BLOOD FEEDING DYNAMICS OF NEWLY EMERGED CAT FLEAS, *Ctenocephalides felis* (Bouché) (SIPHONAPTERA: PULICIDAE) IN CHAMBERS ATTACHED TO INSECTICIDE-TREATED AND UNTREATED CATS

by

CHRISTINE MARIA MCCOY

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Approved by:

Co-Major Professor
Alberto B. Broce
and

Co-Major Professor
Michael W. Dryden

ABSTRACT

The cat flea, *Ctenocephalides felis* (Bouché) 1835, is an important pest of cats and dogs, yet few studies have been able to document the relatively unhindered feeding dynamic. Studies were conducted to determine the amount of hemoglobin carried over from the larval to adult stage. Next, a series of studies determined flea feeding dynamics within confinement feeding chambers placed on cats. The ratio, total number and gender of fleas determined were then used in a 28-day insecticide study, in which blood consumption per cell was assayed. Fipronil, imidacloprid and selamectin were the topical insecticides evaluated, as well as nitenpyram, a systemic insecticide. The results of this study suggest the importance of the dynamics of flea feeding in relation to the effectiveness of insecticides to reduce feeding.

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DEDICATION

This thesis is dedicated to my mother, Bonnie Kay Hunter McCoy, who has supplied me with the strength and determination I need in my studies.

INTRODUCTION AND LITERATURE REVIEW

The cat flea

The cat flea (*Ctenocephalides felis*) (Bouché 1835) is a blood feeding ectoparasite of a wide variety of mammals and birds and is the most common ectoparasite of dogs and cats worldwide (Rust and Dryden 1997). Cat fleas are members of the order Siphonaptera, family Pulicidae and genus *Ctenocephalides*. The family Pulicidae contains only 14 species, *C. felis* being the most economically important species (Borror et al. 1992). There are four subspecies of *Ctenocephalides felis* in the world. The most common pest of cats and dogs and the only subspecies to occur within North America is the cat flea, *Ctenocephalides felis* (Rust and Dryden 1997).

Life cycle of the cat flea

Fleas are holometabolous insects, completing development in four distinct stages. A blood meal is required by both sexes to initiate reproduction. Female fleas will begin mating and oviposition 20 to 36 hours after feeding on a host (Akins 1984; Rust and Dryden 1997). The eggs are oviposited in the host's hair coat, but as they don't have any adhesive properties, they fall into the host's environment (Dryden 1989). Two to ten days later, the eggs will hatch into the first larval instar. The larvae live in the host's environment, usually in the carpet, den, or burrow of the host and feed on partially digested flea feces and on other organic matter. Recent findings have suggested that larvae can feed on viable

and unviable conspecific eggs to successfully complete development. Cat flea larvae can survive by feeding on flea feces or younger or injured larvae alone (Hsu et al. 2002; Lawrence and Foil 2000), but will not develop on decaying vegetable matter, feathers, or cat feces (Strenger 1973; Silverman and Appel 1994). After three larval instars, the larvae spin a silk-like cocoon and pupate in the premises. As early as seven days later, the adults will emerge in response to stimuli that include carbon dioxide, heat, and movement (Benton and Lee 1965; Osbrink and Rust 1985). While adult emergence can occur in as little as a week, emergence may be delayed for more than 200 days if there is no stimulus to trigger adult emergence present in the pupal environment (Rust & Dryden 1997). Once the adult fleas emerge, they immediately begin to display active host-finding behavior. Adults display positive phototaxis, and are most responsive to light with peak transmittance between 500 and 525 nm (Dryden and Broce 1993). Cat fleas orient into the light and then jump on passing hosts as the host's body casts a shadow, temporarily interrupting the light source. Adult cat fleas begin feeding almost immediately once they acquire a host. Flea feces can be observed within 8 to 9 minutes of initiation of feeding (Dryden 1990).

Veterinary importance of cat fleas

Fleas are the cause of the most common skin affliction of cats and dogs, Flea Allergy Dermatitis. Flea Allergy Dermatitis (FAD) is a threshold condition, the severity of the allergy depending upon each individual animal. Clinical signs can include erythema, crusting, alopecia and self mutilation (Halliwell 1983). In

addition to causing FAD, fleas are the intermediate host for the most common tapeworm of dogs and cats, the double-pore tapeworm, *Dipylidium caninum*, (Rust and Dryden 1997) which can infect cats and dogs that ingest fleas infected with the cysticercoid when grooming. The cat flea can also serve as the vector of *Rickettsia typhi*, *R. felis* and *Bartonella henselae* (Azad et al. 1997; Foil et al. 1998; Finkelstein et al. 2002; Rolain et al. 2005). Severe infestations of adult cat fleas can cause anemia and death in a variety of animals, including dogs, cats, lambs, kids and dairy calves (Yeruham et al. 1989; Dryden et al. 1993; Rust and Dryden 1997; Araújo et al. 1998).

Cat fleas are not only a nuisance to animals, but they can also be to humans as well. Flea bites can cause an allergic reaction, papular urticaria, in humans. Humans can also become infected with the double pore tapeworm, *Dipylidium caninum* by ingesting infected cat fleas. Dipylidiasis occurs most commonly in children and the elderly and is often symptomatic and goes unnoticed or unreported (Chappell et al. 1990; Molina et al. 2003).

History and current knowledge of flea feeding dynamics

Many studies have been conducted to examine insecticides' efficacy against adult cat fleas. However, there have been only a few published studies that have investigated blood feeding by adult fleas on cats and the effect of insecticides on the incidence of fleas feeding (Dryden 1998; Franc and Cadiergues 1998). However, the effect of insecticides on the volume of blood consumed by cat fleas has not been determined. Evaluations of blood consumption by cat fleas was

first reported by Dryden and Gaafar (1991) where they used two radionuclide blood tags (^{31}Cr-erythrocyte and ^{125}I-albumin) and a gravimetric method to quantify blood consumed by male and female fleas. They determined that female fleas consumed an average of 13.6 µl (±2.7 µl) of blood per day, an amount 15 times their body weight. Another method that has been used to determine the amount of blood consumed by insects is the Drabkin's reagent method. Drabkin's reagent consists of 1.0 g sodium bicarbonate ($NaHCO_3$), 0.1 g potassium carbonate (K_2CO_3), 0.05 g potassium cyanide (KCN), 0.2 g potassium ferricyanide [$K_3Fe(CN)_6$]; these are dissolved in 1000 ml of distilled water to form the reagent (Legowski and Boroviczeny 1962). The Drabkin's reagent converts all forms of hemoglobin to cyanmethemoglobin and these levels can be analyzed in a spectrophotometer. Sigma Aldrich (St. Louis, MO) carries a pre-mixed Drabkin's reagent. The first group to use the Drabkin's reagent method to quantify blood consumption in insects was Briegel et al. (1979) in the yellow fever mosquito, *Aedes aegypti* (L.). Kern et al. (1992) were the first to use this method to quantify blood consumed by fleas by measuring hemoglobin found in the flea feces, although the purpose was not to quantify blood ingestion by adult fleas; rather, the objective of Kern and co-workers study was to develop a technique to quantify flea feces in the presence of environmental proteinaceous contamination. They found that an average flea-infested domestic cat can produce 23.02 mg of debris (flea feces, cat dander, hair, etc.) per cat per hour, with flea feces accounting for 10.41 mg per cat per hour. In that study, flea feces accounted for 44.28% of the debris.

