Sokoine University of Agriculture

MSc Dissertation

Effect of Pyriproxyfen and Ivermectin on Survival and Reproductive Perfomance of Plague Flea Vectors, *Xenopsylla Cheopis*

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November, 2023

EFFECT OF PYRIPROXYFEN AND IVERMECTIN ON SURVIVAL AND REPRODUCTIVE PERFOMANCE OF PLAGUE FLEA VECTORS, *XENOPSYLLA CHEOPIS*

Dissertation Submitted In Partial Fulfilment of the Requirements for the Degree of Master of Science in Parasitology Sokoine University of Agriculture

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November, 2023

EXTENDED ABSTRACT

One of the primary carriers of plague in many tropical and subtropical regions of the world is *Xenopsylla Cheopis*. Targeting flea vectors by sprinkling or dusting homes and surroundings with chemical insecticides is the primary method for controlling plague. However, the likelihood of insecticide resistance has increased because to the prolonged use and excessive reliance on chemical insecticides. Ivermectin and pyriproxyfen, which target both endoparasites and ectoparasites, will limit their actions and reduce their susceptibility to resistance. These substances have however not tested against plague fleas. The objectives of this study were to evaluate the effects of pyriproxyfen and ivermectin on reproduction and survival, as well as the viability of incorporating them into the current management methods. Two experimental designs were used; the first exposure to pyriproxyfen was investigated for its efficacy against adult, fecundity, and larval stages of the oriental rat flea. The contact bioassays against both the larval and adult stages of fleas conducted through series of laboratory experiments. In adult fleas, treatment and controls groups were tested in four replicates while larvae were six replicates. Exposure time of pyriproxyfen for adult fleas were 30 minutes and none for larvae

Twelve rats were used in total for the second exposure method of the ivermectin and pyriproxyfen . Four rats were given 0.2 ml of ivermectin, four were given 0.3 g of pyriproxyfen powder, and four were left untreated. The adult fleas were exposed for 12 hours, and 10 days of observation followed. The survival curve of adult fleas was produced after data analysis using the R software. Additionally, the least square method was used to calculate the impact of pyriproxyfen and ivermectin on adult mortality. The survival rate of adult Xenopysylla cheopis fleas in a treatment group shown to be 100% at day 0. As time passed, there was a decreasing chance that the treated group would survive, and by day six, every flea in the treated group had died ($P=0.0313$).

In comparison to the control group, the treatment group significantly produced less eggs (<0.0001). In the treatment group of flea larvae,

90% of the larvae died by day eight, and by day 20 of monitoring and observation, there were no cocooned flea larvae. 98% of the larvae in a control group survived and 60% of them formed cocoons. According to the research, adult fleas, egg viability, egg laying, and larval growth are all affected when 2g of pyriproxyfen mixed with sand and dried cattle blood powder is directly applied to them. Pyriproxyfen alone was considerably less toxic to adult fleas during a period of 24 hours, and mortalities began from day 2 and increased. However, similar hormone with other exposure methods in conjunction with ivermectin in action have shown that this is not the case.

Pyriproxyfen was very effective in day four using the least square method, with an LS mean of 3.75 and a 0% egg-laying rate. Maximum mortalities were achieved at day five with an LS mean of 4.5 and a P-value of (0.0001), and 5% less eggs were deposited by adult fleas that were exposed to ivermectin. The combination of pyriproxyfen and ivermectin was effective against adult fleas, with the largest mortalities occurring on days three and four with LS Means of 4.75 and 5, respectively, and no eggs being deposited during the course of the monitoring period of ten days. This combination of pyriproxyfen and ivermectin may be utilized to better control the plague in endemic areas and as flea vector control agents.

IKISIRI KUU

Moja ya wabebaji wa msingi wa tauni katika maeneo mengi ya kitropiki na ya kitropiki ya ulimwengu ni *Xenopsylla Cheopis*. Kulenga vidudu vya viroboto kwa kunyunyizia au kutia vumbi kwenye nyumba na mazingira kwa viua wadudu vya kemikali ndiyo njia kuu ya kudhibiti tauni. Hata hivyo, uwezekano wa kustahimili viua wadudu umeongezeka kwa sababu ya matumizi ya muda mrefu na kuegemea kupita kiasi kwa viua wadudu vya kemikali. Ivermectin na pyriproxyfen, ambayo inalenga endoparasites na ectoparasites, itapunguza matendo yao na kupunguza uwezekano wao wa kupinga. Viuatilifu hizi hata hivyo hazijajaribiwa dhidi ya viroboto wa tauni. Malengo ya utafiti huu yalikuwa kutathmini athari za pyriproxyfen na ivermectin juu ya uzazi na kuishi, pamoja na uwezekano wa kuziingiza katika mbinu za sasa za udhibiti wa ugonjwa na wadudu wanasambaza tauni. Miundo miwili ya majaribio ilitumika; mfiduo wa kwanza wa pyriproxyfen ulichunguzwa kwa ufanisi wake dhidi ya watu wazima, uzazi, na hatua za mabuu ya kiroboto cha panya wa mashariki. Uchunguzi wa kibayolojia wa mguso dhidi ya hatua zote mbili za mabuu na watu wazima wa viroboto uliofanywa kupitia mfululizo wa majaribio ya kimaabara. Katika viroboto wazima, vikundi vya matibabu na udhibiti vilijaribiwa katika nakala nne wakati mabuu yalikuwa nakala sita

Muda wa mfiduo wa pyriproxyfen kwa viroboto wazima ulikuwa dakika 30 na hakuna kwa mabuu.Panya kumi na mbili zilitumiwa kwa jumla kwa njia ya pili ya mfiduo wa ivermectin na pyriproxyfen. Panya nne zilipewa 0.2 ml ya ivermectin, nne zilipewa 0.3 g ya poda ya pyriproxyfen, na nne ziliachwa bila kutibiwa. Viroboto wazima waliwekwa wazi kwa masaa 12, na siku 10 za uchunguzi zilifuata. Njia ya kuishi ya viroboto wazima ilitolewa baada ya uchanganuzi wa data kwa kutumia programu ya R. Zaidi ya hayo, mbinu ya angalau mraba ilitumika kukokotoa athari za pyriproxyfen na ivermectin kwa vifo vya watu wazima. Kiwango cha kuishi cha viroboto wa watu wazima Xenopysylla Cheopis katika kikundi cha matibabu kilichoonyeshwa kuwa 100% kwa siku 0. Kadiri muda ulivyopita, kulikuwa na uwezekano wa kupungua kwa kundi

lililotibiwa lingeishi, na kufikia siku ya sita, kila kiroboto katika kikundi kilichotibiwa alikuwa amekufa. (P=0.0313). Kwa kulinganisha na kikundi cha udhibiti, kikundi cha matibabu kilizalisha mayai kidogo (<0.0001). Katika kundi la matibabu ya mabuu ya kiroboto, 90% ya mabuu walikufa siku ya nane, na hadi siku ya 20 ya ufuatiliaji na uchunguzi, hakukuwa na mabuu ya kiroboto. Asilimia 98 ya mabuu katika kundi la udhibiti walinusurika na 60% yao waliunda vifukofuko. Kulingana na utafiti, viroboto waliokomaa, uwezo wa yai, utagaji wa yai, na ukuaji wa vibuu vyote huathiriwa wakati 2g ya pyriproxyfen iliyochanganywa na mchanga na poda ya damu ya ng'ombe kavu inatumiwa moja kwa moja kwao. Pyriproxyfen pekee ilikuwa na sumu kidogo kwa viroboto wazima katika kipindi cha masaa 24, na vifo vilianza kutoka siku ya 2 na kuongezeka. Hata hivyo, homoni sawa na mbinu nyingine za mfiduo kwa kushirikiana na ivermectin katika hatua zimeonyesha kuwa hii sivyo.

Pyriproxyfen ilikuwa nzuri sana katika siku ya nne kwa kutumia mbinu ya angalau mraba, ikiwa na maana ya LS ya 3.75 na 0% ya kiwango cha kuatamia. Kiwango cha juu cha vifo kilipatikana katika siku ya tano kwa wastani wa LS wa 4.5 na thamani ya P ya (0.0001), na 5% chini ya mayai yaliwekwa na viroboto wazima ambao waliathiriwa na ivermectin. Mchanganyiko wa pyriproxyfen na ivermectin ulikuwa mzuri dhidi ya viroboto wazima, huku vifo vikubwa zaidi vikitokea siku ya tatu na nne na LS Means ya 4.75 na 5, mtawalia, na hakuna mayai yaliyowekwa wakati wa kipindi cha ufuatiliaji wa siku kumi. Mchanganyiko huu wa pyriproxyfen na ivermectin unaweza kutumika kudhibiti vyema tauni katika maeneo hatarishi na kama mawakala wa kudhibiti vekta ya viroboto.

DECLARATION

I, **NICOLAUS ANANIA MWAKALINGA,** do hereby declare to the Senate of the Sokoine University of Agriculture that this dissertation is my own original work and it has neither been nor concurrently been submitted for a higher degree award in any other institution.

_____ _____

Nicolaus Anania Mwakalinga Date
(MSc. Candidate)

The above declaration confirmed by;

_____ _____

Prof. Ladslaus L. Mnyone Date
(Supervisor)

_____ _____

Dr. Jahashi S. Nzalawahe Date
(Supervisor)

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AKNOWLEDGEMENTS

I give all the glory and honor to God Almighty for his grace and favor throughout my MSc studies.

I extend my sincere appreciation to NOVEL COMPOUND PROJECT under COSTECH for sponsoring my entire study

I greatly appreciate the remarkable assistance and guidance of my research supervisors and mentors; Prof. Ladslaus L. Mnyone and Dr. Jahashi Nzalawahe. Their tireless support, time and advice made this research possible. I learnt a lot of things throughout the processes.

