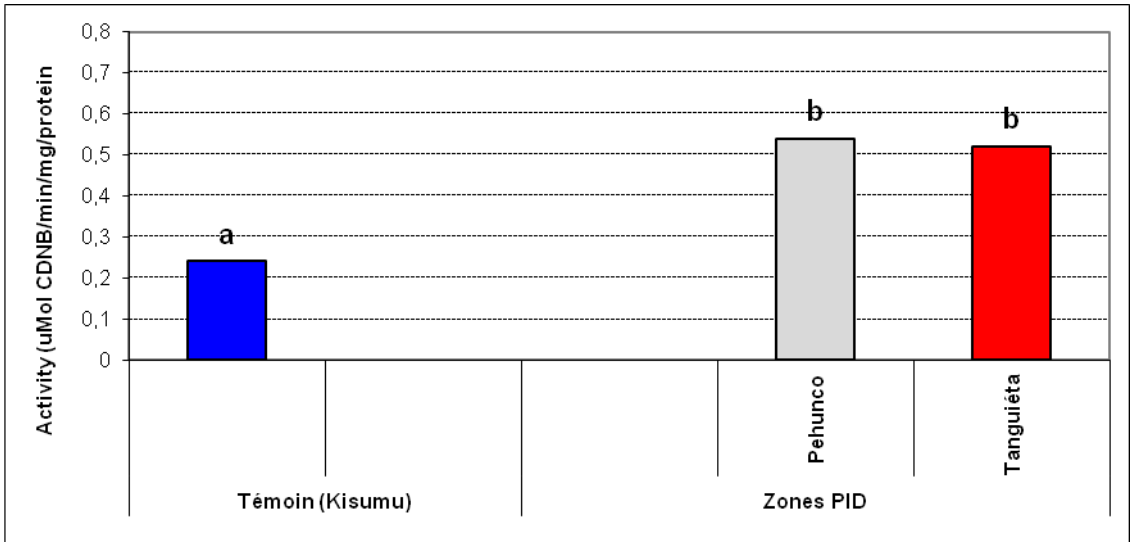


Fig 4 a: GST activity in *Anopheles gambiae* populations from Pehunco and Tanguieta

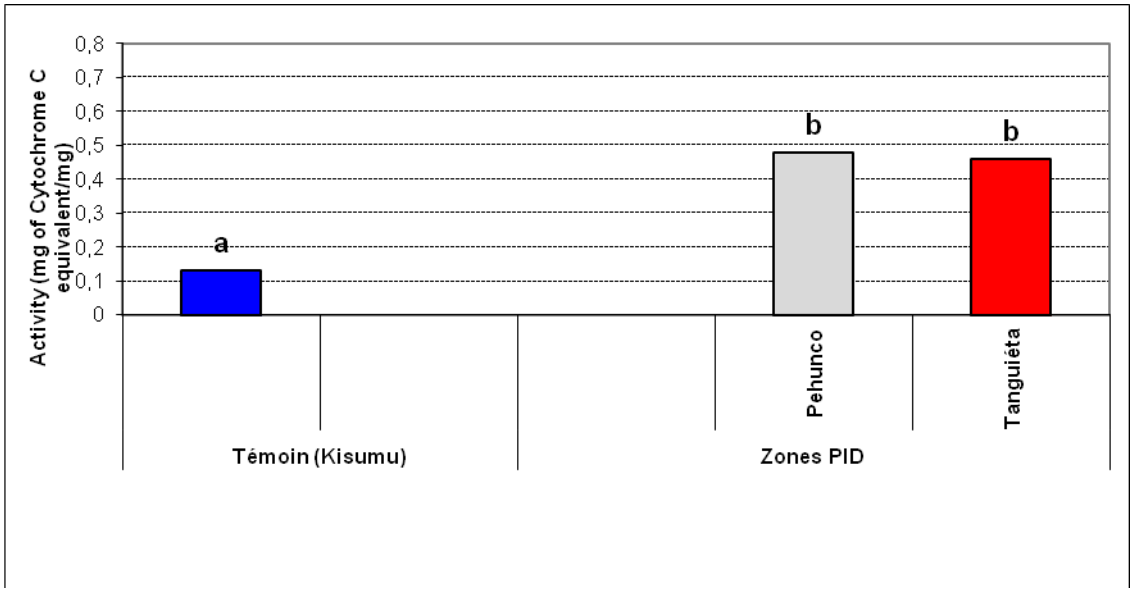
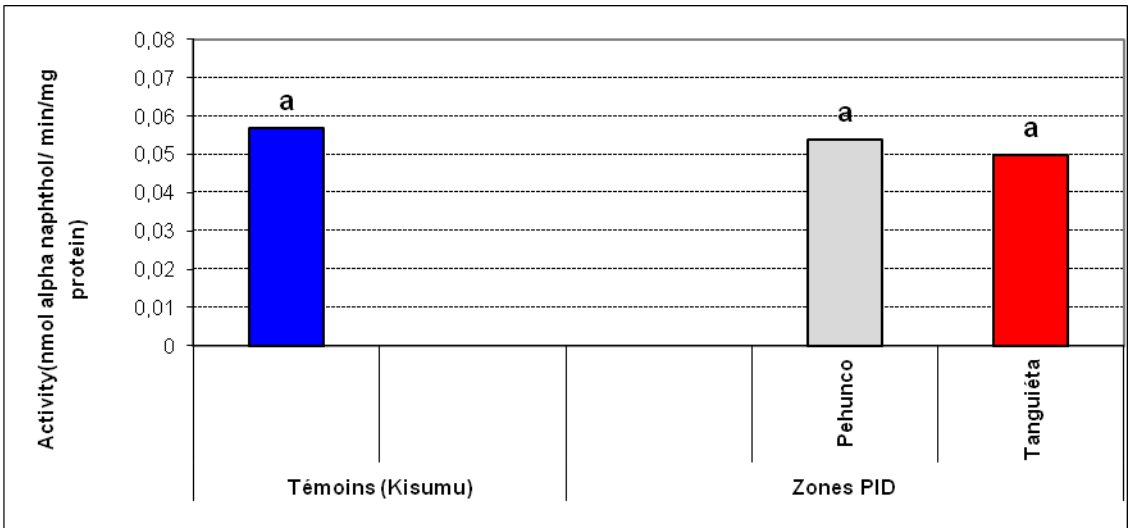


Fig 4 b: Oxydase activity in *Anopheles gambiae* populations from Pehunco and Tanguéta

No significant difference was observed with either α and β-Naphthyl acetate substrates in Pehunco and Tanguieta *Anopheles gambiae* populations compared to the Kisumu susceptible strain (P>0.05)



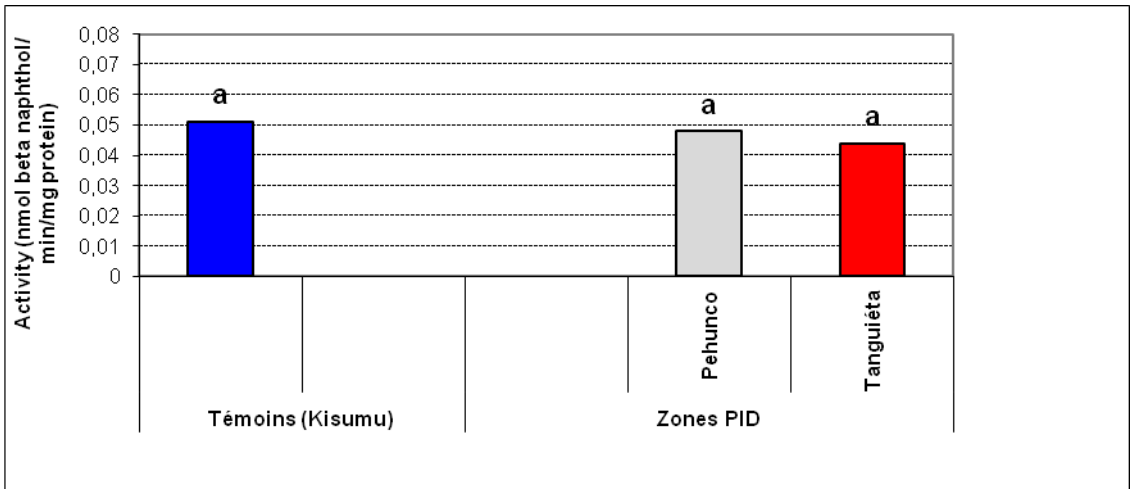


Fig 4 c: Esterase (α and β-Naphthyl) activity in Pehunco and Tanguieta *Anopheles gambiae* populations

One route of metabolic resistance is through up-regulation of detoxification enzymes. Overexpression of enzymes related to insecticide resistance is generally assumed to be associated with cytochrome P450-dependent monooxygenases (P450), carboxylesterases (COE), and glutathione-S transferases (GST). Among these three families, evidence suggests that P450s commonly play a primary role in pyrethroid resistance. To date, out of 111

putative *An. gambiae s.s.* P450s, four have been observed to be overexpressed in adult mosquitoes from colonies characterised as pyrethroid resistant, *namely* CYP6Z1, CYP6Z2, CYP6M2 *and* CYP325.

