

5.3.4. La résistance par modification de la cible

La résistance au DDT et aux pyréthrinoïdes est conférée par une mutation d'un gène codant pour la protéine constituant les canaux à sodium (Davies et al 2007). Plusieurs types mutations synonymes sont impliquées mais la mutation la plus courante est la substitution de la leucine par la phénylalanine, conférant la « knockdown resistance » ou *kdr* (Liu 2015). Ces gènes mutés sont souvent récessifs et la résistance a été décelée chez les insectes homozygotes pour la mutation (Berticat et al 2008). Chez les individus mutés, ces insecticides n'arrivent plus à interférer avec le fonctionnement des canaux sodium (Soderlund and Knipple 2003, Davies et al 2007).

La résistance aux carbamates et aux organophosphorés est associée à une modification de la conformation des sites d'accrochage de l'acétylcholinestérase, modifiant l'affinité pour les insecticides mais permettant une dégradation au moins partielle de l'acétylcholine et un fonctionnement normal de la synapse (Hemingway and Ranson 2000, Nauen 2007). L'insensibilité des diverses espèces résistantes peut être très variable, car différentes mutations peuvent apporter une résistance (Ace 1, Ace 2). Ces mutations sont bien connues chez les moustiques (Weill and Malcolm 2004).

La résistance aux insecticides inhibiteurs du GABA est assez similaire aux mécanismes mis en jeu pour le *kdr* c'est-à-dire par une mutation génétique au niveau du gène codant pour la protéine du récepteur GABA (Bass et al 2004a). La substitution de l'alanine en sérine conduit à une diminution de l'affinité de ces récepteurs aux cyclodiènes et aux phenylpyrazoles (Bass et al 2004a).

5.4. Détection de la résistance aux insecticides

Généralement la détection de la résistance aux insecticides chez une population se divise en trois étapes : les tests insecticides sur les insectes ou détection de la résistance phénotypique, les méthodes biochimiques et les méthodes moléculaires qui visent surtout à caractériser les mécanismes impliqués dans la résistance (World Health Organization 1998).

5.4.1. Détection de la résistance phénotypique

Les protocoles de test insecticide recommandés par les experts de l'OMS ont pour but d'identifier la résistance phénotypique chez une population par rapport à une « ligne de base » (World Health Organization 1970). Pour cela, il est essentiel de déterminer le niveau de résistance chez une population n'ayant pas subi de pression insecticide et où les individus résistants sont rares. Les données obtenues

chez les populations sensibles serviront de référence pour déterminer la sensibilité de la population étudiée.

L'approche « temps d'exposition-réponses » consiste à exposer les insectes à une seule concentration pendant plusieurs intervalles de temps (Temps Létal ou TL); tandis que l'approche « concentrations-réponses » consiste à utiliser plusieurs concentrations et regarder les mortalités correspondantes et faire l'analyse des Concentrations Létales (CL).

Les résultats sont ensuite analysés sous forme graphique avec souvent une droite de régression entre la mortalité et le logarithme du temps d'exposition ou des concentrations utilisées (Hemingway 1995). Il est alors possible de déterminer à partir de ces données la concentration ou le temps au bout duquel 100% de mortalité est obtenu pour une population sensible. C'est le temps ou la concentration diagnostique. On doit soupçonner la présence d'individus résistants aux insecticides si l'écart des CL50 est très important ou si l'écart des CL50 étant faible mais la ligne de régression est beaucoup plus horizontale que la ligne de référence, ou si la ligne de régression se terminant en plateau il est impossible d'obtenir des CL90 ou des CL100 (Hamon and Mouchet 1961). Le même raisonnement peut être appliqué pour l'étude du temps diagnostique (Brogdon and Chan 2010).

Cependant il a été prouvé que l'approche temps-réponse est plus sensible en détectant le changement de la sensibilité aux insecticide dans une population, et correspond plus aux résultats des tests biochimiques (World Health Organization 1970, Brogdon and McAllister 1998a). Une des hypothèses avancées serait que la quantité d'insecticide absorbé par contact, et donc la toxicité de l'insecticide serait fonction de temps d'exposition (Roush and Mckenzie 1987).

Ainsi à part la mortalité finale, la mesure du KD50 ou KD90, qui sont respectivement le temps au bout de lequel 50 ou 90% des individus sont mourants ou paralysés, donnent une idée du degré de résistance dans une population (Kofi et al 1998, Karunaratne et al 2007, Nkya et al 2014). KD vient du terme «knock-down» qui est un effet typique du mode d'action des insecticides qui ciblent les canaux à sodium voltage dépendants (DDT et pyréthrinoides). Cet effet est provoqué par une excitation excessive de la fibre nerveuse qui se traduit par une paralysie puis la mort plus ou moins réversible de l'insecte suivant l'insecticide et la dose utilisée (Haubruge and Amichot 1998). L'effet KD représente la traduction symptomatologique de la sensibilité du système nerveux de l'insecte vis-à-vis du pesticide (Haubruge and Amichot 1998). Il existe généralement une corrélation inverse entre les temps de KD et le taux de mortalité résultant: plus ce temps est élevé, plus le taux de mortalité qui en résultent est faible (Koffi et al 1998).

5.4.2. Méthodes biochimiques pour la détection de la résistance

Les tests biochimiques visent à mesurer l'activité des enzymes de détoxification des insecticides impliquées dans les mécanismes de la résistance métabolique.

Les études de l'action des synergistes ont constitué les prémices des études sur l'implication des enzymes de détoxification dans la résistance métabolique chez le moustique ainsi que chez d'autres espèces d'insecte. Les insectes préalablement exposés à ces molécules perdent leur résistance à un insecticide ou à des groupes d'insecticide si l'enzyme de détoxification recherché est présent chez l'insecte (Picollo and Vassena 2000). En comparant ensuite le niveau de résistance obtenu entre une population exposée ou non avec les synergistes, l'expérimentateur est en mesure de diagnostiquer le mécanisme de détoxification mis en jeu chez l'insecte (Brogdon and McAllister 1998b). Cependant, les résultats de ces tests sont à prendre avec précaution car certains synergistes n'arrivent pas à bloquer efficacement certains enzymes de détoxification (Liu 2015).

Le dosage enzymatique était l'approche la plus utilisée (Adelman et al. 2011, Gnanguenon et al. 2015). Il s'agit de mesurer l'activité enzymatique ou l'augmentation du métabolisme chez l'insecte en faisant des dosages enzymatiques, ciblant les principaux enzymes impliqués. L'extraction enzymatique est effectuée sur des individus résistants et sensibles. Les valeurs obtenues sont ensuite comparées à une valeur standard obtenue chez une souche sensible de référence. Plusieurs techniques ont été développées pour le dosage enzymatique, mais la plus courante est celle utilisant la lecture des plaques par spectrophotométrie (World Health Organization 1998).