I am indebted to Dr. Abdull Katakweba, Mr. Ginethon Mhamphi, Ms. Resta Maganga, Mr. Omary Kibwana, Dr. William G. Mseti, Mr. Godfrey Katusi and all staff at the Sokoine University of Agriculture, Institute of Pest Management for their supports and encouragements through laboratory works, data analysis and reading my dissertation.

I extend my heartfelt gratitude to my mother NERUSIGWE MWABULESI, the late Father Mr. ANANIA MWAKALINGA and for their love, care and loyalty and their costs incurred for my studies Journey to date.

And finally, I would like to thank my Dearly wife LINDA DARLENE MUHOZE for her tolerance, love and care rendered to me throughout the study period.

DEDICATION

This research work is dedicated to my late beloved FATHER ANANIA AMBONISYE MWAKALINGA and all plague endemic communities throughout Africa and beyond.

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LIST OF ABREVIATIONS AND SYMBOLS

COSTECH	Tanzania Commission for Science and Technology
DDT	Dichlorodiphenyltrichloroethane
GABA	γ-Aminobutyric acid
GLM	General Linear Model
GLMM	General Linear Mixed Model
IGRs	Insect Growth Regulators
IPM	Institute of Pest Management
LSM	Leas Square Method
PPF	Pyriproxyfen
SAS	Statical Analysis System
SUA	Sokoine University of Agriculture

STRUCTURE OF THE DISSERTATION

This dissertation consists of FOUR chapters, chapter ONE describes background information on plague fleas' vectors and plague disease, causative agent, its distribution and burden worldwide, Africa and in Tanzania, the information on common vectors, different control agents, problem statement , justification and research objectives. Chapter TWO (Manuscript ONE) this explains the effect of pyriproxyfen on oriental rat fleas' adult survival, fecundity and Larval Development**.** Chapter THREE (Manuscript TWO) deals with the effect of combining of pyriproxyfen and ivermectin on oriental rat fleas' and its reproductive performance. Chapter FOUR consists of general discussion, conclusion and specific recommendations.

CHAPTER ONE

1.0 GENERAL INTRODUCTION

1.1 Background

Many tropical and subtropical regions of the world are home to the flea species *Xenopsylla cheopis*, which is a major carrier of plague (Miarinjara & Boyer, 2016). *Yersinia pestis*, a gram-negative bacterium, is the cause of the highly fatal infectious disease plague (Laudisoit *et al*., 2007). Despite there being only occasional epidemics, the disease is still a serious health concern for the entire world. Currently, most cases and fatalities are found in Africa, with the worst-affected countries being Algeria, Madagascar, the Democratic Republic of the Congo, Mozambique, Uganda, and Tanzania (Miarinjara and Boyer, 2016). Between 2007 and 2011, there were 2409 cases in Madagascar that were confirmed, and in 2012, 67% of all cases worldwide were in that country (Miarinjara and Boyer, 2016).

Given the plague propensity to reemerge after an extended period of dormancy, the likelihood of deadly outbreaks in Tanzania cannot be understated. The Lushoto, Mbulu, and Karatu districts are the most significant disease foci described to date, despite the fact that many areas of Tanzania are susceptible to plague epidemics (Makundi *et al*., 2008; Ziwa et al., 2013). A total of 630 deaths attributed to plague outbreaks were documented in the Lushoto district between 1980 and 2003, out of about 7000 cases (Makundi *et al*., 2008). After roughly 32 years of quiet, the most recent illness outbreak was reported in Mbulu in 2007 (Makundi *et al*., 2008; Ziwa *et al*., 2013). Targeting flea vectors by sprinkling or spraying homes and the neighborhood with chemical insecticides helps to prevent plague (Katakweba *et al.,* 2015).

In Tanzania and other countries where the disease is endemic, this strategy has dramatically reduced the risk of and effects from plague (Laudisoit *et al.,* 2007). But frequent usage and overuse of chemical pesticides has increased the possibility of the emergence of insecticide resistance (Stenseth *et al*., 2008; Boyer *et al*., 2014). In

Madagascar, the first instances of Dichlorodiphenyltrihloroethane resistance in the *Xenopsylla Cheopis* species were originally reported in 1965, and it was first proven in 1981. Malathion, Fenitrothion, and Propoxur resistance development in *Xenopsylla Cheopis* were also reported in 1983 (Boyer *et al*., 2014). The potential impacts of pesticide resistance on the capacity of *Xenopsylla spp*. to transmit plague to make it on and off since precolonial times are one issue that has been widely disregarded. There is a pressing need to develop complementary control tools, particularly those that are less prone to insecticide resistance and allow sustainable applications. Novel compounds, pyriproxyfen and ivermectin, all of which have demonstrated great potential for controlling many arthropod vectors, provide plausible options.

1.2 Plague Vector, Life Cycle and Transmission

Black rat, *Rattus rattus* appears to be a principal host with main species of fleas which are primarily involved in transmission of plague. At least 80 flea species are known to carry the etiological agent of the disease, although the role in disease transmission varies (Gage and Kasoy, 2005). Oriental rat flea *Xenopsylla cheopis* (Siphonaptera: Pulicidae). Rothschild, 1903 is considered to be the most efficient vector as well as the major vector to humans. Other flea species have been identified as vectors in East Africa including the Island of the South West Indian Ocean includes; (*Ctenophtalmus bacopus, C.cabinus, Dinopsyllus, Pulex irritans,* and *Xenopsyla brasiliensis*) (Eisen and Gage, 2012).

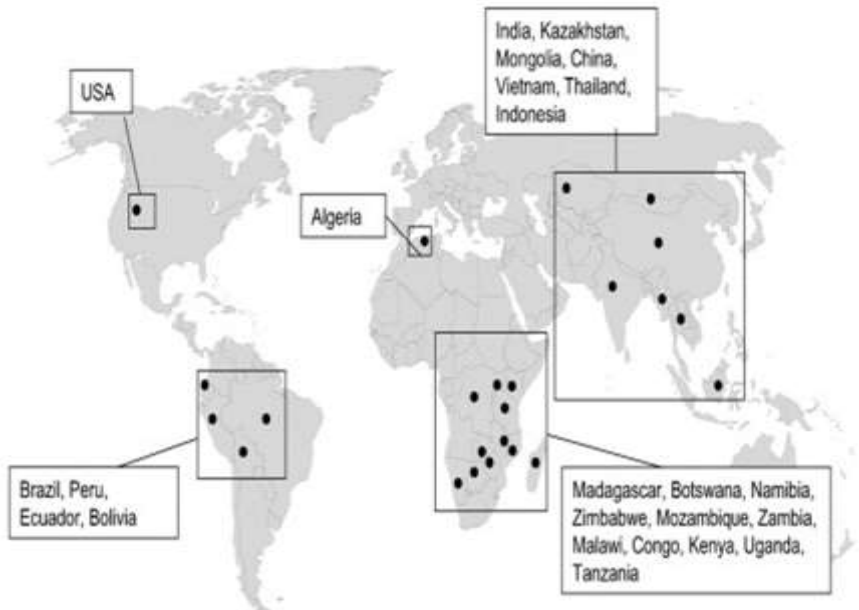

Figure 1.1: Global distribution of natural plague foci as of March 2016
Source: WHO/PED as of 15 March

The plague is foremost a disease of rodents and is transmitted between rodent hosts via fleas. Humans and other mammals are, in fact, completely accidental hosts (Amedei *et al*., 2011; Chouikha and Hinnebusch, 2012; Gage, 2012). Life cycle of *Y. pestis* consists of a cycle between rodents and fleas (Figure1.2). When rodent hosts die, the fleas abandon the corpses and seek new hosts, inadvertently infecting other mammals, such as human (Anismovu and Amoako, 2006). When a flea feeds on an infected rodent, bacteria from the rodent's blood are taken into the flea's midgut. *Y. pestis* does not enter the cells of fleas or adhere to the flea digestive tract, resulting in half of fleas, removing all of the bacteria through their feces.

In order to overcome this, *Y. pestis* must build up an incredibly high density within the blood of its host rodent. When bacteria manage to persist in the flea's midgut, they rapidly reproduce and form clusters that are too large to be excreted. The bacteria proceed to form a biofilm on the proventriculus, a valve in the flea connecting the esophagus to their midgut. This causes the flea to regurgitate when

it attempts to feed, spewing the bacteria into the bite and passing them onto a new host, where they once again begin to proliferate (Anismovu and Amoako, 2006; Chouikha and Hinnebusch, 2012).

Under the natural environment, plague transmission is maintained primarily by the so-called sylvatic cycle, which involves wild rodents and their fleas. Many species of wild rodent and fleas have been implicated in transmission of plague in natural plague foci (WHO, 1997). Human is extremely susceptible to plague and may be infected either directly or indirectly. Indirect transmission through the bite of flea is the most common route transmission between plague infected rodents and humans (Dennis *et al*., 1999). The disease can also be transmitted through contaminated meat example people who use rodent as a source of food, farmers may be exposed to fleas while they plough their fields other reservoirs that can act as source of transmission include cats and dogs. Close contact with animals suffering from plague, or respiratory droplets of an infected animal is another way for transmission of the disease (Chouikha and Hinnebusch, 2012).