Data obtained this year in Pehunco and Tanguiéta are preliminary data on biochemical resistance. As a matter of fact, biochemical assay used presents a limit because of non specific information provided related to specific resistance gene up and down regulation. For this reason, it is important to further investigate Carbamate resistance mechanisms using high technologies like microarray platform or real time quantitative Polymerase Reaction Chain (RT-qPCR) for linked specific resistance gene expression detection for resistance monitoring on field. Investigating in biochemical resistance is an important issue because Ace-1 mutation frequency detected now in Benin, and particularly in Atacora is too low to represent the alone carbamate resistance mechanism. This is why one of the members of CREC will be trained before the end of this year on the use of RT-qPCR technology. The new technology will be used to follow the metabolic resistance in districts of Atacora under pirimiphos methyl (Organophosphate) IRS and those under bendiocarb (Carbamate) IRS in 2013.

5.3. Dynamics of vector populations in districts under IRS versus control districts

5.3.1. Diversity of mosquitoes collected from April to October

Various species of mosquitoes were collected in Atacora. Table I below shows the most of mosquitoes collected. Out of 18 species, *An. gambie* is the most abundant (70%) (table I). The density of this species is lower inside houses (37.3%: 406/1089) than outside (62.7%: 683/1089). Two malaria major vectors were found: *An. gambie* and *An. funestus* (2.6%). *An. nili* a local vector, was also collected, but at very low density (0.2%). *An. pharoensis* (0.4%) and *An. ziemanni* (3.3%) don't transmit malaria in Benin. Anopheles mosquitoes are the main mosquitoes collected (76.9%). *Culex quinquefasciatus* and the other Culex are found at low density, respectively 10.6% and 0.7%. The disparity between the frequency of Anophelinae and Culicinae in Atacora is explained by the ecological caracteristics of the environment. There are no big cities in Atacora and almost areas are small towns and rural areas appropriated for the development of Anopleles mosquitoes.

Anopheles gambiae collected were analysed using PCR technique to identify the two molecular forms involved in *An. gambiae sensu stricto.* In December 2011, January and February 2012, we have analyzed 134 specimens of *An. gambiae s.s.* (see last report: April-August 2012) using PCR technique. In December, the frequency of the S form was high

(81.8%: 27/33). Only 6 specimens of the M form were found among the 33 *An. gambiae s.s.* analyzed, either 18.2%. In January, February and April (dry season), the situation has changed. *An. gambiae* M has become more abundant: 81.7% (152/186) against 18.7% (34/186) for *An. gambiae* S. During the rainy season (May to August), the frequency of M has drastically decreased: 5% (47/ 948) against 95.6% (901/948) (Table II). The increase of the population of *An. gambiae* M during the dry season is probably due to the presence of some larvae breeding sites favourable for the development of this population. This observation is in accordance with what is known about the development of this population. In fact, in dry savannah areas, the larvae breeding sites created during the dry season are best places for the development of the Mopti chromosomal form which is similar to M form in some contexts.

Table I: Mosquitoes species caught from April to October 2012.

Species	**April - October 2012**		
	Indoor	Outdoor	Total
Anopheles gambiae	406	683	1089
Anopheles funestus	19	22	41
Anopheles nili	2	1	3
Anopheles pharoensis	2	4	6
Anopheles ziemanni	10	41	51
Anopheles coustani	0	1	1
Aedes aegypti	2	16	18
Aedes vitatus	3	16	19
Aedes luteocephalus	0	0	0
Aedes gr. Palpalis	0	5	5
Aedes gr. Tarsalis	0	0	0
Culex quinquefasciatus	56	99	155
Culex gr decens	2	2	4
Culex nebulosus	2	0	2
Culex tigripes	0	0	0
Culex annulioris	0	5	5
Mansonia africana	37	109	146
Mansonia uniformis	0	4	4
TOTAL	**541**	**1008**	**1549**

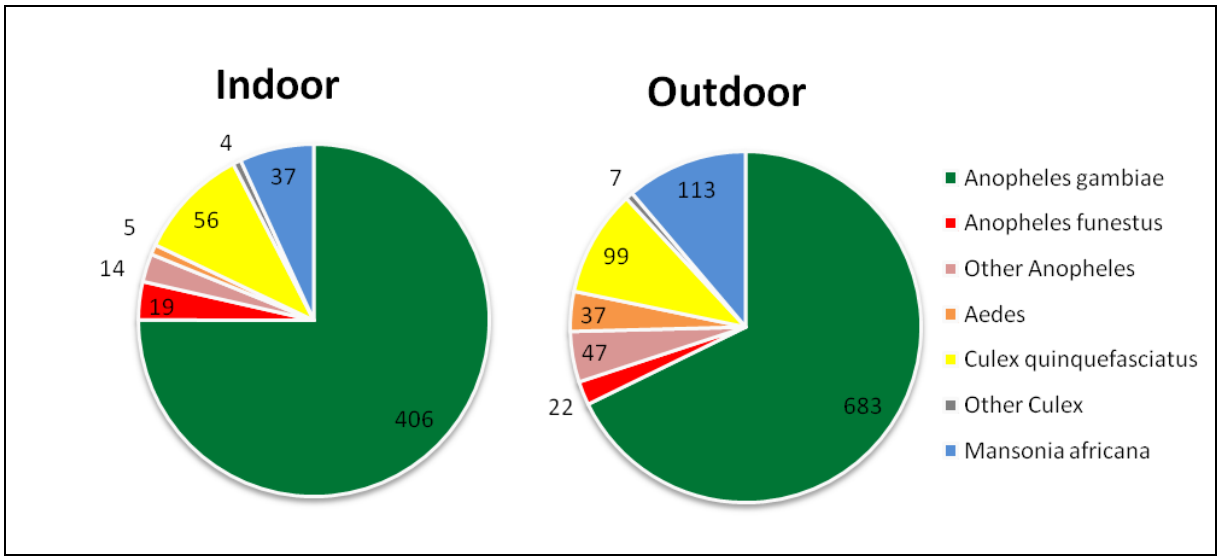


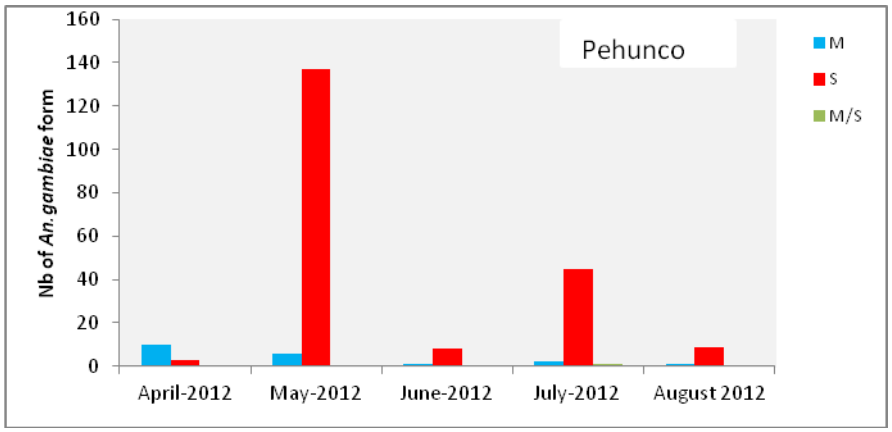
Figure I. Diagram showing species of mosquitoes caught from April to October 2012

Table II: Monthly variation of *An. gambiae* molecular forms

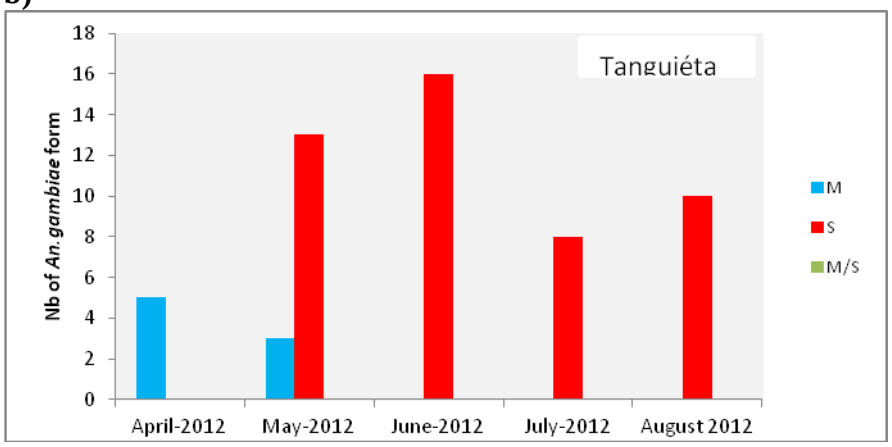
	April-2012				May-2012				June-2012				July-2012				August-2012			
	n	M	S	M/S	n	M	S	M/S	n	M	S	M/S	n	M	S	M/S	n	M	S	M/S
Pehunco	13	10	3	0	143	6	137	0	9	1	8	0	48	2	45	1	10	1	9	0
Tanguiéta	5	5	0	0	16	3	13	0	16	0	16	0	8	0	8	0	10	0	10	0
Matéri	5	5	0	0	33	4	29	0	13	1	10	2	45	0	44	1	22	0	22	0
Natitingou	8	8	0	0	73	12	61	0	10	1	9	0	11	0	9	2	17	6	11	0
Kouandé	4	3	1	0	58	2	56	0	42	1	41	0	7	1	6	0	18	0	18	0
Copargo	51	23	27	1	173	4	169	0	78	1	77	0	50	1	47	2	46	0	46	0

