BIOLOGICAL CONTROL OF THE COMMON HOUSE FLY *MUSCA DOMESTICA* L. IN HORSE STABLES, USING *BACILLUS THURINGIENSIS SEROVAR ISRAELENSIS AND BEAUVERIA BASSIANA*

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PREFACE

I hereby certify that the experimental work described in this dissertation is the result of my own investigation and carried out in the School of Animal and Poultry Science, University of KwaZulu - Natal. Where the work of others was used, it has been duly acknowledged in the text. The results in this dissertation have not been submitted, in whole or in part, for a degree or diploma or any tertiary institution at any other University.

I, **Cheralyn Martins,** declare that

1. The research reported in this dissertation is, except where otherwise indicated, my original research.

2. This dissertation has not been submitted for any degree or examination at any other university.

3. This dissertation does not contain other persons' data, pictures, graphs or other information, unless specifically acknowledged as being sourced from other persons.

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Abstract

House flies (*Musca domestica* L.*)* are common pests affecting horses and their owners. Control of house flies in stable yards is currently based on the use of pesticides. However, the development of resistance by these flies to most pesticide groups has motivated horse owners to seek alternative methods of fly control. An entomopathogenic fungus, *Beauveria bassiana* (*Bb*) and an entomopathogenic bacterium, *Bacillus thuringiensis* var. *israelensis* (*Bti*) are two biological agents known to have activity against house flies. The broad objective of this study was to evaluate the effect of these two biological control agents on house flies in an equine environment.

Using a structured questionnaire, presented in Chapter 2, thirty horse owners in KwaZulu-Natal were asked about the nuisance value of house flies, their current control measures, the potential market for biocontrol agents against house flies, and each owner's perception of biocontrol methods. The horse owners were using three methods of house fly control namely, physical, chemical and biological. Most horse owners (97%) wanted access to effective biocontrol agents for control of house flies. Most horse owners (80%) stabled their horses at night, some or all of the time. The resultant manure piles in the stable yard were considered to be the primary cause of house fly problems. About 64% of the horse owners were dissatisfied with the currently available methods of controlling house flies in this situation.

Chapter 3 covers two observational trials in which varying doses of *Bacillus thuringiensis* var. *israelensis* (*Bti*) were fed to horses, in order to identify a baseline dosage to give to horses in order to adequately control house fly populations growing in horse manure. The bacterium *Bti*, grown on wheat bran, was fed to six miniature horses at doses of 0, 0.125, 0.25, 0.5, 0.75, and 1.0 g per meal in Trial 3a, and at 0, 0.5, 1, 2, 4 and 8 g per meal in Trial 3b. Faeces were collected three times a week for 11 weeks and placed in incubation trays to allow the number of emerging adult house flies and closed pupae to be counted. In Trial 3a, there was a significant reduction in the number of closed pupae with an increase in *Bti* in the feed. The regression equation suggests that there will be 3.1 times as many closed pupae in the faeces when horses are fed 1 g of *Bti* in their feed, than when horses are fed no *Bti*. This dosage is the minimum baseline dosage for future trials.

Using manure from horses dosed in Trials 3a and 3b, the survival of the bacterium through the gut of horses was evaluated using a standard isolation technique. The growth of *Bt* colonies on the manure after the *Bt* isolation technique showed that some of the bacterial cells survived transition through the digestive tract of the horse. This study was qualitative in nature and did not attempt to quantify the level of *Bti* spore survival.
These two observations suggest that *Bacillus thuringiensis* var. *israelensis* has the potential to be used as a biocontrol agent, applied via horse feed, for the control of house flies in stable yards. Future clinical trials, with appropriate replication, should be conducted using 1 g *Bti*/meal as the lowest test dosage.

The objective of Chapter 4 was to determine whether spraying *Bti* or *Bb* on to horse manure is effective in the control of house flies. Over a six week period, two spraying trials were conducted in which increasing doses of *Bb* and *Bti* were sprayed on to 500 g samples of horse manure. Counts of house fly pupae and adults were taken. The doses of *Bb* and *Bti* tested were 0, 1, 2, 4 g in Trial 4a, and 0, 4, 8 and 12 g in Trial 4b. The research reported in Chapter 4 was characterized by the unexpectedly high levels of biological variation in egg, larvae and pupae numbers that were found in samples of horse manure, taken from the same skip two days apart. The statistical design of the two trials conducted was inadequate to cope with the high level of variation about treatment means for fly and larval counts. However, despite the lack of significant differences between treatment means, there is observational evidence that suggests that both *Bb* and *Bti* do have an effect on house fly survival. A simplified statistical model, which compared the number of hatched house flies on untreated manure, with the number on manure treated with any level of *Bb* (1 to 4 g /250 ml water), found a significant reduction in the number of hatched flies on treated manure. There was no significant corresponding reduction in the number of closed pupae, which suggests that *Bb* acts primarily before the larva pupates. The optimal dose of *Bb* and *Bti* to be sprayed on to manure could not be determined because of the high variation about treatment means. It is suggested that, in future trials similar dosages for *Bb* could be tested, but that higher dosages of *Bti* (starting at 2 g/250 ml water) should be used. Trial periods should be extended and replication increased dramatically to reduce variation about treatment means. Transformation of data before analysis may also be necessary to equalize variation about treatment means.

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GENERAL INTRODUCTION

Due to its large population size and high fecundity, the common house fly (*Musca domestica* L.) is recognized as a major pest in livestock communities (Axtell, 1986; Carn, 1996). Apart from their nuisance factor, high numbers of house flies raise health concerns for humans and animals alike (Scott *et al.*, 2000).

The use of pesticides has been the preferred method to control flies for many years (Abate *et al.*, 2000). However, house flies have developed resistance to most of these insecticides (Frazer, 1967; Scott *et al.*, 2000). Furthermore, the use of pesticides is expensive and they may have side effects that have a negative impact on the environment, such as soil leaching and denitrification (Pell *et al*., 1998). For example, the pesticide dichlorodiphenyltrichloroethane (DDT) has a half-life of 57.5 years in temperate soils and is difficult to remove from the environment. This pesticide disrupts endocrine development in humans and animals during organogenesis (early embryonic growth phase), resulting in a deformed foetus (Colborn, 1993).

Besides being a nuisance factor in horse stable yards, house flies are associated, in the minds of owners and riders, with filth and dirt. The presence of house flies in a horse yard reflects negatively on the owner's upkeep of his yard and on the health status of his animals, thus owners make every effort to eliminate them (Carn, 1996).

Given the growing environmental awareness and the resistance problem, many horse owners are searching for alternative methods of control, such as biological control programmes (Kellstedt *et al.,* 2008). Biological control utilizes biological agents, such as bacteria and fungi, to reduce pest populations without damaging the environment. Two biological agents, a fungus known as *Beauveria bassiana* Vuillemin (Bb) and a bacterium known as *Bacillus thuringiensis* Berliner serovar *israelensis (Bti)* and have been used to control a wide range of arthropod pests (Axtell, 1986).

The overall objective of this study was to test the potential of *Bti* and *B. bassiana* and as biological agents against house flies in an equine environment. This research could then

provide the basis for the registration of commercial biocontrol agents that horse owners could use to reduce the house fly population in a stable yard to a manageable level without harming the environment. Two methods of application were tested, namely feeding the bacterium to horses, and spraying the fungus and the bacterium on to horse manure.

The effects of *B. b* and *Bti* against house flies in an equine environment have not previously been reported in the literature. Given the lack of prior information on appropriate doses to work with, the trials in this project were deliberately observational in their design, as discussed by Rayner (1967), aiming to determine suitable starting-point dosages for feeding the biocontrol agents to horses, or treating their manure directly. These treatments will need to be further refined in larger trials, run for longer periods, with greater replication in order to provide satisfactory registration trials, with appropriate statistical analysis.

The referencing style utilized in this dissertation adheres to the referencing structure of the Journal of Animal Science (JAS, 2013)

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CHAPTER 1: LITERATURE REVIEW

1.1. Introduction

Arthropod pests such as insects, mites, ticks and house flies found in livestock and poultry production systems across the planet cause massive economic losses, equating to at least four billion dollars per annum in the USA in the 1980's (Axtell, 1986). Effective pest management has been an important factor in agriculture since the beginning of biocenosis.

One of the greatest arthropod concerns in agriculture is the house fly (Malik *et al.*, 2007). Their detrimental role in agricultural production is caused by their large populations. The earliest recording of house flies' involvement in the transmission of disease was as far back as 1577, when Mercurialis suggested that flies were responsible for the transmission of the plague between two people (Malik *et al.*, 2007). The role of flies as a health hazard to humans and animals has been well documented (Hogsette *et al.*, 2009).

However, the use of pesticides to control house flies can negatively affect the environment; for example through nitrogen toxicity, soil leaching, etc. (Axtell, 1986). Livestock owners have looked at using more "environmentally friendly" products to control house flies, including biological control agents. Biological control is the use of antagonistic living organisms to reduce pest populations to manageable levels (Grønvold *et al.*, 1996). Examples of biological control agents of house flies include parasitoid insects such as wasps; fungi; bacteria and entomopathogenic nematodes (Grønvold *et al.*, 1996; Service, 2000). *Beauveria bassiana* Vuillemin and *Bacillus thuringiensis* Berliner serovar *israelensi*s (*Bti*) are a fungus and a bacterium respectively, with the potential to kill house flies in livestock facilities. These two agents have been used in chicken houses and successfully reduced the number of adult house flies, when applied at low doses to the chicken feed (Mwamburi *et al.*, 2009).

This literature review evaluates the possible use of the fungus *Bb* and bacterium *Bti* as potential biological control agents against house flies attracted to horses.

1.1.1. General information about house flies

House flies are from the order Diptera. Diptera are known for their ability to exploit most ecological niches in any biological environment due to their larvae's ability to evolve (West, 1951).

There are over 3 900 species of flies in the family *Muscidae* and 66 species of flies in the genus *Musca* (Service, 2000). The common housefly, *Musca domestica* L., is a pest of animals and humans. Their primary nuisance value lies in the size of the population of flies (Brown *et al.*, 1995). These pests cause a reduction in livestock productivity by distressing animals during times of feeding, resting and by vectoring pathogens (Moon, 2002; Cheeke, 2005).

Concentrated human or agricultural activity, even at the village level, creates large amounts of organic waste, such as manure, which are attractive breeding and feeding sites for house flies (West, 1951). This causes an exponential increase in the house fly population in these areas (Forse, 1999). Flies are highly adaptive arthropods and their larvae may successfully develop in numerous media, such as horse manure and decaying organic matter.

1.1.2. Habitats of the house fly

Flies can be characterized into either haematophagous (blood sucking) or non-haematophagous (non-blood sucking groups) (Axtell, 1986). *Musca domestica* flies are non-haematophagous and they largely breed in fresh manure (Meyer *et al.*, 1983; Axtell, 1986). Protein is an essential requirement for the house fly's larval stages and is a prerequisite for breeding sites (Glaser, 1923; Oldroyd, 1964). Cow dung, composts and horse manure are the most popular breeding sites for flies (Meyer, 1990). House fly larvae have a higher preference for human, pig and horse manure (Oldroyd, 1964). Flies are thus attracted to manure with high levels of protein. Horses that eat high protein "racing rations" generate higher nitrogen levels in their manure than horses fed on "maintenance rations" and, therefore, provide an ideal site for the breeding of flies (Lawrence *et al.*, 2003).

1.1.3. Species and sex determination of the house fly

The stable fly *Stomoxys calcitrans* (L.) looks similar to the house fly and, for the survey in Chapter 2, a clear differentiation was needed in order to prevent misidentification. These two fly species are often confused because they are commonly found together in larger livestock enterprises, such as dairy farms and livery yards (Kaufman *et al.*, 2005). Stable flies are less abundant than house flies (Watson *et al.*, 1995) and are haematophagous (Kaufman *et al.*, 2005). As with the house fly, their large population sizes cause a nuisance to livestock. Their haematophagous nature results in a painful bite that leaves small calluses on the skin of the animal. Animals are less likely to feed when being pestered by stable flies and therefore these insects can cause considerable weight loss in affected livestock (Campbell *et al.*, 1987; Watson *et al.*, 1995). The morphology of wings is an important taxonomic characteristic feature used to identify fly species. Vein 4 in a house fly's wing bends up sharply towards Vein 3 and joins at the tip of the wing, known as the costa (Service, 2000).

The sex of house flies can be determined by examining the space between the eyes. The terms "dichoptic" and "holoptic" are used to describe female and male house flies, respectively. Dichoptic flies have large spaces between their eyes, while in holoptic flies the compound eyes are contiguous (Walker, 1994).

1.1.3.1 General physical characteristics of the house fly's body

The order Diptera contains insects with two pairs of wings; three pairs of legs and antennae (Service, 2000). The common house fly comprises a head, a thorax and a segmented abdomen. An adult house fly is generally 6 – 9 mm long and coloured from light to dark grey. They have reddish eyes and are positively phototactic; that is, they are attracted towards light (Yoho *et al.*, 1973). It has been found that the photoreceptors in the house fly's compound eyes are receptive to light in the ultraviolet (340-365 nm) and blue-green (450-550 nm) wavelengths (Service, 2000).