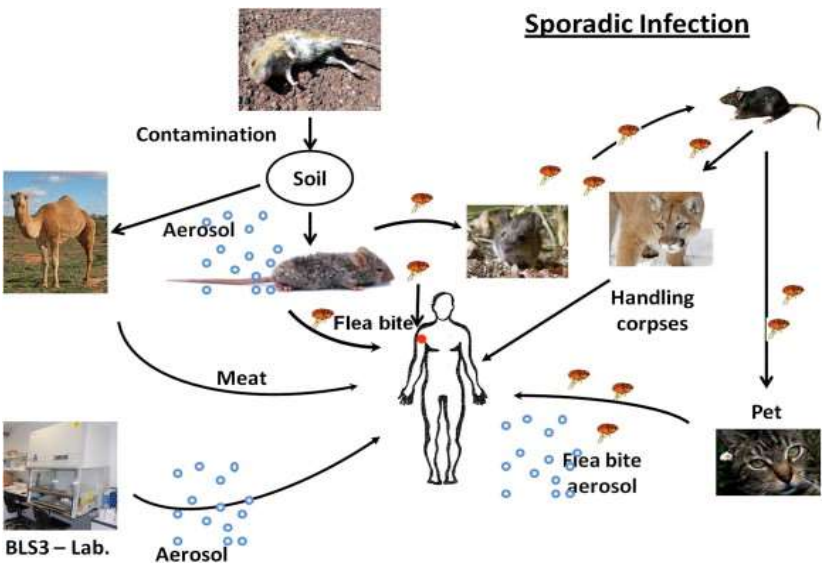

Figure 1.2: Plague transmission
(WHO, 2010)

1.3 Clinical Forms of Plague

There are three forms of plague infections depending on the route of infection: Bubonic plague, septicemic and pneumonic plague. Bubonic plague is mainly spread by infected fleas from small animals. *Yersinia pestis* enters at the bite and travels through the lymphatic system to the nearest lymph node where it replicates, the lymph node becomes inflamed, tense and painful and is called a" bubo" At advanced stages of the infection the inflamed lymph nodes turns into open sores filled with pus (WHO, 2010). Bubonic plague can advance and spread to the lungs which is the more severe type of plague called pneumonic plague.

Pneumonic plague, or lung-based plague is the most virulent, with incubation as short as 24hrs the infection is transmitted via droplets to the other humans, and delay in treatment can be fatal. The symptoms of pneumonic plague include fever, headache, shortness of breath, chest pain and cough (Bertherat, 2016).

Septicemic plague, is the rarest form of a plague in a 100% mortality rate if untreated but 'only' 22% if treatment is available within the first 24 hours. When bacteria proliferate at high levels with the blood, septicemic plague arises. The blood-infection causes small blood clots to occur, cutting off circulation to parts of the body and depleting the clotting molecules in the blood. This leads to uncontrolled bleeding, nausea, fever, vomiting blood, abdominal pain, and diarrhea. The blood clots result in cyanosis and necrosis due to a lack of oxygen reaching the tissues, with gangrene as a secondary effect (Amedei *et al.,* 2011; Anismovu and Amoako, 2006).

1.4 Prevention and Control of Plague

Primary prevention of human plague mainly focuses on vector control or rodent reduction within limited areas affected by plague epizootics (Gratz, 1999). The most widely used method to reduce plague in animal population is to use insecticides to remove fleas from hosts. Fleas are the main vectors for *Y. pestis,* so by removing the vector we should be able to substantially reduce bacterial

transmission among individual (Biggins *et al.,* 2010). Recent studies from the West Nile region have shown that indoor residual spraying (IRS) and insecticide delivery tubes effectively reduce flea loads on rodents in the home environment where most exposures are believed to occur (Eisen *et al.,* 2014; Boegler *et al.,* 2014; Borchert *et al.,* 2010). Also the rodent can be controlled by using lethal trapping and application of rodenticides.

Secondary prevention of plague aims to reduce case fatality rates through education campaigns that emphasize recognition of signs of plague and urging persons with symptoms consistent with plague to seek care without delay (Gratz *et al.,* 1999).

The control also requires investigation of animal and flea species implicated in the plague cycle in region, developing environmental management program to understand thezoonosis of the disease cycle and to limit spread and the surveillance of animal foci (WHO, 2012). Also plague can be controlled by using vaccines. There are two types of vaccines currently used ,live vaccine which is derived from an attenuated strain related to EV76 strain and the killed vaccine uses formalin-fixed virulent strain of *Y. pestis* (WHO, 2010).

1.5 *Xenopsylla Cheopis*

The oriental rat flea, *Xenopsylla cheopis* (Rothschild, 1903), is a member of the family Pulicidae in the order Siphonaptera. Fleas are highly specialized, successful insect ectoparasites of birds and mammals (Marquardt *et al.,* 2000). Of the nearly 2 500 species of fleas known from around the world (Triplehorn and Johnson, 2005), few are as infamous as the oriental rat flea.

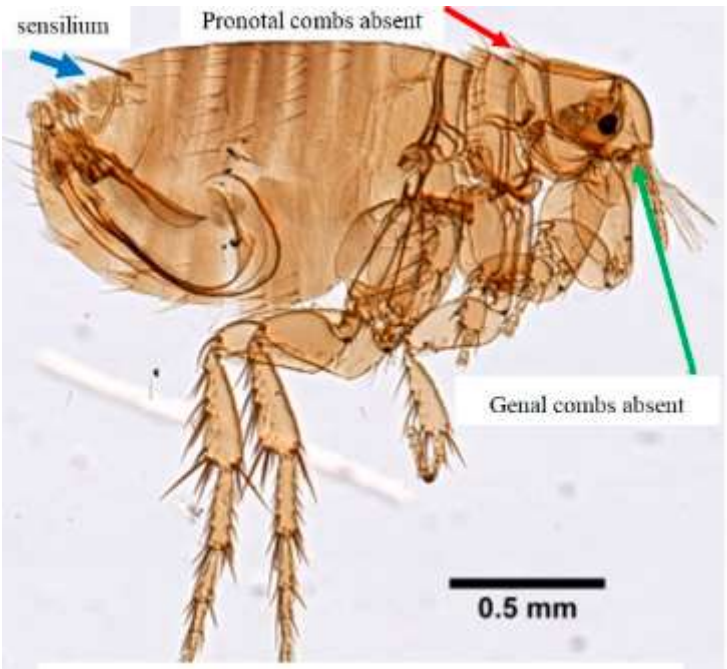

Figure 1.3: *Xenopsylla cheopis* (Triplehorn and Johnson 2005)

LIFE CYCLE AND BIOLOGY

Fleas, including *Xenopsylla cheopis*, are holometabolous and have four distinct life stages: egg, larva, pupa, and adult (Figure 1.4).

EGGS

Flea eggs are ovoid in shape and have a smooth surface. Eggs are laid singly on either the host, or more commonly, in the nest and surrounding environment of the host. The egg stage is generally adapted to relatively high humidity, and females may produce up to six eggs daily and as many as 300-400 eggs during a lifetime (Gage 2005). Eggs are generally fully developed in approximately two weeks.

LARVA

The larval stage hatches from the egg stage. Fleas have three instars of increasing size (ranging from 0.5-3 mm). Larval development takes about two weeks but has been recorded to take as long as 200 days in poor conditions (Trivedi 2003). The larvae live in nests and

surrounding areas associated with their hosts (Marquardt et al. 2000, Trivedi 2003).

PUPA

Larvae develop into pupae, and pupae range from 2-4 mm in length. Pupae are generally pale or light brown in color. Flea pupae are often encased in a silken cocoon adorned with small sand-size particles and dirt from the surrounding environment (Gage 2005). The length of time spent in the pupal stage appears to be directly related to ambient temperature and environmental cues and is suspected to range from 2-9 weeks.

ADULT

Adult oriental rat fleas are obligate blood feeders, and both male and female adults feed on blood. They are telmophagic, meaning that they bite and then feed on blood that pools on the surface as opposed to siphoning it directly from beneath the skin. Blood is kept from coagulating through special enzymes in the saliva. In the flea foregut a muscular valve, the proventriculus, is responsible for breaking down the blood meal for digestion.

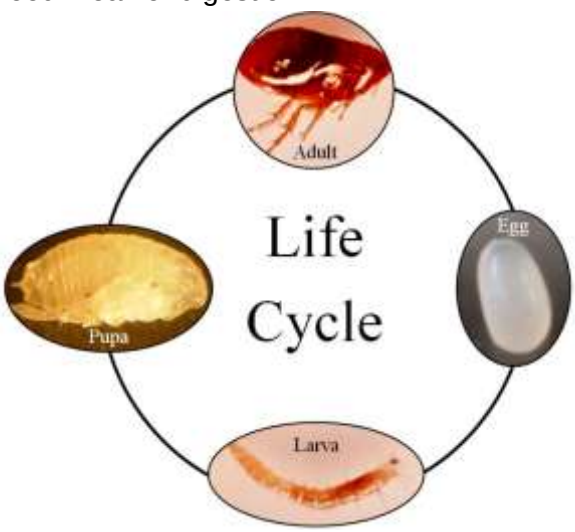

Figure 1.4: Life cycle of *Xenopsylla cheopis*
(Gage 2005)

1.6 Insecticide Resistance in Flea Vectors and the Mechanisms

Resistance to insecticide is defined by WHO as the ability of a strain of insects to tolerate doses of toxicants which would prove lethal to the majority of individuals in a normal population of the same species. Susceptibility is defined as the inability to withstand a pesticide at normal use rate (Biswas *et al*., 2016).

The use of insecticides as the control of flea vectors has led to the great improvement of the fight against disease vectors (Nauen, 2007; Hemingway, 2000). Consequently, they intensive use of these insecticides has caused selection pressure to many populations of insects, which resulted to the development of survival mechanisms in the presence of these recommended insecticides. Currently almost all classes of insecticides are involved in resistance (Miarinjara, 2016). There many reports of *X.cheopis* developing resistance against organochlorine, carbamates, organophosphates, pyrethroids and pyrethrins. In the beginning the rodent fleas were fully susceptible to DDT, but of recent they have developed resistance to most of the commonly used chemical insecticides (Biswas *et al.*, 2008). This is posing a threat and generates a further need to make a prior study on the insecticide susceptibility status of rodent fleas in the plague endemic areas (Gratz *et al*., 1999).

According to Miarinjara *et al*., in 2017, *X. cheopis* populations from Malagasy prisons were resistant to at least seven insecticides out of twelve. Few insecticides namely diedrin, permetrincyfluthrin and fenitrothionwere still effective. Resistance to insecticides could be a serious challenge in plague foci. Finding an effective insecticide is crucial in an emergency context to deal with a potential epidemic occurring in those areas.