5.4.3. Détection moléculaire de la résistance

La détection moléculaire de la résistance aux insecticides permet de voir les changements au niveau génétique (Berticat et al 2008). Les plus facilement détectables sont les mutations impliquées dans la modification des cibles (*kdr*, *Ace 1*) (Weill and Malcolm 2004). La mise en place des méthodes moléculaires incluent l'extraction d'ADN, les réactions PCR (polymerase chain reaction), le séquençage et le clonage. Les détections de routine se font par PCR en utilisant des amorces spécifiques encadrant le gène d'intérêt et permettent d'étudier plusieurs populations à la fois (Adelman et al 2011). Cette technique permet surtout de trouver les gènes responsables de la résistance par modification des cibles.

D'autres techniques visant à étudier les mécanismes moléculaires à la base de la résistance métabolique. Par exemple chez les moustiques, la résistance aux insecticides associés à l'enzyme GST est due à une défaillance de la régulation du gène codant pour cette protéine, provoquée elle-même par une

mutation. Elle se manifeste par une surexpression ou un taux de transcription élevé du gène (Che-Mendoza et al. 2009). La technique de « microarray » ou puce à ADN permet d'analyser en même temps les différents profils de transcription des superfamilles des gènes impliqués dans la résistance aux insecticides (David et al. 2005, Mitchell et al. 2012, Nkya et al. 2014, Thomsen et al. 2014). Les outils de la bioinformatique permettent aussi une analyse poussée des séquences de gènes des populations d'intérêt, générées par les techniques comme le séquençage à haut débit, et ensuite comparées avec les séquences disponibles dans les banques de données (Adelman et al 2011).

6. Objectifs de la thèse

Dans le contexte de la lutte anti-vectorielle et le développement de la résistance des puces aux insecticides, cette thèse a pour principal objectif d'améliorer la lutte contre les puces vectrices de la bactérie de la peste à Madagascar.

Ainsi ce travail a été divisé en 4 grandes parties. Chaque partie est constituée par un résumé de l'étude et un ou plusieurs articles (publié, soumis ou en cours) correspondants. Le résumé comprend le contexte et les objectifs de l'étude, un résumé du matériel et méthode et les principaux résultats. Le ou les articles correspondants à chaque chapitre se trouvent à la suite du résumé.

La première partie est axée sur l'élevage de *X. cheopis*, étape essentielle de la thèse qui nous a permis de réaliser les travaux de laboratoire rapportés dans les parties qui vont suivre. Cette partie correspond à un article en cours d'écriture.

Dans la deuxième partie, la sensibilité de *X. cheopis* aux insecticides à Madagascar a été évaluée. Cette partie comprend trois articles dont la sensibilité de *X. cheopis* à la deltaméthrine (publié), la sensibilité de *X. cheopis* à 12 insecticides (publié) et la sensibilité des puces collectées dans les prisons de Madagascar (accepté).

La troisième partie concerne l'évaluation sur terrain de la lutte insecticide, qui est l'épandage d'insecticide en poudre et l'utilisation des boîtes de kartman. Un article en cours est associé à cette étude.

Enfin, la quatrième partie traite de l'évaluation en laboratoire du fipronil, un insecticide systémique pour lutter contre *X. cheopis*. Un article publié est associé à cette partie.

Suite à ces quatre chapitres, une discussion générale reprendra l'ensemble des points soulevés dans ces travaux de thèse. Dans cette discussion, les limites des méthodes de lutte actuelle vont être discutées et des perspectives sont proposées pour les améliorer. Enfin les limites des études effectuées dans la thèse et des perspectives de recherche à venir dans le domaine y sont également abordées.

II. Optimisation de l'élevage de *Xenopsylla cheopis* en insectarium pour les besoins en expérimentation

1. Contexte

Les puces présentes dans l'environnement immédiat de l'homme sont connues pour les nuisances (piqûre, dermatites) qu'elles occasionnent ainsi que leurs actions spoliatrices en cas d'infestation massive (Franc 1994). L'importance des puces en santé publique est surtout liée à leur capacité à transmettre des microorganismes pathogènes et des parasites aux humains et aux animaux domestiques (Durden and Traub 2002, Gage 2004, Duchemin et al 2006). Les études sur la biologie, la physiologie, et la transmission de pathogène chez les puces ont conduit au développement de techniques de laboratoire permettant l'élevage des puces (Metzger and Rust 2001, Kernif et al 2015). D'ailleurs l'élevage en laboratoire a permis d'observer des aspects de la biologie de la puce qui sont difficilement observables dans les conditions naturelles. En outre, certaines manipulations de laboratoire nécessitent un nombre important de puces vivantes à portée de main (Hudson 1958, Hudson et Prince 1958).

La peste est probablement la plus connue des maladies transmises par les puces, étant responsable de trois pandémies historiques. La puce du rat *Xenopsylla cheopis* est le vecteur majeur de la bactérie de la peste (*Yersinia pestis*) en zone tropicale (Durden and Traub 2002, Gage 2004, Duchemin et al 2006).

Depuis une décennie, Madagascar est devenu le pays qui rapporte le plus de cas de peste à l'Organisation Mondiale de la Santé (Bertherat 2015). Les recherches sur les vecteurs de la bactérie de la peste et les tests de sensibilité aux insecticides nécessitent un nombre important de puces. Cependant, la plupart du temps, il est difficile d'avoir un nombre suffisant de puces vivantes lors des collectes sur terrain. Depuis une vingtaine d'année, plusieurs souches de puces vectrices (*X. cheopis* et *Synopsyllus fonquerniei*) ont pu être maintenues dans le laboratoire d'Entomologie Médicale de l'Institut Pasteur de Madagascar (Ratovonjato et al 2000). La température et l'humidité relative optimales requises pour le maintien des puces en insectarium ont été largement étudiées et fixées pour les colonies (Bacot and Martin 1924, Sharif 1949, Kreppel et al 2016).

Ainsi, pour des besoins précis en test insecticide il était nécessaire d'obtenir un grand nombre de puces de la même souche. Notre objectif est d'étudier les facteurs susceptibles d'optimiser le maintien et la production en masse de *X. cheopis* dans le laboratoire d'Entomologie Médicale de l'Institut Pasteur de Madagascar. Nous avons entrepris des expériences portant principalement sur la fréquence des repas sanguins des puces adultes et la composition du milieu d'élevage.

2. Méthodes

Les puces ont été collectées sur terrain lors de missions de surveillance ou de dératisation dans les foyers de peste. Dès leur capture, les rongeurs sont sacrifiés puis leurs poils brossés dans une bassine afin de récupérer des puces vivantes (Figure 15, A). Les puces sont collectées à l'aide de pompe manuelle et transférées dans des bocaux d'élevage (en verre transparent, 1,5 litre) contenant du son de riz stérile rempli au dixième, pour le transport jusqu'au laboratoire. Chaque bocal est recouvert de voile à maille fine maintenue en place par un élastique (Figure 15, B).