House flies have four longitudinal dark body-length stripes on the dorsal surface of the thorax (Walker, 1994; Service, 2000). The abdomen is a pale yellow colour (Walker, 1994). The house fly has antennae that are separated into three segments. These are hidden in a downward position in front of the face and are not easily seen with the human eye (Service, 2000). The house fly's mouth is known as a proboscis; modified for the sucking up of fluids such as sweat, tears and fluids in horse manure (Oldroyd, 1964; Service, 2000).

1.1.3.2. The complex feeding structure of the house fly's mouth

To understand the feeding of the house fly, its mouthparts need to be explained. When the proboscis is not in use, it is drawn into the head capsule. Before the fly feeds, the proboscis is extended in a telescopic manner onto the substrate (Service, 2000). At the end of the proboscis is a pair of labella. These are fleshy and oval-shaped. The labella are composed of fine channels called pseudotracheae. These are used to draw up fluids such as nasal discharge and broken down faecal particles (West, 1951; Service, 2000). The physical state of available food determines the method of feeding utilized by the fly. Food in a fluid medium allows for the labella to make direct contact with the food. In semi-fluid foods, such as sputum and nasal discharge, the labella turn inside out to allow the food to be sucked up directly into the food channel. Solid foods such as dried blood and faeces cause the labella to be inverted and miniscule prestomal teeth along the food channel scrape away the food. The fly moistens the food particles with its saliva, or it is regurgitated, and then the food is sucked up the food channel (Kovacs *et al.*, 1990). The latter feeding method predominates in the spread of diseases (Service, 2000).

1.1.3.3. The house fly's legs

The house fly has three pairs of legs. Each leg has a pair of claws and pulvilli. Pulvilli are fleshly cushion-like structures with glandular hairs that are sticky and adhere to smooth surfaces, such as mirrors and windows (Service, 2000). It is these glandular hairs that contribute to the capacity of house flies to pick up pathogens (Oldroyd, 1964; Service, 2000).

1.1.3.4. A labeled diagram of the house fly

Figure 1 shows the detailed anatomy of the house fly *Musca domestica* (from Kobayashi *et al*, 1999).

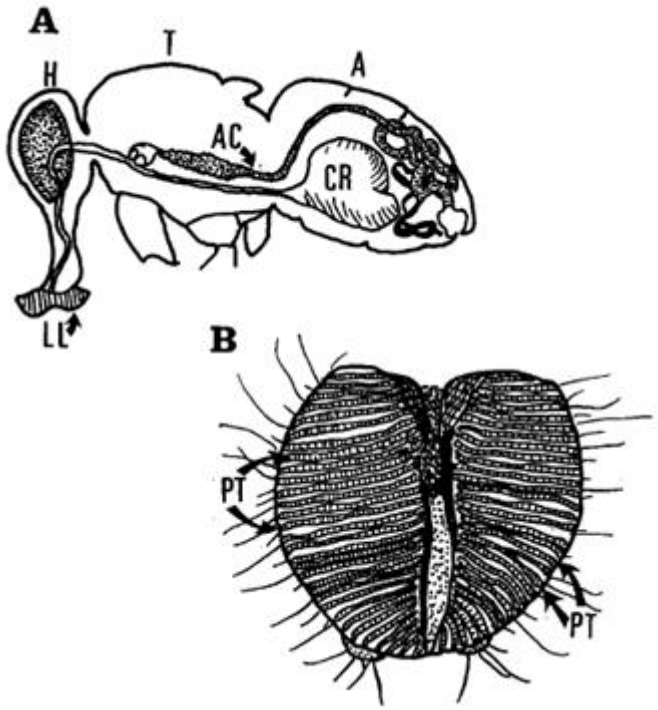

Figure 1. The internal anatomy of the housefly *Musca domestica vinica.* **A**, digestive system. CR = crop; AC = alimentary canal; LL = labellum; H = head; T = thorax; A = abdomen. **B,** detailed structure of the labellum. P = pseuodotrachea. (from Kobayashi *et al.*, 1999)

1.2. House fly life cycle

One of the most significant characteristics of the common house fly is its extreme fecundity. This fecundity is supported by a universal supply of substances suitable for larval nutrition and rapid larval development (Walker, 1994). Female house flies are attracted to numerous types of materials for egg laying. The most common breeding material is decomposing organic matter, such as livestock manure (Eltringham, 1916; Siverly *et al.*, 1955). In the equine environment, stable wear, horse bedding, and manure are favoured breeding sites (Service, 2000). The female lays up to 500 eggs over 3 to 4 days (Brown *et al.*, 1995). The

eggs are laid in batches of 75 to 120 eggs and are deposited in cracks or crevices, or preferably in horse faeces (Hickin, 1974; Service, 2000). Once female house flies have mated, they will only lay their eggs in decaying organic matter that is less than 72 hours old (Hickin, 1974). It is imperative that the female house fly lays her eggs in a moist environment that does not dry out or become too wet, or the eggs will not hatch successfully (Service, 2000).

A house fly's life cycle follows a full metamorphosis (Walker, 1994; McGavin, 2000). The metamorphosis cycle follows a four-step process. The first step is initiated by mating and egg laying, followed by the emergence of the larval stage. The third step is from metamorphosis of larvae to pupae and, lastly, there is the transformation of pupae to adult flies (McGavin, 2000).

House fly eggs have a banana shape and are 1 to 2 mm long and creamy white. These eggs usually hatch within 6 to 12 hours (Service, 2000). Egg hatching may be delayed from eight hours to three days, depending on the weather conditions. Cold weather usually prolongs the hatching period (Axtell, 1986). Hatching occurs when the dorsal concave curve of the egg lifts up and detaches from the egg. Extreme temperatures above 40°C and below 15°C are not conducive to egg survival (Service, 2000). Larvae feed on decomposing material in the environment (Service, 2000).

The larval stage, which marks the "birth" of houseflies, then transforms into a pupa and subsequently into the adult form (McGavin, 2000). Their larvae have no legs and they develop through three stages, referred to as instars (Walker, 1994).

At the start of development, the larvae, also commonly known as maggots, are 1 to 1.5 mm long. The larvae are creamy white and are divided into 11 segments. These "worms" have a small pointed head and a spindle shaped body (Hickin, 1974; Brown *et al.*, 1995; Service, 2000). The small head carries a set of black pincer structures known as mouth hooks that are situated beneath the head (Service, 2000).

By the end of the third instar, the spindle-shaped larvae metamorphose into a muscoid shape with 12 segments (Hafez, 1948). These larvae reach 10 to 12 mm in length. In favourable warm weather, the larval stage may last only three days. However, in cold and unfavourable conditions, the larval period may take up to eight weeks for full development (Hickin, 1974). A waxy ivory colour and the movement of larvae into a cooler, drier site for pupation, characterize full growth of larvae (Hickin, 1974).

In other species of Diptera, such as the mosquito, the pupae remain mobile. However, in flies they are immobile (Walker, 1994). The pupal stage involves the transformation of the larval stage into the adult form. Larvae eat until they are ready to pupate, after which they settle in one place until the transformation is complete (McGavin, 2000). Inside the cuticle, imaginal discs (which are a small collection of cells) assist in developing the broken down larvae tissue into adult organs. Once metamorphosis is complete, the adult fly uses a structure on the head to free itself from the cocoon (McGavin, 2000).

The life cycle of a house fly from an egg to an adult is as short as 7 to 10 days, depending on the temperature. Over winter, flies remain in either a larval or a pupal form, hibernating under livestock manure piles or other protected natural areas (Brown *et al.*, 1995) The adult life span of flies is 4 to 12 weeks (Hickin, 1974).

1.3. The nuisance factor and diseases vectored by house flies

Compact systems of raising livestock in modern agriculture produce large quantities of manure (Hickin, 1974). Closed storage sites, such as dung heaps or manure pools, serve as optimum breeding sites for flies. For convenience of transport, these manure sites are usually found near the housing of the livestock, i.e., near to stables for horses (Hickin, 1974; Axtell, 1986).

House flies have microhabitat associations with other fly species such as the stable fly, as previously mentioned (Rutz *et al.*, 1991). Kühlhorn (1964) found 330 different species of flies from 47 families near horse stables. Neighbouring stable yards and other house fly

breeding sites collectively enhance the size of the fly population. It has not been proven that house flies have a direct negative effect on the performance of horses, possibly because of the difficulty of measuring this accurately. However, their large numbers have been noted to cause annoyance and nuisance (Smallegange, 2004), which can prolong riding or training times in a horse yard. House flies are therefore considered a nuisance in equine breeding yards. Newborn foals are at risks of infection of house flies laying eggs in their navel cords because the amniotic fluid smell attracts house flies (Evans, 1989).

Besides the intense irritation that these flies cause to horses during riding, stabling and feeding, these vectors can introduce viruses to their hosts by feeding on infected substrates such as manure or nasal discharge, that attach to mouthparts of the house fly. The flies pass the disease or virus on to their hosts through cuts and abrasions. Larger flies have coarser mouthparts that allow for heavier contamination of the virus and a higher infection rate (Brown *et al.*, 1995; Carn, 1996). Habronema and draschia (summer sores) are transmitted by *M. domestica* to horses. Houseflies are also the intermediate host for the adult nematodes *Habronema muscae*, *H. majus* and *Draschia megastoma* (Schuster *et al*., 2010).

1.4. Effects of flies on horse temperament

Temperament can be defined as an individual's reaction towards simple challenges and changes within the surrounding environment. Quantifying a horse's temperament is difficult (Visser *et al.*, 2001). However Visser *et al*. (2010) developed a protocol to measure the temperament of sport horses using a Novel Object test and a Handling test. Behavioural characteristics were measured and were rated against a 6 score scale. Some of the behavioural characteristics measured were "fearful or brave", "tense or relaxed", "spooky or non-spooky", "enthusiastic or lazy" and "cautious or not cautious" (Visser *et al.*, 2010). There is a lack of research into the effect of house flies effect on horse temperament. Waran *et al*. (2007) reported that house flies impact on horse temperament by providing a constant source of irritation that can affect even the most placid horse, even the most non-cautious, relaxed, non-spooky horse (Visser *et al.*, 2010).

1.5. The digestion and eating behavior of the horse

The digestive system of a horse may influence the number of cells of *Bti* that survive passage through a horse's digestive tract. It is important to understand the prehistoric background and evolution of the horse in order to grasp the concept of its digestive system. The horse is classified under the Order Perissodactyla which dates back to the "grandparent" animal, the tapirs and rhinoceros (Xu *et al.*, 1996). These herbivores were classified into the family Equidae (Groves *et al.*, 2000).

The digestive system of the horse allows it to consume roughage in bulk (Gibbs, 2005). Horses feed largely on forage, leaves, plant stems and a diverse range of grasses (Hoffmann, 1989) and are hind gut fermenters (Rechkemmer *et al.*, 1988). Rate of passage of feed through a horse will vary according to diet, exercise and health (Ellis *et al.*, 2005). A trial using chromic oxide as a marker tested the rate of passage of alfalfa alone and that of alfalfa and Timothy hay; each supplemented with oats, corn or barley (Vander Noot *et al.*, 1967). The authors reported that, in most horses, 4 days were adequate for a full rate of passage but that some animals needed 5 days to clear the marker from the digestive system.

Wild horses graze between 12 and 16 hours per day (Ellis *et al.*, 2005). Concentrated diets such as horse racing meal have modified the horse's digestion and placed it into an "intermediate" feeding class between herbivores and ruminants (Morrison, 1950). Horses do not have microbial cellulases or hemicellulases to digest forage efficiently, as in a ruminant.

The sequence of digestion in horses is related to the utilization of dietary fibre. Horses host a small population of microbes in their colon and can be classified as hind gut fermenters, or as non-ruminant herbivores. A horse's digestive process involves a sequence of digestion mainly focused on the colon and caecum (Ellis *et al.*, 2005).

Feeding hours have generally been reduced to between 6 to 10 hours per day in a managed stable environment (Ellis *et al.*, 2005). Horses do not use energy and protein in their diets efficiently, therefore higher energy demands associated with show jumping, racing, riding

etc. have introduced a requirement for higher levels of starch and protein in the rations (Tinker *et al.*, 1997), causing higher carbohydrate and protein residues in the manure (Slade *et al.*, 1970). Excessive carbohydrate consumption can result in behavioral changes such as weaving, wind-sucking (McGreevy *et al.*, 1995) and coprophagy (Ellis *et al.*, 2005). High protein levels in the horse manure are a major attractant to house flies seeking breeding sites (Rodriguez *et al.*, 1970).

1.5.1. Gastrointestinal tract (GIT) and chemical digestion in the horse

Ingested food enters the stomach in a circular bolus form through the cardiac sphincter and is coated in many levels of saliva (Ellis *et al.*, 2005). Digesta is exposed to different stomach acids dependent on two factors: i) the length of time the bolus resides in the stomach and ii) the degree of mixing of gastric secretions from the stomach mucosa (Ellis *et al.*, 2005). Volatile fatty acids and lactic acid ensiled by feeds cause the low pH (pH 5 to 5.5) in the hind gut of the horse (Kern *et al.*, 1974; Murray *et al.*, 1996). Varloud *et al*. (2007) discovered fluctuations in the pH of the horses gut, depending on a higher fibre or starch diet. The pH of mixed digesta from the hindgut of the horse was found to be 4.5 and 5.1 for high fibre and high starch feeds, respectively (Julliand *et al.*, 2001; Varloud *et al.*, 2007).