History of Flea Control and Management

Historically, flea control consisted of treating both the animal and the environment with insecticides (Rust and Dryden 1997). With the advent of effective topical spot-on insecticides, the focus of flea control is almost entirely on the host (Dryden and Broce 2002). Modern residual adulticides are used not only to provide animal's relief from fleas, but are also used to manage flea reproduction and FAD (Dryden and Broce 2002). Residual flea adulticides affect reproduction by killing fleas before females consume enough blood to initiate oviposition. FAD is a function of the numbers of fleas and the duration of flea feeding since the allergic reaction results from the injection of salivary proteins during the act of feeding. FAD can therefore be effectively managed with adulticides by the rapid elimination of fleas and/or reduction of flea feeding.

Commonly Used Flea Adulticides

Selamectin

Selamectin is a member of the avermectin family, most closely related to doramectin: its anti-parasitic characteristics were recognized in 1981 (McTier et al. 2000). Selamectin is the first avermectin endectocide labeled for use in dogs and cats, with a remarkable safety profile. It is also safe for use in ivermectin-sensitive Collies, as well as dogs and cats with adult heartworms and microfilariae (Novotny et al. 2000; Krautmann et al. 2000). Selamectin is applied

topically to the skin and, like several other avermectins, is absorbed transdermally. The avermectins are 16-membered macrocyclic lactones which exhibit not only insecticidal, but acaricidal and nematocidal activity. Their anti-parasitic properties are thought to be related to increased membrane permeability through interaction with glutamate-gated chloride ion channels (Schaeffer and Haines 1989). Selamectin is labeled for once a month topical application at 6-12 mg/kg for the control and elimination of fleas.

Imidacloprid

Imidacloprid is a neonicotinoid, first introduced in Europe and Japan in 1990 and registered in the U.S. in 1992. Imidacloprid was first used in the control of phytophagous insects, soil insects, termites, turf insects, and the Colorado potato beetle. Imidacloprid acts on the central nervous system, causing irreversible blockage of postsynaptic nicotinergic acetylcholine receptors. Although it works well on insects, imidacloprid has minimal effect on mites or nematodes (Ware 2000). Imidacloprid is labeled for once a month topical application at 10-20 mg/kg for control and elimination of fleas on dogs and cats.

Fipronil

According to Ware (2000), fipronil is in the class phenylpyrazoles, the only insecticide in this relatively new class. It was first introduced in 1990 and was registered in the U.S. in 1996. Fipronil is used for the control of many crop and foliar insects, as well as formulated as baits for cockroaches, ants and termites.

The mode of action of fipronil is believed to be by blocking the GABA-regulated chloride channel. This allows fipronil to have effectiveness against insects that have resistance or reduced susceptibility to pyrethroid, organophosphate and carbamate insecticides. Fipronil has broad spectrum pesticidal activity being effective against both insects and arachnids. Fipronil is applied topically to dogs and cats once a month at 6.7 – 15 mg/kg for the control and elimination of fleas and ticks.

Nitenpyram

Nitenpyram is a neonicotinoid used as a flea adulticide that is marketed by Novartis Animal Health (Greensboro, NC). It was first discovered by Takeda Chemical Industries (Tinembart and Tashiro 2000). Unlike the previously mentioned insecticides, nitenpyram has a short half life (cats 7.7 hours: Witte and Luempert 2001) and can be administered daily. The minimum recommended dose is 1 mg/kg body weight. A low dosage tablet contains 11.4 mg nitenpyram and is indicated for cats and dogs weighing up to 11 kg (24.2 lb). The higher dosage tablet contains 57.0 mg nitenpyram and is for dogs weighing between 11 and 57 kg (24.2 and 125.4 lb). Puppies or kittens that weigh 1 kg or greater can be treated. Once administered, nitenpyram is rapidly absorbed from the gastrointestinal tract of the host. Fleas begin to die within 30 minutes after nitenpyram administration and complete elimination can be seen within 4 hours (Dryden et al. 2001; Schenker et al. 2001).

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Overall Objectives

Flea infestation of companion animals is an important problem for pet owners and veterinarians. Fleas feed upon the blood of their host, while injecting salivary antigens into the host, that can cause an allergic reaction known as flea allergy dermatitis. This is important because not all insecticides available today have a rapid killing effect which eliminates fleas before they feed. Thus, the importance of knowing the effect of these different chemistries on the feeding dynamics once the flea receives a killing dose. I investigated flea feeding dynamics in the presence of insecticides of different modes of action to better understand the effect of these insecticides on flea feeding. The overall objectives of my work were as follows:

1. Establish baseline levels of hemoglobin and its by-products in unfed fleas
2. Determine the feeding dynamics of unfed cat fleas in confinement chambers on cats
3. Investigate the rate of kill kinetics of nitenpyram
4. Determine the effect of commercially available insecticides on flea feeding

PAPER

BLOOD FEEDING DYNAMICS OF NEWLY EMERGED CAT FLEAS, *Ctenocephalides felis* (SIPHONAPTERA: PULICIDAE) IN CHAMBERS ATTACHED TO INSECTICIDE-TREATED AND UNTREATED CATS

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Introduction

The cat flea *Ctenocephalides felis* (Bouché) is the most common external parasite of cats and dogs worldwide, causing feline miliary dermatitis and flea allergy dermatitis (FAD) (Rust and Dryden 1997). Severe infestations of cat fleas can result in anemia and even death of a variety of hosts (Dryden et al. 1993; Rust and Dryden 1997; Araújo et al. 1998). Cat fleas also serve as vectors for several pathogenic bacteria including *Bartonella henselae*, *R. typhi*, and *Rickettsia felis* (Azad et al. 1997; Foil et al. 1998; Finkelstein et al. 2002; Rolain et al. 2005) and are the primary intermediate host for the most common tapeworm of dogs and cats in North America, *Dipylidium caninum* (Rust and Dryden 1997).

Controlling fleas therefore not only relieves the discomfort caused by fleas but also can prevent the transmission of infectious agents and the production of anemia and FAD. Historically, flea control consisted of treating with insecticides both the animal and the environment (Rust and Dryden 1997). With the advent of effective topical spot-on insecticides, the focus of flea control is almost entirely on the host (Dryden and Broce 2002). Modern residual adulticides are used not only to provide animals relief from fleas and FAD, but are also used to manage infestations of the premises by hindering flea reproduction (Dryden and Broce 2002). Residual adulticides can prevent flea reproduction by killing newly acquired fleas before females can consume enough blood to initiate oviposition.

By preventing reproduction, flea populations within the premises are driven to extinction.

Since the allergic reaction that produces flea allergy dermatitis results from the injection of salivary proteins during the act of feeding, the severity of the disease is a function of the numbers of fleas, the duration of flea feeding and the hypersensitivity of the individual dog or cat. FAD can therefore be effectively managed with adulticides if they rapidly kill fleas and/or prevent or reduce the time of flea feeding.

Adult cat fleas are obligate ectoparasites, requiring a blood meal to initiate reproduction. After approximately 24 hours on a host, females will mate and begin to oviposit (Hudson and Prince 1958; Akins 1984). According to Dryden and Gaafar (1989), a female cat flea can produce approximately 1,745 eggs over a 50-day period. Within minutes to a few hours the eggs fall off the host into the surrounding premises and hatch into larvae. The larvae then feed upon the profuse amount of feces produced by the adult fleas parasitizing the host that also accumulates in the premises (Silverman and Appel 1994; Lawrence and Foil 2000). Flea feces are essentially partially-digested blood. After the larvae complete three larval instars, they spin a silk-like cocoon and later emerge as adults. By feeding continually and excreting large amounts of feces, female fleas are providing a protein food source for larvae. The production of profuse amounts of feces is energetically expensive to the female flea, but the larvae directly benefit from these feces, providing for the next adult generation (Silverman and Appel 1994). Although males contribute to the quantity of fecal

matter, it is likely females produce the majority of the feces. While female cat fleas can consume up to 15 times their body weight in blood per day, the exact volume of blood consumed by males is unknown but is likely less than their body weight daily (Wade and Georgi 1988; Dryden and Gaafar 1991).