Arrivés au laboratoire, les bocaux d'élevage sont placés sur des étagères et maintenus à une température de 23 à 27°C et à une humidité relative de 75 à 85%. Tous les stades de développement de la puce sont maintenus ensemble dans un même bocal et la litière est changée une fois par an. Pour le comptage des puces ou le changement de la litière, le contenu du bocal d'élevage est versé dans une bassine, et les puces adultes sont aspirées une à une avec une pompe manuelle. Après le comptage, la litière est remise en place dans le bocal à l'aide d'un entonnoir en métal galvanisé et les adultes sont remises dans leur bocal d'élevage; sinon lors du changement de litière, les adultes sont transférés sur une litière neuve.

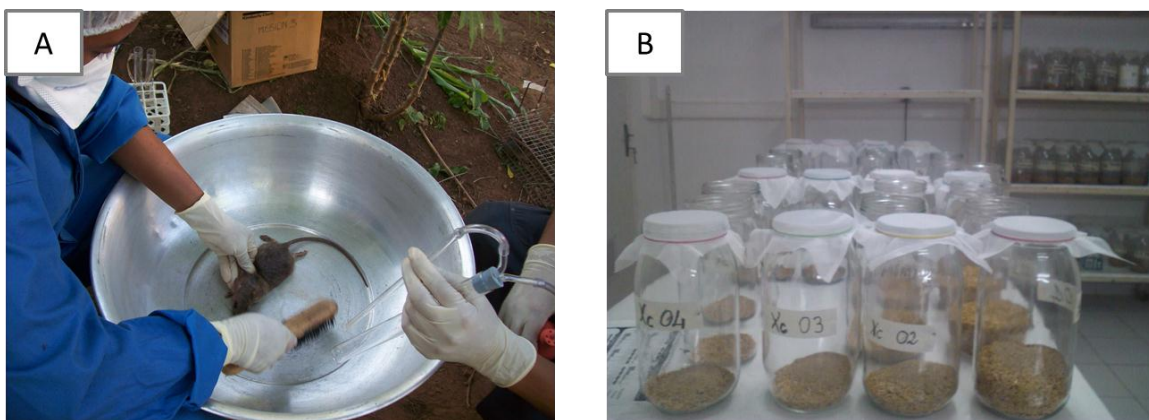


Figure 15: Collecte et élevage de puces

A. brossage du pelage d'un rat et collecte des puces à l'aide d'un aspirateur à pompe. **B.** bocaux d'élevage (Photos : A Miarinjara).

Une cuillerée à soupe de nourriture pour larve est ajoutée à chaque bocal d'élevage. Elle est composée de poudre de biscuit pour souris de laboratoire (75%), de sang de zébu séché en poudre (23%) et de la levure de bière (2%). Les puces adultes sont nourries avec des souriceaux une fois par semaine, à raison d'un souriceau par bocal.

Pour les tests que nous avons effectués, tous les groupes ont été soumis à une condition d'élevage standard et les groupes ne diffèrent entre eux que par les effets à tester qui sont propres à chaque test. Le nombre de puces de départ était maintenu à 20, sans tenir compte du sexe et de l'âge. Une même souche a été utilisée pour le besoin d'un test. Les mêmes températures et humidité relative usuelles ont été maintenues pour tous les tests. Le nombre de puces adultes a été compté toutes les deux semaines.

1) Le premier test porte sur l'effet de la fréquence de repas sanguin des puces adultes, en vue de diminuer l'apport de souriceau. Les nombres de puces obtenus ont été comparés entre trois groupes nourris respectivement chacun une fois par semaine, une fois toutes les deux semaines et une fois toutes les trois semaines. Les groupes ont été suivis pendant 238 jours.

2) Le second test a pour objectif de démontrer l'effet du changement de litière sur la croissance de la colonie. A la fin du premier test, les souches qui ont été utilisées ont été réparties en deux groupes subissant ou non un changement de litière. Après le changement de litière, un troisième groupe a été constitué avec les anciennes litières contenant les stades immatures.

3) Il a été rapporté que des stimuli physiques tels que les vibrations et la pression sont parmi les facteurs contribuant à l'émergence des puces adultes (Durden and Traub 2002, Gage 2004). En effet la méthode utilisée pour le comptage ou les changements de litière fut considéré comme un facteur de stimulation de l'émergence des puces adultes. Quatre groupes ont été constitués avec des fréquences de comptages différents pour voir l'effet de la stimulation de la litière: une fois par semaine, une fois toutes les deux semaines, une fois toutes les trois semaines et enfin un groupe compté une fois après 112 jours d'observation et à la fin du test, après 224 jours.

4) Pour ce quatrième test, le but a été de trouver la proportion de son de riz et de nourriture pour les larves qui donnerait le meilleur rendement. Ces composantes ont été quantifiées et neuf groupes ont été constitués en utilisant trois quantités de son de riz et trois quantités de nourriture pour larve. Le nombre de puces obtenu a été comparé entre chaque groupe.

5) L'objectif ici a été de trouver la bonne proportion pour chacun des trois composantes de la nourriture pour les larves. Des groupes ont été constitués en faisant varier la composition de la nourriture pour

larve, tout en gardant la même quantité de nourriture et de son de riz pour chaque groupe. Un groupe témoin ne recevant aucune nourriture a été inclus dans le test.

3. Principaux résultats

La plus forte densité de puces en élevage a été obtenue avec les groupes nourris une fois toutes les semaines. Hélas la taille de la population a diminué après environ 126 jours (Article 1, Figure 1). Le changement de litière a permis de relancer la croissance en nombre de la colonie (Article 1, Figure 2). Nous avons également observé un effet significatif de la stimulation de la litière : les groupes dont la litière a été stimulée chaque semaine et toutes les deux semaines comportaient un nombre plus important de puces adultes. La quantité de litière la plus importante a aussi permis d'avoir le plus grand nombre de puces. Par contre, nous n'avons pas observé d'effet de la variation de la composition de la nourriture avec les mêmes nombres de puces obtenus avec ou sans nourriture pour larves.

4. Valorisation scientifique

La description complète de la méthodologie ainsi que les résultats de ces travaux sont les objets d'un article en cours d'écriture :

Article 1

Miarinjara Adélaïde, Harimalala Mireille, Rajaonarimanana Mandimby Andriatsiferana, Ramihangihajason Tojo Rindra, Boyer Sébastien. Maintenance and mass rearing of *Xenopsylla cheopis* (Siphonaptera pulicidae) laboratory colonies for experimental purposes.

