348 Table1: Indoor versus outdoor host and flea species number.

Host species	Indoor			Outdoor		
	Host No.	*Xenopsylla cheopis*	*Synopsyllus fonquerniei*	Host No.	*Xenopsylla cheopis*	*Synopsyllus fonquerniei*
R. rattus	252	160	7	272	34	52
M. musculus	149	19	1	0	0	0
S. murinus	3	0	0	3	0	1
Total	404	179	8	275	34	53

349

350 Table 2: Rodents and flea indices for each group and capture session

Treatment	Day 0 (pre-treatment)			Day 2 (post-treatment)			Day 35 (post-treatment)		
	No. rodent	Infested rodent (%)	Flea index	No. rodent	Infested rodent (%)	Flea index	No. Ro-dent	Infested rodent (%)	Flea index
Control	69	18 (26.1)	0.58	63	21 (23.8)	0.54	68	30.9 (33)	0.70
Dust	83	21 (25.0)	0.49	88	9 (10.2)[a]	0.15[b]	70	31.4 (18)	0.65[c]
Bait-box	96	33 (34.4)	1.02	73	22 (24.7)	0.68	69	44.9 (31)	2.30

351 [a] :the proportion of infested rodent was significantly lower when compared with Day 0, with p=0,00078

352 [b]: The flea index was significantly lower than in Day 0, with p=0,0105

353 [c]: The flea index was significantly higher than in Day 2, with p= 0,0016

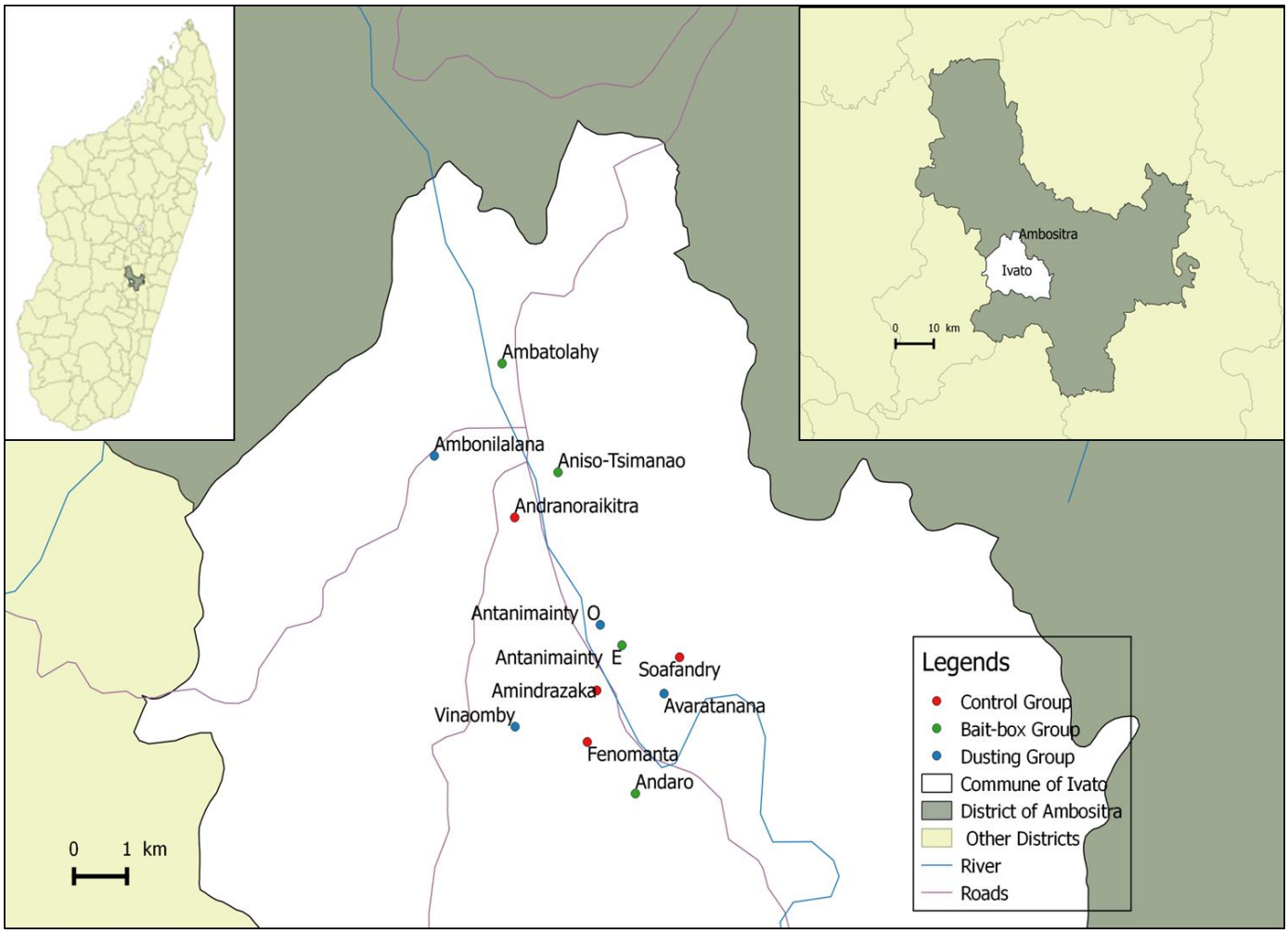
354

355 Table 3: Specific flea index according to groups, day of sampling and trap emplacement

Treatment	Trap emplacement	***Xenopsylla cheopis***		***Synopsyllus sp.***	
		Day 0	Day 2	Day 0	Day 2
Control	Indoor	0.87	0.47	0.06	0.05
Control	Outdoor	0.08	0.16	0.23	0.40
Dust	Indoor	0.60	0.02	0.00	0.02
Dust	Outdoor	0.09	0.11	0.24	0.20
Kartman	Indoor	1.21	0.78	0.03	0.03
Kartman	Outdoor	0.26	0.21	0.29	0.26