There are different mechanisms of insecticide resistance these include target size sensitivity changes. This is based on three main insect central nervous system target sites namely; voltage-gated sodium channel, acetyl cholinesterase (AChE) in the cholinergic synapses and gamma amino butyric acid (GABA) sites in chloride channels at neuromuscular synapses.

The insecticide resistance involving the different target sites can be exemplified as follows. Resistance to Dichlorodiphenyltrichloroethane and pyrethroids is conferred by a mutation in the voltage-gated sodium channel usually by a substitution of the amino acid leucine with phenylalanine at the same or proximate codon position in domain IIS6 of the protein.

These substitutions confer knockdown resistance, *kdr* (Ames, 2011), On the other hand the resistance to organophosphates and carbamates is associated with insensitive AChE. This is achieved by structural modifications of the pesticide binding sites in AChE that allow at least partial binding to acetylcholine, Allowing AChE to break down the buildup of acetylcholine in the synapse and the synapse can function normally (Ames, 2011). Another mechanism is through metabolic resistance, this relies upon alteration of enzyme systems that arthropods use to detoxify foreign materials and/or preventing an insecticide from reaching its site of action. These occurs with esterases, oxidases, oxygenases, hydrolases and glutathione-s transferases (Ferrari, 1996; WHO, 2012). Yet anaother insecticide resistance mechanism is behavioral changes whereby insects develop a tendency of moving away from treated surface or area.. Also can be acquired through cuticular mechanism in which the cuticle reduces the penetration and uptake of an insecticide (Ferrari, 1996).

1.7 Slow Acting Control Agents

Insect growth regulators (IGRs), or juvenile hormone analogues, are compounds that elicit hormonal effects which disrupt or inhibit insect metamorphosis when administered at specific times and/or dosages (Meola *et al.,* 2000). This compounds disrupt and impede the life cycle of insects in the egg and larval stages of development.One example of IGRs is pyriproxyfen (Meola *et al.,* 2000).

1.7.1 Pyriproxyfen

Generally, pyriproxyfen is a broad-spectrum insect growth regulator with insecticidal activity against public health insect pests:

houseflies, mosquitoes, fleas and cockroaches (WHO (2008) . In agriculture and horticulture, pyriproxyfen has registered uses for the control of scale, whitefly, bollworm, jassids, aphids and cutworms (WHO, 2008) Pyriproxyfen degrades rapidly in soil under aerobic conditions, with a half-life of 6.4–36 days. Pyriproxyfen disappeared from aerobic lake water–sediment systems with half-lives ranging from 16 to 21 days (WHO, 2008). Pyriproxyfen is a relatively new pesticide, few environmental data have been collected. It has being reported to inhibit metamorphosis preventing emergence of adults from pupae (Brattoli *et al*., 2011). Also has extremely low toxicity to humans but to insects like mosquito it is reported to be effective at controlling mosquito larvae at very low doses and can persist for up to six months in a variety of aquatic habitat types. In addition, exposure of larvae to sub-lethal doses of PPF affects the adults' egg development, egg production and reduces the hatching of eggs of the mosquitoes (Brattoli *et al*., 2011).

1.7.2 Ivermectin

Ivermectin is a drug of macrocyclic lactones family with exceptional potency against endo- and ectoparasites at extremely low doses. It is highly active against a wide spectrum of nematode species, including most larvae and adult forms, and many arthropod parasites of domestic animal (González Canga *et al*., 2009). The excellent spectrum of activity of ivermectin resulted in the all-embracing name 'endectocide' (Lifschitz *et al*., 1999). The mode of action of ivermectin involves the act on GABA neurotransmission and glutamate gate Cl^-, leading to a flaccid paralysis, death and elimination of parasites (Taylor, 2001). The selectivity of pharmacological action is associated with lack of target glutamate receptors in mammalian species and relative restriction of GABA receptors to the central nervous system in mammals (Taylor, 2001). However, despite this tremendous potency, there are other organisms within these groups that appear to be refractory to ivermectin. The cat flea, *Ctenocephalides felis* (Bouche´), is a clinically relevant example. Ivermectin was orally administered weekly at 0.5 mg/kg or daily at 0.05 mg/kg and observed to be inactive against this parasite on dogs (Blair *et al*., 1984). Banks *et al*.

(2000) and Shoop *et al*. (2001), corroborated independently those results by showing that ivermectin has weak systemic activity against the cat flea in artificial membrane flea feeding assays. But ivermectin has a wide antiparasitic activity with long veterinary use and its activity against *Onchocerca volvulus* was discovered. Animal and *in vitro* experiments have shown that ivermectin has lethal effects on blood-sucking insects when these are fed on treated blood samples (Kavanaugh *et al*., 2020). It increases mortality and reduces the fertility of tsetse flies, triatomine bugs, ticks, and sandflies. Experiments in animal and *in vitro* have shown that ivermectin readily kills adult mosquitoes, including *anopheles*. In human trials of mass drug administration for onchocerciasis and filariasis contro, the effect of ivermectin on mosquitoes seems to be maintained in the field (Kavanaugh *et al*., 2020). These insecticidal properties of ivermectin have led some to suggest that this drug could have a role in the control of arthropod vector-borne diseases. Given this indirect evidence we therefore set out to test directly the effect of ivermectin on *Xenopsylla cheopis* being the major vector of plague in Africa.

1.8 Problem Statement and Study Justification

Historically, plague remains the most fatal pandemic disease ever recorded which resulted in the deaths of 75 – 200 million people from the 6th to 19th Centuries (WHO, 2014). Most such deaths occurred in medieval Europe, where the disease killed 30-50% the entire population (WHO, 2009). Tanzania reported 7000 cases and 630 deaths as a result of plague outbreaks in Lushoto and Mbulu district from 1980 to 2003 (Makundi *et al.,* 2008). However, plague can cause outbreaks that are as devastating as those of the past, if its control challenges are not well addressed. Mainly because the disease can emerge and re-emerge after decades of epidemiological silence. Notably, In Tanzania plague re-occurred about 32 years quiescence (Makundi *et al.,* 2008). One of the major control problems that needs to be addressed is increasing development of insecticide resistance against virtually all classes of insecticides that are currently in use for controlling fleas in plague endemic areas (Ziwa *et al.,* 2013). Besides, there is no any effective

plague vaccine available to date. Therefore, complimentary control strategies based on late acting control agents, that are less vulnerable to resistance, are desirable (Wraight *et al.,* 2000). Insect growth regulators example pyriproxyfen and ivermectin have demonstrated great potential for vector control but are yet to be comprehensively evaluated against survival and reproductive performance plague flea vectors, *Xenopsylla Cheopis.*

1.9 Objectives

1.9.1 Main objective

To determine effects of novel control agents on against survival and reproductive performance of a flea vector, *Xenopsylla Cheopis*

1.9.2 Specific objectives

(i) To determine the effect of pyriproxyfen and ivermectin on survival of immature and mature stages of plague fleas' vectors;
(j) To determine the ovicidal effect pyriproxyfen and ivermectin on the eggs of flea vectors;
(k) To determine the effect combination of ivermectin and pyriproxyfen on survival effect of the flea vectors

1.10 Significance of the Study

These compounds provide non-chemical means of flea control that can be integrated into the existing chemical control methods thus improving control of plague vectors whilst prolonging and/or restoring the effective shelf-life of chemical insecticides.

CHAPTER TWO

Manuscript One

2.0 Effect of pyriproxyfen on Adult Survival, Fecundity and Larval Development of *Xenopsylla Cheopis,* the Plague Flea

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2.1 Summary

Targeting fleas is the key strategy for controlling plague in Tanzania. However, the reliability of this method is being undermined by risk of spread of flea insecticide resistance.Although it is well known that resistance is less likely to develop when insect growth regulators with modest action like pyriproxyfen(PPF) are used, this effect has not been tested with regard to plague vector flea *Xenopsylla Cheopis*. The effectiveness of pyriproxyfen exposure against the adult, fecund, and larval phases of the oriental rat flea, *Xenopysylla Cheopis*, was studied. Through a series of laboratory tests, contact bioassays were undertaken against fleas in both their larval and adult stages. Four replicates, each with 30 adult fleas, was used to test all treatment and control groups.

In the case of larvae, the treatment and control groups underwent testing in six repetitions, each containing 20 second-stage larvae. Pyriproxyfen exposure time for mature fleas were 30 minutes. For larvae, there was no exposure period and the pyriproxyfen was used throughout the entire 20-day rearing period. Adult *Xenopsylla Cheopis* fleas in a treatment group appeared to have a 100% survival rate on day one. The control group's chance of survival appeared to be about 100% for the whole ten days of monitoring, but the treatment group's chance of survival decreased with time and were completely eliminated by day six (P=0.0313).

Average egg production in the treatment group was noticeably lower than in the control group. 90% of the flea larvae in the treatment group died by day eight, and by day 20 of monitoring and observation, none had developed into cocoons.While 60% of the larvae in the control group cocooned, and 98% of the larvae survived. The results have demonstrated that 2 g of pyriproxyfen combined with sand and dried cattle blood powder when applied directly has an impact on adult fleas, egg laying, viability, and larval development. Based on these findings, pyriproxyfen may be utilized to combat the plague flea vector in plague endemic locations.

Key Words *Xenopsylla Cheopis,* pyriproxyfen, adult fleas, egg viability and larvae

2.2 Introduction

Plague is a life-threatening infectious disease caused by the gram-negative bacteria *Yersinia Pestis*. The disease remains a public health threat, particularly in Africa. Although plague has remained quiescent in several endemic foci in Tanzania and elsewhere in the region, the occurrence of severe outbreaks cannot be underestimated.

Tanzania's Mbulu area reported a plague outbreaks following more than 20 years of silence (Makundi *et al.,* 2008). Algeria reported an outbreak following a near-fifty-year period of silence (Stenseth *et al.,* 2008). Following a protracted period of quiescence, plague outbreaks are frequently accompanied by an increase in cases and are generally more deadly. After 63 years without plague, Mahajanga city in Madagascar saw an increase in cases and burden (WHO, 2014). As a result, it is essential to ensure that there are reliable control systems in place. The majority of the plague prevention and control measures in place today are based on using chemical insecticides to combat fleas.