There have been a few published studies that have investigated blood feeding by adult fleas and the effect of insecticides on feeding behavior (Dryden 1991; Kern 1992; Cadiergues and Franc 1998). However, none has investigated the effect of insecticides on the quantity of blood consumed.

Dryden and Gaafar (1991) used two radionuclide blood tags (^{31}Cr-erythrocyte and ^{125}I-albumin) and a gravimetric method to quantify blood consumed by fleas. Another method used to determine the amount of blood consumed by insects is the Drabkin's reagent method (Briegel et al. 1979). The Drabkin's reagent converts all forms of hemoglobin to cyanmethemoglobin and these levels can be analyzed in a spectrophotometer. Kern et al. (1992) were the first to use this method when they quantified flea feces in the presence of environmental proteinaceous contamination.

Since cat flea larvae are consuming adult flea feces, small amounts of hemoglobin and its by-products may still be present in unfed adult fleas. This could interfere with blood consumption determinations based on analysis for hemoglobin (and its by-products by flea digestion). Therefore a baseline of hemoglobin or other compounds that might react with the Drabkin's reagent in unfed fleas should be established. The first objective of this study was to establish baseline levels of hemoglobin and its by-products in unfed fleas. Then a

series of studies was conducted using the Drabkin's reagent method to determine the effect of flea gender, density, gender ratio, and age post-emergence on the quantity of blood consumed by fleas feeding in confinement chambers attached to cats. In evaluating each variable, the optimal feeding rate (volume of blood consumed and excreted/unit of time) was determined, as quantified by the Drabkin's reagent method (Kern 1992).

Once the effect of these variables was established, studies were undertaken to determine the effect of various insecticides on blood consumption by cat fleas.

Materials and Methods

Cat Fleas

In all experiments, cat fleas used were from the KSI colony, which was first colonized at Kansas State University in 1990 from cat fleas collected at a local animal shelter and maintained on cats in the Department of Diagnostic Medicine and Pathobiology, Kansas State University. Eggs were collected daily from pans beneath stainless steel cages housing individual cats. While cats were infested with fleas, an Elizabethan collar was placed on the cats to reduce grooming activity. Flea eggs were placed in Petri dishes containing ground dog chow, dried bovine blood and brewer's yeast and were reared according to procedures detailed by Dryden (1989).

Cats

Purpose-bred domestic, short hair cats were obtained from an USDA approved vendor, Liberty Research (Waverly, NY). Cats were housed in individual stainless steel metabolic cages equipped with stainless steel litter pans and ¼ inch corn cob litter. Cats were given commercial dry cat chow and water *ad libitum*. All care and handling of the cats conformed to the Kansas State University Institutional Animal Care and Use Committee guidelines (protocol #2053.2).

Cat Hemoglobin Standard Calibration Curve

Blood was drawn from three cats to develop a standard calibration curve to describe the correlation between the optical density, hemoglobin and blood volume. This calibration curve was then used to quantify hemoglobin and estimate the quantity of blood in fleas and their feces. Blood was drawn from the cephalic vein of the cats with a 22 gauge needle into each of two Vacutainer purple top tubes (Becton Dickinson, Franklin Lakes, NJ). One tube was used to determine the amount of cyanmethemoglobin in the blood according to the Drabkin's reagent method and the other tube was sent to the Clinical Pathology Lab at Kansas State University for complete blood count (CBC). The CBC was used to determine if the cats used for the calibration curve were within normal parameters for hemoglobin.

The Drabkin's reagent method used to quantify the amount of hemoglobin in fleas converts all forms of hemoglobin to cyanmethemoglobin, which is then quantified spectrophotometrically (Briegel et al. 1979; Burtis and Ashwood 1999). Feline whole blood in quantities of 1, 5, 10, 20, 40, and 80 µl were tested. First the blood was pipetted into a 15 ml borosilicate tube containing five ml of 1.0 *M* sodium phosphate buffer, pH 7.0. Five ml of the Drabkin's reagent (Sigma Aldrich, St. Louis, MO) were then added. The tube was vortexed for one min. and incubated at room temperature (approximately 25° C) for 30 min. After incubation, the mixture was filtered through a #1 Whatman filter paper and 500 µl were removed after filtration and placed into a crystal cuvet. The sample's absorbance was read in a spectrophotometer at 540 nm (Genesys™ 8, Thermo

Spectronic, Rochester, NY). The process was repeated for whole blood samples from three cats. The mean absorbance values were then compared to the various blood volumes analyzed and regression analysis was conducted on the raw data to yield a standard calibration curve.

Baseline level of hemoglobin in unfed fleas

The following numbers of unfed female and male fleas were analyzed to determine the baseline levels of remaining hemoglobin, its by-products or other compounds that might react with the Drabkin's reagent in unfed adult fleas: 1, 5, 10, 25, 50, 75, and 100. Three replicates of each series were conducted. The results of these experiments were subtracted from subsequent hemoglobin determinations to account for hemoglobin present in unfed fleas. Males and females were analyzed separately. First, fleas were rinsed in distilled water to eliminate debris from their bodies and homogenized in 20 µl of sodium phosphate buffer. The homogenate was then transferred into a 15 ml borosilicate tube containing 5 ml of 1.0 *M* sodium phosphate buffer, pH 7.0. Five ml of Drabkin's reagent were added to the buffer and homogenized flea mixture. The tube was vortexed for one minute; then incubated for 30 min. at room temperature and filtered through a #1 Whatman filter paper (Middlesex, UK). The samples, in 500 µl quantities, were placed in cuvets and absorbance read as described previously. Regression analysis was performed on both values for unfed female and male fleas. These values were compared to the standard

calibration curve to obtain correction equations for both unfed female and male fleas in subsequent experiments.

Preparation of experimental cats

Cats were shaved on the lateral aspect of the rib cage with a #10 clipper blade against the lay of the hair (Oster Cryotech™, Niles, IL). The inner and outer edges of a clear Plexi-glass confinement chamber with an inner diameter of 5.0 cm (1.97") and an outer diameter of 6.0 cm (2.36") (Mobile Mechanix, Marathon, NY) was traced using a permanent marker and the circular outline was clipped with a #40 blade (Oster Cryotech™, Figure 1). The area was cleaned with 70% isopropyl alcohol and air dried. An adhesive foam gasket with the same inner and outer diameter as the chamber (Converters, Inc., Huntingdon Valley, PA) was attached to the base of the chamber and then affixed to the cat, using a surgical adhesive (Vetbond™ 3M, St. Paul, MN) to ensure attachment to the cat (Figures. 2, 3). To further prevent the chamber from being dislodged, the cat was fitted with a full body stockinette (Surgi-Sox®, Campbell Pet Company, Brush Prairie, WA; Figure 4).

Blood consumption by fleas as a function of gender

Eight chambers (two per cat) were attached to four cats and either ten male or ten female unfed fleas from the KSI strain, 96 – 120 hours post-emergence were placed into one of four chambers. Each cat had fleas placed in a chamber for feeding times of 5, 60, 240 and 1440 min., following a 4 x 4 Latin Square Design.

Fleas and feces were then extracted from chambers by vacuum aspiration from a selected cell following each feeding duration. Fleas and feces collected from each chamber were analyzed using the Drabkin's reagent method and absorbance values compared to the standard blood volume curve (using correction equations for background absorbance of unfed fleas). Data were analyzed with the SAS® v. 8 (Cary, NC) program using the GLM procedure (least square means).