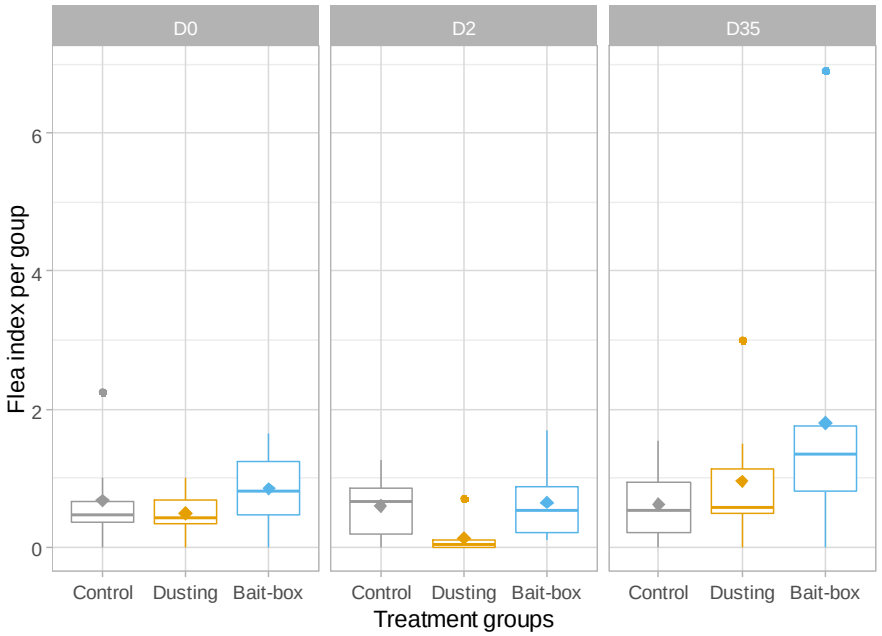
356

Figure1: Study sites.



Upright corner: map of Madagascar showing the location of the district of Ambositra. Upleft corner: the location of the commune of ivato in the district of ambositra.

Figure 2: Comparison of flea index, between treatments for each session capture.

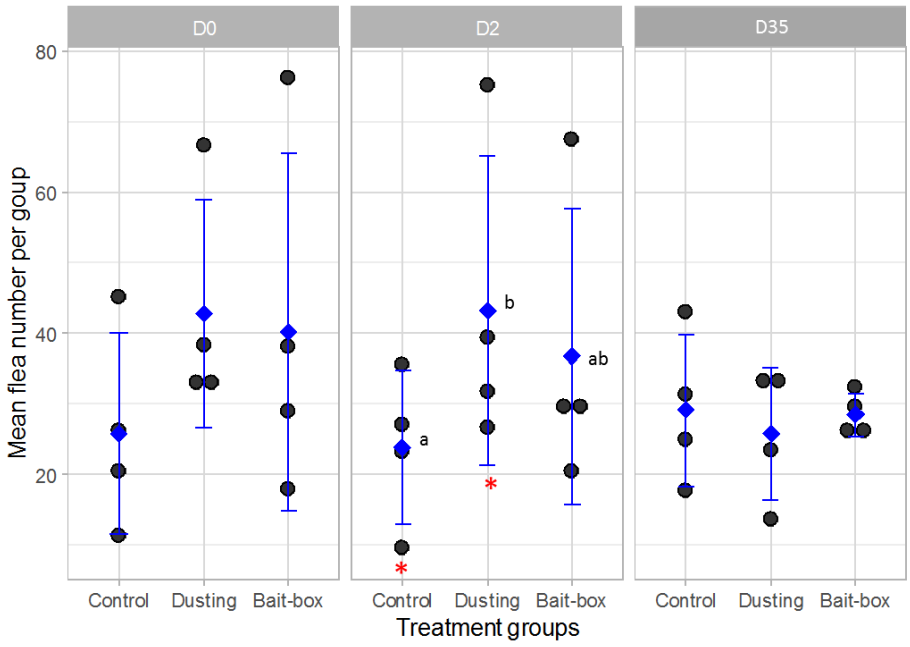


363

364 D0, D2 and D35 are the three capture sessions, corresponding to preliminary capture, two days and one
365 month post-treatment, respectively. For each boxplot, diamond-shaped dot represent the mean flea
366 index for each group. Outlier dots are uncommon value of flea index.

367

368 Figure 3: Comparison of the mean house index (house fleas) between treatments for each session
369 capture.



370

D0, D2 and D35 are the three capture session. Blue dots and blue lines represent the mean house index and standard error for each group. Grey dots are the mean house index for each village in the group. Red asterisk indicate the group where there was “hamlet effect”. The same letter (a, b and ab) found in a group indicates the absence of significant difference.

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V. Evaluation en laboratoire du fipronil, insecticide systémique, pour lutter contre *Xenopsylla cheopis*

1. Contexte

La méthode utilisant l'épandage d'insecticide en poudre à l'intérieur des habitations a été utilisée à Madagascar depuis 1947 pour lutter contre les puces de rat (Brygoo 1966). Les résultats de nos études précédentes ont montré une résistance des puces *Xenopsylla cheopis* aux familles d'insecticides habituellement utilisés en lutte anti-vectorielle (articles 2, 3 et 4). Pour gérer l'expansion de la résistance aux insecticides, il est primordial d'explorer d'autres alternatives ayant un mode d'action ou une formulation différente. L'utilisation d'un insecticide systémique pourrait être un alternatif pour réduire la densité des puces de rat. Un insecticide systémique, par opposition aux insecticides qui agissent par contact, agit sur l'insecte par voie orale. L'efficacité des insecticides systémiques a été déjà évaluée au cours de plusieurs études pour contrôler les puces de rongeurs dans les foyers de peste (Bennington 1960, Clark and Cole 1968a, 1968b, Miller et al 1972, Mbise et al 1994, Borchert et al 2009, Jachowski et al 2012). L'insecticide doit être ingéré par l'hôte pour agir sur les insectes hématophages tels que les puces.

L'utilisation d'insecticide systémique n'a jamais été évaluée à Madagascar, alors que l'association étroite entre *X. cheopis* et les deux espèces de rongeurs réservoirs de la peste (*Rattus norvegicus* et *R. rattus*) pourrait favoriser une approche systémique de la lutte. Comparée aux méthodes de traitements actuelles, un insecticide systémique qui ciblerait directement la puce sur son hôte pourrait offrir de meilleures perspectives en terme d'efficacité, tout en limitant les effets toxiques sur les insectes non-cibles et le contact avec l'homme.