Figure 2: Monthly variation of *An. gambiae* molecular form

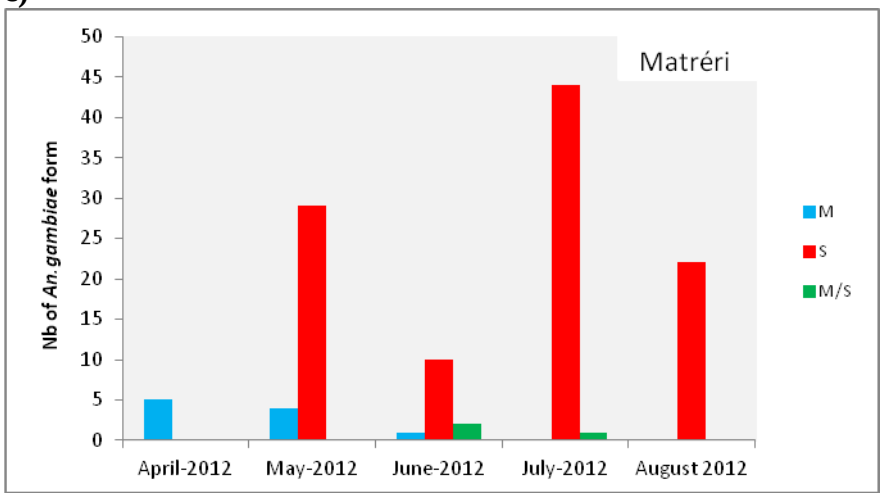
a)



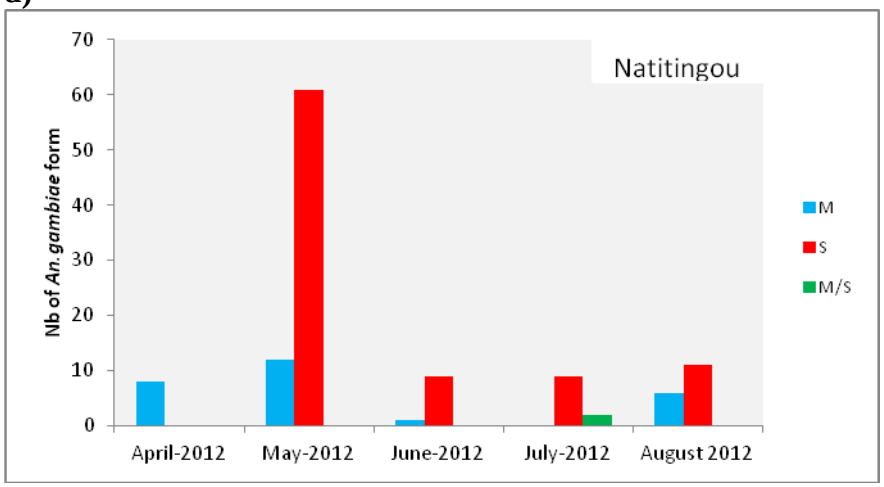
b)



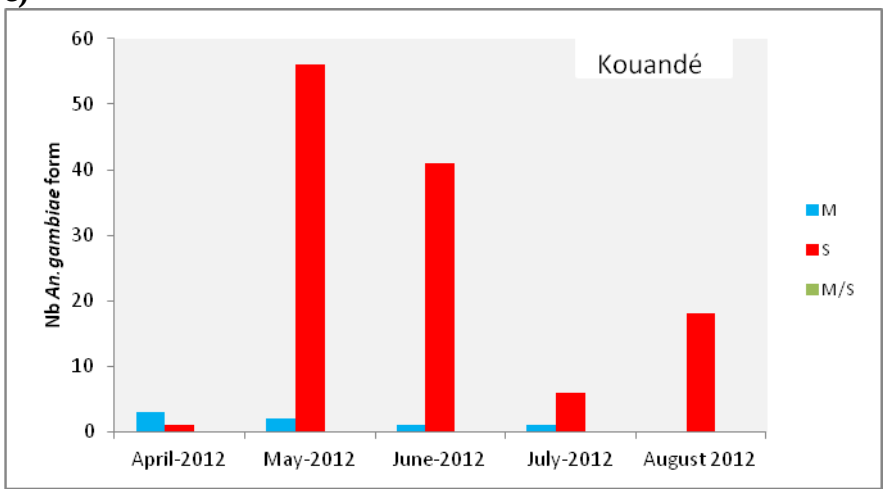
c)



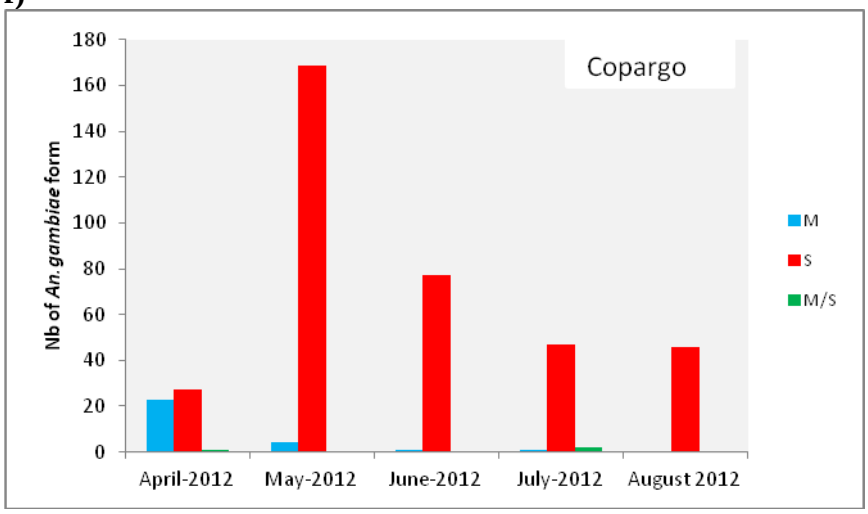
d)



e)



f)



<u>Figure 2</u>: Monthly variation of *An. gambiae* molecular form.

5.3.2. Decrease of density of *An. gambiae* in districts under IRS 5-6 months after intervention.

Table I below shows the densities of *An. gambiae s.l* caught indoors from April to October 2012. The data of April represent the results of mosquitoes collected during the dry season before the second round of IRS; those of May represent results recorded at the beginning of the spray. They represent the first data on the density of *An. gambiae s.l* at the beginning of the rainy season and while IRS was ongoing.. The data recorded from June to October were the results after IRS. They are, actually, the results that reflect the impact of IRS on the density of *An. gambiae s.l* if any. In reality, the densities reported here represent the number of *An. gambiae* s.l caught on Atacora aboriginal volunteers by landing catch after 16 night catches. In total, 234 *An. gambiae s.l* were caught in Copargo in the control area from June to August after 48 night catches, which adds up to 4.9 *An. gambiae s.l* per night catch. In the districts under IRS, the man landing rate of *An. gambiae s.l* was much lower: 1.4 (66/48) in Pehunco, 0.64 (31/48) in Tanguiéta, 1.6 (76/48) in Materi, 0.75 (36/48) in Natitingou and 1.4 (67/48) in Kouandé. The impact of IRS seemed to be much more remarkable on indoor vector-man contact.. In total, 245 *An. gambiae s.l* were caught inside the houses in the control district from May to August; this is equivalent to 7.65 mosquitoes per night catch against an average of 0.75 (121/160) in the districts under IRS altogether, which represents an 90.2% reduction.

Regarding data obtained in September and October, the drastic reduction observed between June and August (90.2% reduction) was not observed. During these 2 months, 5.9 *An. gambiae* were caught per night catch in the control distric against 3.4 in Tanguiéta, 2.6 in Matéri, 3.4 in Natitingou, 3.2 in Pehunco and 2.6 in Kouandé, that means a mean rate of 3.1 *An. gambiae* per night catch in September and October in the districts under IRS *versus* 5.9 for the control district (47.4% reduction). By other hand, contrary to what was observed in June-August, the frequency of *An. gambiae* is higher inside houses (3.5 per night catch: 281/80) than outside: (2.8: 221/80). These results show beyond 4 months (May-August), bendiocarb treated walls are no more effective against wild mosquitoes.