Passage retention time is the time taken for the retention of feed in the animal's stomach. Larger particles such as long fibres are retained for a longer period of time (Kaske *et al.*, 1990). The composition of the diet will affect the microbial population and mean retention time (Julliand *et al*., 2001). Ground grains are commonly used in animal feeds. Whole grains have a longer retention time than extruded or micronized grains (Rosenfeld *et al.*, 2009). The dry matter (DM), organic matter (OM) and total fibre content will affect the digestibility and retention of food in the horse's stomach (Cuddeford *et al.*, 1995). Five days is a good adaptation period for horses to adjust to a new feeding regime (Rosenfeld *et al.*, 2009).

Horses are known to have an abundant microbial colonization of the digestive tract (Varloud *et al.*, 2007). Julliand *et al*. (2001) found various types of bacteria in the horse's digestive tract. Cellulolytic bacteria were present at relatively low levels. Extensive populations of

lactobacilli, lactate-utilizing bacteria and streptococci bacteria were found to predominate (Julliand *et al.*, 2001).

1.6. Control measures for house flies

Concentrated livestock systems increase the number of arthropods in an agricultural environment (Axtell, 1986). Examples of a concentrated livestock system are chicken houses, pigsties and livery yards. In any livestock system, the management of arthropod pests is imperative to reduce pest vectored diseases (Axtell, 1986). Control of arthropod pests by their natural enemies through multitrophic interactions, e.g. wasps and fly eggs or genetically modified organisms (Bottrell *et al.*, 1998), has become unpredictable in agricultural systems. Agriculture systems are man-made, making them unnatural habitat systems that are unsuitable for the living requirements of the natural enemies of pests that develop abnormal populations (Landis *et al.*, 2000).

There are three primary ways to control pests: 1, using pesticides (or chemical control), 2, physical control and 3, biological control.

1.6.1. Pesticides (chemical control)

Chemical methods of house fly control require the use of synthetic insecticides commonly known as pesticides. These are usually harmful to the environment (Grønvold *et al.*, 1996) . The majority of pesticides are used on commercial crops such as cotton, coca and vegetables (Abate *et al.*, 2000).

Pesticides used to be considered more cost-effective, efficacious and generally more competitive than other means of disease pest control (Frazer, 1967). However, these chemical are hazardous and often harmful to the environment (Axtell, 1986). Roberts and Andre (1994) noted that insect resistance to pesticides is an increasing problem. They suggested that resistance is either physiological, biochemical or behavioural. Mosquitoes showed a strong behavioural resistance to dichlorodiphenyltrichloroethane (DDT) by avoiding sprayed rooms or rapidly exiting these rooms. Similarly, pyrethroids stimulated a behavioural resistance in

arthropods (Roberts *et al.*, 1994). Owners have tried to use multiple pesticides to reduce resistance. However, multiple pesticides can encourage resistance in secondary pests, disrupt biological control measures and generate cross-resistance (Tabashnik, 1989).

Examples of chemical control used in a stable yard are Agita® fly traps (Greenberg, 1959; Grønvold *et al.*, 1996), which contain a pesticide, and an insecticidal spray that has thiamethoxam as an active agent. Thiamethoxam is a neonicotinoid insecticide that targets the nervous system of the animal. House flies are attracted to this pesticide by the use of a pheromone called tricosene (Howard and Wall, 1998).

1.6.2 Physical control

Examples of physical control measures relevant to horse management include manure removal, fly masks, fly traps and general sanitation in Southern Africa. Physical control of house flies may be combined with pesticides.

It is important to note that no single physical control measure will work for all geographical areas (Maunsell *et al.*, 2008). Geographical areas differ in terrain, topography temperature, and climate. The success of physical control measures may therefore be limited by the environment.

1.6.2.1 Removal of manure from the stable yard

Systematic removal of horse manure from the stable yard and surrounds is a commonly practiced physical method of controlling house flies, by reducing the number of breeding sites (Greenberg, 1959; Thomas et al., 1996). Generally, a livery yard stables horses overnight and returns them to pasture during the day. Groomsmen clean out or "muck out" manure from stables in the morning. The manure is often stored for compost (Mathews *et al*., 2003). Forse (1999) showed that, in small chicken houses, removal of manure reduced the population of house flies. Flies are also attracted to left over horse meal and therefore feeding horses outside the stable is another physical method of fly control (Greenberg, 1959).

1.6.2.2. Fly masks and fly traps

A second physical method of fly control is the use of fly masks. Evans (1989) suggested fly masks could be used to prevent flies from agitating horses' face areas. Fly masks are worn over the ear area and hang over the eyes, ending just above the nostrils. Other than house flies, horses are agitated by a host of other flies from other Dipteran families (Evans, 1989), such as stable flies, screwworm flies, tsetse flies (Forse, 1999) and, most commonly, bot flies (Evans, 1989). Fly masks can protect the horse's eyes from many of these flies.

"Red top®"[1] fly traps are see-through bags with a red lid filled with a fishmeal attractant. These traps can trap up to 20 000 house flies and the attractant can remain effective for up to 12 weeks (Anonymous, 2010). The traps are reusable if replacement attractant is purchased.

1.6.2.3. Sanitation

Musca domestica flies are also known as filth flies due to their preference for decaying matter as breeding sites (Oldroyd, 1964). Theoretically, sanitation should reduce the available areas for breeding and thus reduce the number of house flies (Quarterman *et al.*, 1949). House flies are known vectors of the disease shigellosis among children. Cohen *et al.*, (1991) found that improved sanitation reduced the number of house flies within the population and, therefore, reduced the outbreak of shigellosis.

Sanitation of a livery yard can be improved by good management. The yard consists of individual stables, the stable yard, the feed and tack rooms, as well as a number of horses. Individual stables should be mucked out daily (Forse, 1999). The feeding room should be kept clean, tidy and shut because the smell of rich horse food attracts flies (Cheeke, 2005). Feeding basins should be washed and dried daily (Cooke, 1989; Forse, 1999).

1.6.3. Biological control

The widespread resistance of house flies to pesticides (Roberts *et al.*, 1994) and an increasing awareness of the effect of pesticides on the environment (Axtell, 1986) has caused horse owners and farmers to search for alternative methods of fly control (Scott *et al.*, 2000). Biological control (or biocontrol) is defined as a method implemented by man in order to reduce a parasitic or pest population to a non-harmful level by utilizing antagonistic living organisms (Grønvold et al., 1996).

Advantages of biological control include the lack of chemical residues (Khan *et al.*, 2004) and sustainability (Larsen, 1999). There are three major groups of natural enemies that attack house flies, namely, fungi, bacteria and other insects (Roberts, 1986). Currently wasps are sold that parasitize the larvae of house flies. No other biochemical agent is registered in South Africa for the control of house flies on horses. A biocontrol product has been developed to control house flies in poultry, and has been registered for commercial use. This involves adding *Bti* to chicken feed at low levels (1g per kg) (Mwamburi *et al.*, 2009). Feeding *Bti* to horses has not yet been investigated by others.

1.6.3.1 Bacteria

There are many bacteria which are effective against house flies, but the most widely commercially used biocontrol agent is *Bti*.

1.6.3.1.1 *Bacillus thuringiensis*

Bacillus thuringiensis Berliner *(Bt)* is a leading bio-insecticide used to control insects and nematodes (Indrasith *et al*., 1992; Schnepf *et al.*, 1998; Mwamburi, 2008; Fang *et al*., 2009). The species *Bt* is an aerobic, gram-positive, spore-forming bacterium (Mwamburi *et al.*, 2009). *Bacillus thuringiensis* can be isolated from many environmental sources such as soil, insects and coniferous and deciduous leaves (Schnepf *et al.*, 1998).

The most common formulation of *Bt* as a bio-pesticide is in the form of concentrated lipids, oil-based powders, water dispersible granules and dust (Boyetchko *et al.*, 1999).

1.6.3.1.2 Mode of action of *Bacillus thuringiensis*

During the stationary growth phase, the bacterium forms a proteinaceous parasporal crystal (Gill *et al.*, 1992; Schnepf *et al.*, 1998). This parasporal body is unique to *Bt* and is the primary taxonomic feature to distinguish *Bt* from other *Bacillus* species such as *B. cereus* (Mwamburi, 2008). The parasporal body is comprised of protoxins made up of the precursors of crystal (Cry) proteins and other insecticidal particles such as vegetative insecticidal protein (VIP) toxins that may be lethal to more than one insect species.

Upon oral consumption of the protoxins, digestion in the gut of the target insect results in the release of the toxins *per se*. These toxins then attack the gut lining of the target pests, causing peritonitis, septicaemia and death (Guo *et al*., 2009). Septicaemia is caused by the crystalline body. The pro-toxins are solubilised inside the insect gut to release proteins called δ-endotoxins. These toxins interact with the epithelial lining of the midgut, creating a cation-selective channel within the membrane, and ultimately leading to the insect's death (English *et al.*, 1992; Gill *et al.*, 1992).

Bt endotoxins are encoded by active proteins Cry (crystal) genes (Guo *et al*., 2009). Cry I to Cry VI proteins are known to target certain insect species; namely, Lepidoptera, Diptera, Coleoptera and Hymenoptera, and also the Nematoda (Schnepf *et al.*, 1998; O'Grady *et al.*, 2007.)

1.6.3.2. Fungi

The entomopathogenic fungus species *Culicinomyces clavisporus* has a considerable larvicidal effect against *C. nubeculosus*, a well-known pest that belongs to the 'midge' or house fly family. However, the most commonly known insecticidal fungus against house flies is *B. bassiana* (*Bb*) (Clarkson *et al.*, 1996; Mwamburi *et al.*, 2009). Although *B. bassiana* can attack a wide range of insect hosts, individual isolates may be species-specific (Clarkson *et*

al., 1996). In an experiment performed by Kaufman *et al*. (2005), the efficiency of *B. bassiana* against house flies was tested against a well-known pyrethroid treatment. It was reported that treated areas sprayed with *B.bassiana* had less than half the number of house flies found in pyrethroid treated areas. In addition, the number of adults and larvae of the predatory beetle (*Carcinops pumilio*) recovered by 43 - 66% in *B. bassiana* treated areas, greater than the population found in pyrethroid treated areas (Kaufman *et al.*, 2005).

1.6.3.2.1 Mode of action of *Beauveria bassiana*

The mycoinsecticide *Beauveria bassiana* is an omnipresent fungus (Roberts, 1986; Clarkson *et al.*, 1996). Conidia sporulate within approximately 18 hours, grow over the surface of the host before forming an appressorium. An appressorium is a swollen hyphal structure that assists in attachment to the cuticle of the insect (Roberts *et al*., 1986).These conidia are fragile and short-lived, yet they germinate rapidly and aid in host attachment. They are covered with mucus which is relatively sticky (Pell *et al.*, 2003). The penetration of the host insect is by a peg that develops at the base of the appressorium. Once this has penetrated through the proteinaceous cuticle of the insect, the fungal mycelium enters into the host tissues and rapidly ramifies throughout the insect (St. Leger, 1995; Clarkson *et al*., 1996). The fungus may also enter through the respiratory system or orally through the gut of the insect's larvae (Pekrul *et al.*, 1979).

Once inside the host, the fungus produces hyphae in a sponge-like network, and produces a range of metabolites. These metabolites are significant in initiating three processes within the insect; 1) inhibition of the insect immune system; 2) changing insect behavior; and 3) acting as an antibiotic against competing micro-organisms (Griesch *et al.*, 1998). The fungus emerges through the host cuticle and sporulates when environmental conditions are favourable. Sporulation is usually initiated 4 to 6 days after infection by *B. bassiana* (Pell *et al.*, 2003). Post death, the fungus decomposes the insect's internal organs, leaving the outer chitin/protein exoskeleton (Griesch *et al.*, 1998).

In an attempt to understand the mode of action of *B. bassiana*, Pekrul *et al*,(1979) tested the virulence of *B. bassiana* against the maize earworm (*Heliothis zea*). In this experiment, *B. bassiana* was found to produce conidia which are attracted to the protein found in the chitinous cuticle of the host (Bidochka and Khachatourians, 1992). Once the hyphae burrow holes through the cuticle, *B. bassiana* releases enzymes which degrade the cuticle of the host (Clarkson *et al.,* 1996). Steinkraus *et al*. (1990) reported that, under favourable conditions for *B. bassiana bassiana* in chicken houses, 1% of house fly adults were infected.

Beauveria bassiana can be used in combination with insecticides to reduce the level of pesticide usage. Anderson *et al*. (1989) found that there was no adverse effect on *B. bassiana* when one specific formulation was mixed with four well known insecticides. The lethal effects on the pests were significantly higher when *B. bassiana* was mixed with an insecticide than when the insecticides were used on their own (Anderson *et al.*, 1989).