Le fipronil s'avère alors être un bon candidat. Appartenant à la famille des phenylpyrazoles, c'est un insecticide qui est toxique par contact et par la voie systémique. Il a été largement utilisé pour contrôler les infestations de puces de chat, *Ctenocephalides felis*, en exploitant ses deux voies d'administration (Dryden et al 2000, Schenker et al 2003, Rust 2016). Son utilisation en tant qu'insecticide systémique

contre les puces de rat a donné des résultats prometteurs (Borchert et al 2001, Leirs et al 2001, Santora et al 2002, Poché et al 2017). L'intérêt du fipronil réside aussi dans le fait qu'il appartient à une famille d'insecticide qui n'a pas encore été utilisé pour la lutte contre les puces vectrices à Madagascar. Son mode d'action est similaire aux cyclodiènes (Sparks and Nauen 2015), limitant ainsi le phénomène de résistance croisé entre les insecticides utilisés actuellement contre les puces de rat.

Ainsi cette étude a pour objectif de déterminer l'efficacité du fipronil en tant qu'insecticide systémique contre *X. cheopis.* Les DL50 chez les puces exposées par contact et par la voie systémique vont être déterminées. L'hypothèse étant que le fipronil utilisé comme insecticide systémique soit plus efficace dans le contrôle des puces de rongeurs.

2. Méthode

Des puces résistantes à la deltaméthrine ont été utilisées pour les tests. Nous avons utilisé le fipronil sous forme d'une émulsion liquide, vendu dans le commerce sous la marque Termidor 25 EC (BASF, Operation Crop Protection, Ludwigshafen, Germany). Premièrement, des tests de toxicité aiguë ont été effectués avec *X. cheopis* se gorgeant sur un dispositif d'alimentation artificiel contenant du sang mélangé à des doses connues de fipronil (Figure 17). Deuxièmement, les puces ont été gorgées sur les deux espèces de rongeurs ayant consommé des appâts contenant des doses connues de fipronil. Enfin, des puces ont été exposées aux papiers imprégnés de fipronil, selon le protocole de test insecticide préconisé par l'OMS, afin de comparer la toxicité du fipronil par voie systémique et par contact. Pour tous les tests, la mortalité a été enregistrée pour les puces gorgées. La DL50 a été évaluée en utilisant un modèle (GML : generalized linear model) qui estime la dose correspondant aux mortalités.

3. Principaux résultats

Le fipronil a entraîné une mortalité chez les populations étudiées, pourtant résistantes à la deltaméthrine et à d'autres insecticides. Aucune résistance croisée à cet insecticide n'a été observée. Aucun problème d'appétence ni avec les rats vis-à-vis des appâts traités ni avec les puces avec le sang traité en gorgement artificiel n'a été rencontré. Néanmoins le taux de gorgement des puces avec cette méthode restait très faible. Cet insecticide était neuf fois plus toxique pour les puces par voie systémique que par contact. La toxicité du fipronil consommé par les rats augmente avec le temps avec une DL50 observée chez les puces qui diminue régulièrement pendant les 72 heures suivant la consommation de l'insecticide.

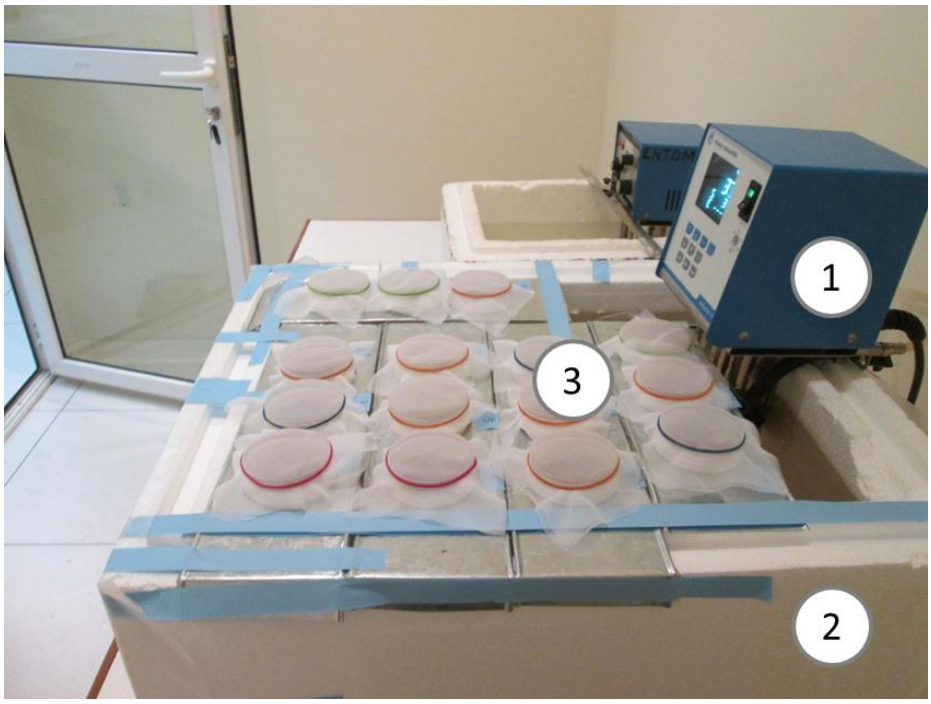

Figure 17: Dispositif de gorgement artificiel.
1. dispositif de chauffage. **2.** bac contenant de l'eau chauffée, **3.** cellule de gorgement (voir détail dans l'article). (Photo : A Miarinjara)

4. Valorisation scientifique

Les détails de la méthodologie ainsi que les résultats de ces travaux sont parus dans l'article suivant :

Article 6 :

Rajohnson Dora Murielle, **Miarinjara Adélaïde**, Rahelinirina Soanadrasana, Minoarisoa Rajerison, Boyer Sébastien. (2017) Effectiveness of Fipronil as a Systemic Control Agent against *Xenopsylla cheopis* (Siphonaptera: Pulicidae) in Madagascar. Journal of Medical Entomology. doi: 10,1093/jme/tjw200,