Gender ratio and density effects on flea blood consumption

Two chambers were attached to each of three cats and 20 fleas (10 females; 10 males), 96 – 120 hours post-emergence, were placed into each chamber. In the initial trial, a 1:1 gender ratio and 20 total fleas were used. Fleas and feces were extracted from chambers by vacuum aspiration from two randomly selected chambers at 5, 60, and 240 min. Volume of blood consumed and excreted per cell was then determined by the Drabkin's reagent method as described previously. Process was repeated for 2:1 and 1:2 female: male ratios. In addition, each gender ratio was evaluated not only using 20-21 fleas, but also 42 and 84 fleas. Each cat had each combination of gender and density ratios at each time interval (a Latin Square Design). The combination of density and female:male ratio that produced the greatest volume of blood per cell / unit time was used in subsequent experiments. Linear regressions and slopes of the lines were obtained for comparison.

Age post-emergence

Three chambers were attached to each of three cats, and fleas (1:1; 42 total fleas) of specific ages post-emergence were placed in each chamber. Age of fleas post-emergence that were evaluated were 24 – 48 h, 48 – 72 h, 72 – 96 h, and 96 – 120 h post-emergence. Fleas and feces were vacuum aspirated from chambers from each cell at 240 minutes. Volume of blood consumed and excreted per chamber was then determined by the Drabkin's reagent method as previously described. The flea age post-emergence that produced the steepest slope (volume of blood per cell / unit time) was then used in subsequent experiments.

Speed of flea kill of orally administered nitenpyram

The speed of kill of nitenpyram on established flea infestations was determined by placing unfed fleas into confinement feeding chambers on cats and then treating cats with nitenpyram. On day zero, two chambers were attached to each of 12 cats. Unfed fleas (21 female and 21 male fleas) 96 – 120 hours post-emergence were placed into the chambers. Approximately 24 h later, an 11.4 mg nitenpyram (Capstar®, Novartis Animal Health, Greensboro, NC) tablet was orally administered to six cats. At 15, 30, 60, 120, 240, and 480 min post-treatment, fleas were aspirated from four randomly selected chambers (two treated and two control cats) and assessed as being either live, dead or moribund. Mean and standard deviation were calculated for each treatment group at each time.

Effect of nitenpyram on blood consumption by fleas placed on treated cats

On day zero, six cats were orally administered an 11.4 mg nitenpyram tablet. Three additional cats served as untreated controls. One hour after treatment with nitenpyram, two confinement feeding chambers were attached to each of the twelve cats. Unfed fleas (21 female and 21 male fleas) 96 – 120 hours post-emergence were placed into the chambers. At 15, 30, 60, 120, 240, and 480 min post-treatment, fleas and feces were aspirated from four randomly selected chambers (two treated, two control cats). The amount of hemoglobin (contained within fleas and feces) was quantified by the Drabkin's reagent method and the blood volume consumed and excreted by fleas was determined at each time period using the equation determined by the standard curve.

Comparative effect of four commercially available insecticides on flea blood consumption

Twenty cats were weighed and randomly distributed into five treatment groups (four cats each). Cats were housed in individual stainless steel cages that were equipped with stainless steel litter pans with corn cob litter. Cats were given commercial cat food and water *ad libitum*. The cats were dosed topically with either imidacloprid, fipronil or selamectin on Day 0, while cats in Group 2 received one nitenpyram tablet one hour prior to flea infestation.

All insecticides were applied according to label instructions based upon the cat's weight.

Treatment groups were as follows:

Group 1. Untreated controls; no placebo treatment was used in this study

Group 2. Nitenpyram; an 11.4 mg tablet. (Capstar®; Novartis Animal Health, Greensboro, NC)

Group 3. Imidacloprid; 9.1% w/w spot formulation (Advantage™ Flea Adulticide; Bayer Animal Health, Shawnee Mission, KS)

Group 4. Fipronil; 9.7% w/w spot formulation (Frontline® Top Spot™; Merial Animal Health, Duluth, GA)

Group 5. Selamectin; 60mg/ml spot formulation (Revolution®; Pfizer Animal Health, Exton, PA)

All cats had one confinement feeding chamber attached 7 days post-treatment, and then 42 fleas (21 female, 21 male) KS1 Strain, 96 - 120 hours post-emergence) were placed into the chamber. All cats were fitted with a surgical stockinette (Surgi-Sox®, Campbell Pet Company, Brush Prairie, WA) while chambers were attached to help minimize chamber dislodgment. Twenty-four hours after chamber attachment and flea infestation the quantity of blood consumed and excreted was determined by removing fleas and feces by vacuum aspiration. Quantity of blood consumed and excreted was determined spectrophotometrically using the Drabkin's reagent method described previously. Once fleas and feces were removed the confinement feeding chamber was also removed. To evaluate the residual effect of insecticides on flea feeding, chambers were reattached and reinfested with fleas on days 14, 21, and 28 and collection and blood analysis determined as previously described. Data were

log-transformed to normalize them and were analyzed using Proc GLM (least square means), to make pair-wise comparisons.

Results

Cat Hemoglobin Standard Calibration Curve

The various quantities of blood treated with Drabkin's reagent were plotted against the absorbance (optical density) at 540 nm. A linear regression was calculated, forming a calibration equation: $Y = 0.0084X + 0.0042$, ($R^2 = 0.994$) where Y equals optical density and solving for X yields blood volume (Figure 5). This equation was used to estimate the volume of blood consumed by fleas in all subsequent experiments.

Unfed fleas

The amount of hemoglobin or other material carried over into the adult stages of unfed fleas that may have reacted with the Drabkin's reagent were significantly different for males and females ($r^2 = 0.76$, $p = 0.0004$, Figure 6). Twenty-five unfed female and male fleas gave the equivalent optical density as 0 and 0.135 µl of blood, respectively in the standard calibration curve. Even though unfed fleas had very low optical densities, all subsequent blood feeding data were corrected for this background using the formulas $Y = 0.0184X - 0.2041$ and $Y = 0.0403X - 0.4301$ for male and female fleas, respectively.

Flea feeding dynamics as a function of gender

After five minutes on the host, male and female fleas had consumed equivalent amounts of blood. Ten adult female fleas consumed and excreted 1.73 µl blood,

while males consumed and excreted 1.17 µl blood (Table 1), but by 60 minutes, females had consumed significantly more blood than males. At one, four and 24 hours post-infestation, females had consumed 1.48, 2.82 and 8.46, times as much blood as the males, respectively (Table 1).

Gender ratio and density effects on flea feeding dynamics

Fleas placed in confinement feeding chambers for 240 minutes at a 1:1 gender ratio consumed similar amounts of blood regardless of the density. Fleas placed in confinement feeding chambers at densities of 10:10, 21:21 and 42:42 consumed 0.648, 0.543 and 0.698 µl of blood per female/male pair unit. Since density had no effect on blood consumption within the ranges evaluated, a density of 21 female and 21 male fleas was chosen. Lower numbers of fleas (10:10) could potentially have more background influence (OD) while the higher number of fleas were more difficult with which to work.

When evaluating gender ratios, the largest numeric volume of blood was consumed by fleas having a 1:1 gender ratio at a density of 84 fleas. However, when blood volume (hemoglobin assayed) was calculated based on a per minute basis, it was determined that there was no difference in blood consumption by fleas relative to the gender ratios evaluated. Fleas fed for 240 minutes with gender ratios of 1:1, 2:1 and 1:2 (female:male) consumed 0.066, 0.066 and 0.051 µl of blood per minute. Therefore, since density difference (within the range evaluated) was not significant ($p < 0.05$) and gender ratio was not different

(within the feeding duration evaluated) a 1:1 female:male gender ratio with 42 total fleas was used for all subsequent studies.