Article 1

Maintenance and mass rearing of *Xenopsylla cheopis* (Siphonaptera pulicidae) laboratory colonies for experimental purposes

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Abstract

Xenopsylla cheopis, the rat flea is the main vector of *Yersinia pestis*, the plague bacterium in Madagascar. Maintaining *X. cheopis* colony in laboratory is necessary for experimental purposes. Here we study some aspects of the factors which may improve the maintenance and the mass production of *X. cheopis* in the laboratory of Medical Entomology, at the Institut Pasteur de Madagascar. We undertook laboratory experiments, focusing mainly on adult blood meal frequency and the composition of rearing medium. According to the manner that fleas have been maintained in our insectarium, we found that a larger number of adults were obtained with a weekly fed colony, the highest quantity of rearing medium, and frequent disturbance or shaking of the rearing medium. Furthermore, sterilized rice bran layer, our rearing substrate, was suitable for larval development, without the need to add additional larval diet powder to the rearing medium. We also observed that even under optimal conditions, the number of fleas in each colony should decline after reaching the highest number. However, transferring the colony to a new rearing medium permitted to induce further growth in the size of the colony.

Background

Fleas are holometabolous insects. The life cycle undergoes four life stages as eggs, larvae, pupae, and adults. Flea larvae are legless, eyeless, and worm-like (Rust and Dryden 1997, Durden and Traub 2002). They feed on organic solid particles they have found in the environment, mainly dried blood provided by adult droplets (Silverman and Appel 1994). Flea larvae are negatively phototactic and very susceptible to

desiccation (Sharif 1949); and they burrow inside the host liter. The larvae undergo two molting, and the mature larvae spin a silky cocoon incorporating available debris in the immediate environment, in order to pupate inside. Depending on environmental conditions and species, adult fleas will emerge from the cocoon after few days or months (Durden and Traub 2002). Appropriate signals from the host or the environment stimulate the emergence of adult fleas (Durden and Traub 2002, Krasnov 2008). Adults are laterally compressed, wingless and chitinized. Males and females are mandatory haematophagous, taking their blood meal on living host. Some species may spend most of their lifetime in host's fur, others will only be present at the moment of the blood meal and burrow in the liter, and females of some species attached or buried themselves with mouthparts on their host skin (Krasnov 2008). Typically, the mating occurs during or immediately after a blood meal (Durden and Traub 2002). The lifespan of adult fleas will varies depending on environmental conditions, species blood meal and host behavior (Krasnov 2008). Temperature and relative humidity are playing an important role in the development and the survival of all flea stages (Bacot 1914, Bacot and Martin 1924, Sharif 1949, Kreppel et al 2016).

Fleas present in the immediate human environment have been implicated in the transmission of several pathogens and parasites to humans (Gage 2004). In addition, they are notorious for their biting and burrowing habits of some species (*Tunga sp.*) that cause intense itching and annoyance (Bitam et al 2010). The cat flea, *Ctenocephalides felis* gathered much attention because it is the most widely distributed ectoparasite in domestic animals (Silverman and Appel 1994, Rust and Dryden 1997). In addition to its annoyance for pets and humans, *C. felis* is responsible for flea bites, allergy dermatitis and the transmission of dog tapeworm (Rust and Dryden 1997). Plague could be the most known diseases transmitted by fleas, provoking historically three great pandemics (Duplantier et al 2005). The oriental rat flea, *Xenopsylla cheopis*, has been the subject of a great amount of studies due to its role in the transmission of *Yersinia pestis* (Bacot and Martin 1915, Burroughs 1947, Eisen et al 2007, Aoyagi et al 2015, Miarinjara and Boyer 2016, Rajohnson et al 2017).

Madagascar is a country where plague remains a public health concern. Since the last decade, most of worldwide cases have been reported from Madagascar (Bertherat 2015). Researches have been conducted to better understand the persistence of this disease and to find more appropriate techniques for plague control and risk management. The history of plague introduction, the epidemiology, and the principal antagonists of plague cycle have been widely described (Duplantier et al 2005, Andrianaivoarimanana et al 2013). However, little is known regarding the vectors: the species involved in the transmission, their vectorial capacity, their bionomic, their distribution and the suitability of vector control policies against them. It has been admitted that the cosmopolitan rat's flea *X. cheopis* and an

endemic species, *Synopsyllus fonquerniei* are vectors of *Y. pestis* in Madagascar (Andrianaivoarimanana et al 2013). Yet, the role played by *Pulex irritans*, the human flea and *Xenopsylla brasiliensis* recently discovered in Malagasy plague focus is highly suspected, but still unknown (Ratovonjato et al 2014, Miarinjara et al 2016).

The need for a better understanding of vector biology, physiology and their transmission of pathogen has led to the development of laboratory techniques for flea rearing (Metzger and Rust 2001, Kernif et al 2015). Furthermore, the laboratory rearing can provide important information about the bionomic of vectors, hardly visible on the field. In addition some laboratory bioassays require important number of living flea (Hudson 1958, Hudson and Prince 1958). The study of the vectors of *Y. pestis* in Madagascar required the development of rearing methods suitable for obtaining a large number of rat fleas (Ratovonjato et al 2000). For instance, at least 60 individuals from the same population are required to perform one insecticide susceptibility test (Boyer et al 2014, Miarinjara and Boyer 2016). However, most of the time, it is difficult to complete this number, relaying only on field collection. In addition, many individuals may die during transport because the collection site may be located far away from the laboratory. For the purpose, two vector species (*X. cheopis* and *S. fonquerniei*) have been reared at the Institut Pasteur de Madagascar (Medical Entomology Unit) insectarium for two decades. Standard rearing conditions have been described elsewhere and roughly maintained (Ratovonjato et al 2000), although some improvements have been brought throughout the years. The optimal temperature and humidity required for *X. cheopis* maintenance have been determined (Bacot and Martin 1924, Sharif 1949, Kreppel et al 2016).

Here we describe some experiments carried out at identical temperature and relative humidity conditions, in order to confirm the hypothesis made about the factors which may influence *Xenopsylla cheopis* rearing in the insectarium. These observations will help to optimize the flea rearing, in order to fulfill particular need (flea number or generation) for biological tests. We studied the feeding frequency in order to determine the most appropriate frequency to optimize the colony. Then, we wanted to verify if the renewal of the rearing medium allowed having more individuals. We evaluated the shaking or disturbing effect of the rearing medium, generated by the counting procedure. We also tested the effect of variations on the composition of the rearing medium (nutritive medium and the substrate); and finally, the effect of several variations in the composition of larva nutritive medium.