Article 6

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Research article

Vector Control, Pest Management, Resistance, Repellents

OXFORD

Effectiveness of Fipronil as a Systemic Control Agent Against *Xenopsylla cheopis* (Siphonaptera: Pulicidae) in Madagascar

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Abstract

Fipronil was evaluated as a systemic control agent for the rat flea *Xenopsylla cheopis* (Rothschild), the main vector of *Yersinia pestis* (Yersin), the causative agent of plague, in Madagascar. The effectiveness of fipronil as a systemic control agent against *X. cheopis* was assessed by determining the toxicity values of the "Lethal Dose 50" (LD_{50}). Two techniques were used to evaluate the systemic action of the insecticide on the vector: 1) an artificial feeding device filled with blood–fipronil mixture from which *X. cheopis* was fed and 2) rodent hosts, *Rattus norvegicus* (Berkenhout) and *Rattus rattus* (L.), which fed on fipronil-treated bait. As a standardized control method, the susceptibility of *X. cheopis* to fipronil was evaluated by exposure to impregnated paper within World Health Organization (WHO) insecticide test protocol to compare its effect to the systemic activity of the studied insecticide. Results showed that when administered in a systemic way, fipronil appears to be more effective: the toxicity level was evaluated to be ninefold higher compared with the WHO test. Compared with other methods, which require indiscriminate dusting of rodent burrows and human dwellings, fipronil applied in a systemic way enables the direct targeting of the plague vector. Thus, this method appears to be a superior alternative to fipronil-dusting for the control of the main plague vector in Madagascar. However, subsequent tests in the field are necessary to confirm the suitability of fipronil administration in a systemic way on large scales.

Key words: plague, fipronil, *Xenopsylla cheopis*, *Rattus* sp., systemic insecticide

Plague is an infectious disease present in Madagascar since its introduction in the harbor city of Toamasina in 1898 (Brygoo 1966). Recently, Madagascar reported 1,359 human plague cases between 2011 and 2015 (WHO 2011, 2013; Bertherat 2015). In 2015, 93% of the reported cases were of the bubonic form (Bertherat 2015), a clinical form of the disease that is most often acquired through the bites of infectious fleas. Many efforts have been made for plague vector control against *Xenopsylla cheopis* (Rothschild) (Siphonaptera: Pulicidae), the main vector for *Yersinia pestis* (Yersin) in Madagascar (Brygoo 1966, Duplantier et al. 2005). Insecticidal dusting, applied to rodent burrows and human dwellings (Chanteau 2006), still the most commonly used method. It was linked to the introduction of dichloro-diphenyl-trichloroethane (DDT) in the country in 1947 (Chanteau 2006). But since 1965, many cases of *X. cheopis* resistance to insecticides were reported, especially to DDT (Ratovonjato et al. 2000, Coulanges et al. 1982). With the exception of dieldrin, recent trials following World Health Organization (WHO) insecticide test protocols (WHO 1970) performed with some insecticide families used in vector control have proven the resistance to many insecticides. *Xenopsylla cheopis* showed resistance to 12 insecticides belonging to organophosphate, organochlorine, carbamate, and pyrethroid families (Boyer et al. 2014, Miarinjara and Boyer 2016). As *X. cheopis* has already manifested resistance to most insecticide families used in vector control, it is crucial to conduct research on insecticide efficacy, longevity, and application mode, so as to prevent future development of resistance.

Fipronil, a phenyl pyrazole insecticide, has been shown to have a broad-spectrum insecticidal activity against a large number of veterinary and agricultural pest species and is used in >30 countries (Simon-Delso et al. 2015). In addition to its effectiveness in controlling insect pests of crops and animals, the use of this insecticide for public health purposes has been investigated (Rojas de Arias and Fournet 2002, Xue et al. 2009). It was found effective both as larvicide and adulticide against insect species of economic and medical

importance (Xue et al. 2009, Poché et al. 2013, Diaz 2005). Moreover, in the past decade, its adulticidal efficacy against fleas on cats and dogs has been widely shown (McCoy et al. 2008; Dryden et al. 2000, 2005). In Madagascar, from 1997 to 2000, fipronil has already been used in a locust control program, but it also killed nontarget insects, in particular, termites and also vertebrates and consequently, its use was prohibited (Peveling 2000, Peveling et al. 2003). Since then, no investigation was made regarding the efficacy of this insecticide in vector control policy.

The effectiveness of fipronil was also evaluated against house flies *Musca domestica* L. (Diptera: Muscidae) and the malaria vector mosquito-species, *Anopheles gambiae* Giles (Diptera: Culicidae) and *Anopheles stephensi* Liston (Diptera: Culicidae). This was done using bioassays with toxic sugar bait containing fipronil (Scott and Wen 1997) and tests using impregnated paper as per WHO protocol (Kolaczinski and Curtis 2001). Furthermore, fipronil was previously tested against plague vector fleas with in vitro assays as systemic insecticide incorporated in rodent bait (Leirs et al. 2002). In the case of Madagascar, this host-targeted technique should involve *Rattus rattus* (L.) (Rodentia: Muridae), the main plague reservoir in rural foci, and *Rattus norvegicus* (Berkenhout) (Rodentia: Muridae) in urban foci (Andrianaivoarimanana et al. 2013).

Here, we determine the effectiveness of fipronil against *X. cheopis* following three different methods. First, acute toxicity tests were performed with X. *cheopis* feeding on an artificial feeding device with blood containing fipronil. Second, fipronil used in rodent bait was tested with fleas feeding on *R. norvegicus* and *R. rattus*. In order to compare the toxicity of fipronil by systemic ways, susceptibility to fipronil by contact was carried out according to the WHO impregnated paper protocol. The hypothesis is that fipronil used as systemic insecticide is more effective in controlling rodent fleas in Madagascar.