Age post-emergence

No male fleas emerged until approximately 72 hours post-emergence of the first female flea. Thus no males were included in the confinement feeding chambers until the 72 – 96 hour experiments. Twenty-one female fleas, 24 – 48 hours post-emergence consumed and excreted a mean of 11.54 µl blood in 240 minutes (4 hours). Twenty-one female fleas 48 – 72 hours post-emergence consumed and excreted 10.26 µl of blood. Forty-two fleas (21 males and 21 females) consumed 19.61 µl blood in a four hour period.

Speed of flea kill of orally administered nitenpyram

No fleas were observed to be either dead or moribund until 60 minutes post-dosing with nitenpyram (Table 2). However, by 60 minutes, 38% of the fleas were classified as either dead or moribund. At 240 minutes, 100% of fleas recovered from nitenpyram treated cats were dead or moribund (Table 2).

Effect of nitenpyram on blood consumption of fleas placed on treated cats

The amount of blood consumed by fleas exposed to nitenpyram and those having no exposure to insecticides were not different at 15, 30, or 60 minutes post-infestation on the host (Table 3). At 120 minutes post-infestation (180 minutes post-dosing with nitenpyram), blood consumed by control fleas was

significantly greater than blood consumed by fleas feeding on nitenpyram treated cats. The amount of blood consumed by fleas placed on cats dosed with nitenpyram was not different at any time point at which fleas were recovered (15, 30, 60, 120, 240 or 480; Table 3).

Comparative effect of four commercially available insecticides on flea blood consumption

Forty-two C. *felis* (1:1 gender ratio) feeding on control cats for 24 hours consumed from 244 to 302 µl blood, while the fleas feeding on cats dosed with nitenpyram consumed blood quantities ranging only from 0.31 to 3.99 µl blood during the same period of time (Table 4). Fleas feeding on the selamectin treated cats had significant reductions in blood consumption though day 28. The amount of blood consumed by fleas exposed to selamectin throughout the experiment was not different than blood volume of fleas exposed to nitenpyram (Table 4). Fleas feeding on fipronil-treated cats had a significant reduction in blood consumption on Day 7. By day 14, the amount of blood consumed and excreted by fipronil-treated cats was not significantly different than that of the control group (Table 4). Fleas feeding on imidacloprid-treated cats had a significant reduction in blood consumption on Days 7 and 14, but by Day 21 post-treatment, the amount of blood consumed was not significantly different than that of the control group. Once the blood consumption of fleas in these two groups reached statistical equivalency to fleas feeding on untreated controls, cats in these treatment groups were no longer infested.

Discussion

It was determined that unfed fleas did contain a small quantity of hemoglobin or some other compound that reacted with the Drabkin's reagent. However, the level of background absorbance was extremely small when compared to fed fleas. Twenty-five unfed female fleas gave an absorbance value equivalent to 0.135 µl whole cat blood, whereas 10 female fleas feeding for only five minutes were determined to have consumed 1.73 µl blood. Even though the amount of background was extremely small, a formula was derived and used in all subsequent blood feeding evaluations to account for background absorbance of unfed fleas.

In these evaluations, ten female fleas were determined to have consumed 10.36 µl of blood within the first 24 hours post-infestation. A previous investigation had shown that females could consume up to 13.6 µl blood per day (Dryden and Gaafar 1991). In that investigation, the estimated quantity of blood was determined during the peak reproduction phase of a cat flea, while in this investigation, blood consumption of fleas was determined during their first 24 hours of infestation. In the current investigation, it was determined that male fleas consumed 1.25 µl of blood in a 24 h period. This is the first reference to the amount of blood consumed by males.

In the investigation of gender ratio and density effect, there did not appear to be any gender ratio or density effects on flea feeding in the time frames and numbers of fleas evaluated. However, it seems probable that if fleas were allowed to feed for longer periods of time, the gender ratio of 2:1 f:m would

consume and excrete larger quantities of blood than a 1:1 ratio. Since females consume and excrete more blood than the males, doubling the numbers of female fleas could be expected to result in larger quantities of blood being consumed and excreted.

As there were no male fleas available in sufficient numbers until 96 hours post-emergence of the first female fleas, fleas used in all experiments were 96 – 120 hours post-emergence. These results were not completely unexpected, since it has been previously documented that cat fleas are protogynous, that is, females emerge before the males (Hudson and Prince 1958; Dryden and Smith 1994).

In the investigation on the speed of flea kill of nitenpyram, the first noticeable effect of nitenpyram was observed at 60 minutes. Within one hour of nitenpyram administration, 38% of the fleas were either dead or moribund and 100% were dead or moribund within four hours. In a previous investigation, dead fleas were recovered in trays beneath cats one hour after administration of nitenpyram (Rust et al. 2003). Similarly, Dryden et al. (2001) observed 100% flea mortality at 4 hours post-treatment with nitenpyram in beagles, and Schenker et al. (2003) reported 100% mortality at 3 hours on cats. In the speed of kill evaluations by Dryden et al. (2001), Schenker et al. (2003) and Rust et al. (2003), fleas were classified as being either live or dead, whereas the fleas observed in this study were classified as being either dead, live or moribund.

In the evaluation of the effect of nitenpyram on blood feeding, nitenpyram was orally administered weekly one hour prior to placing fleas in the confinement feeding chambers. This was done because the maximum plasma concentration

of nitenpyram in cats has been shown to occur within 0.6 hours of administration, while the half life in cats is only 7.7 hours (Witte and Luempert 2001). In this evaluation fleas were first sampled for consumed and excreted blood at 15 minutes after infestation. The amount of blood detected in fleas and the feces that accumulated within the confinement feeding chamber over the next eight hours never increased beyond the 15 minute evaluation. This indicates that the blood consumption of fleas placed on nitenpyram-treated cats was suspended within at least 15 minutes post-infestation. Further investigations into the effect of nitenpyram on blood feeding will need to examine shorter exposure durations. In the investigations of the effect of insecticides on flea feeding, nitenpyram and selamectin were effective as antifeedants throughout the duration of the comparative study. However, fipronil only produced a significant reduction of blood feeding at day 7 when compared to controls, while imidacloprid produced a significant reduction of blood feeding on both days 7 and 14. Fleas placed on imidacloprid- and fipronil-treated cats seven days post-treatment had reductions in blood consumption of 90.8 and 69.8%, respectively, as compared to fleas feeding on control cats. At 14 days post-treatment, there was no significant difference in blood consumption between fleas feeding on fipronil-treated and untreated cats, whereas fleas on imidacloprid-treated cats consumed 55.7% less blood compared with untreated controls. Previous studies have investigated the effect of topical insecticides on reducing the percent of fleas feeding on treated cats, but none had looked at reductions in blood volume consumed. In a previous study conducted in this laboratory, when cats were treated with a fipronil

spray formulation at 15 mg/kg and topical imidacloprid spot-on at 20 mg/kg, 89 and 92% of unconfined fleas placed on cats 6 days post-treatment consumed blood, respectively (Dryden 1998). In another investigation, unconfined fleas placed on cats topically treated with imidacloprid and fipronil had reductions in the percent of fleas blood feeding of 49.6 and 39.5%, respectively, on day 7; while reductions in percent of fleas blood feeding on day 28 was 0 and 3.4%, respectively (Cadiergues and Franc 1998). While the use of a confinement feeding chamber may not appear appropriate to evaluate the antifeeding properties of topical insecticides that are not absorbed transdermally, such as fipronil and imidacloprid, these data are not disparate from the data in previous studies where fleas were unconfined.