Figures 1a to 1f show the number of *An. gambiae s.l* caught inside and outside the houses in each district under IRS as well as in the control district. They also show the monthly change. It follows then that the anopheline man landing rates was much lower over the study period inside the houses in the districts under IRS except September-October (figures 1 a-e). But, the

control district (Copargo) rather showed the contrary. Here, the anopheline man landing rate was higher inside than outside except September-October (figure 1f). It can then be concluded from this observation that the interior of the houses was uncomfortable for the *An. gambiae s.l* to have a rest in, after the 4 months following IRS.

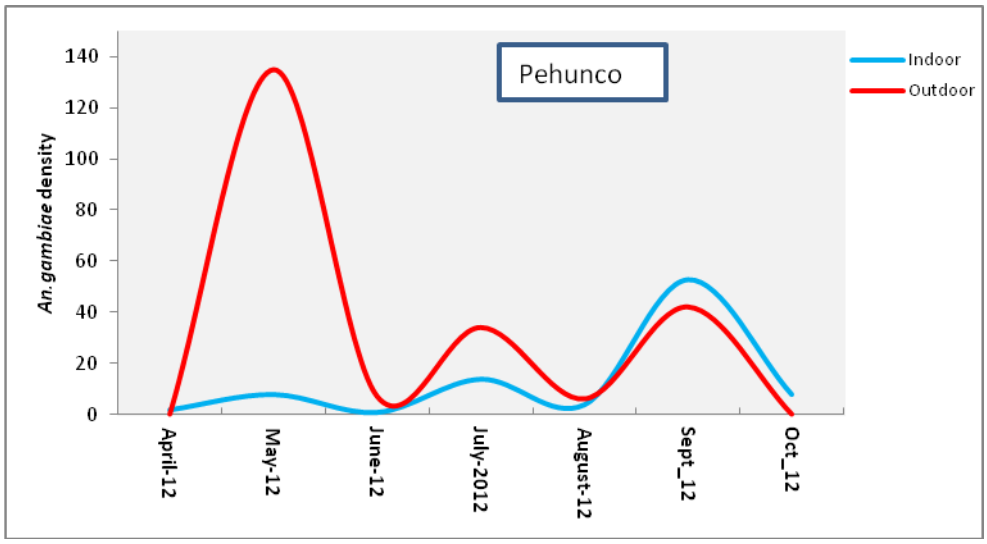
In 2011, the houses of Pehunco were not treated. Then, some data of Pehunco 2011 were used as control compared to those of Pehunco 2012. But, in 2011, the M&E was done each 2 months. Then, only data registered in July and September are available. Frequencies of indoor/outdoor *An. gambiae* in July and September 2011 *versus* 2012 were illustrated in figure 1 bis. These results confirm the drastic reduction of *An. gambiae* biting inside houses in districts under IRS (figure 1 bis)

Table I: Monthly variation in vector density from April to October 2012.

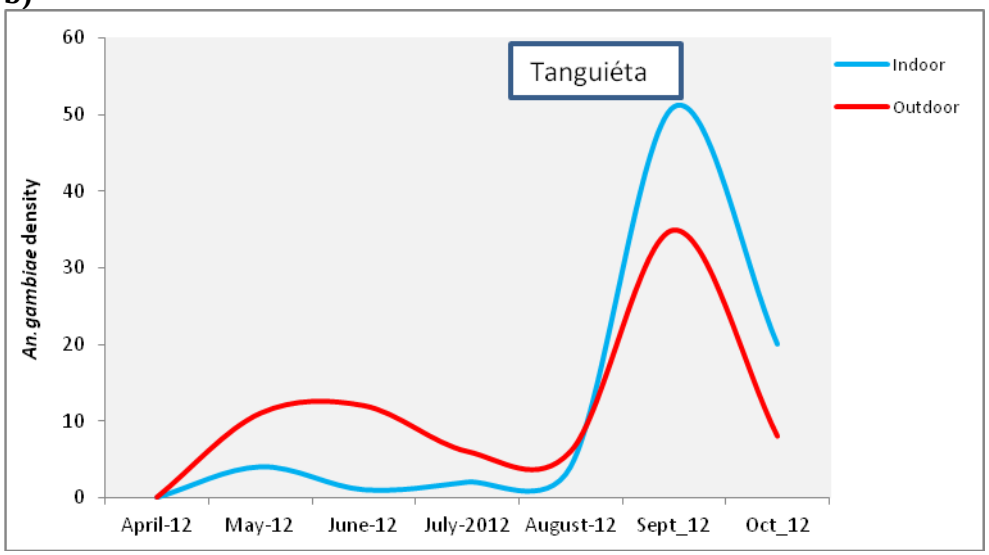
		April-2012	May-2012	June-2012	July-2012	August-2012	sept-2012	oct-2012
Pehunco	Indoor	2	8	1	14	4	53	8
	Outdoor	0	135	7	34	6	42	0
Tanguiéta	Indoor	0	4	1	2	4	51	20
	Outdoor	0	11	12	6	6	35	8
Matéri	Indoor	0	2	1	15	6	26	36
	Outdoor	0	26	8	30	16	5	16
Natitingou	Indoor	0	24	2	3	6	19	36
	Outdoor	1	48	7	7	11	36	19
Kouandé	Indoor	2	11	5	1	7	24	8
	Outdoor	0	46	37	6	11	49	1
Copargo	Indoor	23	79	50	67	26	86	44
	Outdoor	7	45	20	51	20	44	15

Figure 1: Monthly variation in Vectors density from April to October 2012.

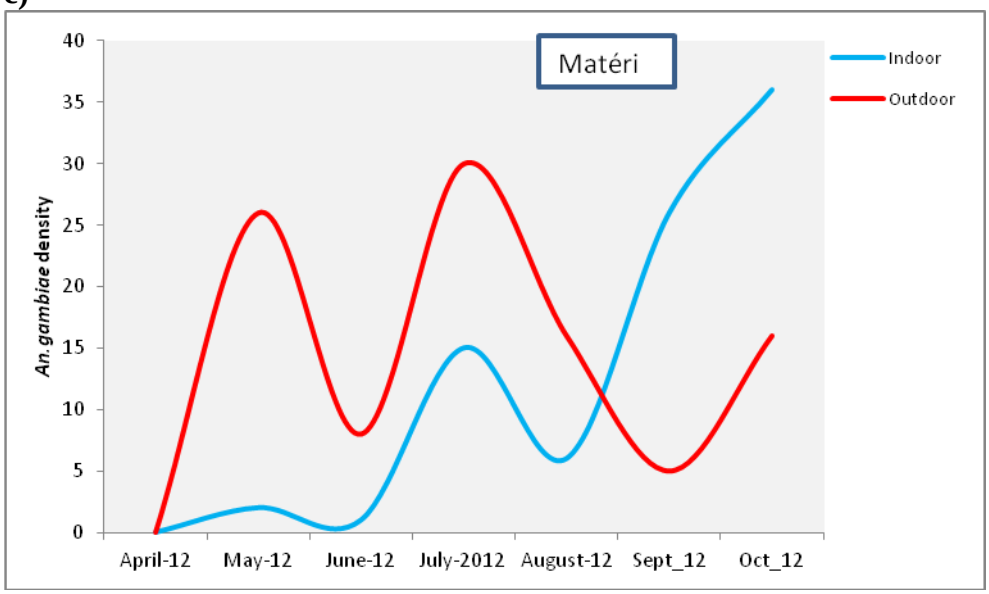
a)



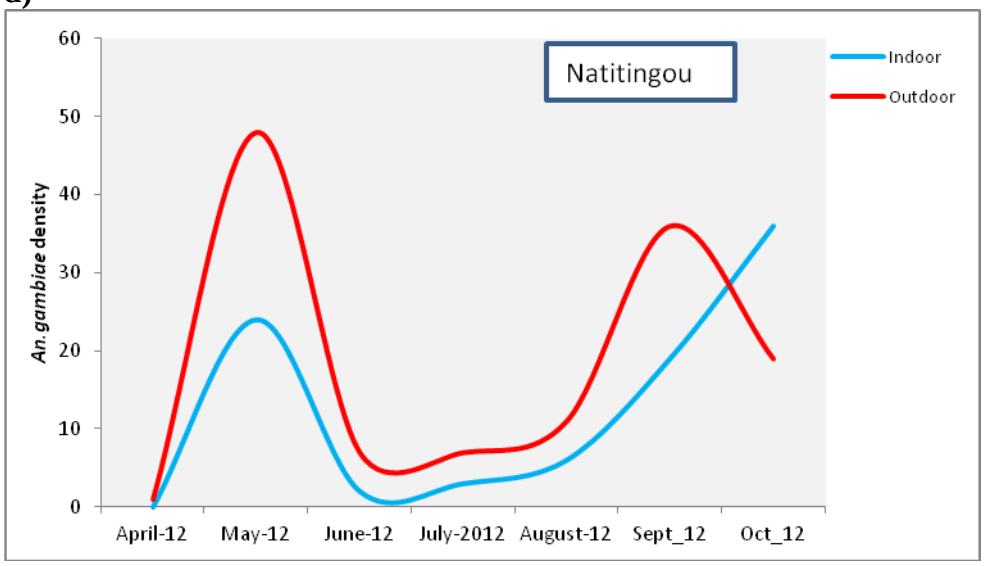
b)