1.6.3.3. Entomopathogenic nematodes (EPN)

Entomopathogenic nematodes can serve as alternatives to broad-spectrum chemical insecticides. Their advantages include their safety for humans and other non-target organisms, reduction of pesticide residues in food, preservation of other natural enemies and increased biodiversity in managed ecosystems (Grewal *et al.*, 2001)

Entomopathogenic nematodes (EPN) are approximately 1 to 3mm in size. These parasitic roundworms are most efficient in their control of soil-inhabiting insect pests. Biologically, they exhibit simple life cycles, developing from an egg through four larval stages to the adult phase. They originate from the Phylum Nematoda and are differentiated into two primary genera, namely *Steinernema* (family: Steinernematidae) and *Heterorhabditis* (family: Heterorhabditidae) (Mwamburi *et al.*, 2009).

These nematodes have a symbiotic relationship with bacteria that exist in the nematodes' alimentary tracts. Specific bacteria have evolved to form relationships with specific nematodes species (Ciche *et al.*, 2006). An example is the symbiosis between the nematode

Steinernema carpocapsae and the bacterial species *Xenorhabdis nematophilis* (Kaya *et al.*, 1993).

1.6.3.3.1 Mode of action of entomopathogenic nematodes

The nematodes enter the insect's body where pathogenesis occurs relatively quickly due to the bacteria inside the nematodes' tract. Bacteria utilize nematodes as a mode of transport inside the insects haemocoel, where pathogenesis occurs (Ciche *et al.*, 2006). The nematode retains the bacterium in its intestine until it regurgitates the bacterium once inside the haemocoel of the host insect (Ciche *et al.*, 2003). The bacteria quickly germinate and colonize the insect, with pathogenesis occurring within 1 to 3 days (Ciche *et al.*, 2006).
Different routes of entry are used by the entomopathogenic nematodes according to the sex of the host insect. After 2 hours of exposure of female house flies to nematodes, the nematodes were found to enter through the cloaca, along the oviduct and then the ovaries. In male house flies, the nematodes entered the cloaca and then moved through the wall of the ejaculatory tract and finally into the haemocoel. All larvae were found to have been penetrated via the anus. Male nematodes only penetrate the larvae after 10 female nematodes have successfully paralyzed the larvae (Renn, 1998).

1.6.3.3.2 Entomopathogenic nematodes as a possible biological control agent

Nematodes are found in a vast array of ecological niches including forestry, grasslands, oceans and deserts (Hominick *et al.*, 1996). The efficacy of entomopathogenic nematodes as an alternative to chemical control was tested by Renn (1998b). Two known entomopathogenic nematodes, namely *Steinernema feltiae* and *Heterorhabditis megidis*, were tested against a well-known pesticide formulation (methomyl) on house flies in pig farrowing pens in the U.K. It was found that there were significantly fewer house fly counts in farrowing pens sprayed with the nematode species than in those sprayed with the commercial bait methomyl (Renn, 1998b). The potential in commercializing EPNs as biological control agents rests in the fact that they are safe to non-target organisms, easy to apply, safe to the environment and are compatible with most agricultural chemicals (Kaya *et al.*, 1993).

1.6.3.4. Natural enemies of the house fly

In a study by Legner and Dietrick (1989) on ten poultry farms around California, a significant negative correlation was found between the numbers of adult predatory insects and their prey's population size. Examples of predator species are two flies known as *Ophyra aenescens* (Wiedemann) and *Muscina stabulans* (Fallen). These two species are known to attack *Musca domestica* L., *Fannia femoralis* (Stein) and *F. canicularis* (L) (Legner *et al.*, 1989).

Another major predator of the common house fly larvae is the wasp parasitoid *Muscidifurax raptor* (Ruiu, L., *et al*., 2007). Other known parasitoid species are *Spalangia cameroni* Perkins, *Spalangia endius* Walker, *Spalangia gemina* Boucek, and *Dirhinus himalayanus* (Masi) (Geden, 1999). The parasitoid wasp lays her eggs in manure where the larvae grow and eat house fly eggs. Geden (1999) tested the effect of different moisture levels of chicken manure – (45, 55, 65, 75, and 85%) - on the production of a parasitoid wasp, *M. raptor*. The preferred moisture was 75% or less. However, the result of the experiment also suggested that multiple species released under heterozygous moisture conditions are far more effective than the release of a single parasitoid (Geden, 1999).

Ruiu *et al*. (2007) fed three toxic bacterial strains to house flies, in order to test their effects on the parasitoid wasp *M. raptor*. These three bacterial strains were *B. thuringiensis* subsp. *kurstaki* (*Btk*) strain HD1, *B. thuringiensis* subsp. *israelensis* (*Bti*) strain ONR60A, and *Brevibacillus laterosporus*. A high concentration of each bacteria was fed (>10^9 spores/g of diet). *Bti* was the most toxic bacterium to the wasp. *Bti* reduced reproductive potential and caused significant mortality in the adult phase, and caused significant losses and reduced adult emergence in the larval phase (Ruiu *et al.*, 2007).

1.7 Summary

There is little literature covering the biological control of flies in the environment of horses, despite a move to "greener living" and more demand for environmentally friendly products. Horse riding is an expensive recreational sport in which substantial expenditure is spent on feed, facilities, veterinary costs, transport costs, and breeding. The health and peak performance of horses is of utmost importance, so there is a clear need for effective, affordable biological control agents of house flies in their stabled environment. The objective of this study was to quantify the demand for biological control; to assess the effects of feeding increasing concentrations of *Bti* in the feed; and to assess the effect of spraying either *Bti* or *Bb* directly on to the manure; and to analyse, by *Bt* isolation, the amount of *Bti* that survives after digestion in the horse's tract.

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CHAPTER 2: PERCEPTIONS OF HORSE OWNERS ON THE EFFECT OF HOUSE FLIES IN AN EQUINE ENVIRONMENT IN KWAZULU-NATAL

Abstract

There is very little information in the literature regarding the use of biological control of house flies in the stable yard. Despite a lack of research in this field, house flies are presumed to be an important threat to horses because these pests occur in large numbers and horse owners spend heavily to try to control the flies. A questionnaire was prepared to assess the response of horse owners to the idea of using biological control on house flies in a horse environment. The questionnaire was constructed to support research into the feeding of *Bacillus thuringiensis israelensis* to horses. The main information captured included the perceived importance of house flies as a nuisance pest, the current methods of fly control in and around KwaZulu–Natal and the horse owners' perceptions of biological control agents. Overall three categories of house fly control were found: chemical, physical and biological. Physical control methods predominated. Most horse owners (97%) were agreeable to the idea of trying methods of biological control, with over 74% describing themselves as environmentally aware, to varying degrees. Most horse owners (83%) would prefer to apply biological control agents against flies in the horse feed rather than by spraying the products on to the horse, or in and around the stable environment. Around 80% of owners stables some or all of their horses, all or some of the time. The survey results supported the need for research leading to the development of biological control agents to control house flies in the environment of horses.

2.1 Introduction

Arthropod pest problems are associated with man-made livestock systems, which have evolved into two types of farming; either intensive (indoor) farming or extensive (outdoor) farming. Intensive or constricted farming is often associated with the problem of disposing of

manure. Either type of farming holds farm owners responsible for manure management and disposal. Examples of intensive farming systems where manure deposition is a problem are poultry houses, piggeries and stables. Thousands of tons of manure are available to be utilized as fertilizer. However, the supply of manure exceeds the area of land that would be needed to dispose of it (Anonymous 2, 2010). In addition to negative outcomes such as nitrate leaching into soils, these manure piles are attractive breeding and feeding sites for house flies *Musca domestica* (L.) and stable flies *Stomoxys calcitrans* (L.).

House flies are known as universal pests to both humans and livestock in urban and farming communities (Axtell, 1986). Their high fecundity and biotic potential makes them economically important in enclosed livestock environments (Du Rand, 2009). Farm owners spend heavily on pesticides to reduce the house fly population. However, many house flies have evolved resistance against the organophosphate insecticides used in controlling house flies (Kellstedt *et al.*, 2008). Livestock owners are therefore forced to use a range of high cost methods in order to reduce their house fly populations.

Horse owners are in a competitive, cosmopolitan business in which their horses are rated by their aesthetic appearance as well as their performance. A premium is placed on the horse's overall appearance. House flies are commonly known as filth flies (Levine *et al.*, 1991) and are generally related to poor, dirty conditions. The removal of house flies in the equine environment is therefore highly desirable and owners go to great trouble in order to remove them. Due to a dearth of information on the use of biological control in the equine world, a study was conducted to establish the relationship between horse owners and the arthropod pest, the house fly.

2.2 Materials and methods

Five regional areas in and around Pietermaritzburg, South Africa, were chosen for the distribution of a questionnaire. In total, fifteen smaller areas were analyzed. At least two horse owners from each area were interviewed. Ethical clearance was obtained from the Ethical Clearance Committee of the University of KwaZulu-Natal (Attachment 1).

A questionnaire was constructed using a similar design to that employed by Dutton *et al*. (2003). Qualitative and quantitative answers were solicited. Indirect questions were asked using a 1 to 5 scale scoring system. Horse owners were contacted either telephonically or in person. A minority of the questionnaires were answered by email by the owners.

The objective of this study was to find out if there is a market for biological control agents against house flies, and to determine the level of support for testing of *Beauveria bassiana (Bb)* and *Bacillus thuringiensis* var. *israelensis (Bti)* for the biological control of the house fly. Three specific objectives were targeted in the questionnaire:

1) To capture the current methods of fly control used in and around KwaZulu- Natal.
2) To understand the effect of house flies on horse owners - (i.e. nuisance factor, distress).
3) To determine horse owners' perceptions of biological control.

Candidates for the questionnaire were horse owners owning one or more horses, who knew about the types of fly control currently practiced and had at least 2 years' experience with horses.

The answers to 30 questionnaires were captured into a spreadsheet Excel (2007). The horse owners' answers were presented graphically as pie charts, using Microsoft Excel.

Data were analyzed using the Chi-square Test, to test whether data sets were independent or associated.

$$\chi^2 = \frac{(Observed - Expected)^2}{(Expected)}$$

2.3 Results

2.3.1. Methods of fly control currently used in KwaZulu- Natal by horse owners

Three types of fly control were practiced by horse owners - physical, chemical and biological (Figure 2.1). However, the majority of horse owners only used physical and chemical treatments to control house flies. Only 3% used biological control measures.

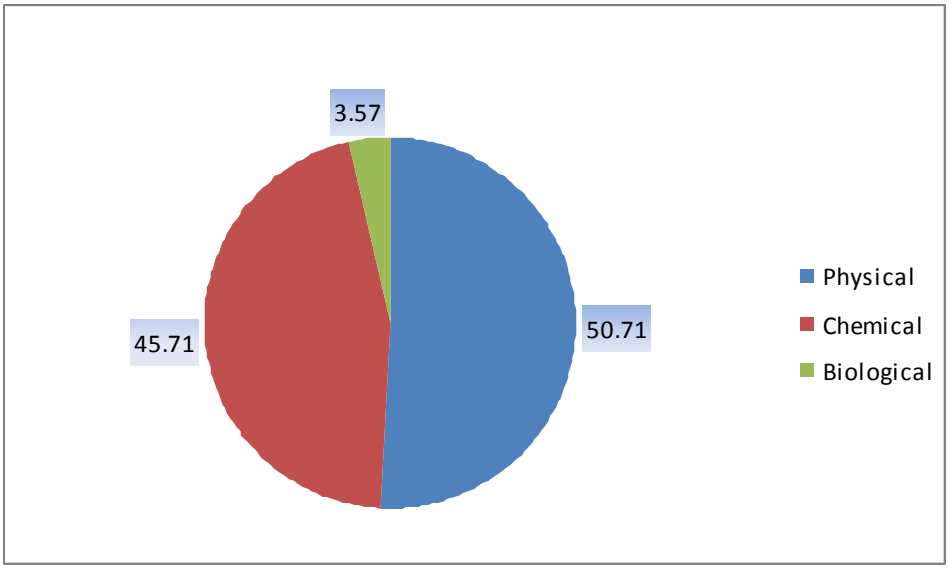

Figure 2.1: Methods of house fly control used by surveyed horse owners in KwaZulu-Natal

In Figure 2.2, the common methods of fly control practiced by horse owners in KwaZulu-Natal are captured. Some of the commonly used "physical" house fly control methods are fly swatters, fly masks and manure removal. Twelve percent of horse owners also used chemical control methods such as Agita®[1] (Novatis, 2011) which uses Thiamethoxam as an active ingredient (Novatis, 2011) (Figure 2.2).

2.3.2. The effect of manure removal on house fly populations

[1] **AGITA® 1 GB a** registered product brought in local stores such as Allison's, Pietermaritzburg

Most horse owners believed that there was a strong relationship between the efficient removal of manure from the stable and the control of house flies (χ^2 = 9.01, where the $\chi^2_{(3, 5\%)}$ = 7.81 for p-value is less than 0.05).