Orally administered nitenpyram dramatically reduced blood consumption by fleas. Due to its short half-life, nitenpyram was administered once a week. Its duration of activity against fleas infesting cats has previously been shown to be approximately 72 hours (Rust et al. 2003). Fleas placed on nitenpyram-treated cats never consumed more than 1.6% (98.4% reduction) as much blood as fleas placed on control cats. Topically applied but transdermally absorbed selamectin also significantly reduced blood consumption. The greatest quantity of blood ever consumed by fleas placed on selamectin-treated cats occurred 28 days post-treatment when blood consumption was reduced by 88.9%. It must be noted that this last investigation only evaluated blood consumption by fleas and did not evaluate product efficacy. Even though fleas on imidacloprid- and fipronil-treated cats consumed large quantities of blood, they may have in fact

ultimately died. Values above the standard calibration curve were obtained in this investigation and the amount of blood consumed and excreted by fleas was extrapolated beyond the standard curve. The fact that both nitenpyram and selamectin greatly reduce flea feeding implicates the use of these compounds as therapies for indications of FAD.

The confinement feeding chamber coupled with the Drabkin's reagent method is an accurate way to examine the dynamics of cat flea feeding.

Figure 1. Shaving technique used to trim the cat's hair coat before attachment of the confinement feeding chamber.

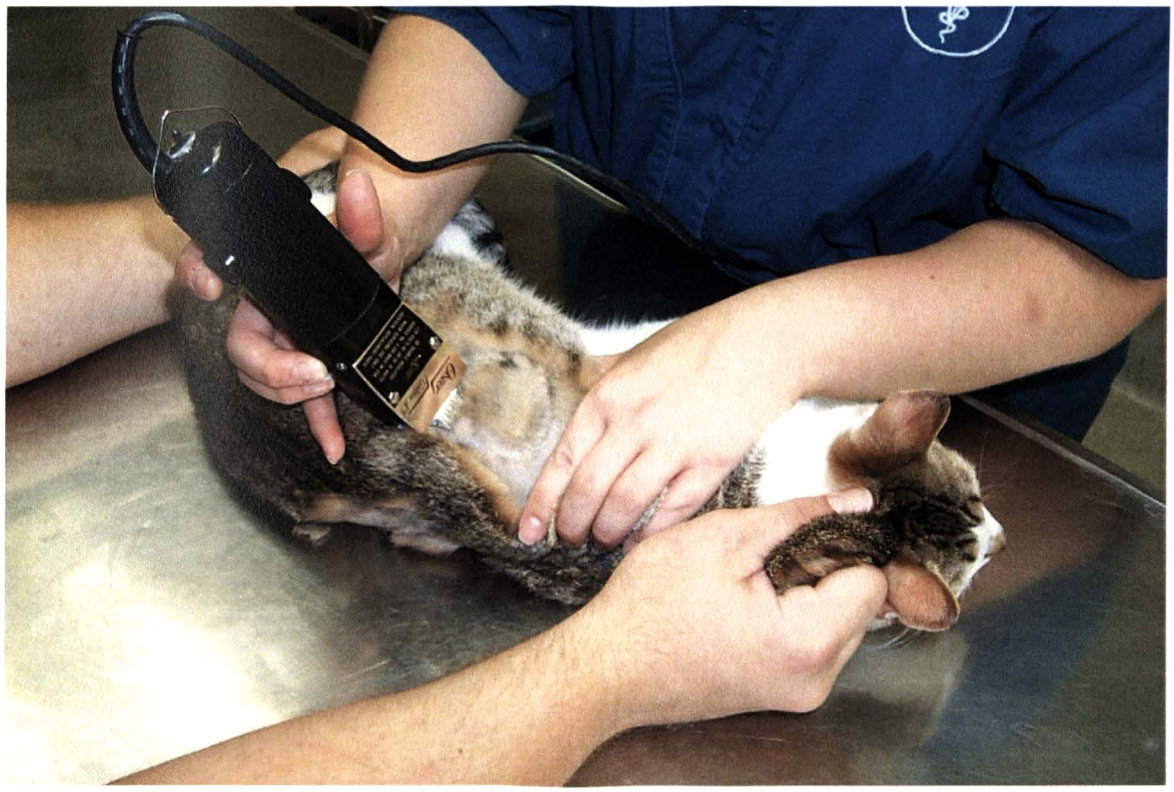

Figure 2: Materials needed to attach the confinement feeding chamber to the cat: Surgi-Sox®, Vetbond™, foam gasket and chamber.

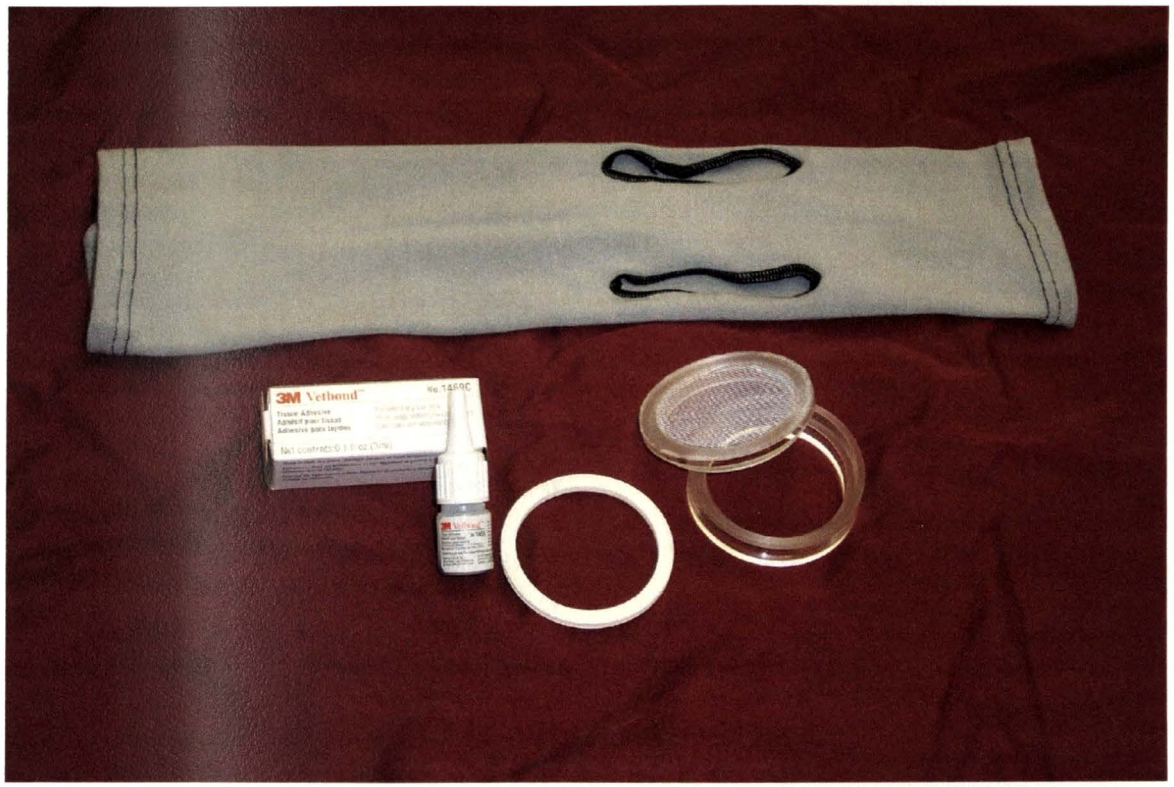
3M Vetbond
No.1469C

Figure 3: Cat with attached confinement flea feeding chamber apparatus.

Figure 4: Cat with attached confinement feeding chamber held in place with Surgi-Sox®.