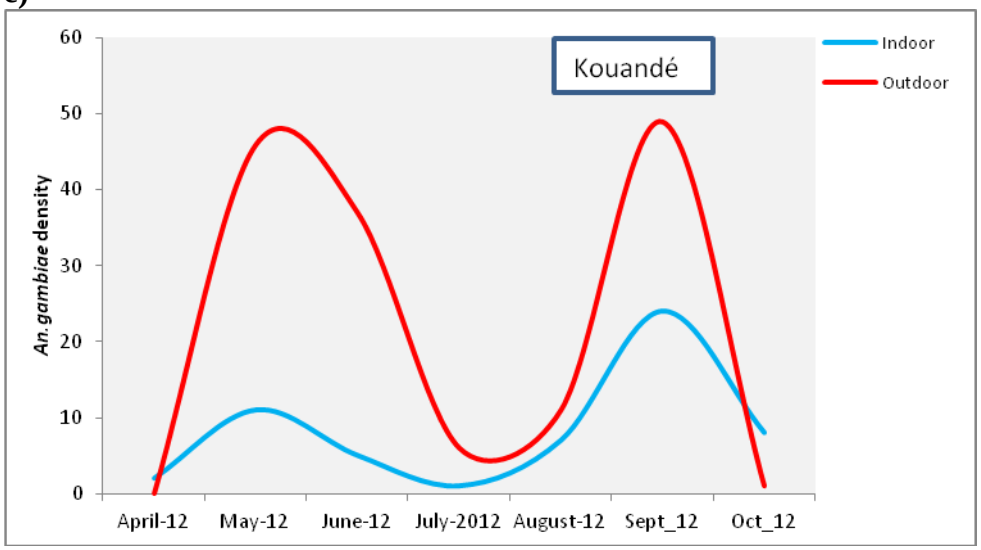
c)



d)



e)



f)

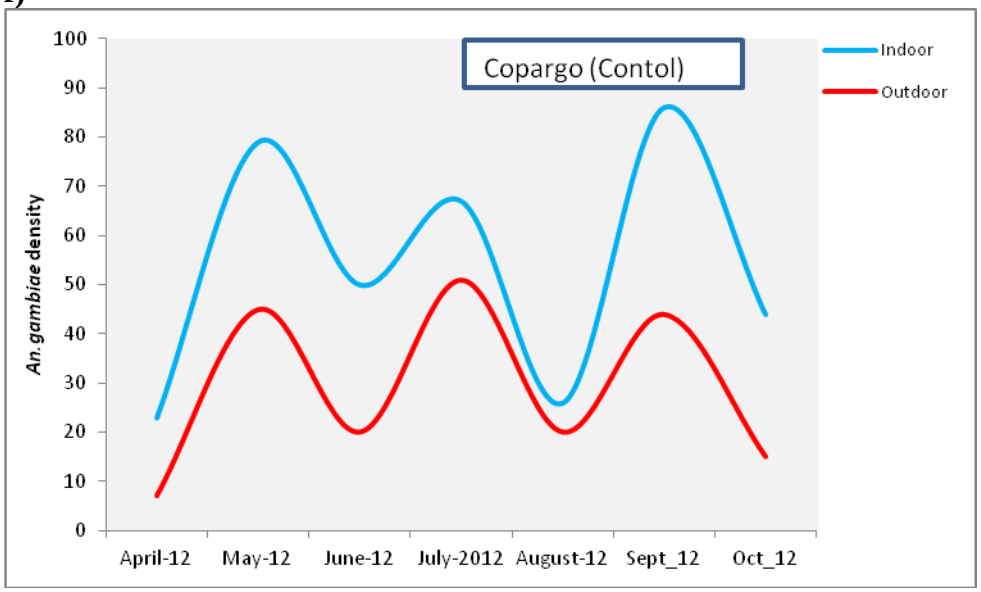


Figure 1: Monthly variation in Vectors density from April to October 2012.

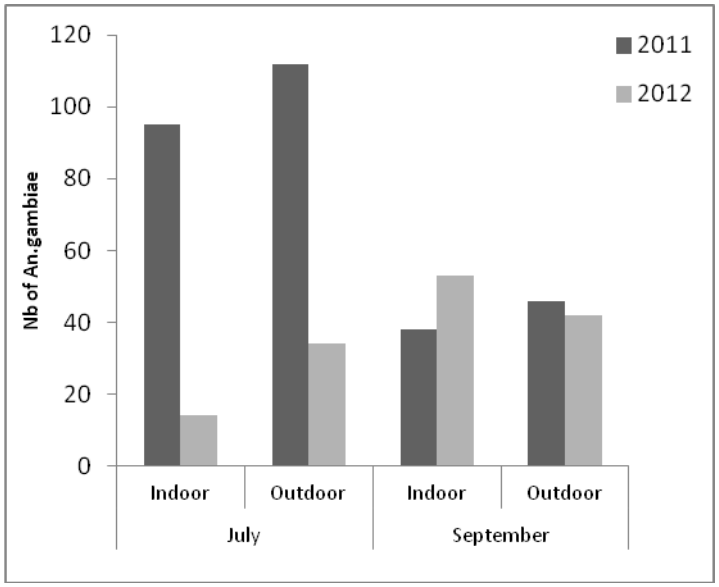


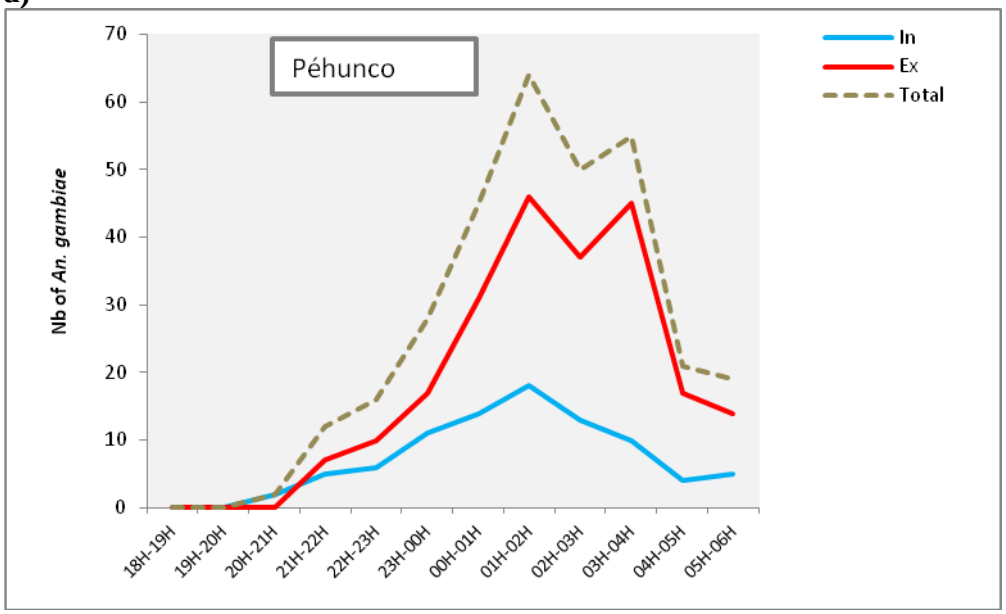
Figure 1 bis. Frequencies of indoor/outdoor *An. gambiae* in July and September 2011 and 2012 in Pehunco

5.3.3. Noctural biting cycle of An. gambiae

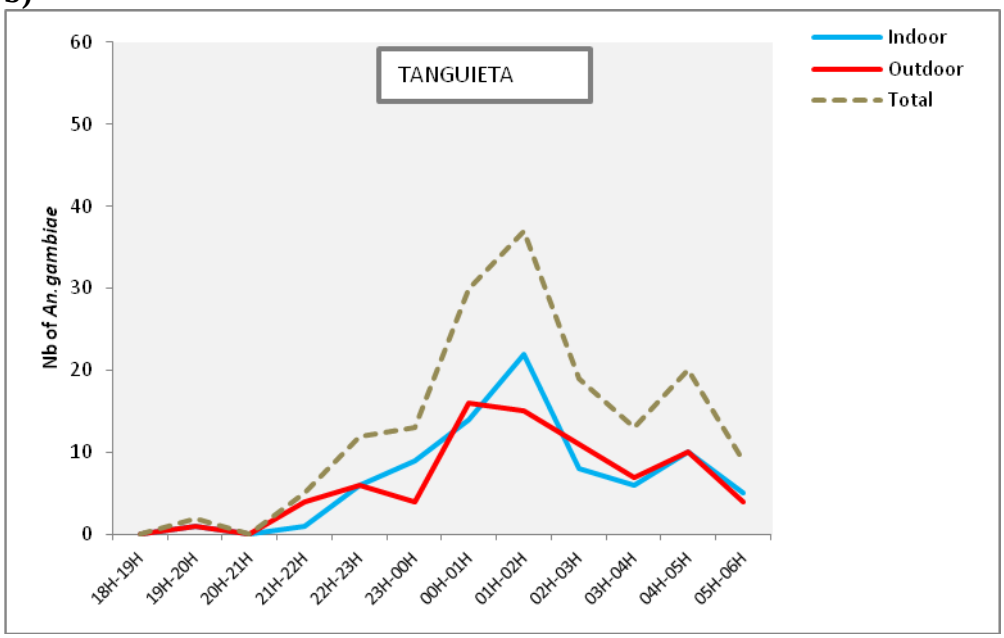
The nocturnal biting cycle shows 2 peaks. The biting frequency is high around 1 AM which is the first peak. Another peak less important is situated around 4 AM. This feeding behavior is the same for mosquitoes feeding inside and outside.