Materials and Methods

Xenopsylla cheopis Populations

Xenopsylla cheopis previously reported to be resistant to deltamethrin (Boyer et al. 2014) was chosen to perform all bioassays in this study. The strain was collected during plague surveys. Live fleas were collected from live-trapped rats in Sherman traps (H.B. Sherman Trap. Inc, Tallahassee, FL) and wire-mesh BTS traps (Besancon Technical Service, Besancon, France). Flea populations were maintained at the Medical Entomology Unit (Institut Pasteur de Madagascar, IPM) under laboratory conditions (24–27 °C, 75–80% relative humidity [RH]). Eggs, larvae, nymphs, and adults were kept together in clear 2-liter glass jars covered with muslin, containing 35 g of sterilized rice bran as litter. The litter was renewed when the flea population reached >200 adults, allowing a more rapid increase. Immature stages were fed with 8.5 g of larva diet, 75 g of sterilized dried oxblood, 5.5 g of dried yeast, and 200 g of laboratory animal-diet powder, and adult fleas were fed by placing live young mice from the IPM animal breeding facility into each jar for 3 h each at a time, 3 d per week. Young mice were not purchased or donated, but were bred for this purpose.

Rattus Species

Adult rats, *R. norvegicus* and *R. rattus*, weighing 110–250 g and 90–140 g, respectively, were used for the host-targeted technique. *Rattus norvegicus* were captured in the field using wire-mesh and Sherman traps, and were housed at laboratory condition (20–25°C, 70–75% RH) with food and water ad libitum for 15 d before the test (acclimatization). Conversely, *R. rattus* was bred at the Plague Unit of IPM. The rats were caught either during a collaborative research project or an epidemic response on the basis of a National Health Priority.

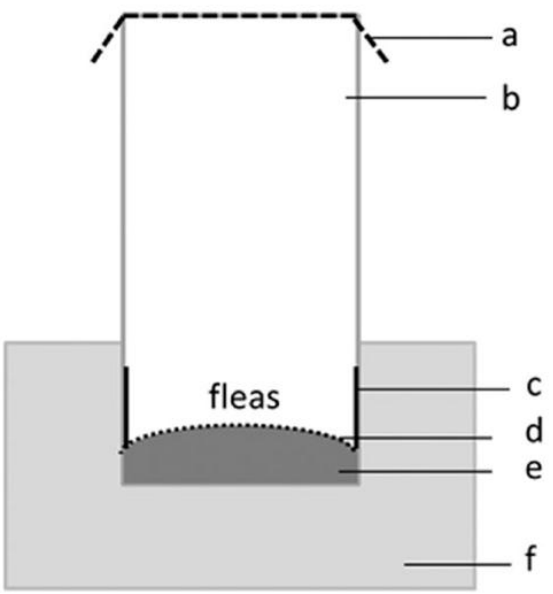

Fig. 1. Diagram of the artificial blood-feeding device. a: fine mesh cloth; b: plastic beaker (52 by 74 mm); c: plastic petri dish with the bottom removed; d: parafilm membrane; e: blood; f: heated water bath.

Artificial Blood-Feeding Bioassays

Acute toxicity tests bioassays were performed with an artificial blood-feeding device, modified from the method developed by Bar-Zeev and detailed in Fig. 1 (Bar-Zeev and Sternberg 1962). It consisted of placing the fleas in a cylindrical tube (plastic beaker) closed at one end with a membrane (parafilm); this end was partly submerged in a container of blood heated in a water bath (Fig. 1). Five milliliters of sheep blood treated with fipronil (Termidor 25 EC; BASF, Operation Crop Protection, Ludwigshafen, Germany) at five levels of concentrations (0, 0.5, 5, 50, and 500 mg of fipronil/liter blood (ppm)) were prepared by mixing the required volume of insecticide directly into the blood. Twenty unfed fleas of both sexes were placed in each beaker and three replicates per concentration were used. Fleas were allowed to feed on the entire device for 4 h maintained at 37 °C by a water bath. The bioassay was carried out under laboratory conditions (22–25 °C, 70–75% RH).

At the end of the acute toxicity test, the beaker was removed from the water bath and placed in a large pale-colored basin, the fine mesh cloth was removed, and remaining live fleas were caught with a manual pump aspirator. Feeding status of the fleas was determined by observation under binocular magnifier. Only blood fed flea mortality rate was considered. Number of both alive and dead blood fed fleas was recorded.

Rodent Bioassay

The bioassays were carried out under laboratory conditions (20–25 °C, 70–75% RH) according to the technique of rearing oriental fleas as described by Smith and Eddy (1954). Treatment baits (containing fipronil) and control baits were prepared as following: about

Table 1. Fipronil concentration in baits administrated to rats

Species	Replicates	mg of fipronil/1 kg of bait					
R. norvegicus	4[a]	0	0.5	5	50	500	_
R. rattus	2[b]	0	1.5	3	5	7.5	10

[a] Two males and two females per fipronil concentration.
[b] One male and one female per fipronil concentration.

1 kg of bait was prepared using 350 g of wheat flour, 300 g of peanut butter, 50 ml of water, and 2.5 ml of vegetable oil (Borchert et al. 2011). All ingredients were mixed manually to obtain a homogeneous paste and shaped in 5-g pellets. For each 5-g pellet, fipronil was added to the bait by the required volume in order to meet required concentrations (Table 1). The insecticide was injected into the pellet using micropipette in small drops until it was fully absorbed. The acute toxicity test was performed on 12 *R. rattus* and 20 *R. norvegicus*. Tests were first performed on *R. norvegicus* and the results obtained were used to redefine concentrations used for *R. rattus* tests (Table 1). The study was conducted in accordance with the Institut Pasteur (Paris) guidelines (https://www.pasteur.fr/sites/www.pasteur.fr/files/charte_ethique_fr_oct2012.pdf, accessed 12 December 2016) for animal use and was approved by the institutional ethics committee of the Institut Pasteur de Madagascar.

Rats were housed individually in cages (9 by 8 by 23 cm, metal mesh with cells 1 by 1 cm). The cage was then set on a 12- by 27-cm steel tray with blotting paper in the bottom to absorb urine and spilled water. The cage and tray were placed in a galvanized iron pan (35 cm in diameter and 40 cm height; Fig. 2). The rats were starved (without food but had an access to water) for 24 h before fipronil-treated bait administration in order to ensure total consumption on the first day of the test. During the test, they were exposed to the fipronil-treated bait for 24 h and were infested with 10 randomly selected unfed fleas of both sexes. After the fleas were introduced on the rat's fur, the pan was sealed with a fine mesh cloth held in place with an elastic band to prevent fleas from jumping out.