The primary method of preventing plague is still flea control, which involves applying chemical insecticides to homes and the surrounding area. Initially, practically all endemic countries including Tanzania, employed chlorinated hydrocarbons like Dichlorodiphenyltrichloroethane. Later,carbamates, pyrethroids, and organophosphates took their place. In endemic African countries, pyrethroids and carbamates are still applied whenever outbreaks occur (Miarinjara *et al.,* 2016). Tanzania currently favors the insecticide carbamate (5% carbaryl dust) (Rugalema *et al.*, 2020). High operational costs, poor compliance, and—most significantly—insecticide resistance limit the strategy as the case with. *Xenopsylla Cheopis* which was noted to be resistant to various insecticides in Tanzania (Rugalema *et al.*, 2020). Recently, Madagascar reported resistance to alphacypermethrin, lambdacyhalothrin, deltamethrin, etofenproxy, bendiocarb and propoxur(Boyer *et al.*, 2014). The fact that resistance can be driven by use of chemicals against other pests many countries remain vulnerable to consequences of flea

insecticide resistance. Clearly, complementary tools particularly those that are less vulnerable to resistance are needed. The use of Insect growth regulators (IGRs), or juvenile hormone analogues as complimentary control method is expected to increase efficiency of control method and lessen development of insecticide resistance (Meola *et al.,* 2000). Insect growth regulators (IGRs), or juvenile hormone analogues, are compounds that elicit hormonal effects which disrupt and inhibit insect metamorphosis at specific times and/or dosages (Meola *et al.,* 2000). Pyriproxyfen is an insect growth regulator formulated forspot-on topical treatment and for prophylactic flea control on dogs, cats and other domesticated animal including sheep and goats (Brattoli *et al*., 2011). Fleas can absorb pyriproxyfen by direct contact as well as by taking a blood meal from the host. Currently their two juvenile hormone mimics, Methoprene and pyriproxyfen, are used to inhibit reproduction and growth development in fleas (Bouhsira *et al.,* 2012). Residues of these compounds are highly effective against both the egg and larval stages of fleas (Olsen, 1985; Marchiondo *et al*., 1990; Donahue and Young, 1992, Meola *et al*., 1993). Eggs are particularly vulnerable because they are thin, single-layered gelatinous chorion with porous surface that is penetrated easily by these lipophilic chemicals (Meola *et al*., 1993). Adult fleas die due to membrane disruption and destruction of the fat body, salivary glands, gut epithelial cells, oocytes, and other internal tissues (Meola *et al*., 1996). The use of pyriproxyfen has been limited primarily to residual treatments, either as premise sprays applied to the microenvironment of the flea (Hinkle *et al*., 1995: Kawada and Hirano, 1996) or as on-animal treatments applied to the hair coat of the pet (Donahue and Young, 1992). The present study were conducted to explore the effect of pyriproxyfen hormone mixed with sand, cattle dried blood meal and the mixture allowed to contact adult fleas and larvae of plague flea vectors to see if would prevent hatching of eggs, reduction number of eggs laid, or any effect on larvae and survival of adult fleas so as to be used as complimentary control strategies that is less vulnerable to resistance and desirable.

2.3 Materials and Methods

2.3.1 Study area

This study was conducted at the Institute of Pest Management laboratory of Sokoine University of Agriculture which is located at the slopes of the Uluguru mountains 6°51'5"S37°39'26"E coordinates. At the Institute there is insectary with fresh colonies of *Xenopsyalla cheopis* established from field collected eggs preferentially from Mbulu Tanzania being one of the plague endemic areas.

2.3.2 Fleas rearing

Flea colony was maintained in a room with a controlled temperature (28^0C -+2^0C) and relative humidity ranged from 80 to 90. The rearing pan was made of transparent plastic container of 35 cm in diameter x 37 cm height. Adult fleas were fed on white rats, and the larval rearing medium was a mixture of sand and powdered cattle-blood. Because the gravid fleas leave the host to oviposit and return for additional blood-meals, the infested rat was confined in a screen cage, kept in a container withthe larval medium.

2.3.3 Experimental design

The study was conducted exclusively through a series of laboratory experiments. The contact bioassays against both the larval and adult stages of fleas were conducted using slightly modified WHO standard procedures (Aïzoun *et al.,* 2013). This world health Organization explain that the Standard Operating Procedure (SOP) describes the process to follow for evaluating the susceptibility of adult insect vectors to insecticides using the WHO tube test. This bioassay is a direct response-to-exposure test, measuring insect mortality 24 hours after exposure to a known standard concentration of an insecticide (e.g. the discriminating concentration) for a period of 1 hour. This procedure should be followed for testing insect susceptibility to insecticides.

2.3.4 Experimental setup

All treatments and control groups were tested in four replicates with each replicate being allocated 30 adult fleas.. In the case of larvae, treatment and control groups were tested in six replicates, with each

replicate being allocated 20 larvae of the second stage since I used them after a week of the emergency. In contrast to the case with adult flea larvae in the treatment group were reared with the PPF throughout the experiment (Figure 2.1).

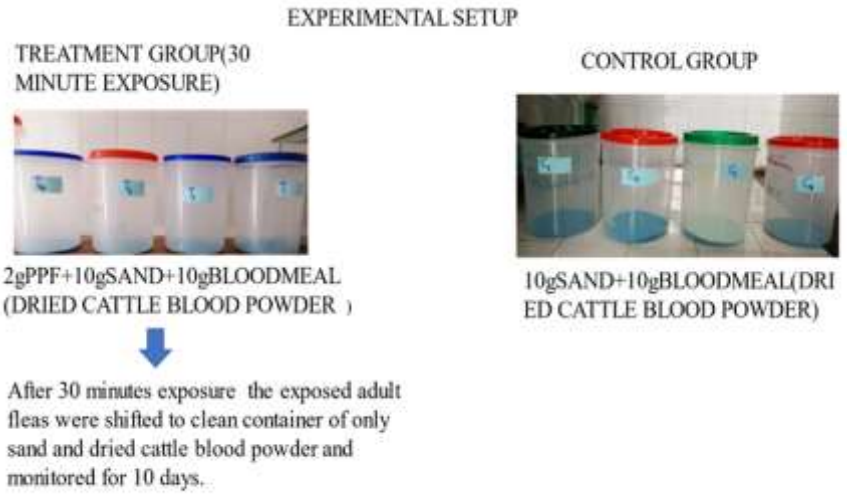

Figure 2.1: Showing experimental setup of the adult flea to pyriproxfen

2.3.5 Effect of pyriproxyfen on adult fleas survival and egg viability

A total of 240 adult fleas that were one week old were counted with and used to determine the effects of pyriproxyfen exposure for 30 minutes on adult fleas. They were kept in eight container. Glass tubes were placed in each container according to the treatments and controls. In order to give the adult fleas a place to rest, a piece of paper was placed upright in each tube. As shown in the experimental setup, prepared containers labeled treatment (1-4) contained a mixture of 10 grams of sand, 10 grams of host blood meal, and 2 grams of pyriproxyfen, whereas control containers labeled control (1-4) contained only a mixture of ,10grams of bloodmeal and 10grams of sand.

Eggs were examined and counted using a dissecting microscope and thereafter confirmed with a compound microscope. Treatment

and control containers were maintained for 10 days, during which the fecundity and survival of the adult fleas were monitored

2.3.6 Effect of pyriproxyfen on larva

A total 240 flea larvae were utilized in the study to examine the survival effect of pyriproxyfen on the flea larvae stages. To create the full mixture utilized for the treatment group, 10 grams of sand, two grams of host blood meal, and two grams of pyriproxyfen powder were mixed. For the control group, mixture of 10grams sand and 10grams of bloodmeal were used. Twenty flea larvae were put in a single container. Two hundred and forty larval altogether in twelve distinct containers, were used in the entire experiment. To determine survival and transit from larval to cocoons, the experiment was followed up for a total of 20 days.

2.3.7 Data analysis

Data was entered into MS Excel for cleaning, arranging, and analysis using R statistical software version 3.6.2. The survival package was used to analyze the survival status of the adult fleas and Kaplan-Meier was used to determine the survival probability and survival curve of the treated and control group over time. The t test and the Chi square test were used to test the different means and proportions of the larvae that survived, cocooned, and emerged to adult fleas.

2.4 Results

2.4.1 Survival of adult fleas exposed to pyriproxyfen-treated sand and bloodmeal

The results of the experiment showed that adult *Xenopsylla Cheopis* fleas in a treatment group appeared to have a 100% survival rate at day 0. The control group's survival probability were 100% for the entire ten days of monitoring, but the treated group's survival probability gradually fell until by day six, all fleas in the treated group died ($P = 0.0313$) (Figure 2.2).

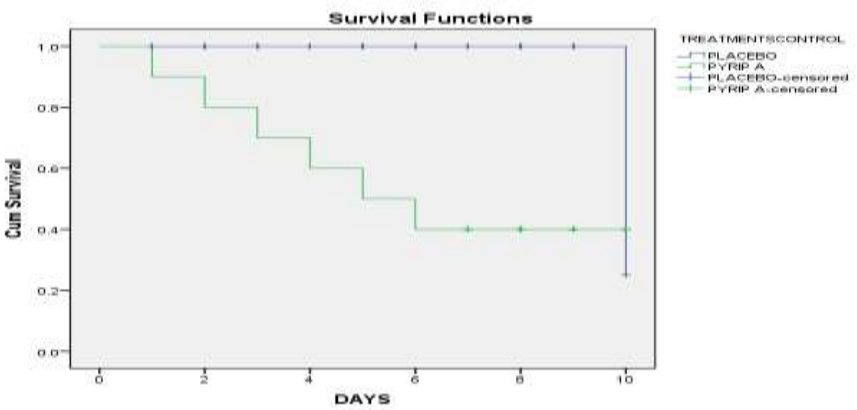

Figure 2.2: Kaplan-Meier probability survival curve of the treated and untreated group (placebo)

2.4.2 Effect of pyriproxyfen on flea egg production and flea egg hatching

When exposed adult *Xenopsylla Cheopis* fleas were observed for ten days, the treatment group mean egg production was significantly lower(P value= 0.001) (mean = 3.300) than that of the control group (mean = 31.700). The sample treated with pyriproxyfen differed significantly from the sample not treated in the control group, according to a sample t test that compared the mean difference with a p value of 0.001. Ninty percent of the eggs laid by fleas exposed to pyriproxyfen in the treatment group were laid as shells that had collapsed or were empty, incontrast to 8% in the controls.