In Atacora, *An.gambiae* begins its blood feeding from 8-9 PM to 6 AM. Before, no Anopheles was caught. For the next mosquito landing catches, we suggest to begin at 9 PM to 6 AM.

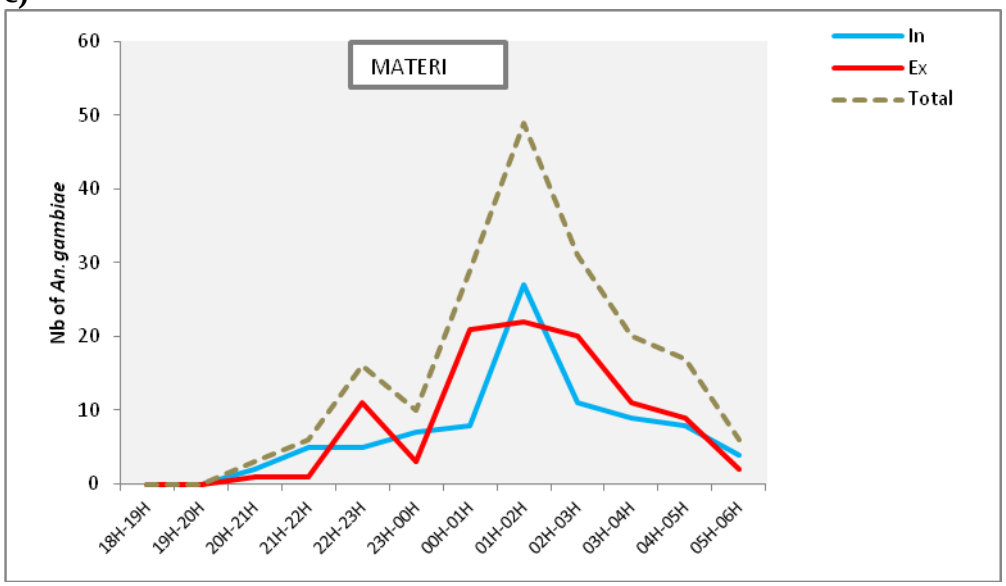
a)



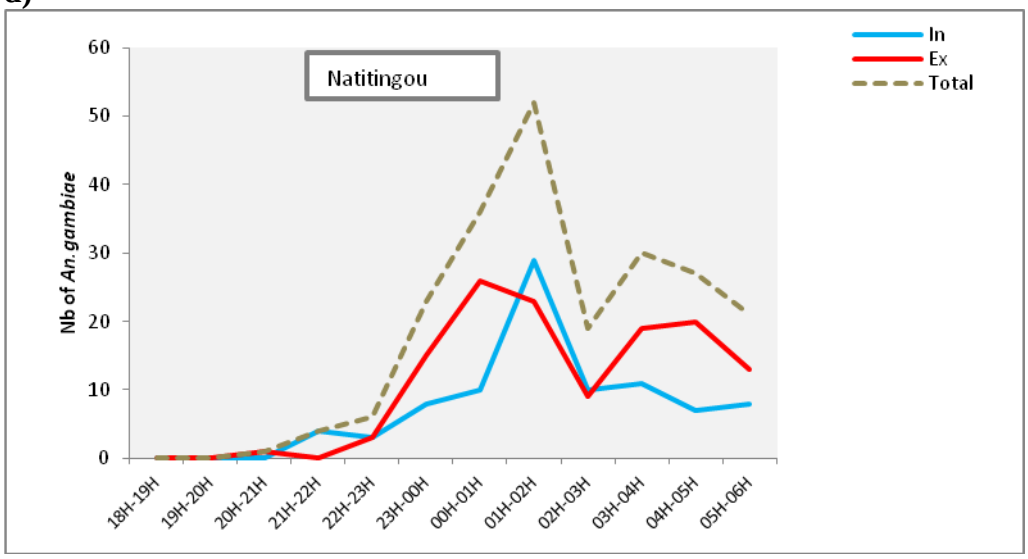
b)


TANGUIETA
Indoor
Outdoor
Total
Nb of An.gambiae
60
50
40
30
20
10
0
18H-19H
19H-20H
20H-21H
21H-22H
22H-23H
23H-00H
00H-01H
01H-02H
02H-03H
03H-04H
04H-05H
05H-06H

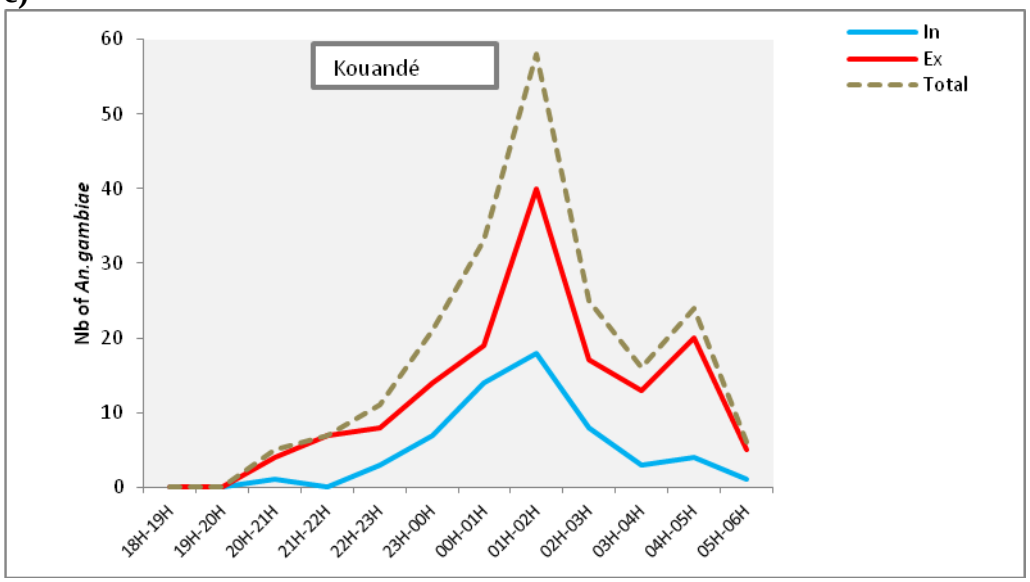
c)


MATERI
In
Ex
Total
Nb of An.gambiae
60
50
40
30
20
10
0
18H-19H
19H-20H
20H-21H
21H-22H
22H-23H
23H-00H
00H-01H
01H-02H
02H-03H
03H-04H
04H-05H
05H-06H

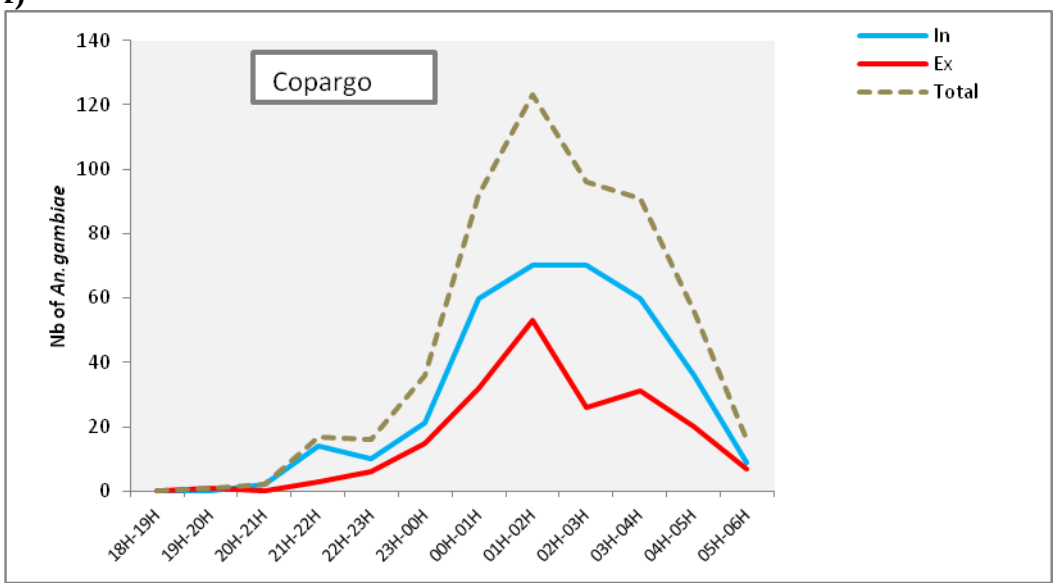
d)


Natitingou
In
Ex
Total
Nb of An.gambiae
60
50
40
30
20
10
0
18H-19H
19H-20H
20H-21H
21H-22H
22H-23H
23H-00H
00H-01H
01H-02H
02H-03H
03H-04H
04H-05H
05H-06H

e)



f)



<u>Figure</u>: The nocturnal biting cycle of *An. gambiae* from April to October 2012.