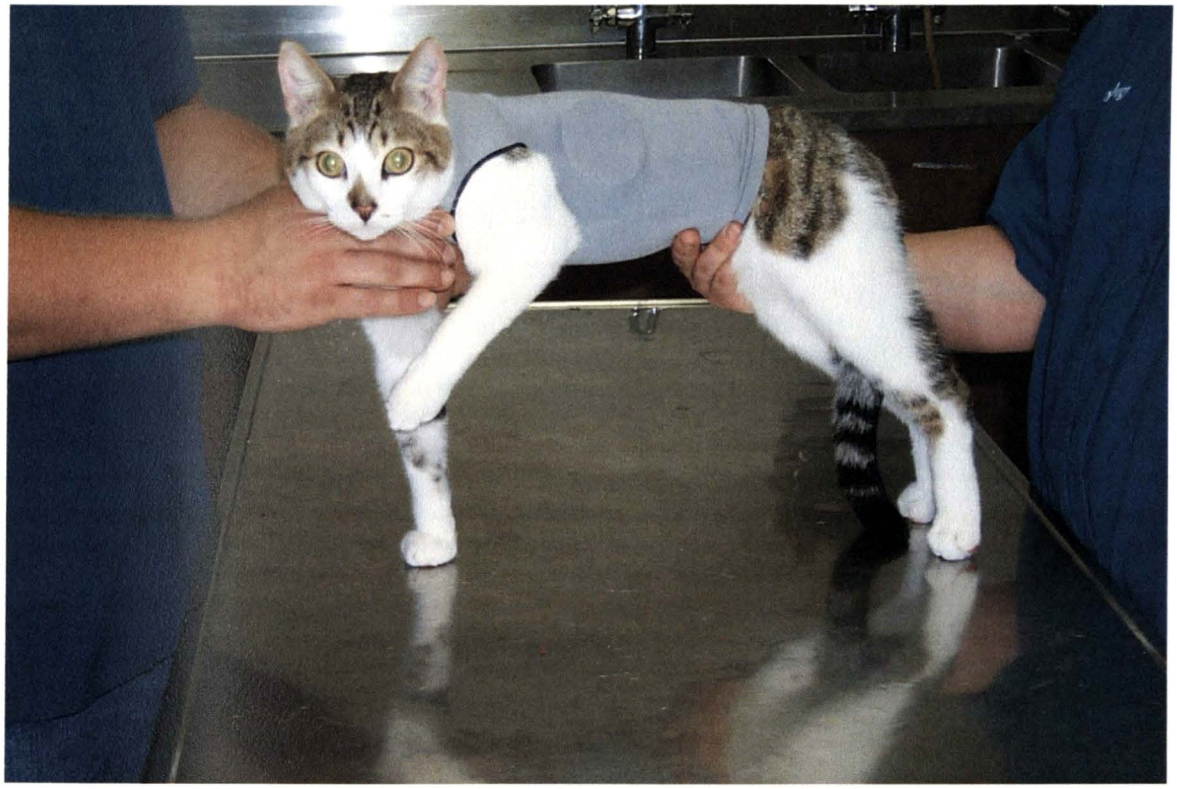

Figure 5: The relationship between quantity of blood (μL) and the absorbance (optical density) at 540 nm.

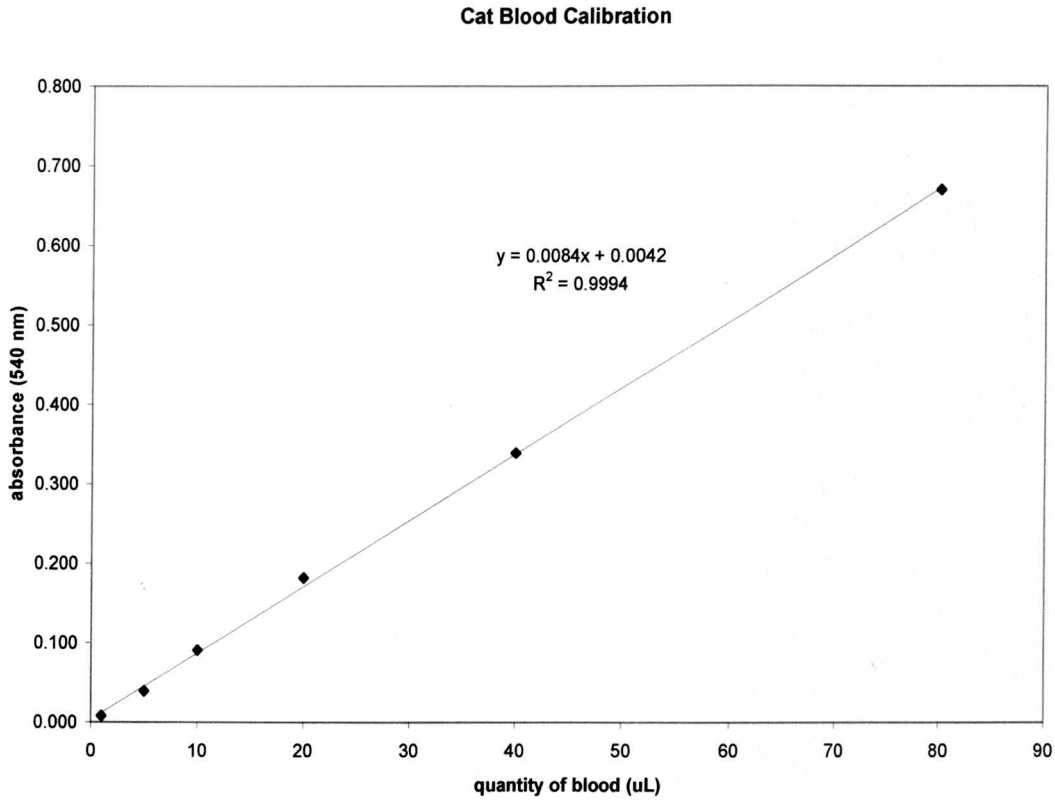
Cat Blood Calibration
0.800
0.700
0.600
0.500
0.400
0.300
0.200
0.100
0.000
absorbance (540 nm)
y = 0.0084x + 0.0042
R2 = 0.9994
0
10
20
30
40
50
60
70
80
90
quantity of blood (uL)

Figure 6: Baseline levels of hemoglobin found in unfed fleas.