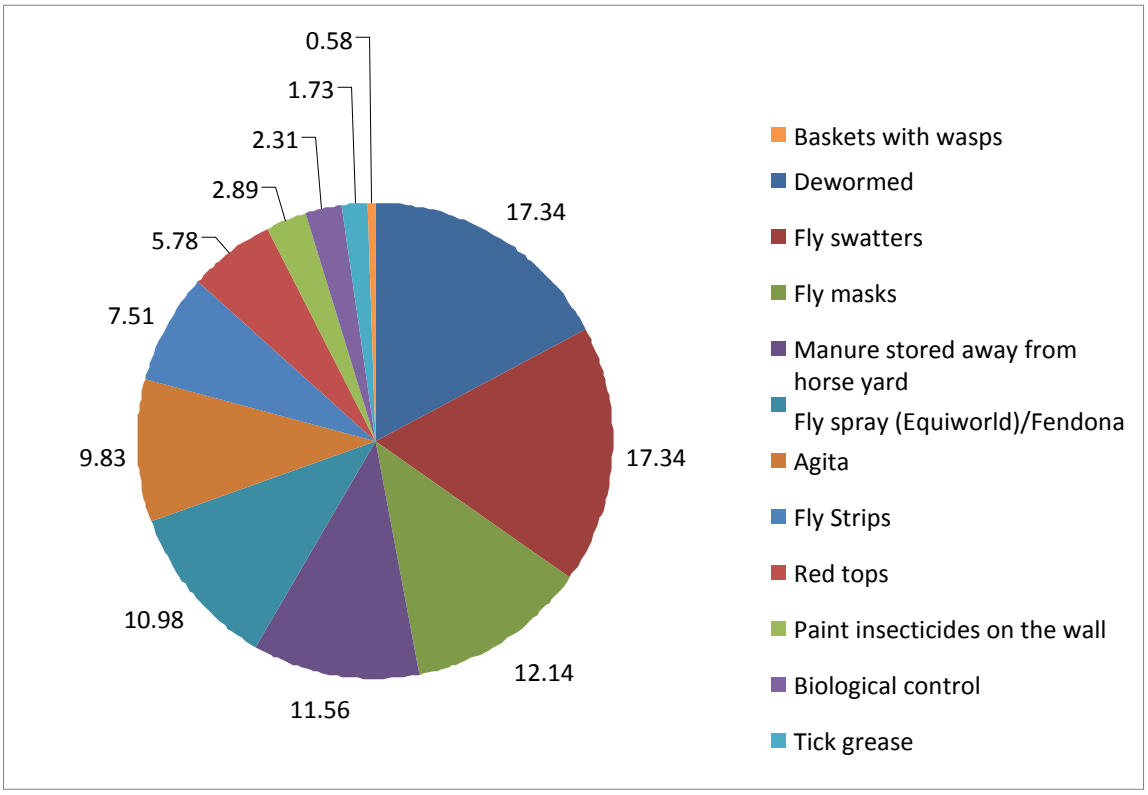

Figure 2.2: Current methods of house fly control used in KwaZulu–Natal

2.3.3. The number of horse owners stabling their horses.

This question is important in determining the potential market for biocontrol products which can be sprayed on to manure heaps, or in the stable itself. The size of this potential market will determine the direction of research into the integrated control of house flies in equine environments (Chapter 4). Over 70% of horse owners use stables to house their horses, some or all of the time (Figure 2.3). However, most horse owners did not consider the stabling of horses to be correlated with house fly problems (χ^2 = 0.641, which is less than $\chi^2_{(1,5)}$= 3.84 for p≤ 0.5).

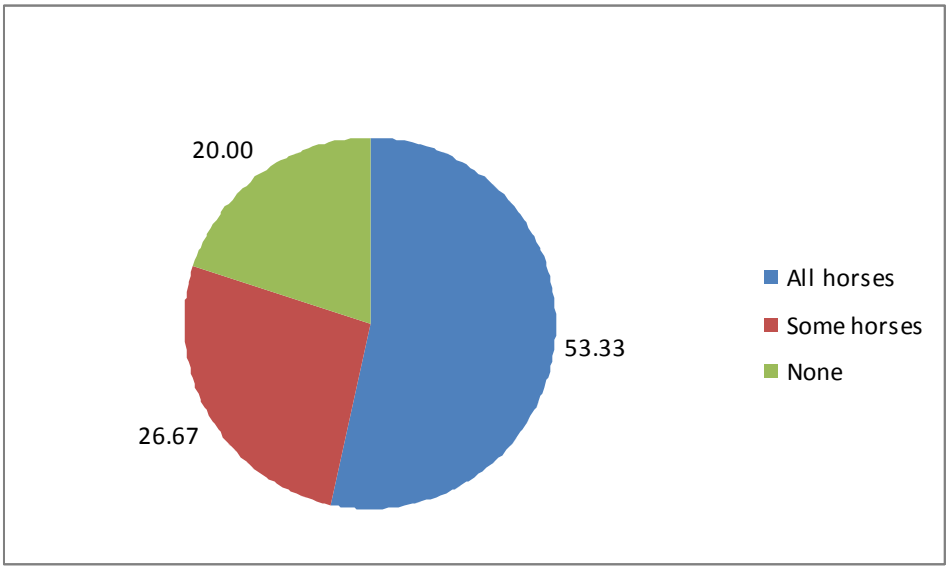

Figure 2.3: Percentage of horses stabled by survey respondents

2.3.4. The relative importance of house flies as pests of horse and horse owners

In South Africa, most of the research into the relationship between horses and insects has been focused on the *Culicoides* midge species because they transmit the deadly *African horse sickness* virus. There are no reports in the literature on the importance of house flies as nuisances or health threats in stable yards in South Africa Therefore, horse owners were asked to identify their top four nuisance pests and rank these pests in order of seriousness. House flies were considered to be the most serious nuisance pest (Figure 2.4), with more than 60% of respondents identifying flies as the top nuisance. Surprisingly, given the deadly nature of the virus they carry, midges were only ranked third in importance.

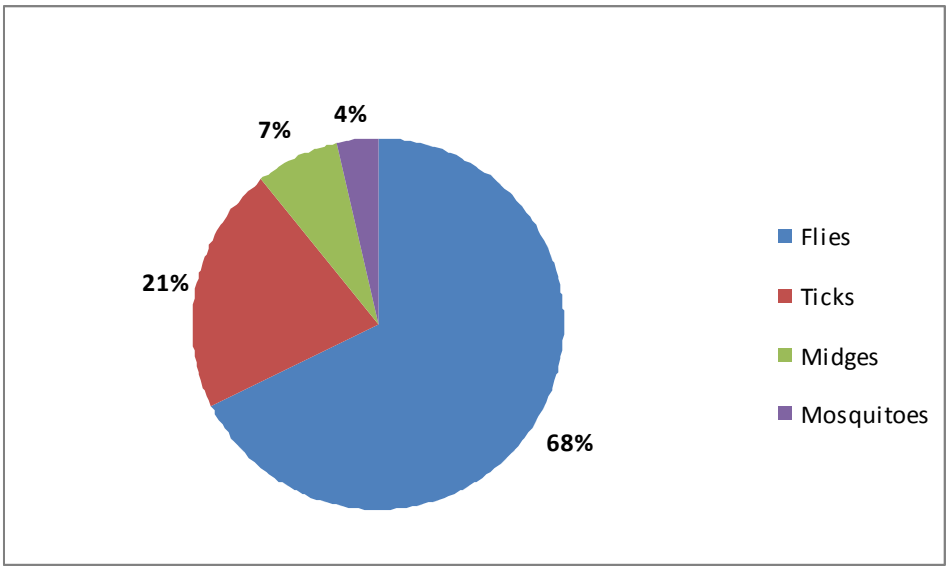

Figure 2.4: Number of respondents (% total) ranking flies, ticks, midges or mosquitoes as the worst arthropod nuisance around horses

Horse owners were also asked to rate the success of their own house fly control programmes. A minority (30%) were satisfied with their fly control measures. However, the majority (64%) felt that their fly control measures were inadequate and should be improved. A small number of respondents (3%) found the number of house flies around their horses to be unbearable and feel they need a drastic improvement in their control measures. Conversely, there was a small group of owners (3%) who report seeing flies only "now and then" (Figure 2.5).

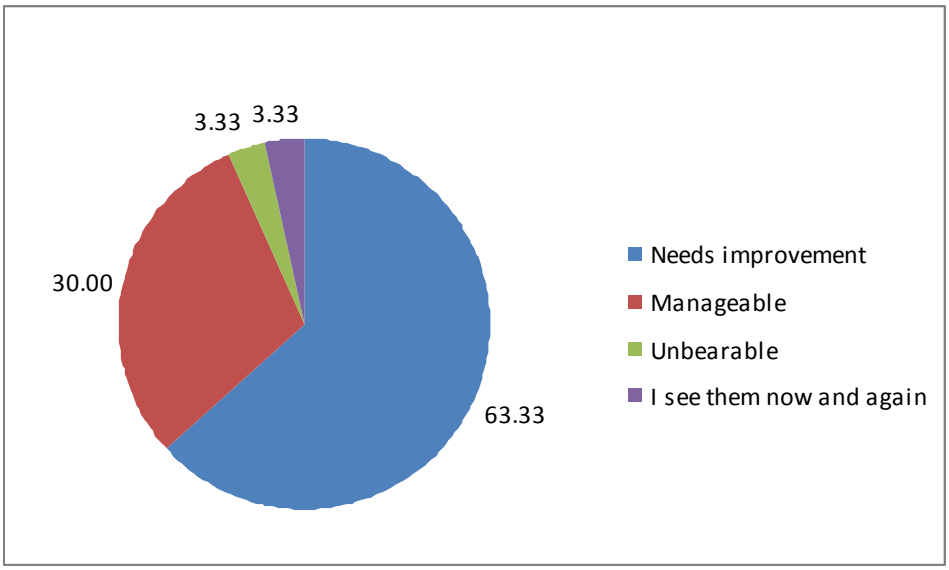

Figure 2.5: Number of respondents (%) rating the level of house fly control in their stable yards

2.3.5. Role of smell in enhancing the house fly problem

Horse owners are of the strong opinion that smelly stables contribute to a house fly problem. The results suggest that there is a perceived association between the smell of the environment and the house fly pest problem in an environment (χ^2 = 26.77, with a $\chi^2_{(3, 5)}$ = 7.81 for $p \leq 0.05$).

2.3.6. Other factors affecting house fly populations

Horse owners believe that contributing factors affecting the fly population in KwaZulu-Natal may include nearby piggeries, dairies, chicken houses and sewage-holding facilities (31%) (χ^2= 8.14, which is greater than $\chi^2_{(3, 5\%)}$ = 7.81 for $p \leq 0.05$; Figure 2.6).

Most horse owners did not think that feeding high protein concentrates to horses inside the stables contributed to large house fly populations The calculated chi-squared was as low as 0.01, where the $\chi^2_{(1,5\%)}$= 3.84.

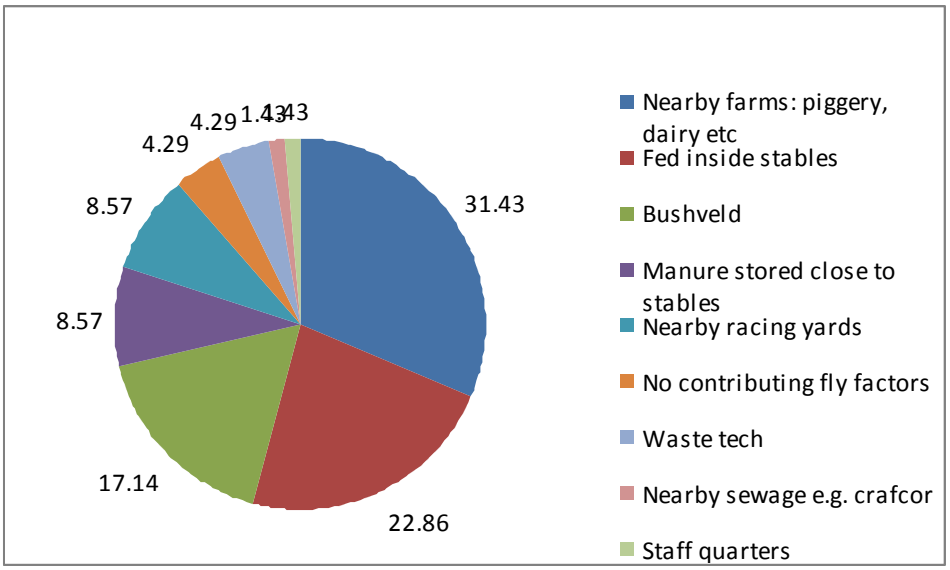

Figure 2.6: Percentage of respondents identifying contributing factors to house fly infestations

2.3.7. Horse owners' perceptions of their own environmental consciousness

The majority of horse owners were supportive of environmentally friendly fly control measures (Figure 2.7). Forty percent of horse owners considered themselves to be "environmentally friendly" and would like access to biocontrol agents in order to mix them with chemical controls. A further (32%) rated themselves as "tree-huggers"; i.e., they were keen to introduce environmentally friendly means of fly control and would prefer to use biological control products exclusively (Figure 2.7).