5. 4. Human Biting Rate (HBR) in districts under IRS

5.4.1. Decrease of HBR until 4 months after IRS

Table I below shows the number of bites of *An. gambiae* that a person receives per night each month since the implementation of IRS. The HBR registered in Copargo (control) are 6.25, 8,38 and 3.25 bites respectively in June, July and August, either an average rate of 178.7 bites (50+67+26/24x30) (Table I below) of *An. gambiae* inside houses for one month during the period June to August. Outside houses, each man receives 113.7 bites per month for the same period. In the districts under IRS, the HBR is lower during all the period after IRS. As a matter of fact, inside houses, the HBR decreases from 178.7 in the control district to 23.74 in

Pehunco (86.7% of reduction), 8.7 in Tanguieta (95.13% of reduction), 27.5 in Materi (84.6% of reduction), 13.7 in Natitingou (92.3% of reduction) and 16.2 in Kouande (90.9% of reduction). The reduction of the HBR is not as high outside houses : 113 bites per man per month in Copargo (control) against 31.24 in Natitingou (72.5% of reduction) and 58.5 in Pehunco (48.5% of reduction). In conclusion, in the districts under IRS, the HBR has drastically decreased inside houses.

5.4.2. Increase of HBR 5-6 months after IRS

Until 3-4 months after IRS, each inhabitant in districts under IRS received 16.7 *An. gambiae* bites inside houses for one month. Five and six months after IRS (September-October), the HBR increases to 105 bites/per man/month. By other hand, during this period, the HBR is higher inside houses (105 bites) than outside (79 bites/man/month).

Table I. Monthly variation in *An.gambiae* biting rate in the control and intervention districts from April to October 2012.

			April-2012	May-2012	June-2012	July-2012	August-2012	September-2012	October-2012
Pehunco	Inside	Total Mosquitoes	2	8	1	14	4	53	8
		nb human cathes	8	8	8	8	8	8	8
		nb night catches	2	2	2	2	2	2	2
		HBR/night	**0,25**	**1,00**	**0,13**	**1,75**	**0,50**	**6,63**	**1,00**
	Outside	Total Mosquitoes	0	135	7	34	6	42	0
		nb human cathes	8	8	8	8	8	8	8
		nb night catches	2	2	2	2	2	2	2
		HBR/night	**0,00**	**16,88**	**0,88**	**4,25**	**0,75**	**5,25**	**0,00**
Tanguiéta	Inside	Total Mosquitoes	0	4	1	2	4	51	20
		nb human cathes	8	8	8	8	8	8	8
		nb night catches	2	2	2	2	2	2	2
		HBR/night	**0,00**	**0,50**	**0,13**	**0,25**	**0,50**	**6,38**	**2,50**
	Outside	Total Mosquitoes	0	11	12	6	6	35	8
		nb human cathes	8	8	8	8	8	8	8
		nb night catches	2	2	2	2	2	2	2
		HBR/night	**0,00**	**1,38**	**1,50**	**0,75**	**0,75**	**4,38**	**1,00**
Matéri	Inside	Total Mosquitoes	0	2	1	15	6	26	36
		nb human cathes	8	8	8	8	8	8	8
		nb night catches	2	2	2	2	2	2	2
		HBR/night	**0,00**	**0,25**	**0,13**	**1,88**	**0,75**	**3,25**	**4,50**
	Outside	Total Mosquitoes	0	26	8	30	16	5	16
		nb human cathes	8	8	8	8	8	8	8
		nb night catches	2	2	2	2	2	2	2

		HBR/night	**0,00**	**3,25**	**1,00**	**3,75**	**2,00**	**0,63**	**2,00**
	Inside	Total Mosquitoes	0	24	2	3	6	19	36
		nb human cathes	8	8	8	8	8	8	8
		nb night catches	2	2	2	2	2	2	2
		HBR/night	**0,00**	**3,00**	**0,25**	**0,38**	**0,75**	**2,38**	**4,50**
Natitingou									
	Outside	Total Mosquitoes	1	48	7	7	11	36	19
		nb human cathes	8	8	8	8	8	8	8
		nb night catches	2	2	2	2	2	2	2
		HBR/night	**0,13**	**6,00**	**0,88**	**0,88**	**1,38**	**4,50**	**2,38**
	Inside	Total Mosquitoes	2	11	5	1	7	24	8
		nb human cathes	8	8	8	8	8	8	8
		nb night catches	2	2	2	2	2	2	2
		HBR/night	0,25	1,38	0,63	0,13	0,88	3,00	1,00
Kouandé									
	Outside	Total Mosquitoes	0	46	37	6	11	49	1
		nb human cathes	8	8	8	8	8	8	8
		nb night catches	2	2	2	2	2	2	2
		HBR/night	**0,00**	**5,75**	**4,63**	**0,75**	**1,38**	**6,13**	**0,13**
	Inside	Total Mosquitoes	23	79	50	67	26	86	44
		nb human cathes	8	8	8	8	8	8	8
		nb night catches	2	2	2	2	2	2	2
		HBR/night	**2,88**	**9,88**	**6,25**	**8,38**	**3,25**	**10,75**	**5,50**
Copargo (Control)									
	Outside	Total Mosquitoes	7	45	20	51	20	44	15
		nb human cathes	8	8	8	8	8	8	8
		nb night catches	2	2	2	2	2	2	2
		HBR/night	**0,88**	**5,63**	**2,50**	**6,38**	**2,50**	**5,50**	**1,88**

5.5. Decrease of sporozoitic index of P.falciparum in An. gambiae in districts under IRS

In the control area, 383 thoraces were analyzed by ELISA CSP from May to October. The sporozoitic index is high: %CS+ = 10.4. (40 thoraces+ for circum-sporozoitic antigen). This index is lower in the districts under IRS during the same period: 3% (28 th+/935).

5.6. Entomological Inoculation Rate (EIR) in the districts under IRS

5.6.1. Drastic decrease of EIR until 3-4 months after IRS

Table I below shows the Entomological Inoculation Rate (EIR) from April to August for each district. In the control area, the mean HBR is 150.7 bites for one month and the mean sporozoitic index is 0.082, either 49.7 infected bites of *An. gambiae* per man for the total of the 4 months of the study (May to August). In the districts under IRS, the EIR has drastically decreased: 4 infected bites of *An. gambiae* per man for the 4 months (0.024 x 41.6 x 4) in

Kouande, 3.2 in Natitingou, 1.6 in Materi, 4.64 in Tanguieta and 2.28 in Pehunco, either respectively 92%, 93.5%, 96.8%, 90.6% and 95.4% of reduction compared to the control. Except Pehunco, 2 and 3 months after IRS, the EIR was negative in all districts under IRS.

5.6.2. Increase of EIR 5 months after IRS

The EIR is higher in September-October (3.7 infected bites per month = 0.04 x 92.25) than in May-August (2.5: 0.023 x 110.8) in districts under IRS. But, during the same period, the EIR has increased from 14.6 infected bites per month (0.082 x 178.7) in May-August to 30.1 (0.17 x 177.1) in September-October in the control.

Table I : Infection rate for *P.falciparum* calculated by circumsporozoite protein (CSP) ELISA from the head and thoraxes of *An.gambiae* from April to October 2012.