Table 2.1: Eggs laid in different groups at different days post contamination of the treatment groups with pyriproxyfen

Days	t1	t2	t3	t 4	c1	c2	c3	c 4
0	0	0	0	0	0	0	0	0
1	6	5	4	4	8	6	8	14
2	2	3	2	1	6	7	8	6
3	0	1	1	1	6	7	10	8
4	0	0	0	0	3	4	8	7
5	0	0	0	0	2	3	4	4
6	0	0	0	0	1	2	3	4
7	0	0	0	0	0	1	1	3
8	0	0	0	0	0	0	0	2
9	0	0	0	0	0	0	0	1
10	0	0	0	0	0	0	0	0

t=Treatment ,c=Control

2.4.3 Effect on larva development

In this experiment, it was found that the treated *Xenopsylla Cheopis* larvae failed to reach pupation by day 10 of the monitoring period, and by that time, all of the larvae died. In contrast, the control group larvae started to pupate on days 9 and 10, and by days 16, 17, and 19, they had all emerged as new adult fleas. Ninty eight percent of the larvae in the control group survived on all days, which is significantly higher than that of the treatment group (χ_1^2= *1270.5*, P <0.001). Sixty percent of the larvae in the control group cocooned within 20 days; and Twenty five percent (χ_1^2= *412.14*, P <0.001) of the cocooned larvae eventually emerged as adults. In the treatment group, 90% of the flea larvae died by day eight, and 0% survived by day 20 of monitoring and observation.

2.5 Discussion

The present studies aimed to determine whether combined application of ivermectin and the insect growth regulator hormone pyriproxyfen is more efficacious against the plague vector flea *Xenopsylla Cheopis* than application of ether ivermectin ofr pyriproxyfen alone.This combined use of an insecticide and an

insect growth regulator provides a complimentary control tool to existing chemical methods (Wraight *et al.,* 2000). According to this study, pyriproxyfen when directly in contact with adult fleas (*Xenopsylla Cheopis)* for thirty minutes caused the plague vector flea population in the treated group to gradually decline over time until by day six, all of the treated group fleas died (P = 0.0313). For the entire 10 days of monitoring, their survival chance seemed to be practically one compared to the control group. This is as a result of the pyriproxyfen making the adult flea lipophilic and interfering with sodium channels on its body (Meola *et al.,* 1996). The number of mortalities rose on days two, three, and four after exposure. This is supported by a previous study that tracked fleas exposed to dog hair treated directly on the spot with pyriproxyfen and found mortality rates of 90% and 100%, respectively, over the same time period (Meola *et al.,* 1996). According to Meola *et al.* (1996), the loss of the fat body, salivary glands, intestinal epithelial cells, oocytes, and other internal structures results in the death of the flea.

A large proportion of eggs from adults exposed to two grams of pyriproxyfen-treated sand and bloodmeal in this experiment collapse, and failed to hatch. With eggs collected in bioassays using juvenile hormone mimics, this appears to be a typical insect growth regulator action (Marchiondo *et al.,* 1990; Palma *et al.,* 1993; Meola *et al.,* 1993; Donahue and Young, 1996). Meola *et al.* (1996) demonstrated that the collapsed egg is a result of the oocyte degenerating during maturation and entering oviduct with a weak chorion in pyriproxyfen-treated flea. The egg seems to be crushed b oviduct's muscular action during ovulation, leaving behind a fractured empty shell. Blagburn (1996) reported failure of flea eggs to hatch after the dogs treated topically with pyriproxyfen.

It was demonstrated in this study that the pyriproxyfen killed the larvae and prevented any of the larvae from completing metamorphosis. The observed mortality of larvae may be due to intestinal rupture (Marchiondo *et al*., 1990). The majority of earlier studies showed that flea larvae fed on adult feces and ingesting either one or 10 ppm pyriproxyfen died in the first, second, or third

instar, indicating that higher concentrations of pyriproxyfen in flea' feces were toxic to larvae or prevented larval-pupal or pupal-to-adult metamorphosis (Chamberlain *et al.,* 1988).

Irregular changes in the conditions at the insectary accounted for the failure of larvae in a control group to develop into cocoons and some very little mortality. This chemical could be applied through the spot-on application method of preference for flea control on dogs, cats, and livestock.

2.6 Conclusion

From the present studies, it can be concluded that pyriproxyfen boost insecticide efficacy. It protracted biological activity makes it an effective tool for reducing flea breeding in the domestic environment. As with other insect growth regulators, pyriproxyfen use necessitates use of an additional insecticide to preclude development of resistance. The deposition of trace amounts of pyriproxyfen in domestic environment where fleas are breeding will result to control.

Ethical Considerations

Ethical clearance was obtained from the Sokoine University of Agriculture Research and Publication Committee reference number SUA/DRRTC/R/13/2021 Morogoro, Tanzania.

Acknowledgements

We extend our sincere gratitude to the sponsor, the Commission of Science and Technology in Tanzania (COSTECH). Institute of Pest Management for providing the necessary facilities and space to conduct this research.

Competing interests

The authors declare that they have no competing interests.

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CHAPTER THREE

Manuscript Two

3.0 *Xenopsylla Cheopis* reproductive performance and survival after combined treatment with pyriproxyfen and ivermectin

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Submitted to journal of parasitology research| HINDAWI

3.1 Abstract

A tropical and subtropical region of the world, Xenopsylla Cheopis is one of the primary vectors of plague and murine typhus. Plague control primarily involves application of chemical insecticides to houses and surrounding environment to kill the vector fleas. In countries in which plague is endemic, risk and effect have been greatly reduced by this strategy. However the possibility development of insecticide resistance has increased due to prolonged uses and overreliances on chemical insecticides. To

minimize risk of development of resistance, intergrated pest management which employs system approach is applied.. Combining systemic insecticides with long-acting medications like pyriproxyfen improves control of plague vectors and prevents resistance development. We investigated the combined effect of pyriproxyfen and ivermectin on adult fleas and their reproductive performance. An experimental study design involving three treatments and four control groups each comprising four replicates was used. Using least square method to establish the effectiveness of pyriproxyfen, ivermectin and combination Pyriproxyfen alone shown to be less effective on adult fleas within a period of 24hours and mortalities started from day 1 and increased significantly (<0.0001) byday six. On day four, Pyriproxyfen waseffective(least square mean 3.75) causing mortality and no eggs laid. The number of eggs laid by the adult fleas subjected to ivermectin decreased to 5%, and maximum mortalities were seen at day five with an LS mean of 4.75 and a P value of 0.0001. The combination effect on adult fleas appears to begreater, with the highest mortalities at day three and four, with LS Mean 4.75 and 5, respectively, and 0% flea reproductive performance *Xenopsylla Cheopis*. 10 days of monitoring. This dual mode of pyriproxyfen and ivermectin could be used as control agents of plague flea vectors and improve plague control in endemic areas.

Keywords: Pyriproxyfen, Ivermectin, adult fleas, mortalities and eggs laid

3.2 Introduction

3.2.1 Background information

Xenopsylla cheopis (oriental rat flea) is the most importantvector for human plague and is found worldwide in association with its primary hosts*, Rattus spp* (Amatre *et al.,* 2005).. This flea usually inhabits tropical, subtropical, temperate and rarely colder areas. Parasites living in host nest and clothing beds,couches make perfect home for them.They attach to host only when sucking blood. Other times they are free-living in the host's nest (Duplantier *et al.,* 2005). Adults of both sexes feed on blood. The most commonway of controlling the plague vector, *Xenopsylla Cheopis* with insecticides is applied to rat

burrows and human houses. It is connected to the 1947 introduction of dichloro-diphenyl-trichloroethane (DDT). however since 1965, numerous instances of *Xenopsylla cheopis* insecticide resistance, particularly to DDT, have been documented (Ratovonjato et al., 2000). Current tests perfomed with some insecticide families used in vector control,in accordance with WHO insecticide test guidelines have demonstrated resistance to numerous insecticides with exception of dieldrin. *Xenopsylla Cheopis* was shown to be resistant to 12 insecticides from the organophosphate, organochloride, carbamate and pyrethroid groups (Miarinjara and Boyer, 2016).

The early detection and monitoring of insecticide resistance in a vector population and the use of long-acting compounds like pyriproxyfen in combination with systemic drugs like ivermectin may positively impact intervention strategies. Also, pyriproxyfen has been reported to inhibit metamorphosis to prevent the emergence of adults from pupae (Brattoli *et al.,* 2011) and has extremely low toxicity to humans, howeverit is effective at controlling mosquito larvae at very low doses and can persist for up to six months in a variety of aquatic habitat types. In addition, exposure of larvae to sub-lethal doses of PPF affects the adults' egg development, egg production, and reduces the hatching of eggs (Mbare *et al.,* 2014). Similarly, ivermectin is a macrocyclic lactone drug with exceptional potency against endo-and ectoparasites at extremely low doses, and it is highly active against a diverse range of nematode species, including most larvae and adult forms, as well as many arthropod parasites of domestic animals (González Canga *et al.,* 2009). The excellent spectrum of activity of ivermectin resulted in the all-embracing name 'endectocide' (Lifschitz *et al.,* 1999).