During the remainder of the acute toxicity test, rats were provided with the usual laboratory food (biscuit, dried cassava, corn) and tap water. Both types of baits (treated and untreated) were weighed 24 h after exposure in order to determine the amount consumed. The blotting paper was changed only once during the acute toxicity test carried out with *R. norvegicus*, but daily during the test using *R. rattus*. For the latter, we evaluated the flea mortality rate by calculating the LD_{50} value after each change of the blotting paper. For *R. norvegicus*, flea mortality rate was evaluated at the end of the acute toxicity test, 3 d after the bait consumption, by collecting and counting the number of live and dead fleas inside the nest material and on the rats by brushing with an adapted hard-bristled brush. Dead fleas were checked carefully inside removed blotting paper before they were discarded. All recovered fleas were observed under binocular magnifier for their blood-feeding status before each evaluation of the flea mortality rate. A chronogram of the test schedule is represented in Fig. 3. At the end of experiment, rats were euthanized using CO_2.

Fipronil Bioassays

Bioassays were conducted according to the WHO impregnated paper protocol (WHO 1970) and carried out in 18-cm glass test tubes covered with fine mesh cloth, under laboratory conditions (20–25°C, 70–75% RH). Each tube containing a group of 10 randomly selected fleas of both sexes was exposed for 8 h to filter paper (Whatman #1, 1.5 cm wide by 6 cm long) previously impregnated with fipronil at the following concentrations: 0.5, 5, 50, 100 mg of fipronil/liter (ppm). The impregnation was carried out with 2 ml of acetone–silicone solution of the corresponding fipronil concentrations. Four replicates of the test were performed for each fipronil concentration where a total of 180 fleas of both sexes have been used. Batch of fleas exposed to paper impregnated only with acetone–silicone oil solution was used as negative control (three replicates). The number of dead fleas was counted after 10, 20, 30, 40, 60, 120, 180, 300, and 480 min. Flea mortality was scored after 24-h exposure. The susceptibility status was assessed after this exposure time: mortality rates of 98–100% were considered to indicate susceptibility; 80–98%, tolerance; and <80%, resistance (WHO 1970). The test was not validated, and the data not included, if the control mortality rate was over 20%. Control mortality was corrected using Abbott's formula (Abbott 1925) when control values mortality were between 5 and 20%.

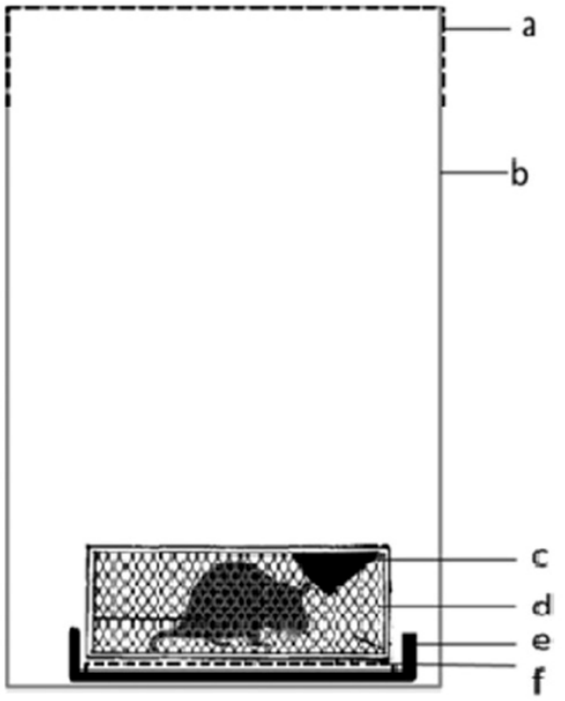

Fig. 2. Diagram of the equipment used to hold rodents and fleas during acute toxicity tests. a: fine mesh cloth; b: galvanized iron pan; c: bait compartment; d: cage; e: tray; f: blotting paper.

Statistical Analysis

Flea mortality rate evaluated in each toxicity test was analyzed using a generalized linear model (glm) with binomial distribution and logit-link function. The logit function estimates fipronil concentration response of exposed fleas and allows the determination of the $LD_{50} \pm SE$ and its standard error with the representative exposure. The analysis was done using the R software (R version 3.1.2, 2014).

Results

Susceptibility of *X. cheopis* Fed With Fipronil-Treated Blood

Among the 300 tested unfed fleas, the feeding rate with the artificial device was 15%, of which 41% survived and 59% died. The feeding

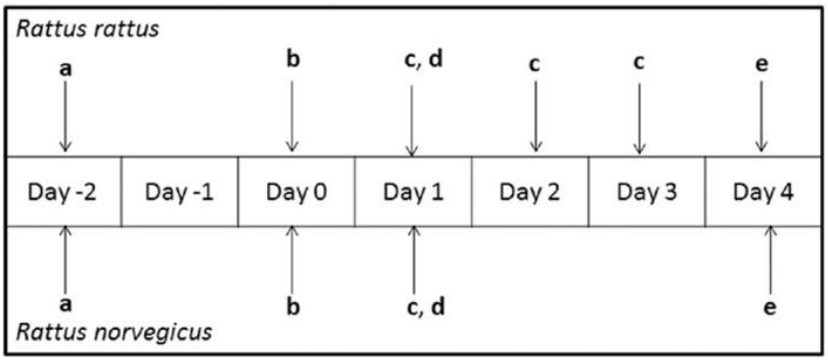

Fig. 3. Chronogram of acute toxicity test carried out with *R. rattus* and *R. norvegicus*. a: acclimation of the rats; b: flea infestation and bait application; c: renewal of blotting paper; d: weighing of nonconsumed bait; e: final evaluation of flea mortality.

rate was 4% in the control group and 11, 13, 50, and 22% for the each fipronil concentration (0.5, 5, 50, and 500 ppm), respectively. It was observed that 72% of blood fed fleas were recorded at the 50 ppm and 500 ppm concentrations. Hence, the increase in fipronil concentration did not affect the palatability of the blood. The LD_{50} was 0.4 ± 147.6 ppm.

Susceptibility of *X. cheopis* Fed on Fipronil-Treated Rodents

All formulations of bait applied were consumed without any consumption difference being observed between the applied fipronil concentrations. Observation of the blood-feeding status before each evaluation of the flea mortality rate revealed that all fleas, dead or alive, were fed. No effect of rat gender was observed ($P > 0.05$).