Materials and methods

Standard rearing condition

To raise a new colony, adult fleas were collected in the field during plague surveillance programs. Mammal hosts were euthanized and their fur was brushed inside a basin to collect fleas with manual pump. Adult fleas were caught alive and transported to the insectarium inside a jar with a layer of rice bran. Fleas were identified at the species level under binocular magnifier: the adult fleas are secured inside petri dish placed on crushed ice or on chill table placed directly under the magnifier. Species were reared separately. Eggs, larvae, nymphs and adults were maintained together in clear glass jars (1500 ml) covered with fine mesh white muslin held in place with an elastic band. The sterilized rice bran has been used as substrate for adults, about 1:10 of the jar volume and a tablespoon of dried ox blood was added as larval food. Dried yeasts and laboratory animal diet powder were added to the larva nutritive medium. The litter was renewed once a year, as it seems that frequent change of rearing medium may influence positively the growth of the population. To feed adults, each jar was provided with a young mouse for three days a week, but more frequent feeding seems to improve positively slow growing strain (Ratovonjato et al 2000). The mouse (*Mus musculus*) used in the experiments and colony maintenance was obtained from a mouse colony maintained in the laboratory animals breeding facility of the Unité de Virologie, Institut Pasteur de Madagascar.

Reproduction and survival of fleas is achieved by maintaining fleas at constant temperature and humidity (Sharif 1949). The insectarium has been installed in a chamber kept under a temperature of 22–27°C and 75–85% relative humidity using radiator and adjustable humidifier. The atmospheric parameters are recorded daily using thermo-hygrometers.

Counting adult fleas

As all life stages and generations are kept together within the same container (jar), the effect of each parameter was evaluated by counting the number of adult fleas. For this purpose, the contents of the rearing jar were poured inside a basin and the adult fleas were counted one by one using a manual pump. After that, the liter was returned to the the initial rearing jar using metallic funnel.

Data analysis

Statistical analyses were performed using R Studio (R Core Team and R Development Core Team 2014). The flea number obtained for each group was compared to each other using parametric and non-parametric test, according to suitability of the dataset (ANOVA test, pairwise Tukey test, Kruskal and Wallis test, Welch's t test and pairwise Wilcoxon test). The differences were considered statistically significant at a $p < 0,05$.

Feeding frequency

Three flea populations were used and divided into 3 groups, which were subjected to the same standard rearing conditions, except the feeding frequency. One group consisted of three replicates of each population (nine rearing jars), and each replicate consisted of one jar containing 20 *Xenopsylla cheopis* adults randomly collected from established colonies. The first group were provided with a young mouse every week, the second group once every two weeks and the third group, once every three weeks. The number of fleas in each jar was counted and recorded every two weeks and followed during 238 days.

Rearing medium renewal

At the end of the previous experiment, we evaluated the effect of rearing medium renewal by comparing flea number obtained between the groups (Table1). It consisted on removing all adult fleas from the previous experiment rearing jars and transferring them to a clean jars containing fresh standard rearing medium (Group A4 and A5). The ancient medium was verified for immature stages presence (group A2 and A3) and the emergence of adults was followed during the study. A control colony was left as it was, and the rearing medium was left unchanged (A1). The rearing conditions, the counting process and the comparison of flea number between groups were the same as before. Day 0 corresponds to the following week after the last counting day (238th) of the previous experiment. Counting was done every two weeks for 70 days.

Table 1

Groups	Replicate	Medium renewal	Feeding frequency	Mean flea number per group	SE
A1	3	No	7 days	46,33	16,07
A2*	3	No	7 days	0	0
A3*	3	No	14 days	0	0
A4	3	Yes	7 days	67	38,35
A5	3	Yes	14 days	99,33	62,23

*Adults from A2 and A3 were transferred in A4 and A5 respectively.

Shaking effect

As the immature flea stage inside the cocoon has been reported sensitive to physical stimuli (Rust and Dryden 1997, Durden and Traub 2002), the movement of the liter containing the immature stages from one container to another and the sorting of adults during the counting process were considered as physical stimulation that could influence the adult emergence. Then we set up an experiment to evaluate the “shacking effect”. One flea population was used and divided into four groups (3 replicates

per group) and each group was counted at different time intervals. The first group was counted every week, the second group was counted once every two weeks, the third group was counted once every month and the last group was counted on day 112 and at the end of the experiment (day 224). The number of adult fleas was recorded and compared between groups every 84th, 112th and 224th days. The standard rearing conditions were followed.

Rearing medium composition

We tested nine types of rearing medium (Table 2) by varying the quantity and proportion of rice bran (substrate) and the larva diet. 35 grams of rice bran corresponded to the quantity which fills 1:10 of the rearing jar volume; and 8.50 grams of larva food corresponded to one tablespoon used for standard rearing protocol (group 4). Those quantities were halved, divided in four or doubled depending on experiment requirement (Table 2). One flea population was used and divided in order to have two replicates (one replicate= one jar containing 20 adult fleas) for each group. Adult fleas were counted once every two weeks for 112 days. The same standard rearing condition was followed.

Table 2: quantity in grams of larva diet and rearing substrate (rice bran) provided to each group (1 to 9).

Group	1	2	3	4	5	6	7	8	9
Larva diet	17.00	17.00	17.00	8.50	8.50	8.50	4.24	4.24	4.24
Rice bran	70,00	35	8.75	70	35	8.75	70	35.00	8.75
Total	87	52	25.75	78.5	43.5	17.25	74.24	39.24	12.99

Larva diet composition

The standard diet contained mouse chow powder, blood powder and dried brewer's yeast at 73%, 25% and 2%, respectively. We tested seven larva diet compositions (group A to G in table 3) by removing some of the components and keeping the same quantity of larva diet (15 grams) and substrate (70 grams); and the control group where no larva diet was added. A flea population was used and separated in 27 rearing jars, in order to have 3 replicates for each larva diet composition. Each rearing jar contained 20 adult *X. cheopis*. The test was carried out for 84 days. Fleas were counted on day 42 and at the end of the test.

Table 3: quantity in grams of each component of the larva diet per group (A to G and the control)

	Control	A	B	C	D	E	F	G
Brewer's yeast (L)	0	0	0	15	0	0,40	1.11	0,30
Blood powder(S)	0	0	15	0	3.83	0	13.8	3.75
Mouse chow powder(B)	0	15	0	0	11.1	14.6	0	10,95
Total	0	15	15	15	15	15	15	15