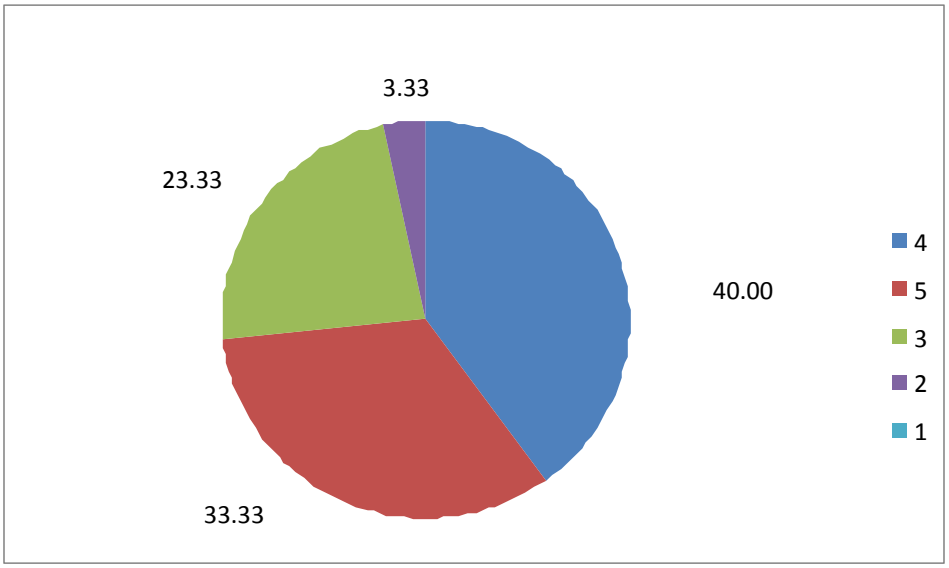

Figure 2.7: Self-assessment score (1 – 5) by horse owners of their own environmental consciousness

2.3.8. Horse owners desire to use biological control agents for fly control

Horse owners were asked whether they preferred biological control or chemical pesticides. Figure 2.8 illustrates that 97 % of horse owners are "pro"-biological control and would like to have access to effective biocontrol agents for fly control.

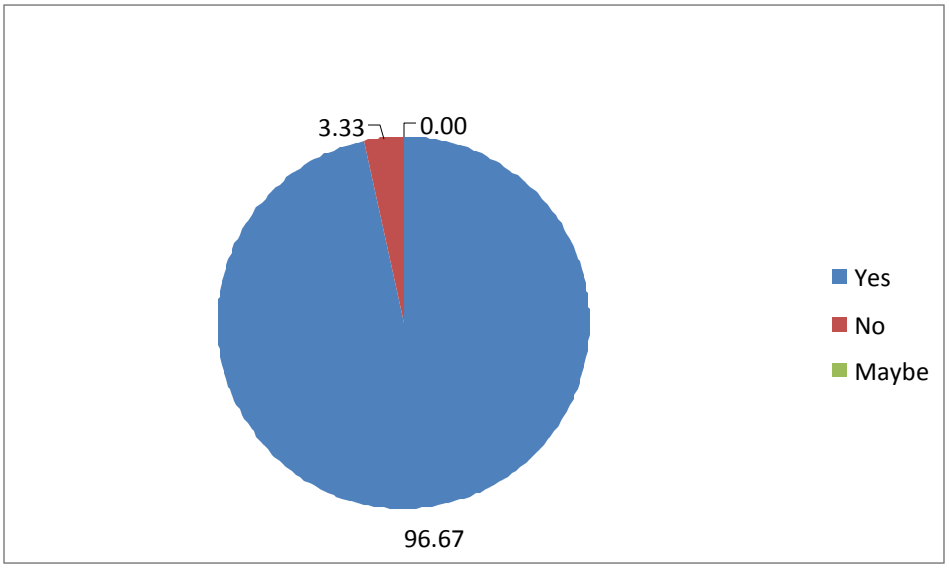

Figure 2.8: Percentage of horse owners who expressed a desire to use biological control agents

2.3.9. Application of biocontrol agents for fly control.

In order to design an appropriate method of biological control application, it would be useful to assess how horse owners would like a biocontrol agent (such as the fungus (*Bb*) or the bacterium (*Bti*)) to be applied. The majority of horse owners (83%) would prefer to feed the biological control agent in the horse feed, while 17% would prefer to spray the product on to the animal or around the environment (Figure 2.9).

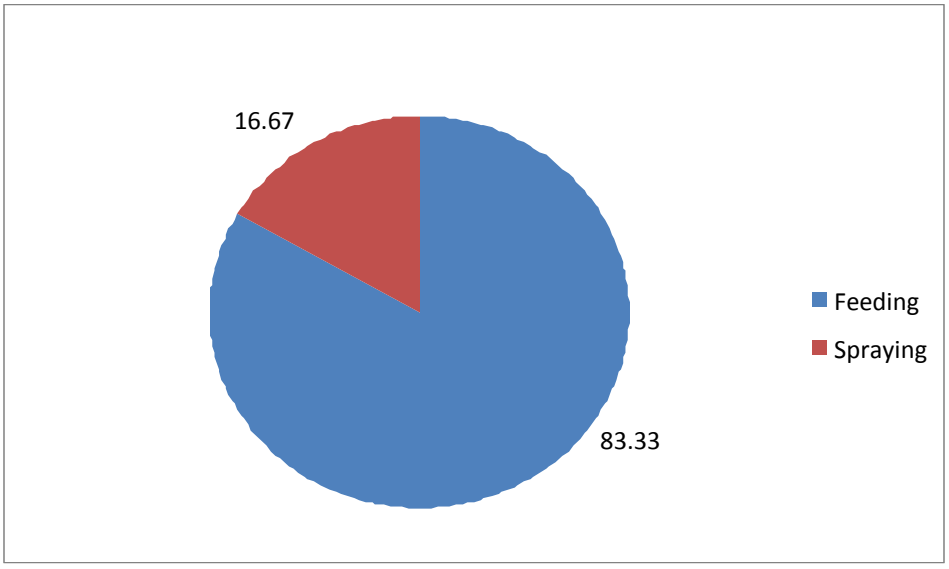

Figure 2.9: Percentage of respondents preferring to feed biological control agents or spray the agents on to the animal or surrounding environment.

2.4 Discussion

Types of fly control can be broadly classified into three categories; namely biological, chemical and physical control (Figure 2.1). It can be argued that there is a degree of overlap between these categories. For example, the use of diatomaceous earth in the feed could be considered a chemical control but, for the purposes of this study, it was considered a "biological" control, as the earth is an inert, environmentally friendly product. According to horse owners, chemical control of horse flies centres on the use of any form of pesticide or insecticide. Physical control involves using different non-chemical management procedures

to control flies, such as removing horse manure from the stable or using fly masks. “Biological control”, according to horse owners surveyed in this study, involves the use of non-chemical products such as utilizing predator-prey relationships, herbs or environmentally friendly products. Some examples of biological control used in KwaZulu-Natal are garlic (in the feed), wasps (against fly pupae) and diatomaceous earth (Rigaux *et al.*, 2001).

Of the fly control methods presented in Figure 2.2, the majority are physical in nature (51%; including the use of fly swats, masks and removal of horse manure from the stabled environment). This may be because successes in controlling house flies are passed down from generation to generation and these strategies may have worked previously (Hofte *et al.*, 1989). However, Figure 2.5 shows that 64% of horse owners were not satisfied with their control measures, and were looking for novel approaches to improve house fly control. This is reflected in Figure 2.8, which shows that 97% of horse owners were interested in testing biological control agents.

Figure 2.3 illustrates the percentage of horse owners that stable their horses. Spraying the stables and the bedding would reduce the number of house flies (Indrasith *et al.*, 1992). In this study, 80% of the owners stabled all or some of their horses, all or some of the time. Designing a product that could be sprayed into the stable environment could conceivably appeal to the 80% of horse owners making use of stabling.

Other factors, besides horse manure, may contribute to the house fly problem in a stable yard. Thirty one percent of horse owners felt that their neighbouring farms may contribute to their house fly populations. House flies are attracted to malodorous smells and are therefore encouraged to breed in areas where there are piggeries and poultry houses (Quinn *et al.*, 2007). Most owners do not realize the association between high levels of protein in the feed and the size of fly populations.

Only 3% of horse owners currently use environmentally friendly products (such as diatomaceous earth and garlic). However, most horse owners were receptive to the use of biological control (Figure 2.8) and 74% would describe themselves as environmentally aware or as "tree-huggers" (Figure 2.7).

Many farmers and livestock owners have historically been committed to pesticides for pest control. However, the development of resistance to pesticides has compromised their confidence in chemical control and farmers in general, and horse owners in particular, are looking for alternative methods of pest control (Dutton *et al.*, 2005). Ninety seven percent of horse owners were "pro"-biological control (Figure 2.8), which indicates a large potential market for biological control products. The widely-held view of manufacturers that farmers and horse owners are not interested in biological control is clearly a myth.

Figure 2.4 shows that house flies were considered to be the worst pests affecting horses. When questioned about *Culicoides* midges, the majority of horse owners felt that their control of the midge was sufficient and they did not consider them to be an important pest. Although house flies have been considered pests for many centuries (Axtell, 1986), no fully effective control measure has been developed. This may be because house flies are not a major vector threat (Strong, 1993); because of development of resistance to pesticides (Liu, 2000); or because of incorrect management procedures (Indrasith *et al.*, 1992). Whatever the reason, no 100% effective house fly control measure has been found to date. The findings of this survey may assist in promoting research into the biological control of house flies.

There are a number of different ways to administer or apply biological control agents, depending on the pest and the environment (Abate *et al.*, 2000). Figure 2.9 illustrates that 83% of horse owners would prefer feed add-ins, while 17% said that they would prefer to use a spray. Therefore, the experimental work reported in this thesis focuses on finding effective feeding and spraying concentrations to control house flies.

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CHAPTER 3: CONTROL OF HOUSE FLIES (*MUSCA DOMESTICA* L) USING *BACILLUS THURINGIENSIS* VAR. *ISRAELENSIS* WITH BRAN MEAL AS A FEED SUPPLEMENT IN STABLED HORSES

Abstract

Biological control of pests utilises living organisms *in lieu* of potentially harmful chemical products such as pesticides. Two experiments were performed to evaluate the control of house flies in the stables of six miniature horses, using the bacterium *Bacillus thuringiensis* Berliner var. *israelensis* (*Bti*) mixed with bran. This was fed to the horses in a two stage observational trial, with a range of concentrations, namely 0, 0.125, 0.25, 0.5, 0.75, and 1.0 g/meal in Trial 3a, and 0, 0.5, 1, 2, 4 and 8g/meal in Trial 3b. The horses were allocated to individual stables, as in a normal stable yard. Two faeces samples per horse were collected three times a week for 11 weeks and placed in incubation trays, to allow the number of adult house flies and closed pupae to be counted. Random samples of manure were taken to quantify the output faecal levels of *Bti* through a *Bt* isolation process. Although no significant difference in the number of adult flies hatching on faecal samples was found, a significant increase in the number of closed pupae was found as the concentration of *Bti* increased in Trial 3a. The regression equation suggests that there would be 3.1 times as many closed pupae when horses are fed 1 g of *Bti* in their feed, than when horses are fed no *Bti*. Growth of *Bt* colonies on the horse manure was found after *Bt* isolation, indicating that *Bt* does indeed survive passage through the horse's digestive tract. These two results provide a starting point for future research into the use of *Bacillus thuringiensis* var. *israelensis* as a biocontrol agent against house flies in stable yards.

3.1 Introduction

Research conducted on species-specific strains of *Bacillus thuringiensis* to control arthropod pests in livestock (Table 3.1). *Bacillus thuringiensis* Berliner var. *israelensis* (*Bti*) is specific

to diphtheria, including house flies, and has been fed to and applied to many invertebrates (Blaustein *et al.*, 1991), micro-invertebrates (Hershey *et al.*, 1998), and other targeted insects such as mosquitoes (Merritt *et al.*, 1992) and black flies (Wipfli *et al.*, 1994) as a larvicide. However, as Table 3.1 illustrates, there is a dearth of information regarding the feeding of the *Bti* strain to horses (Gough *et al.*, 2005).

Table 3.1: Pests controlled by *Bt strains* in livestock and poultry (Mwamburi *et al.*, 2009)

Known strain of *Bacillus thuringiensis*	**Targeted pest**	**Application method**	**Result**	**References:**
Bt. in sheep	Effect of *Bt*	Oral	*Bt* has no adverse effects on sheep	(Hadley *et al.*, 1987)
Bt. subsp. *kurstaki* strain WB3S16	Louse in sheep (*Bovicola ovis*)	Oral	Louse death occurred 8 – 12h post feeding.	(Hill *et al.*, 1998)
Bt	Effects on mammalian tissue	Oral, pulmonary and intravenous	Non-toxic to the mammalian tissues	(McClintock *et al.*, 1995)
Multiple isolates of *Bt*	*Lucilia cuprina* (sheep blowfly)	Oral	All isolates were toxic to feeding larvae in *in vitro* and *in vivo* trials on sheep	(Gough *et al.*, 2005)
Bt. in cattle	Horn fly *Haematobia irritans* (Linnaeus1758) (Diptera Muscidae) in cattle	Oral	Potential harm against the blowfly – probable 9 isolates toxic to larvae	((Oyarzun *et al*., 2008)

Mwamburi *et al.* (2009) used *Bti* as a larvicide for house flies in poultry housing by feeding it to chickens in the feed concentrate. The two treatments tested by these researchers were a known biological control strain of *Bti* (Howarth, 1991*)* and a commercial agrochemical known as Larvadex®[3] (an insect growth regulator). The *Bti* was fed to the birds with wheat bran. Wheat bran is a suitable medium for dispensing large quantities of *Bti* in feed. It is inexpensive, available locally and it does not compromise the sporulation, toxicity and biomass activity of *Bti* (West, 1951). Mwamburi *et al*. (2009) found that *Bti* was effective

against house fly larvae when applied at 250 mg/kg or 500 mg/kg of chicken feed. Larvadex®[2] also significantly reduced larval density and numbers of adult house flies. By the end of the 6-week trial, larval to adult emergence at 250 mg and 500 mg/kg of *Bti*, and Larvadex® were 56 and 66% for *Bti* and 57 and 67% for Larvadex®, respectively. Despite these promising results, no similar trials have been conducted on horses.