The daily renewal of blotting paper for *R. rattus* allowed us to calculate the LD_{50} value every 24 h. The value was 13.13 ppm, 24 h after the bait application, and then decreased to 6.47 ppm after 48 h, 2.0 ppm after 72 h, and reached 0.16 ppm (96 h) at the end of the acute toxicity test. All nonrecovered fleas were considered alive at the time of computing the LD_{50} values prior to the end of the test. Thus, the bioavailability of fipronil in the rodent body increased with time.

Since only one change of blotting paper was effected during the test with *R. norvegicus*, only the LD_{50} value 1.8 ± 0.3 ppm obtained at the end of the test after 96 h was taken into account. This value was 10-fold higher than the LD_{50} obtained by the test carried out on *R. rattus* (0.16 ± 24.0 ppm). The LD_{50} value for the artificial device ranked between values obtained using *R. norvegicus* and *R. rattus*, respectively; this LD_{50} value was fourfold less compared with the value for *R. norvegicus* and fourfold high compared with that of *R. rattus*.

Susceptibility of *X. cheopis* to Fipronil-Impregnated Paper

After 24-h exposure, *X. cheopis* did not show any susceptibility at 0.5 ppm and 5 ppm fipronil concentrations, with mortality rates of 20% and 33%, respectively. Tests with fipronil-treated paper at 50 ppm, however, indicated susceptibility with a mortality rate of 93%. No survivors were recorded at 100 ppm and no mortality was observed in the control. The LD_{50} obtained was 16.4 ± 2.9 ppm.

LD_{50} and LD_{90} values with the representative exposure for each test undertaken are given in Fig. 4.

Discussion

With the WHO trial, where *X. cheopis* fleas were in contact with insecticide-impregnated paper, no resistance to fipronil was observed with the tested concentration and strain. As the used flea strains were described resistant to deltamethrin, which belongs to the pyrethroid insecticide family, the lack of cross-resistance between fipronil and pyrethroids (Davari et al. 2007) favors its use, which is a rather promising observation.

The low toxicity of fipronil by contact application is confirmed again by the WHO procedure. 16.4 ppm was needed to kill 50% of fleas. Metzger and Rust 2002 found that adult cat flea susceptibility was 58.8% mortality at 10 ppm to fipronil-treated animal bedding (Metzger and Rust 2002), which remains in the range of our value. On the other hand, exposure of mosquitoes to paper impregnated with 0.25% fipronil in WHO test kits showed a delayed action of fipronil by tarsal contact (Kolaczinski and Curtis 2001). Its low ability to cross the cuticular barrier is due to the low permeability of insect skin (Scott and Wen 1997, Kolaczinski and Curtis 2001). Due to this delayed action, Scott and Wen (1997) used 72-h exposure time as a standard to determine fipronil toxicity by topical application for German cockroaches (Scott and Wen 1997). We did not adopt this exposure duration because any appropriate strategy for flea vector control during plague outbreaks must act rapidly.

As fipronil adopts a digestive route (Chaton et al. 2001), low concentrations cannot act by contact with an impregnated surface: ingestion through treated-blood remains the most selective way of fipronil administration to fleas. Hence, fipronil can potentially be used for the vector control program in Madagascar.

Fipronil as a Systemic Control Agent Against *X. cheopis*

All LD_{50} values obtained in this study with systemic application are consistent with previous studies using fipronil-treated baits with *R. rattus* against *X. cheopis* (Leirs et al. 2002) and with laboratory rats against *Ctenocephalides canis* (Curtis) (Siphonaptera: Pulicidae) (Borchert et al. 2003). Leirs et al. 2002, observed 100% mortality rate in fleas, with the lower dose that they tested (5 ppm). In this study, 90% mortality is achieved only at 0.30 ± 43.1 ppm, with the acute toxicity test carried with the fipronil-treated rodent *R. rattus*. The high standard error reflects the relatively small sample size. The defensive grooming behavior by the hosts in reaction to flea infestation (Hawlena et al. 2007, Mears et al. 2002) may be a nonnegligible factor, which might explain the difference in LD_{50} values observed between acute toxicity test using *R. rattus* and *R. norvegicus*. As *R. rattus* was from parasite-free laboratory rodent colonies,

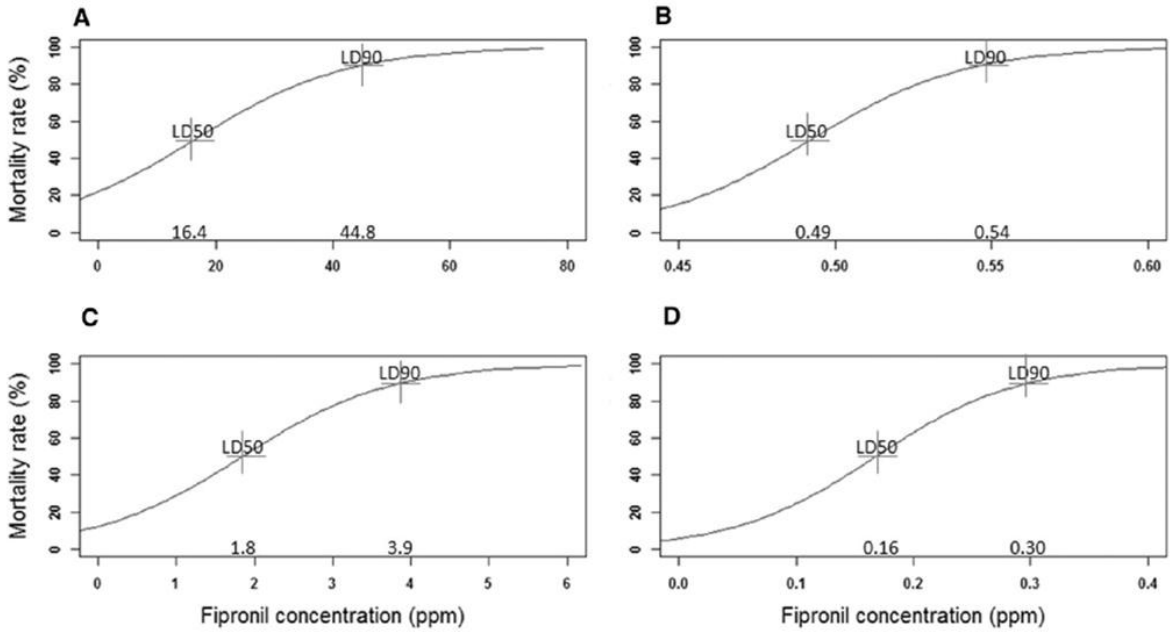

Fig. 4. (**A**) Percentage mortality of *X. cheopis* bioassayed according to the WHO protocol. (**B**) Percentage mortality of *X. cheopis* feeding on the artificial blood feeding device. (**C**) Percentage mortality of *X. cheopis* post bloodmeal on *R. norvegicus*. (**D**) Percentage mortality of *X. cheopis* post bloodmeal on *R. rattus*.