The mode of action of ivermectin involve the act on GABA neurotransmission and glutamate gate Cl^-, leading to a flaccid paralysis, death and elimination of parasites (Taylor, 2001). The selectivity of pharmacological action is associated with lack of target glutamate receptors in mammalian species and relative restriction of GABA receptors to the central nervous system in mammals. However, despite this tremendous potency, there are

other organisms within these groups that appear to be refractory to ivermectin. The cat flea, *Ctenocephalides felis*, is a clinically relevant example. Ivermectin was orally administered weekly at 0.5 mg/kg or daily at 0.05 mg/kg and observed to be inactive against this parasite on dogs (Blair *et al*., 1984). Banks *et al*. (2000) and Shoop *et al*. (2001) corroborated independently those results by showing that ivermectin has weak systemic activity against the cat flea in artificial membrane flea feeding assays. However ivermectin has a wide antiparasitic activity with long veterinary use and its activity against *Onchocerca volvulus* was discovered(Egerton *et al.,* 1979). Animal and *in vitro* experiments have shown that ivermectin has lethal effects on blood-sucking insects when these are fed on treated blood samples (Kavanaugh *et al*., 2020).

It increases mortality and reduces the fertility of tsetse flies, triatomine bugs, ticks, and sandflies. Experiments in animals and *in vitro* studies have shown that ivermectin readily kills adult mosquitoes, including *Anopheles*. In human trials of mass drug administration for onchocerciasis and filariasis control, the effect of ivermectin on mosquitoes seems to be maintained in the field (Kavanaugh *et al*., 2020). These insecticidal properties of ivermectin have led some to suggest that this drug could have a role in the control of arthropod vector-borne diseases. Given this indirect evidence we therefore set out to test directly the effect of ivermectin on *Xenopsylla cheopis* the major vector of plague in Africa, in a controlled study.

The use of insecticide and insect growth regulator in combination, rotation and mosaic would synergize and safeguard their effectiveness and slow action of these compounds prone to resistance development. We aimed to explore their lethal and sub-lethal effects on the oriental rat fleas and integrate them in a manner that they can improve control while limiting evolution of resistance.

3.3 Material and Method

3.3.1 Study location

The study was conducted against laboratory-reared flea species, Xenopsylla cheopis, at the Sokoine University of Agriculture, Institute of Pest Management at Morogoro. The Institute of Pest Management was selected because it has an insectary room which provides a conducive environment for growth and reproduction of various insects, including fleas.

Animal laboratory room

This room comprises white rattus and mice. housed with clean environment, light, own cages. The laboratory animal are given food and water adlibitum each and everday. The design of the housing provides the physiologica condition and habitat appropriate for the species.

3.3.2 Study design

Experimental design was used and the setup was as follows;

Three treatments, four control groups, each with four replicates, were employed in an experimental study design.

Effects of pyriproxyfen and ivermectin on Xenopsylla cheopis mortality and reproductive performance (Figure 3.1). Treatment 1, ivemectin alone (R1, R2, R3, R4)

Treatment 2, pyriproxyfen alone (R1, R2, R3, R4)

Treatment 3, combination (ivermectin +pyriproxyfen) (R1, R2, R3, R4)

Control group (R1, R2, R3, R4)

Where by R stands for replicate.

Treatment 1: Ivermectin alone

Four rats were each subcutaneously injected 0.2 ml of ivermectin at a dose of 0.02 mg/kg, housed in their own cages, and given access to food and water adlibitum. After 24 hours, 20 fleas were fed for 12 hours on each ivermectin treated rats and then the fleas thoroughly brushed off with application of ethyl ether to immobilize them.. Dead fleas were recorded and removed, and live fleas were added to a

container with a non-treated rat so they could be allowed to feed on blood. This allowed the drug to distribute well into the body circulation based on its pharmacodynamic indication. while observing on dead fleas. For ten days, the number of eggs laid was recorded. Fecundity, or the number of eggs, was recorded.
Treatment **2. pyriproxyfen alone**

Fleas fed for 12hours on 4 rats that had been exposed to 0.3g of pyriproxyfen. After this time, dead fleas were counted and removed, and live fleas were transferred to clean, labeled containers with filter paper at the bottom. The daily survival and egg-laying rates of these fleas were then observed. There were four replicates of this experiment, with 20 fleas utilized in each. Filter paper was placed in petri dishes, and the eggs were counted (fecundity) under a stereo microscope.

Treatment 3: combination ivermectin pluspyriproxyfen
Four rats that received 0.2 ml of ivermectin injections and were contaminated with 0.3 g of pyriproxyfen were exposed to fleas for12 hours. After 12hours live fleas were transferred to clean, marked containers and monitored for daily survival for at least 10 days while dead fleas were recorded and discarded after 12 hours. With each replication using 20 fleas, this was done four times.

3.4 Control
Four untreated rats were exposed control fleas to feed on for 12 hours, after which the dead fleas were counted and removed. Twenty (20) live fleas were then transferred to clean containers and their survival and fecundity were observed. There were 20 fleas used in each of the four replicates.

Monitoring containers

Figure 3.1: Showing containers where adult fleas placed after exposure to ivermectin, pyriproyfen and control

3.4.1 Data Analysis

Data was entered in MS Excel for cleaning, arranging and analysis was done using SAS software version 1994. All the data collected were analyzed using the General Linear Model Procedure (GLMP) and means were compared using the Least Square Means method (SAS, 1994).

3.5 Results

3.5.1 Mortalities of adult fleas exposed to combination of ivermectin and pyriproxyfen versus individual compound

The results of the experiment showed that other chemicals administered alone have the same effect at day zero theiran adult flea combination of ivermectin and pyriproxyfen, but therapy at day zero was not significant. Ivermectin or pyriproxyfen were the next most effective treatments after the first day combo, whereas the control group experienced no change. Even while ivermectin and pyriproxyfen were having the same effect, the combination from day three still seemed to have more of an effect. Pyriproxyfen and ivermectin both showed the same effect on day four, which was the combination that had the highest effect during monitoring. Ivermectin and pyriproxyfen had the same effect on days five and six, but pyriproxyfen was less effective than the other two on day seven.

Table 3.1: Combination survival effect of the ivermectin pyriproxyfen over 10days

Days	Control	Ivermectin	Pyriproxyfen	Ivermectin+ Pyriproxyfen	R-Square	SEM	P.value
0	0^{b}	0^{b}	0^{b}	0.5^{a}	0.429	0.144	0.728
1	0^{b}	2.25^{a}	2.5^{a}	3.25^{a}	0.783	0.367	0.0003
2	0^{d}	2.75^{c}	3.75^{b}	4.75^{a}	0.921	0.297	<0.0001
3	0.75^{c}	3.25^{b}	3.25^{b}	4.75^{a}	0.868	0.322	<0.0001
4	0^{c}	$4.25b^{a}$	3.0^{b}	5.0^{a}	0.844	0.473	<0.0001
5	1^{b}	3.5^{a}	3.0^{a}	1.5^{b}	0.739	0.353	0.0008
6	1^{b}	3.0^{a}	3.25^{a}	0.25^{b}	0.826	0.338	<0.0001
7	0^{b}	1.0^{ba}	1.25^{a}	0^{b}	0.522	0.314	0.0268
8	0.75^{a}	0^{b}	0^{b}	0^{b}	0.692	0.125	0.0021
9	0^{a}	0^{a}	0^{a}	0^{a}	0	0.125	-
10	0.75^{a}	0^{b}	0^{b}	0^{b}	0.692	0.125	0.0021

SEM=Standard Error Mean

Super script a, b, c, d, means within each row bearing same letter are not significantly different at $P<0.05$

3.5.2 Number eggs laid by adult fleas after exposure to ivermectin, pyriproxyfen alone and combination

Figure 3.2, ivermectin alone, pyriproxyfen alone, and the combinationof these two significantly reduced adult flea egg laying. Adult fleas exposed to ivermectin laid only 5% of the eggs, while t adult fleas exposed to pyriproxyfen and a combination of pyriproxyfen and pyriproxyfen laid 0% of the eggs. A much greater rate of egg laying and hatching was observed in the control group (88.1%). In the untreated group, 100% of the exposed eggs hatched.

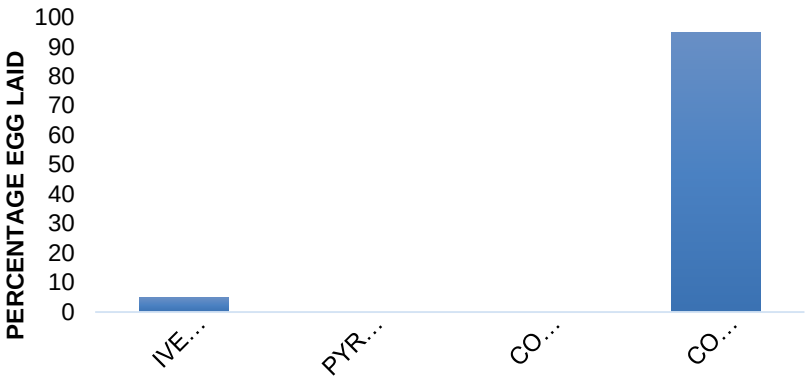

Figure 3.2: Effect of ivermectin, pyriproxyfen and combination on the eggs lain by *Xenopsylla Cheopis*

3.6 Discussion

The results of the current study indicate that exposure of adult fleas, *Xenopysylla Cheopis,* to the tested compounds may slow the spread of this species and also control it since they reduced egg laying, hatching, and eventually killed almost all adult fleas in several days. According to our results, ivermectin and pyriproxyfen combinations were by far the most effective insecticides against *Xenopysylla cheopis*. Starting with the effect of pyriproxyfen alone, it was observed to cause mortalities significantly on day six and at the same time affect the reproductive performance of the adult fleas. Meola *et al*. (2000) explain that pyriproxyfen causes the death of insects by inhibiting embryo development, interfering with molting and blocking metamorphosis. It has been reported that pyriproxyfen can make females lay fewer eggs or none at all and has strong egg-killing activity. The pyriproxyfen hormone is present on the adult flea body, making it lipophilic, and it interferes with sodium channels to disrupt the function of neurons, causing paralysis and death (Dryden *et al*., 2011).