Horses are non-ruminant herbivores, specifically hind gut fermenters (Rechkemmer *et al.*, 1988). Domestication of the horse has affected the times available for grazing and therefore its food intake behaviour. The physical demands of show jumping, racing, and daily riding have necessitated higher energy and protein intakes (Tinker *et al.*, 1997) causing higher nutrient residues in horse manure (McGreevy *et al.*, 1995). This may be one of the main causes of the large numbers of house flies in horse yards. Excessive nutrient residues can result in behavioral changes such as weaving, wind-sucking (McGreevy *et al.*, 1995) and coprophagy (Ellis *et al.*, 2005). Rate of passage of food in a horse varies with diet, exercise and health status (Ellis *et al.*, 2005). Their digestive system combines the function of a short monogastric foregut, together with bacterial fermentation in an extended caecum and colon. This allows for utilization of both high value foods and low value forages. In the large caecum and dual chamber colon, digestion of cellulose and hemicelluloses fibres is facilitated by colonies of micro-organisms (Bradley, 1981). In one study, a passage rate marker of chromic oxide was present in the manure after five days when mixed with diets of lucerne alone or lucerne and timothy hay, each supplemented with oats, maize or barley (Vander Noot *et al.*, 1967). In this study, the survival of *Bti* in the horse's tract after bacterial fermentation, coprophagy and a five day retention time, was tested by counting *Bt* colonies of samples of manure from the treated horses.

Bacillus thuringiensis (*Bt*) Berliner is a gram positive spore forming bacterium that is known for its bio-insecticidal characteristics (Khachatourians, 1991). It has the ability to produce insecticidal endotoxins in the form of parasporal bodies (crystals) (Indrasith *et al.*, 1992). There are many factors that may affect the survival of *Bti* in the digestive tract of the horse. Diet influences the pH in the stomach which may affect the survival of *Bti*, with a pH of 4.5

[2] Larvadex® -

found in high fibre diets and of 5.1 in high starch diets (Julliand *et al.*, 2001; Varloud *et al.*, 2007). Increased volatile fatty acids and lactic acid ensiled by feeds cause a low pH (pH 5 to 5.5) in the hindgut of the horse (Kern *et al.*, 1974; Murray *et al.*, 1996). The success of trials with chickens proved that *Bt* can survive the hostile conditions of the intestine of a monogastric (Mwamburi *et al.*, 2009). However, there appears to have been no prior research to test the survival of *Bt* after passage through the horse's gastrointestinal tract. Therefore post-sampling *Bt* isolation was performed on random samples of horse manure in this trial to test whether the bacillus can survive passage through the equine digestive tract.

In the equine environment, there are many areas which attract house flies, such as stable wear, horse bedding, and dead or rotting vegetable matter, such as manure compost areas (Service, 2000). These provide large breeding grounds for house flies. Interest in controlling these pests has been brought about by their nuisance factor, uncontrollable numbers, and their role as diseases vectors (Axtell, 1986).

House flies have evolved resistance against many pesticides (Liu, 2000). In contrast, *Bti* is a potential biological control agent that can be used as an alternative to pesticides in the equine environment (Howarth, 1991; Mwamburi *et al.*, 2009). In the current study, *Bacillus thuringiensis israelensis* (*Bti*), combined with bran, was fed to horses to determine if it could successfully control house flies. The second objective was to determine whether *Bti* fed to horses survived, as it can only be effective in the manure after passage through the horse gastrointestinal tract.

The hypotheses tested were that the inclusion of *Bti* in horse rations would reduce the amount of adult house flies and increase the number of closed `pupae in the stable environment in a dose-dependent manner. In addition, it was believed that *Bti* would survive passage through the equine gastrointestinal tract and that colonies of *Bti* would be visible in collected faeces samples.

3.2 Materials and Methods

3.2.1 Ethical clearance

Before the trial commenced, ethical clearance was received from the Animal Ethics Subcommittee of the UKZN Ethics Committee in November 2009 (Appendix 3.1). In addition, a veterinary check was undertaken to ensure that the 6 miniature horses were in satisfactory health to be used in the experiment. All the horses were dewormed and treated for ticks. The horses were fed an 11 % protein Maintenance Diet (Meadow Feeds) Bran meal treated with *Bti* was obtained from Plant Health Products (PHP)[#4] (Pty) Ltd, Nottingham Road, South Africa.

Six miniature horses were stabled at the Ukulinga Research Farm, KwaZulu-Natal, Pietermaritzburg Four females (3 adult females and one yearling) and two males (a stallion and a gelding) were used. Body weights ranged from 150 to 350 kg and their ages ranged from 1.5 to 6 years. A general stable management procedure was followed, with stabling of the horses overnight, and release of the horses on to pastures during the day (Evans, 1989; Groves *et al.*, 2000).

3.3 Horse management

The horses were fed at 07.30 before they were led out to pasture, and then fed at 15.00 as they were stabled. Each day, the 6 miniature horses were stabled for 15 hours and were on pasture for 9 hours. Over-night, the horses were supplied with water and eragrostis hay *ad libitum*. The 6 miniature horses had previously been eating off bushveld and kikuyu pastures and therefore they were given 7 days to adjust to the new diets.

[#4] Plant Health Products (Pty) Ltd. P.O. Box 207, Nottingham Road, South Africa

3.4 Experimental procedure

Six concentrations in each experiment were used in Trial 3a and Trial 3b. In Trial 3a, the six horses were provided with bran containing *Bti* at levels of 0 (control); 0.125, 0.25, 0.5, 0.75 and 1g /meal. In Trial 3b, the horses were fed 0 (control); 0.5, 1, 2, 4 and 8 g/meal. These treatments were allocated at random to the horses. The treatments in Trial 3a were fed for 6 weeks and in Trial 3b for 3 weeks.

Stables were colour coded and cleaned every day, and the manure- and urine-saturated piles from each stable were placed into colour coded bins. These bins were designed to represent dung skips and were placed 10 m away from the stables, in direct sunlight. Dung skips are usually found within 30 m of the stables and are one of the strongest attractants of house flies to a stable yard (Coffey, 1966).

The bins encouraged high temperatures which enhances the growth of house fly maggots. Two 500 g samples of manure from each bin were collected every Monday, Wednesday and Friday at 14.00 for the duration of each experiment (2 x 6 x 3 = 36 samples every week). Each sample was placed in a clear rectangular plastic container. Each container was covered with a perforated nylon sheet to ensure that light and air could flow into them. They were then placed inside a temperature- and humidity-moderated glasshouse. Temperature and humidity was recorded at 30 min intervals. Temperature and humidity ranged from 23°C – 47°C and 23% RH – 50% RH (Figure 3.1 and Figure 3.2).

The manure containers were housed in the glasshouse for 21 days. On every second day the trays were sprayed with water to soften the organic matter and to provide water for the house flies. After 21 days, the trays were then placed inside a freezer (-0ºC) to kill any house flies. The period of 21 days was enough time for a full life cycle of flies to complete from collection date. *Bti* growth was also reduced during freezing post 21 days.

3.5 House fly and pupal counts

The trays were kept in the freezer for three days and then thawed for two days. The perforated nylon screen was cut and the number of house flies and pupae were counted using dissecting tweezers. Random samples of the manure were taken to determine the amount of *Bti* present in the manure.

3.6 Statistical analysis

It is always difficult to obtain sufficient numbers of horses for equine research purposes. In this trial, only six animals were used to test six treatments, so there could be no replication of treatment effect, without using a complicated, extended experimental design. In addition, the animals were of different age, sex and physiological state. Collecting two samples from each horse is also not a true replication of treatment effect. However, this trial was, by necessity an "observational experiment". Unreplicated experiments may be termed "observational experiments" but are a legitimate experimental design to be used under specific conditions (Rayner, 1967). Experiments of this nature are intended to provide answers through observation rather than through statistical analysis and are appropriate in situations where the researcher has no documented evidence or peer experience to draw on in deciding on test levels for a ground-breaking experiment. In this trial, there were no previous scientific papers available on the use of *Bti* as a biocontrol agent in horses, and therefore there was no information about appropriate the doses to work with. An observational experiment, as conducted here, was thus appropriate as a preliminary step to identify dosage levels which showed the most promise, which would allow the researcher to suggest suitable ranges for future research, to be conducted with a narrower range of doses, allowing for replication of treatments (Rayner, 1967). With only dosage levels in poultry as a guideline, the experiments reported here were the first steps in identifying the optimum dietary dosages of *Bti* for the control of the common housefly in the equine environment

Once a narrower range of dosages has been identified through an observational experiment, future experiments would need greater levels of replication. A larger number of animals

would have to be sourced, or, with the six animals available, each treatment (*Bti* dosage level) would need to be applied to each animal sequentially over time, in repeated experiments, and analyzed appropriately. The Results and Discussion below should be read with these statistical considerations and limitations in mind.

The area under pest curve (AUPC) was calculated and compared between treatments, with time (days) on the x-axis and either number of adult house flies or closed pupae on the y-axis. This analysis results in a single value (e.g. number of adult house flies) for each treatment level. Differences between the treatment means were tested using the Studentized Fisher's LSD unprotected test at $\alpha = 0.05$. Data were transformed into natural log (Ln) where the coefficient of variation (CV) was too high. The CV% was too high in both Trials 1a and 1b. Significant results were analyzed with linear regression (number of house flies/pupae (y-axis) against *Bti* concentration (x-axis), as proposed by Sokal and Rohlf (1995) to test for any trends.

3.7 Results

The range of temperatures recorded in the University of KwaZulu-Natal glasshouses were recorded from the 13th January – 19th February 2010 is given in Figures 3.1 and 3.2. Temperatures and humidity ranges were appropriate to support adult house fly growth.

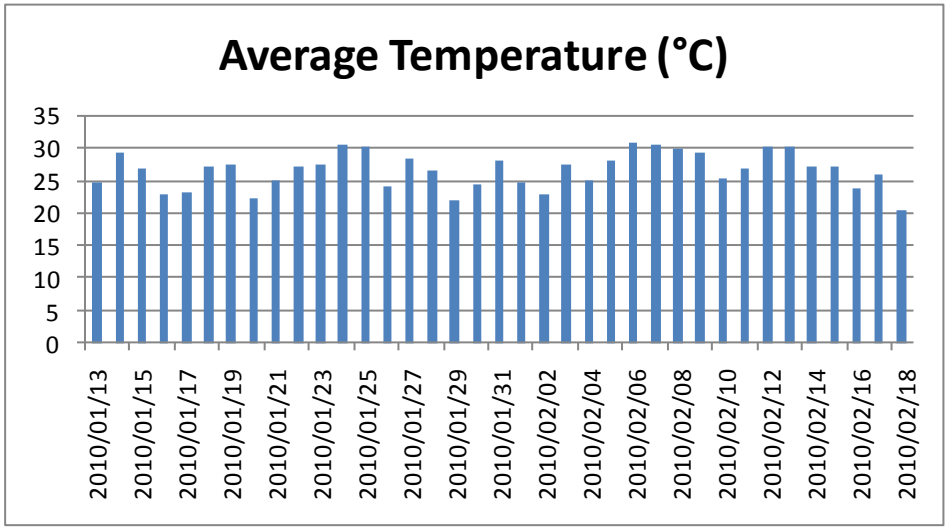

Figure 3.1: Mean temperature range in Glasshouse 2 and 4, Controlled Environment Facility, UKZN for the period 13th January to 18th February 2010

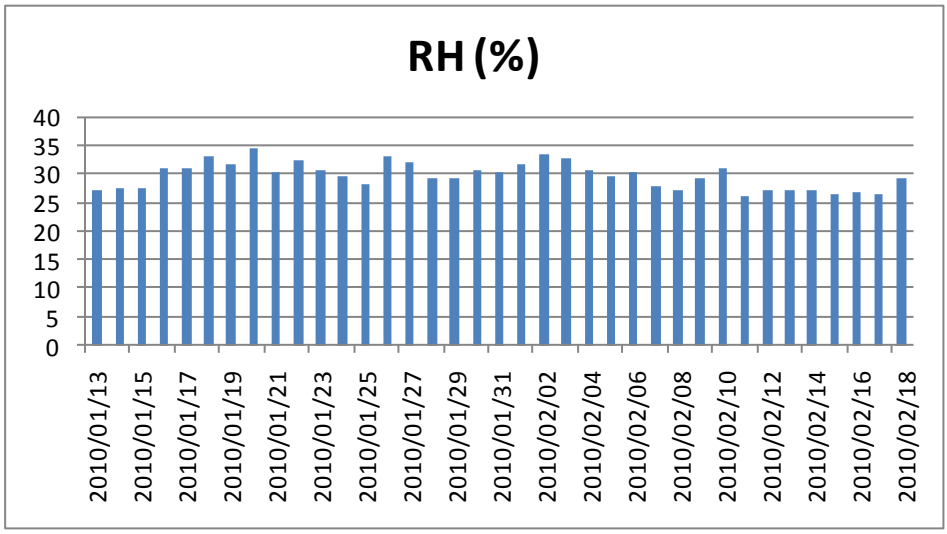

Figure 3.2: Mean relative humidity range in Glasshouse 2 and 4, Controlled Environment Facility, UKZN for the period 13th January to 18th February 2010

The cumulative numbers of adult house flies found in Trial 3a were evaluated using the AUPC values. With untransformed data, no significant difference was found in adult fly AUPC between the treatments in Trial 3a ($p > 0.05$). However the CV was 39 % and therefore the data were transformed using Ln before re-analysis. However, there was still no significant difference between treatment means for Ln (AUPC) for the number of adult flies ($p > 0.05$) (Table 3.2). Therefore, the data was not subjected to further analysis by linear regression.