flea infestation seems to rapidly trigger a severe grooming behavior compared with *R. norvegicus*, which was cleared of their fleas only 15 d before the test. However, taking into account the defensive grooming behavior, we recommend the LD_{90} value 3.9 ± 0.7 ppm obtained with *R. norvegicus* as the concentration to be tested in the field when targeting both *R. rattus* and *R. norvegicus*.

Systemic lethal effects of fipronil were confirmed equally for *X. cheopis*, which consumed blood on rats fed with fipronil-treated baits and fipronil-treated sheep blood using an artificial feeding device. The systemic application has decisive advantages compared with others. First of all, results clearly showed that fipronil appeared to be more toxic when applied in a systemic way. The acute toxicity was ninefold higher with the fipronil-treated rodent *R. norvegicus* compared with the contact application value: the consequence of this difference is a decrease in the quantity of insecticide that needs to be used. Furthermore, despite the low feeding rate, the acute toxicity obtained with artificial feeding device attested the effectiveness of fipronil at low concentration. Measurement of systemic efficacy of Nodulisperic Acid A against fleas with the same method led to 90% mortality at 1 ppm (Shoop et al. 2001), reflecting the good results obtained with fipronil.

The palatability of bait can potentially limit the performance of the systemic action of insecticides in some cases (Mbise 1994). In our study, fipronil did not show any adverse effect on the palatability of the bait. Hence, the fipronil-treated bait formulated in this study was palatable to *R. rattus* and *R. norvegicus* and the concentrations were safe for rats. Trials using this bait formulation can be adopted for large-scale field application.

Effectiveness of insecticides when applied in a systemic way to warm-blooded organisms depends on other factors, such as its enzymatic detoxification, deposition rate in the subcutaneous fat, and physico-chemical characteristics (contribution of lipophility and hydrophility for penetration into the blood; Cochet et al. 1997, Poché et al. 2013, Simon-Delso et al. 2015). In addition to its systemic property, fipronil binds itself to the sebaceous glands (Smith et al. 2000, Beugnet and Franc 2012), with the rat skin becoming its reservoir, and it is not necessary to repeat the administration. Concerning the residual effects, fipronil was reported to have 10–30-wk residual effect for *Oropsylla montana* (Baker) (Siphonaptera: Ceratophillidae) in California ground squirrels *Spermophilus beecheyi* (Richardson) (Rodentia: Sciuridae) at doses of 15 ppm (Metzger and Rust 2002) and more than 30 d in mice against *X. cheopis* at doses of 100 ppm (Eremina et al. 2010). Once such data are available, the use of fipronil in a systemic way may prove to be an effective future means for long-term vector control. It can be thus applied before the plague season in plague-endemic areas.

Considerations of environmental contamination by insecticides require that compounds need to be safer, more efficient, and cost effective to apply, while retaining good control qualities. In addition to its effectiveness at low concentrations in systemic application, fipronil limits environmental contamination, as it is bioaccumulated by rats (Chaton et al. 2001, Agence Française De Sécurité Sanitaire Des Aliments [AFSSA] 2005). Our results confirm that fipronil is more effective in a systemic application. Hence, the use of fipronil in the context of plague outbreak control can be proposed to the National Plague Control Program in Madagascar. Other systemic insecticides such as imidacloprid or lefuneron may also be considered as alternatives to fipronil (Borchert et al. 2003, Davis 1999). The use of arthropod development inhibitors also remains a possible way for controlling rodent fleas (Slowik et al. 2001).

Evaluation of the Fipronil Effectiveness Methods

The rather minor difference of LD_{50} values between artificial blood feeding and live rat bioassays indicates that the former can replace the use of animal hosts, thereby eliminating all biological factors

involved when live hosts are used. However, because of the low feeding rate, the use of artificial blood-feeding device needs improvements. The experimental blood device can facilitate the testing of other systemic insecticides such as imidacloprid (Borchert et al. 2003); however, using hosts-targeting technique is recommended especially when certain insecticides have systemic activity in vitro but not in vivo, such as ivermectin (Zakson-Aiken et al. 2001). Blood-feeding techniques, however, do not correspond exactly to a systemic application method, as insect feeding behavior in this case only plays the role of connector between the insecticide and the targeted insect. Hence, the fact that mixing fipronil with blood in this study did not really present the principle of systemic administration of insecticide. The WHO method, on the other hand, requires significantly higher insecticide concentrations especially in the case of fipronil. The application method of insecticides can really influence the effectiveness of the insecticide and its toxicity. Hence, this parameter needs to be carefully considered for studies that aim to test the effectiveness of insecticides.

Fipronil is less toxic to mammals than to insects (Narahashi et al. 2007); it has also specific, chronic, lethal effects on rodents, where the LD_{50} for rats is 97 mg/kg by body weight (Chemical WATCH Factsheet FIPRONIL). The combination of a slow-acting rodenticide with the formulation described in this study would allow rapid flea control, while resulting in the death of the rodent host after its fleas have died. Leirs et al. 2002 have investigated the efficacy of a combination of fipronil in a rodenticide bait formulation under laboratory conditions without adversely affecting its insecticidal effect.

In conclusion, results of this study are encouraging for using fipronil in a systemic approach for vector control programs in Madagascar. It enables direct targeting of the plague vector, which is advantageous compared with the current vector control method, indiscriminate dusting of rodent burrows, and human dwellings. Field tests need to be further carried out to confirm its suitability for large-scale and natural condition use.

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