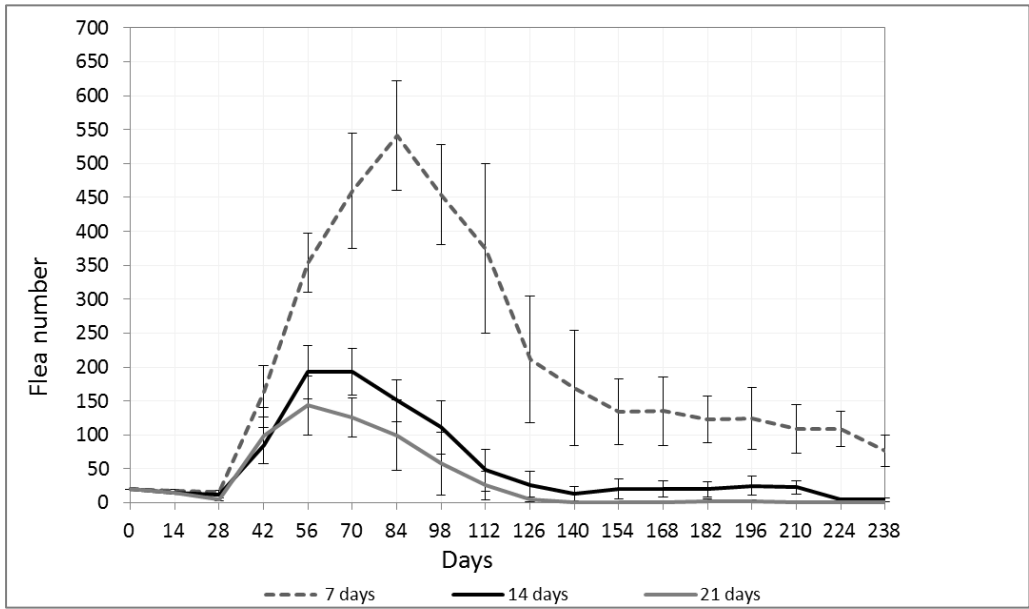
Results

Feeding frequency

The effect of blood feeding frequency was apparent after day 28, with a significantly higher flea number obtained in the group fed once a week ($F=103.6$, $p<0,001$). The mean number of fleas increased from 16.1 (SE= 1.90) on day 28 to 164 (SE=80,25) on 42 and a higher number of fleas was obtained in group fed each week ($F=7.237$, $p<0,01$). No significant difference was obtained between the group fed once every two weeks and that fed every three weeks.

The figure 1 shows that the dynamics of the number of *X. cheopis* as a function of time was quite the same, independently of the population tested and the frequency of blood feeding. First, no increase in the number of fleas was observed from the beginning of the test until day 28. This period was followed by a progressive increase in the number of fleas to a plateau, between day 70 and 98. The maximum number of fleas was found around day 84 in group fed every week. Then the flea number decreased gradually and stabilized around day 154.

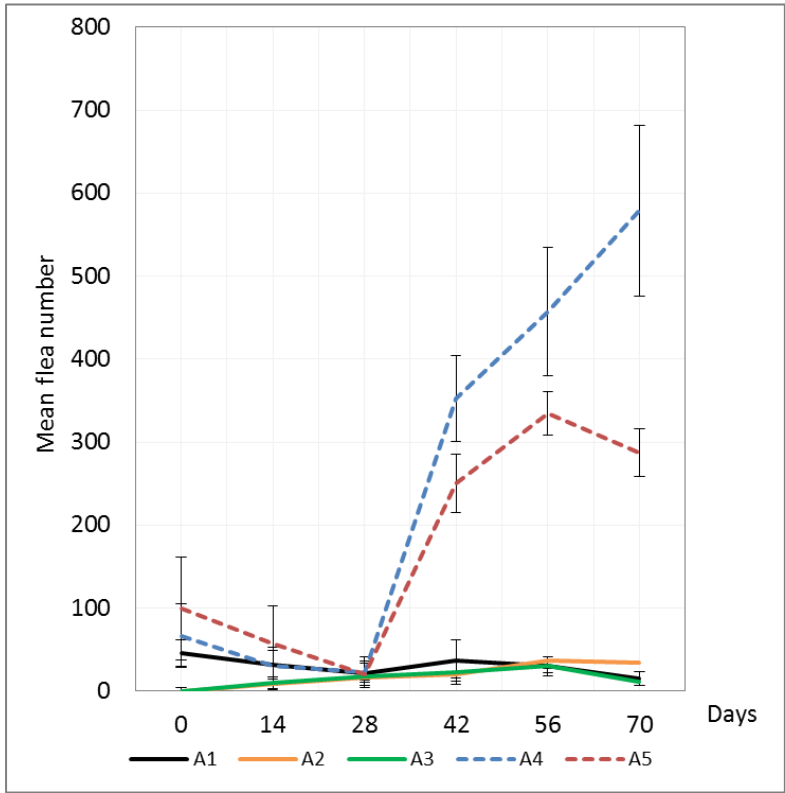
Figure 1: effect of adult flea feeding frequency



Rearing medium renewal

In figure 2, there was a strong effect of rearing medium renewal ($W=270,5$, $p<0,000001$). Groups transferred to a new rearing medium started a new cycle after 28 days and reached the peak around day 70 (A4 and A5), but in the control group (A1), no significant increase was noticed. In A2 and A3, newly emerged adults were produced from the ancient rearing medium, but no peaks were observed.

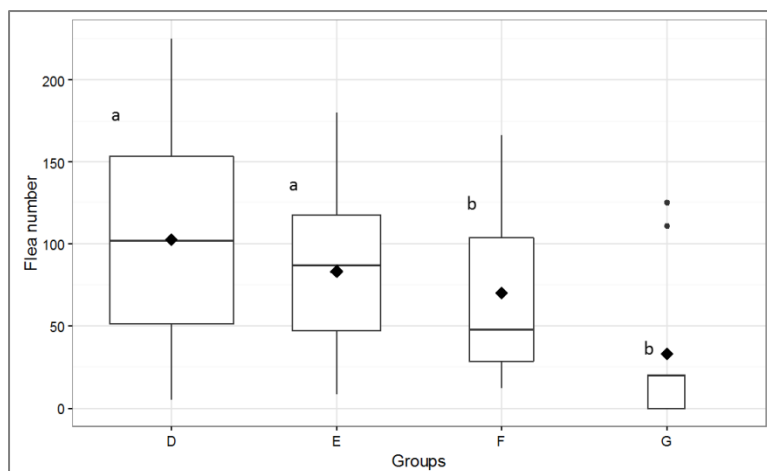
Figure 2: effect of rearing medium renewal



Shaking/counting effect

The shaking or counting effect was observed when the average number of fleas for each group was compared ($F = 6.4747$, $p < 0.01$). The groups counted every week and every two weeks were found with a higher average number of fleas than the two other groups that were counted every three weeks or at the end of the experiment (Figure 3). However this difference was not found when comparing mean flea number obtained at different intervals of time (day 84, day 112 and day 224). It is important to note that in group G, no adult fleas were found in 2 out 3 replicates at the end of the test (day 224).