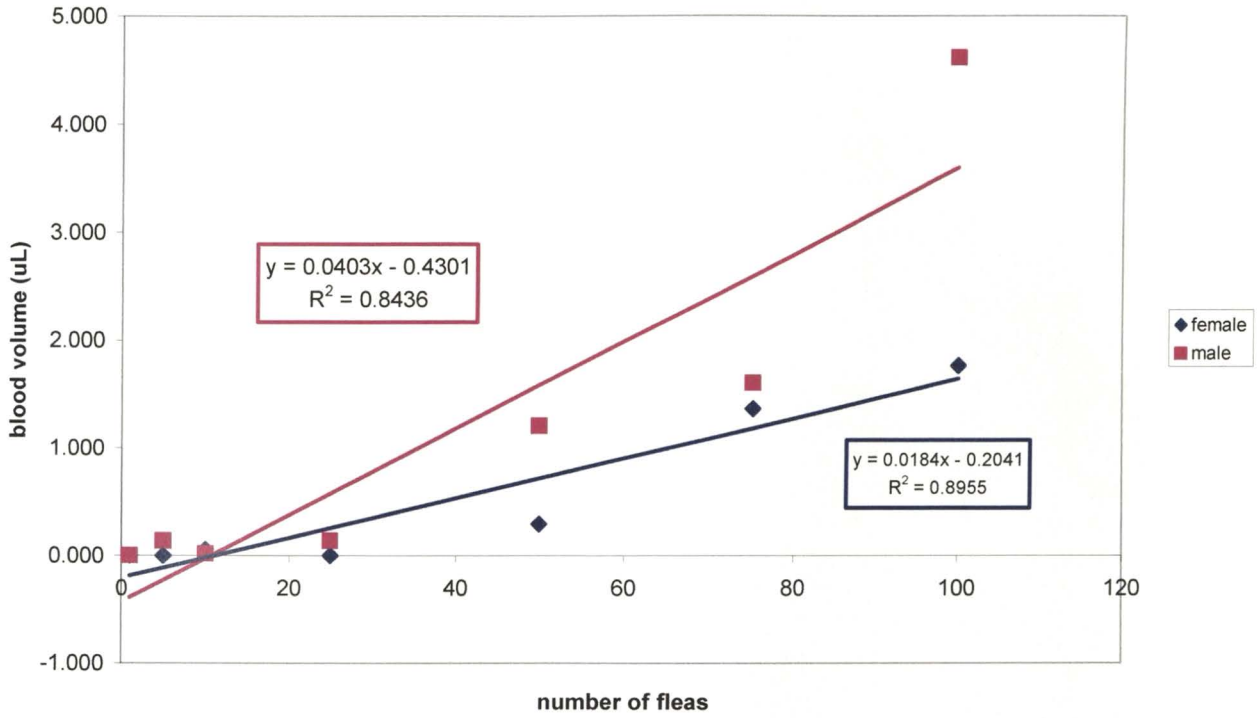
5.000
4.000
3.000
2.000
1.000
0.000
-1.000
blood volume (uL)
0
20
40
60
80
100
120
number of fleas
y = 0.0403x - 0.4301
R2 = 0.8436
y = 0.0184x - 0.2041
R2 = 0.8955
female
male

Table 1: Blood consumption of male and female cat fleas (*Ctenocephalides felis*) in confinement feeding chambers on cats.

	Blood volume µl	
minutes	Females	Males
5	1.73 a,c,d	1.17 c
60	2.45 a,d	1.65 c,d
240	7.33 b	2.60 a
1440	103.64 e	12.25 f

[1] Each cat was fitted with a confinement feeding chamber and then 10 unfed male or female *C. felis* were placed into confinement feeding chambers at time 0.
[2] Fleas and feces were vacuumed aspirated from confinement feeding chambers at 5, 60, 240 and 1440 minutes after infestation and analyzed for hemoglobin using the Drabkin's reagent method and absorbance values compared to the standard blood volume equation y = (0.0084x + 0.0042, where y = optical density. In addition, values were corrected for amount of hemoglobin in both unfed males and females.
[a.b,] Values in columns and rows with unlike superscripts are significantly different ($p < 0.05$).

Table 2. Speed of flea kill of nitenpyram against established adult cat flea (*Ctenocephalides felis*) infestations in confinement feeding chambers on cats.

	Mean number of fleas recovered alive, moribund or dead at each time period[1]						
Minutes post-exposure[2,3]	mean number alive (%)	St Dev	mean number dead (%)	St Dev	mean number moribund[4] (%)	St Dev	mean number dead + moribund (%)
15	39 (100)	4	0 (0)	0	0 (0)	0	0 (0)
30	40 (100)	1.83	0 (0)	0	0 (0)	0	0 (0)
60	24.5 (62)	19.98	0.75 (2)	1.5	14.5 (36)	18.29	15.25 (38)
90	14.25 (35)	15.84	0.25 (1)	0.5	26.25 (64)	17.33	26.5 (65)
240	0 (0)	0 (0)	16.5 (43)	19.07	22 (57)	20.25	38.5 (100)
480	0 (0)	0 (0)	22.5 (58)	9.29	16.25 (42)	9.5	38.5 (100)

[1] On day zero, two chambers were attached to each of 12 cats. Unfed fleas (21 female and 21 male fleas) 96 – 120 hours post-emergence were placed into the chambers. Approximately 24 h later, an 11.4 mg nitenpyram (Capstar®, Novartis Animal Health, Greensboro, NC) tablet was orally administered to each of six cats.

[2] At 15, 30, 60, 120, 240, and 480 min post-treatment, fleas were aspirated from four randomly selected chambers (two treated and two control cats) and assessed as being either live, dead or moribund. Process was repeated such that 4 chambers were evaluated at each time point.

[3] No control mortality was observed at any time tested.

[4] Fleas were classified as moribund if they had limb movement, but were unable to walk, jump or right themselves from lateral recumbancy.

Table 3. Effect of orally administered nitenpyram on blood feeding by cat fleas (*Ctenocephalides felis*)

		Minutes post infestation[2,3]					
		15	30	60	120	240	480
Groups	*n*	Blood volume µl (St Dev)					
Nitenpyram[1]	4	3.52a (1.16)	3.69a (0.72)	4.35a (1.60)	3.49a (2.97)	3.28a (3.51)	1.19a (0.48)
Controls	3	3.07a (1.24)	4.06a (1.00)	6.52a (2.11)	7.60b (1.19)	20.57b (1.34)	53.96b (2.95)

[1] Each cat in the nitenpyram group was treated with an 11.4 mg tablet 1 hour prior to placing fleas in the confinement feeding chambers.
[2] Each cat was fitted with a confinement feeding chamber and then 42 unfed C. *felis* (1:1 gender ratio) were placed into theconfinement feeding chambers at time 0.
[3] Fleas and feces were vacuum aspirated from the confinement feeding chambers at 15, 30, 60, 120, 240 and 480 minutes after infestation and analyzed for hemoglobin using the Drabkin's reagent method and absorbance values compared to the standard blood volume equation $y = (0.0084x + 0.0042)$, where y = optical density and x = blood volume.
[a.b,] Values in columns within the same day with unlike superscripts are significantly different ($p < 0.05$).

Table 4. Effect of various insecticides on blood feeding by confined cat fleas (*Ctenocephalides felis*)

		Day 7		Day 14		Day 21		Day 28	
Groups	*n*	Mean BV µl	St Dev	Mean BV µl	St Dev	Mean BV µl	St Dev	Mean BV µl	St Dev
Control	4	244 a	38.2	285 a	48.7	302 a	79.9	268 a	62.8
Nitenpyram	4	3.99 b	3.99	0.31 b	0.23	0.54 b	0.42	2.41 b	4.25
Imidacloprid	4	22.5 b	9.03	162 c	73.5	243 a	73.7	*	*
Fipronil	3	73.9 c	31.0	307 a,d	39.0	*	*	*	*
Selamectin	3	6.44 b	3.59	6.92 b	1.55	12.9 b	2.52	29.7 b	7.22

[1] Each cat in the control group received no treatment. Each cat in treatment groups received a topical minimum unit dose of selamectin (6 mg/kg), imidacloprid (10 mg/kg) or fipronil (7.5 mg/kg), respectively on Day 0. Each cat in the nitenpyram group was treated with an 11.4mg tablet 1 hour prior to placing fleas in the confinement feeding chamber.

[2] Each cat was fitted with a confinement feeding chamber and then 42 unfed *C. felis* (1:1 gender ratio) were placed into confinement feeding chambers on days 7, 14, 21 and 28.

[3] Fleas and feces were vacuum aspirated from confinement feeding chambers 24 hours after infestation and analyzed for hemoglobin using the Drabkin's reagent method and absorbance values compared to the standard blood volume equation $y = (0.0084x + 0.0042)$, where y = optical density and x = blood volume.

* Confinement feeding chambers were not placed on cats since previous weekly values were equivalent to controls.

[a.b,c,d] Values in columns within the same day with unlike superscripts are significantly different ($p < 0.05$).

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