		Apr_ 2012	May_ 2012	June_ 2012	July_ 2012	August_ 2012	Sept_ 2012	Oct_ 2012
Pehunco	Thorax	2	143	8	48	10	40	8
	Thorax +	0	0	1	2	0	0	0
	IS	0	0	0,13	0,04	0	0	0
	HBR/night	0,13	8,94	0,50	3,00	0,63	5,94	0,50
	EIR	**0**	**0**	**0,06**	**0,13**	**0**	**0**	**0**
Tanguiéta	Thorax	0	15	13	8	10	40	28
	Thorax +	0	1	2	0	0	0	6
	IS	0	0,07	0,15	0	0	0	0,2143
	HBR/night	0	0,94	0,81	0,5	0,63	5,38	1,75
	EIR	**0,00**	**0,06**	**0,12**	**0,00**	**0,00**	**0,00**	**0,38**
Matéri	Thorax	0	28	9	45	22	31	52
	Thorax +	0	0	1	0	0	0	4
	IS	0,00	0,00	0,11	0,00	0,00	0,00	0,08
	HBR/night	0	1,75	0,56	2,81	1,38	1,94	3,25
	EIR	**0**	**0**	**0,06**	**0**	**0**	**0**	**0,25**
Natitingou	Thorax	1	72	9	11	17	40	55
	Thorax +	0	4	0	0	0	0	4
	IS	0,00	0,06	0,00	0,00	0,00	0,00	0,07
	HBR/night	0	4,5	0,56	0,63	1,06	3,44	3,44
	EIR	**0,00**	**0,25**	**0,00**	**0,00**	**0,00**	**0,00**	**0,25**
Kouandé	Thorax	2	57	42	7	18	40	9
	Thorax +	0	1	2	0	0	0	0
	IS	0	0,02	0,05	0	0	0	0
	HBR/night	0,13	3,56	2,63	0,44	1,13	4,56	0,56
	EIR	**0,00**	**0,06**	**0,13**	**0**	**0,00**	**0,00**	**0,00**
Copargo (Contol)	Thorax	30	124	70	50	46	40	53
	Thorax +	4	9	6	7	2	3	13
	IS	0,13	0,07	0,09	0,14	0,04	0,08	0,25
	HBR/night	1,88	7,75	4,38	7,38	2,88	8,13	3,69
	EIR	**0,25**	**0,56**	**0,38**	**1,03**	**0,13**	**0,61**	**0,91**

5.7. Physiological Age Grading ??????????? (me voir)

Table II below shows the parity rate of *An. gambiae* collected inside and outside houses by human landing catch and pyrethrum spray catch (PSC) + Exit Window Trap (EWT) for each month and each district. From May to October, in total 325 ovaries were dissected and examined in the control district (Copargo) from which 208 were found parous; the parous rate is 63.1%. In the districts under IRS, the parous rate is significantly lower: 27.7% (212/767) ($p< 0.05$).

5.8. Exophily

Very few mosquitoes were trapped using window traps during the study to provide a good estimation of the exophily rate as shown on table III. However, the table shows an increase of exophily in districts under IRS (40.2%: 35/87) from June to October compared to the control district (22.2%: 12/54).

5.10. Blood feeding rate of mosquitoes caught in exit window traps and by PSC

The blood feeding rate of *An. gambiae* is high in Copargo (control). It varies from 87% to 100% during the IRS period. During the same period, the rate is lower than 50% in the districts under IRS.except August where the three *An. gambiae* collected were found fed

Table II: Parous rate of *An.gambiae* catch by HLC (indoor and outdoor) and by PSC and EWT from April to October 2012.

		April_2012			May_2012			June_2012			July_2012			August_2012			Sept_2012			Oct_2012		
		N Tested	Parous	Parous rate (%)	N Tested	Parous	Parous rate (%)	N Tested	Parous	Parous rate (%)	N Tested	Parous	Parous rate (%)	N Tested	Parous	Parous rate (%)	N Tested	Parous	Parous rate (%)	N Tested	Parous	Parous rate (%)
Pehunco	Indoor	2	2	**100**	8	1	**12,50**	1	0	**0**	14	6	**42,86**	4	1	**25,00**	15	4	**26,7**	8	3	**37,5**
	Outdoor	0	0	**_**	91	19	**20,88**	6	1	**16,67**	25	14	**56**	6	2	**33,33**	15	7	**46,7**	0	0	**_**
	PSC + EWT	11	11	**100**	1	0	**0**	1	0	**0**	9	3	**33,33**	2	0	**0,00**	0	0	**_**	0	0	**_**
Tanguiéta	Indoor	0	0	**_**	4	0	**0**	1	0	**0**	2	0	**0**	4	1	**25,00**	15	5	**33,3**	10	3	**30**
	Outdoor	0	0	**_**	11	3	**27,27**	9	2	**22,22**	3	2	**66,67**	6	1	**16,67**	14	4	**28,6**	5	1	**20**
	PSC + EWT	5	5	**100**	1	0	**0**	3	0	**0**	3	1	**33,33**	1	0	**0,00**	0	0	**_**	0	0	**_**
Matéri	Indoor	0	0	**_**	2	0	**0**	1	0	**0**	15	5	**33,33**	6	1	**16,67**	16	4	**25**	9	4	**44,4**
	Outdoor	0	0	**_**	26	4	**15,38**	4	1	**25**	23	10	**43,48**	16	4	**25,00**	15	8	**53,3**	6	3	**50**
	PSC + EWT	5	5	**100**	5	0	**0**	4	0	**0**	7	2	**28,57**	0	0	**_**	0	0	**_**	0		**_**
Natitingou	Indoor	0	0	**_**	24	2	**8,33**	2	0	**0**	3	1	**33,33**	6	1	**16,67**	15	6	**40**	10	5	**50**
	Outdoor	1	1	**100**	48	9	**18,75**	6	1	**16,67**	3	2	**66,67**	11	4	**36,36**	15	13	**86,7**	6	2	**33,3**
	PSC + EWT	7	7	**100**	1	0	**0**	1	0	**0**	5	1	**20**	0	0	**_**	0	0	**_**	0	0	**_**
Kouandé	Indoor	2	2	**100**	11	1	**9,09**	5	1	**20**	1	0	**0**	7	2	**28,57**	15	8	**53,3**	8	3	**37,5**
	Outdoor	0	0	**_**	46	8	**17,39**	37	3	**8,108**	5	3	**60**	11	3	**27,27**	17	10	**58,8**	1	0	**0**
	PSC + EWT	2	2	**100**	1	0	**0**	0	0	**_**	0	0	**_**	0	0	**_**	0	0	**_**	0	0	**_**
Copargo (Control)	Indoor	23	22	**95,7**	29	27	**93,10**	44	18	**40,91**	20	15	**75**	26	11	**42,31**	17	11	**64,7**	9	6	**66,7**
	Outdoor	7	6	**85,7**	27	22	**81,48**	20	14	**70**	20	10	**50**	20	9	**45,00**	17	7	**41,2**	8	3	**37,5**
	PSC + EWT	21	21	**100**	49	36	**73,47**	8	6	**75**	10	6	**60**	11	6	**54,55**	0	0	**_**	1	1	**100**

Table III: Mosquitoes exophily rate observed from April to October 2012.

Districts	April-2012			May-2012			June-2012			July-2012			August-2012			September_2012			October-2012		
	EWT	PSC	Exophily rate (%)	EWT	PSC	Exophily rate (%)	EWT	PSC	Exophily rate (%)	EWT	PSC	Exophily rate (%)	EWT	PSC	Exophily rate (%)	EWT	PSC	Exophily rate (%)	EWT	PSC	Exophily rate (%)
Pehunco	1	10	**9,09**	0	1	**0**	0	1	**0**	8	1	**88,89**	2	0	**100**	3	7	**30**	0	0	_
Tanguiéta	0	5	**0**	1	0	**100**	3	0	**100**	0	3	**0**	1	0	**100**	2	3	**60**	2	9	**18,18**
Matéri	0	5	**0**	4	1	**80,00**	2	2	**50**	6	1	**85,71**	0	0	_	0	3	**100**	0	8	**0**
Natitingou	0	7	**0**	0	1	**0**	0	1	**0**	1	4	**20**	0	0	_	2	2	**50**	3	6	**33,33**
Kouandé	0	2	**0**	0	1	**0**	0	0	**0**	0	0	_	0	0	_	0	0	_	0	1	**0**
Copargo	3	18	**14,29**	37	12	**75,51**	7	1	**87,5**	0	18	**0**	3	8	**27,27**	2	5	**71,43**	0	10	**0**

Table IV: Blood feeding rate of *An. gambiae* collected by EWT and PSC from April to october 2012.

Districts	April_2012			Mai_2012			June_2012			July_2012			August_2012			Sept_2012			oct-2012		
	Total (PSC+ EWT)	N .feed	Blood feeding rate	Total (PSC+ EWT)	N .fed	Blood feedig rate	Total (PSC+ EWT)	N. Fed	Blood feedig rate	Total (PSC+ EWT)	N .fed	Blood feedig rate	Total (PSC+ EWT)	N .fed	Blood feedig rate	Total (PSC+ EWT)	N .fed	Blood feedig rate	Total (PSC+ EWT)	N. fed	Blood feedig rate
Pehunco	11	11	**100**	1	0	**0**	1	0	**0**	9	4	**44,44**	2	2	**100,00**	1	0	**0**	0	0	_
Tanguiéta	5	0	**0**	1	0	**0**	3	1	**33,33**	3	2	**66,67**	1	1	**100,00**	1	0	**0**	11	7	**63,64**
Matéri	5	0	**0**	5	3	**60**	4	1	**25**	7	3	**42,86**	0	0	_	5	3	**60**	8	8	**100**
Natitingou	7	4	**57,14**	1	0	**0**	1	0	**0**	5	5	**100**	0	0	_	1	0	**0**	9	9	**100**
Kouandé	2	0	**0**	1	0	**0**	0	0	**0**	0	0	_	0	0	_	1	0	**0**	1	1	**100**
Copargo	21	5	**23,81**	49	43	**87,75**	8	7	**87,5**	18	18	**100**	11	11	_	49	43	**87,75**	10	10	**100**