Figure 3: shacking/counting effect

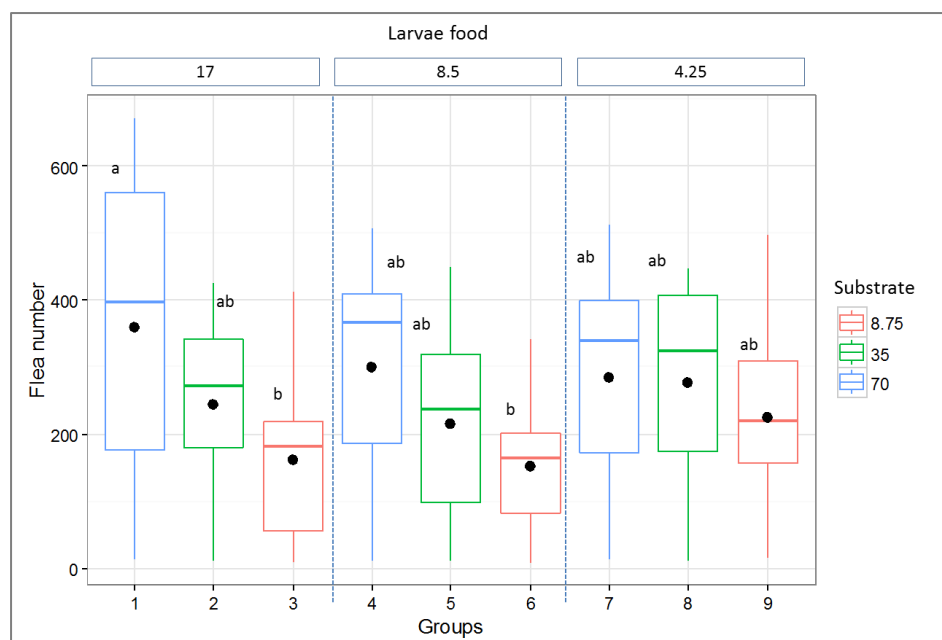


The width of each boxplot is proportional to the number of observation per group. No significant difference in mean flea number was found between groups having the same letter (a or b).

Rearing medium composition

No effect of variation of larvae diet was found, however differences in the average number of fleas were observed according to the substrate quantity (Kruskal-Wallis $\chi^2 = 16.0896$, $df = 2$, $p < 0.001$). The result is a difference in the average number of fleas between nine groups (Kruskal-Wallis $\chi^2 = 20.4213$, $df = 8$, $p < 0.01$). The combined effect of larva food and substrate quantity is showed in Figure 4. Pairwise comparison between groups showed significant differences between group 1 and 3, and 1 and 6. For the group supplied with 17 grams of larvae food; flea number was significantly lower when associated with the minimal substrate quantity. The same observation was done in groups fed with 8.5 grams of larva food, however no difference was found in group supplied with 4.25 grams of larva diet, independently of substrate quantity. Indeed, for the groups raised with the same substrate quantity, no difference in mean flea number was found whatever the larva food quantity (Figure4).

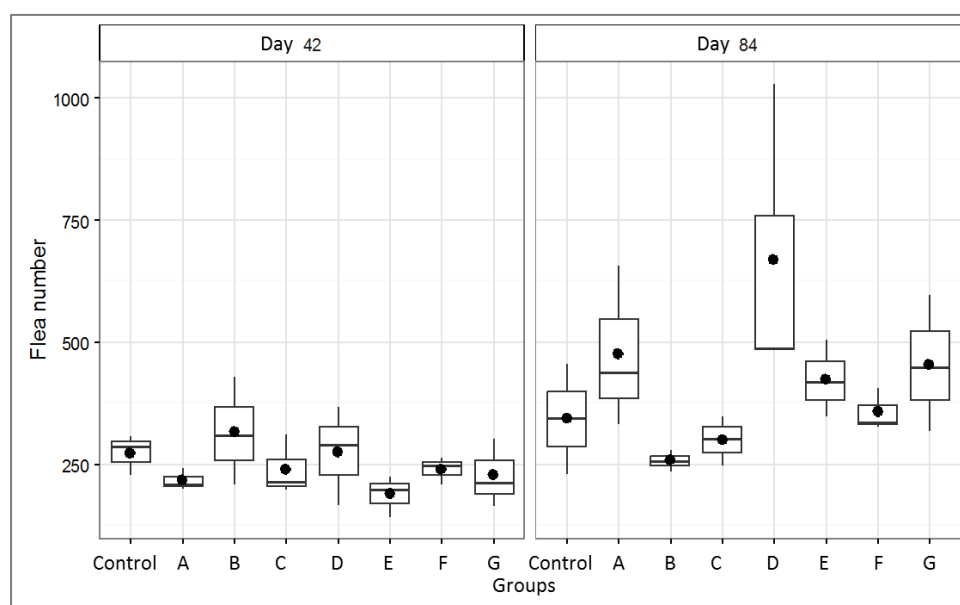
Figure 4: effect of rearing medium composition.



Larva food composition

The average number of fleas observed in each group was the same regardless of the composition of the larvae (ou larval) food. The observation in the control group was significantly the same as observed in groups supplied with larva food. The flea number was significantly higher in day 84, but no difference was found between groups.

Figure 5: effect of larva food composition



Discussion

Adult fecundity and blood meal requirement

The physiology of *X. cheopis* made this species closely dependent to its host by taking intermittent small amount of blood meal (less than 1µl) (Prasad 1987). It was advised to feed rat's flea twice a week (Bland et al 2015), but our results suggested that once a week will be better: Feeding our colony once a week resulted in the highest number of fleas after about two months of rearing.

It may be obvious that the higher number of fleas could be obtained, depending on the initial number that was allowed to colonize each jar. Furthermore, it was recommended to start a colony with hundred individuals to fasten the colonization. Eddy and Smith began their *X. cheopis* colony with 500 newly emerged fleas within a 35 cm diameter and 20 cm deep rearing pan (Smith and Eddy 1954). In our case, this number was rarely reached when beginning colony from field caught rat's fleas. A reasonable flea colony has been obtained with 20 fleas after five weeks, depending on the feeding frequency. Our observations showed that a low feeding frequency was inadequate to maintain a population at the insectary, and the risk of losing the population is high after few months.

Female *X. cheopis* can lay hundreds of eggs during its lifespan and about 2-6 eggs per day (Gage 2004). Generally, a blood meal is required to complete ovary development in female rat flea (Kamala Bai and Prasad 1979) and ovipositions occur at least two days after the first blood meal (Pillai and Prasad 1988). In blood-sucking arthropods, feeding and reproduction are largely interconnected and the blood meal frequency can impact the number of offspring (Prasad 1985). In *X. cheopis* fed females, blood proteins have been shown to be a limiting factor for vitellogenesis, although in males and female tryglycerides are the main source of energy (Prasad 1987, Pillai and Prasad 1988).

However, in the case of fleas, no feeding rhythm in relation to egg maturation has been demonstrated (Pillai and Prasad 1988). On the other hand, feeding can influence the survival of adult fleas and indirectly the number of eggs that the female can produce. Adult off-host *X. cheopis* survival study showed that once fed *X. cheopis* have extended longer lifespan than those that have not been fed (Gage 2004).