Also, Pyriproxyfen has extremely low toxicity to humans however is reported to be effective at controlling mosquito larvae at very low

doses and can persist for up to six months in a variety of aquatic habitat types (Dryden *et al*., 20011). In addition, exposure of larvae to sub-lethal doses of PPF affects the adults' egg development, egg production, and reduces the hatching of eggs (Meola *et al.,* 2000). The results indicated that the number of eggs and the hatching rate of eggs decreased after pyriproxyfen exposure, and the concentration effect can be seen from the results.

Ivermectin alone caused an effect on mortality on the adult fleas and reduced the number of eggs laid. Our study results differ mostly from the other studies, which explain why the use of ivermectin in dogs when given orally is observed to be inactive against flea parasites on dogs (Blair *et al*., 1984). Banks *et al*. (2000) and Shoop *et al*. (2001) independently corroborated those results by showing that ivermectin has weak systemic activity against cat fleas. In contrast to our study, ivermectin was injected into rats, and the drug was given subcutaneously at a dose of 0.02 mg/kg. The effect of ivermectin was significantly delayed from day two through day six. One study in the Ibina region, So Paulo, found naturally infected dogs were treated with two doses of Ivermectin subcutaneously at 0.3 mg/kg with an interval between doses of 15 days and had a total absence of ticks after a week of treatment (Dias *et al*., 2005). The latter study shows death of the ticks, which is one of the ectoparasites, when ivermectin is given subcutaneously. Hence, this shows using ivermectin may cause the death of the ectoparasite.. The death of adult fleas as found in our study was explained by Abbott *et al*. (1925) who discovered inhibition of pupation and adult development in *Chrysomya bezziana* and *Calliphora vomitoria* exposed to sub-lethal doses of ivermectin. As observed by Freitas *et al*. (1996), Ivermectin caused paralysis in *Culex quinquefasciatus* larvae, which could be a result of the activation of chlorine canals as suggested by Banks (2000). Ivermectin binds to GABA, increasing the likelihood of GABAa activating chloride canals in postsynaptic membranes. This promotes an influx of chloride ions and irreversible hyperpolarization with consequent inhibition of transmission signals (Campbell, 1989).

From the results section above, the treatment of rats with Ivermectin and pyriproxyfen at the same time seems to offer better protection from adult fleas than a single use of either ivermectin alone or pyriproxyfen alone. Since the combination provided an insecticide efficacy of between 90% (day 3) and 100% (day 7). This combined effect accounted for both the modes of action of the ivermectin and pyriproxyfen hormones in insects. Delaying resistance development for the control of flea species and other insects was done in integrated pest management (IPM) with consideration of chemical(s) nature, mixture, rotation, or mosaics, and insecticide(s) compatibility with biological agents (Gregory *et al*., 19995). The application frequency, related to the resistance development, was influenced by insecticide activity from potentiation, residual period, and the vulnerability to resistance development of chemicals with secondary pests. Chemicals affect feeding, locomotion, flight, mating, and predator avoidance. Insecticides with negative cross-resistance by the difference in target sites and mode of action would be adapted to mixture, rotation, and mosaic.. The prospects for successful use of a mixture depend on each component killing a very high percentage of the exposed insects which are genetically susceptible to it .Resistance to both components of the mixture is too rare, and also, Curtis et al. pointed out that the mixture strategy was more effective against resistance development at 97% of the control.

The advantage of the combination for the fleas is that the ivermectin activates chloride canals of postsynaptic membranes. At the same time, this promotes an influx of chloride ions and irreversible hyperpolarization with consequent inhibition of transmission signals. Pyriproxyfen elicits hormonal effects and disrupts or inhibits insect metamorphosis when administered, hence impeding the life cycle of insects in the egg and larval stages of development.

3.7 Conclusions

This study found that combining pyriproxyfen and ivermectin was effective in killing and affecting the reproductive performance of the flea species, *Xenopsylla Cheopis*. The dual mode of action of pyriproxyfen and its prolonged duration of action make it a powerful

tool for suppressing developing fleas in the domestic environment and improving flea control programs while reducing the threat of plague reemergence.

Ethical Considerations

Ethical clearance was obtained from the Sokoine University of Agriculture Research and Publication Committee reference number SUA/DRRTC/R/13/2021.

Acknowledgements

We extend our sincere gratitude to individuals and/or groups whose contributions made this study possible: staff/students at the Pest Management Centre (Mr. Ginethon Mhamphi, Mrs Resta Maganga, Ms. Stella Kessy and Mr. Omary Kibwana) and Commission of Science and Technology of Tanzania (COSTECH) for funding the study.

Competing interests

The authors declare that they have no competing interests.

Author's Contributions

NAM, LLM and ASK conceptualized and designed the study. NAM prepared and conducted experimentation. LLM, AASK and NAM conducted data analysis and interpreted the results. NAM wrote first draft of the manuscript. NAM,LLM, JSZ and AASK conducted a series of revisions on the manuscript and produced the version submission. All authors read and approved final version of the manuscript.

Data availability

Additional data related to the published paper are freely accessible upon request.

Funding statement

This study was funded by the Commission of Science and Technology in Tanzania (COSTECH) and the funder was not involved in the manuscript writing, editing, approval, or decision to publish.

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CHAPTER FOUR

4.0 General Discussion, Conclusions and Recommendations

4.1 General discussion

In general, the study was able to report on the efficacy of pyriproxyfen alone in a variety of exposure scenarios as well as when combined with ivermectin against *Xenopsylla Cheopis*. This outlines how the substance may be utilized to overcome insecticide resistance issues and control flea plague vectors in regions where the disease is endemic. When in direct contact with adult fleas *Xenopysylla Cheopis* for thirty minutes, pyriproxyfen s lower survival probability twice compared to the group that was not exposed. This caused by, lipophilic on flea body and interferes with sodium channels to impair neurons' ability to function and cause paralysis and death, is present on the flea's body (Meola *et al.,* 1996).

. This is confirmed by some of the earlier research, which found that fleas exposed to dog hair treated with 12.5 and 125 ppm pyriproxyfen during the same time period died at rates of 90 and 100%, respectively (Meola *et al.,* 1996). According to Meola *et al*. (1996), the loss of the fat body, salivary glands, intestinal epithelial cells, oocytes, and other internal structures results in the death of the flea (Meola *et al.,* 1996). Additionally, we noticed that eggs taken from adults who had been given 2g of pyriproxyfen-treated sand and bloodmeal had discolored and collapsed, preventing them from hatching. This appears to be a typical impact of an insect growth regulator on eggs that were collected in In bioassays using juvenile hormone mimics, eggs were collected, and this action of an insect growth regulator appears to be typical (Marchiondo *et al.,* 1990; Palma *et al.,* 1993; Meola *et al.,* 1993; Donahue and Young, 1996). Meola *et al.* (1996) demonstrated that the collapse eggs laid by pyriproxyfen-treated fleas was e caused by the oocyte's degeneration during maturation and entry into the oviduct with a compromised chorion. In addition, the pyriproxyfen killed the larvae when they were directly exposed to a 2g mixture of sand and dried cattle bloodmeal, and none of the larvae underwent full metamorphosis. Within ten days, every larva in six replicates

exposed died. Given that all of the observed larval deaths seemed to be dried up and void of internal contents, the cause of death may have been a gut rupture.

Ivermectin alone in this study appeared to have an effect at day one, with the least square mean of 2.75 at day two and three, and the highest effect recorded at day four, with a least square mean of 4.25. As observed in adult fleas, they appeared to stop moving and later die due to paralysis, as suggested that ivermectin binds to GABA and then increases the possibility of the GABA in activating chloride canals of postsynaptic membranes. This promotes an influx of chloride ions and irreversible hyperpolarization with consequent inhibition of transmission signals (Campbell, 1985).

The combination survival impact seems to be more efficient than using pyriproxyfen or ivermectin alone just once, because the mixture had an insecticide efficacy of 90% (day 3) to 100%. (day 7). Both the ivermectin and pyriproxyfen hormones' mechanisms of action in insects were explained by this combination effect. Insecticide compatibility with biological agents and chemical nature, mixture, rotation, or mosaics were taken into account when delaying the development of resistance in the management of flea species and other insects.

4.2 Conclusion and Recommendation

4.2.1 Conclusion)

Little information is available on the effects of pyriproxyfen and ivermectin on the plague flea vectors. This study showed pyriproxyfen can be applied directly and even topically to increase its efficiency since we saw significant proof in the *Xenopsylla Cheopis* species adult fleas and larvae that there was mortality in adult fleas treated directly with pyriproxyfen and the treated group of larvae died and those remained failed to reach pupation. Ivermectin alone has a mortality effect, which means that using pyriproxyfen and ivermectin together is effective at killing adult fleas but has an effect on the reproductive performance of flea species like *Xenopsylla Cheopis*, as no eggs were laid on all days of monitoring. The dual

mode by which pyriproxyfen and ivermectin exert biological activity and their prolonged duration of action provide a powerful tool for suppressing developing fleas in the domestic environment and improving control. Reduced plague transmission risk and burden will, among other things, become reflected in improved survival, less expenditure on disease control and treatment, and more ample time to engage in profitable socio-economic activities.

4.2.2 Recommendations

The use of insecticides should be done complementarily with other action compounds so as to lessen the development of insecticide resistance.. Further studies are recommended on the efficacy and effectiveness of ivermectin combined with pyriproxyfen hormone against the plague vector fleas.

Similar studies would be conducted in the field of endemic plague areas because this study was conducted entirely in the laboratory, simulating the field environment. The government should support development of tools like automated contamination devices, which will be simple and less costly to allow delivery of this compound directly to rats in endemic plague areas.

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