The complete development of *X. cheopis* takes approximatively 4-5 weeks at 24-25°C with 75% (Bacot 1914). This was consistent with our observation and the latency period that has elapsed since the beginning of the rearing until the 28th day can be attributed to the moment when females lay eggs and immature stages start to develop. Under suitable conditions, female fleas can deposit about 90% of their eggs after 21 days and adults of the second generation are almost ready to emerge (Smith and Eddy 1954, Cole et al 1972). During this time, some adults may die because their age and sex were not

checked at the beginning of the test. It was observed in cat flea colony that after 22 days, only 40% of females and 24% of males survived (Hudson and Prince 1958). A high increase in the number of fleas was observed when all adults emerged from cocoons, and the increase was maintained when the next generation continued to reproduce. Plateau was reached when the maximum number of fleas in each jar was reached, after four or five generations. At that point, the population decreased slightly and collapsed. It has been estimated that the maximum capacity of a *X. cheopis* colony with all generations maintained will be reached after 2 to 5 months (Bland et al 2015). Even in the group fed once a week, the number of adults decreased, probably due to blood meal shortage: one baby mouse is not enough for the size of the colony. The young mouse was immediately reported exsanguinated within five to ten minutes (Bland et al 2015), knowing that this period varies over the number of fleas in the jar. Secondly, the number of fed adults may decrease and thus impact the population growth. Additionally, a high adult density in certain mammal host species has been observed to impact negatively the number of eggs and the survival of the offspring of some *Xenopsylla* species (Khokhlova et al 2007, Krasnov et al 2008). One of the factors that explain is the competition for blood resource, which results in less or poor food quality (Krasnov et al 2008). This may be one of the explanations of the progressive decrease in the number of fleas after reaching the plateau.

Effect of shaking and renewing the rearing medium

The rearing medium plays an important role in the development of immature stages of fleas. The rearing medium is the principal larval source of food and provides materials that will be incorporated into the silky cocoon. The presence of debris appeared to be a necessity for the normal formation of the cocoon and so long, contributing to the preservation of moisture inside the cocoon (Sharif 1949). In addition, rearing larvae without medium increases their mortality by the sticky nature of their feces (Sharif 1949). However, through the time and in hot and humid environment, the rearing medium may accumulate conditions which may limit the flea normal development. We observed that transferring the colony in new rearing medium permitted to increase significantly the colony size. Changes in the physical properties of the rearing medium have been described to impact directly the larval lifespan and development (Sharif 1949). At high relative humidity, the larval medium may undergo texture changes by forming a crust or firm mass, thereby reducing aeration of the larvae. Ideally, the rearing medium should fall in grains to avoid moisture excess, which is harmful for larvae development (Sharif 1948). In addition to medium renewal, shaking or disturbing the rearing medium optimized the number of fleas in the colony. Here, the “shacking” effect can benefit the colony not only by favoring adult’s emergence, but also by affecting the texture of the rearing medium by improving aeration and prevent compaction.

Counting could be the occasion to check the “health” of a colony by locating the presence of immature stage or parasites. The rearing medium could be parasitized by mites which can spoil larval food or directly parasitize the larvae themselves, and the rice bran can also be contaminated by beetles (Ratovonjato et al 2000). Fungi proliferation is common in humid and warm environments: even if it is not to harmful to flea stages but may favor the formation of firm mass (Sharif 1949). A change of color in the medium accompanied by unexpected odors may indicate that it is contaminated. This can be avoided by using sterilized materials. Renewal of the rearing medium and frequent counting seem to be crucial for maintaining a healthy *X. cheopis* colony, by removing factors that could trigger the normal development of immature stages. This should be done periodically or according to the need for laboratory experiment.

Flea larva diet requirement

The structure of flea’s larvae mouthparts allowed them to feed on dried particles (Kapadi 1908, Durden and Traub 2002). Assuming that flea larvae fed mainly adult faeces containing undigested blood particles, many methods of laboratory breeding used at least dried blood as component of larval diet (Smith and Eddy 1954, Hudson and Prince 1958, Ratovonjato et al 2000, Metzger and Rust 2001, Bland et al 2015). In previous studies, blood and yeast appeared to be an ideal food for larvae of three *Xenopsylla* species (Sharif 1948). Breeding on rearing medium composed by only blood powder may fail due to the lack of nutrients such as vitamin B that flea larvae can find in its natural environment provided by microorganism and fungi (Sharif 1948, Silverman and Appel 1994). The addition of yeast and dog show to dried blood as larval diet for *C. felis* showed significant improvement in larval survival (Silverman and Appel 1994).

However, according to species, flea larvae have different food requirement (Pollitzer 1954). Here, we found that *X. cheopis* larvae can survive and complete life cycle without supplementary diet. Studies on the nutritional requirement of *X. cheopis* larvae and concluded that complete development of larvae could be achieved on relatively low nutritional quality food (Sharif 1948) because larvae received important nutriment reserve during embryonic stage, as the egg is relatively large and the yolk very rich (Pausch and Fraenkel 1966, Prasad 1987). In addition *X. cheopis* species, probably due to its frequent feeding, is closely attached to its host (Pollitzer 1954, Prasad 1987), so that adults may lay eggs and droppings in different places with a fair chance of the resulting larvae suitable breeding conditions (Bacot 1914). Thus, the larvae might find suitable diet outside adult droplets in places where *R. rattus* often occurs, such as granary and warehouses. Furthermore, *X. cheopis* strains have been maintained on

ground laboratory animal chow only and claim that beef blood occasionally causes high mortality from contamination (Cole et al 1972).

In our experiment, the sterilized rice bran used alone as a rearing medium gave the same result as the addition of a supplementary component such as brewer's yeast, dried blood or mouse chow. Comparing breeding success of *X. cheopis* using different substrates such saw dust or sand showed that better results were obtained with rice bran (Ratovonjato et al 2000). Estrade has advanced the possibility of plague transmission between man in Madagascar by observing off-host *X. cheopis* on the floor of human house accumulating dust containing rice debris, and taking their blood meal on human (Estrade 1934). As result we can say that can rice bran particles can fulfill the dietary need of *X. cheopis* larvae. This finding was consistent with the observation on the effect of rearing medium composition. However it would be interesting to see in which extent the rice bran alone, without adding other nutriment source will be sufficient to maintain healthy colony for a longer period.

To summarize, the objective was to determine the factors that may improve the *X. cheopis* rearing; concretely obtaining as much adults as possible for laboratory experiments need. We found that the higher number of adults was associated with weekly feeding, highest substrate quantity, and frequent rearing medium disturbance. Also, the addition of larval diet to the rearing medium did not induce significant increase in adult flea number. The renewal of the liter or the rearing medium improved positively the increase of the adult population of the colonies. These results will help to optimize the care and maintenance of flea colonies raised in medical entomology insectary.

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