Table 3.2: Trial 3a ANOVA of Ln Transformed AUPC (Area Under Pest Curve) values for adult fly counts

Treatments (g of/*Bti*/meal/day)	AUPC Natural Log
0.000	5.999
0.125	5.491
0.250	6.498
0.500	6.321
0.750	6.500
1.000	6.417
F Test value	2.41
P-value	0.098
CV %	8.46

In Trial 3a, numbers of closed pupae were counted at the different levels of *Bti* and were consolidated into single AUPC values. The CV was high at 32.8 %, therefore the Ln of the data was analyzed (Table 3.3). There were significant differences between treatment means in this analysis, so the data were subjected to linear regression analysis (Table 3.4). A regression analysis on the transformed data confirmed that the AUPC values for the number of closed pupae increased with an increase in *Bti* concentration, with an r^2 value of 0.368 (Figure 3.3). The regression equation suggests that there will be 3.1 times as many closed pupae (dead) when horses are fed 1 g of *Bti* than when horses are fed no *Bti* (169 at 0 g and 518 at 1g).

Table 3.3: Trial 3a – ANOVA of Ln Transformed AUPC (Area Under Pest Curve) values for counts of closed pupae (dead)

Treatments (g of /*Bti*/meal/day)	AUPC Natural Log
0.000	5.255
0.125	5.398
0.250	5.648
0.500	6.099
0.750	4.764
1.000	6.525
P-value	<0.001
CV%	5.1

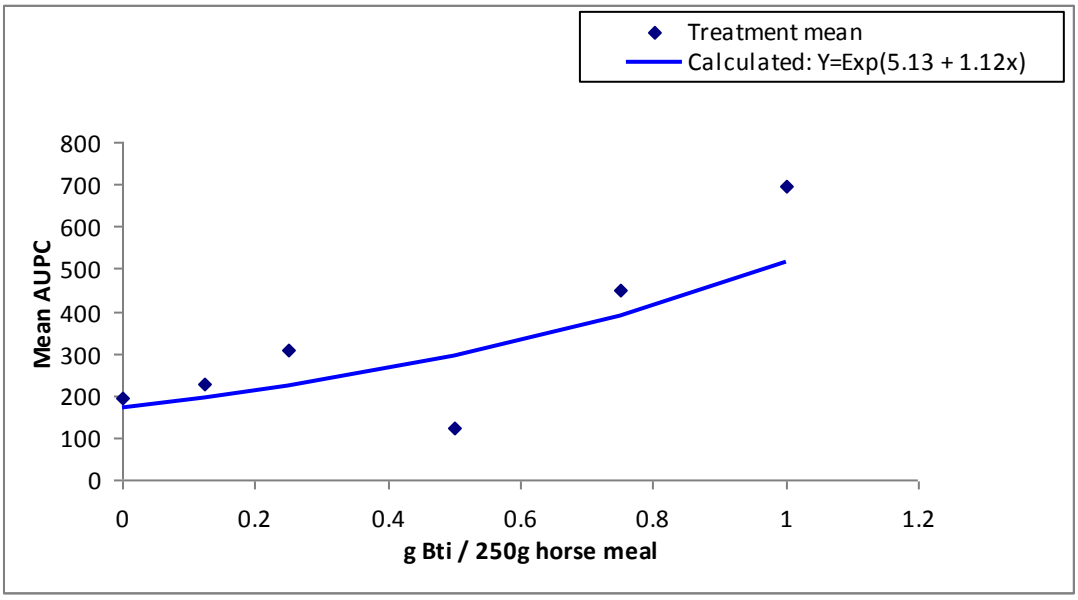

Figure 3.3: Trial 3a: - Regression (y = exp (5.13 + 1.12x) of AUPC (Area Under the Curve) Means for counts of closed pupae, with actual values shown

Table 3.4: Trial 3a - Linear Regression Table for Ln Transformed AUPC of Closed Pupae Counts

Pupae			
Treatments	Estimate	F test	P value
Dosage	5.13	10.9	0.005
Constant	1.12		
(r^2)	0.368		

Analysis of variance was conducted on the AUPC of the number of adult house flies found in Trial 3b but the F test was not significant ($p > 0.05$). However the CV% was high, so the ANOVA was run again on the Ln transformed AUPC value, but the F test remained non-significant for the Ln (AUPC) for the number of adult flies ($p> 0.05$) (Table 3.5). No significant biological conclusion could be drawn from the results and therefore the data was not analyzed using linear regression.

Table 3.5: Ln of the AUPC (Area under pest curve) of adult flies from Trial 3b

Treatments (g/*Bti*/meal/day)	Ln AUPC of adult fly counts
0.0	4.06
0.5	4.44
1.0	4.92
2.0	3.49
4.0	3.88
8.0	3.73
F value	
p-value	0.129
LSD	1.096
CV %	15.10

Analysis of variance was used to analyze the AUPC values of closed pupae counts in Trial 3b. Results were not significant at p-value = 0.824 ($p>0.005$) and the CV% (80.3) was too high for interpretation of the data. Therefore the natural log of the data was used (Table 3.6). The Ln AUPC results of closed pupae Trial 3b were non-significant $p = 0.984$ ($p>0.05$) (Table 3.6) and numbers of closed pupae were thus not affected by the levels of *Bti*.

Table 3.6: Ln (Area under pest curve) of closed pupae from Trial 3b

Treatments (g of/*Bti*/meal/day)	Ln of AUPC of Closed Pupae Counts
0.0	4.69
0.5	4.72
1.0	4.28
2.0	4.12
4.0	4.72
8.0	4.45
F value	
p-value	0.984
LSD	2.215
CV%	27.70

To test for further significance in Trial 3b, data was simultaneously tested for heterogeneous variances and equality of means. Both tests were found to be not significant. Non-parametric methods such as Friedman and Kruskal-Wallis which rely on chi-square approximation were considered to test for significance. However, the sample size was too small for non-parametric analysis.

A *Bti* isolation process was run on random samples of horse manure used in the trial, in order to determine whether any *Bti* was present in the faeces, after digestion. The *Bti* isolation technique used was the process proposed by Travers *et al.* (1987) and modified by Kaske *et al.* (1990). Colonies of *Bti* were successfully isolated and observed using electron and light microscopes.

Colonies of *Bt* with the parasporal protoxin crystal were counted to confirm that neither the horse feed nor the digestive system of the horse destroyed all the *Bti* fed to the horses. In addition, the presence of *Bt* crystals would indicate that the bacterium could be used as an entomopathogenic agent (Figure 3.4) (Travers *et al.*, 1987; Du Rand, 2009).

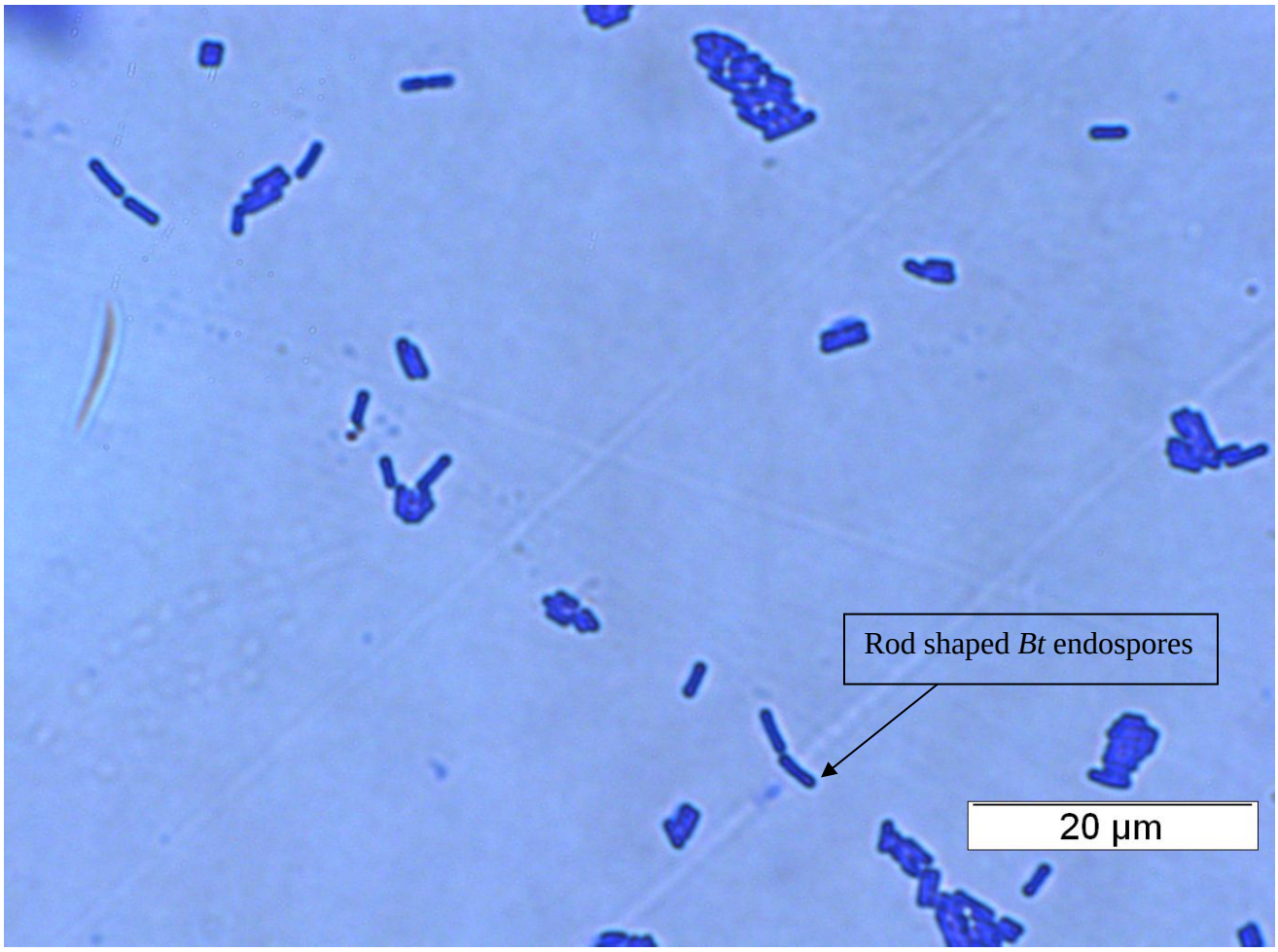

Figure 3.4: Comassie blue stained *Bti* cultures of horse manure

3.8 Discussion

The null hypothesis for these two trials was that the horses' digestive processes would kill all the *Bti* cells. However, it was hoped that the converse would apply and that *Bti* cells would survive passage through the gut of horses, to the extent that they could kill fly larvae and pupae in horse manure. In Trial 3a, the counts of adult flies, consolidated using the AUPC values, were not significantly different between treatments (dosage level in the feed). However, the F test for Ln transformed AUPC values for closed (dead) pupae was significant. Furthermore, a linear regression of the Ln transformed AUPC values against Bti level showed a dose response, in which higher doses of *Bti* killed more pupae. At a dosage level of 1 g of

Bti in the feed, the regression equation suggests that there will be 3.1 times as many closed (dead) pupae in the faeces as when horses are fed no *Bti*. This result reflects the findings of Mwamburi (2009) who fed *Bti* to chickens, and found a significant dose: pupal death response. The count of dead pupae is more important than the count of adult flies because it reflects the property of *Bti* as a larvicide.

With the doses of *Bti* administered to the horses, pupal deaths continued to increase with increasing *Bti* doses, i.e., no plateau was reached. Therefore, it is suggested that in further trial work, the feeding of higher concentrations of *Bti* to horses may cause higher counts of dead house fly pupae. This approach was attempted in Trial 3b, in which the dosage rates were increased from 0.125, 0.25, 0.5, 0.75 and 1.0 g *Bti* to 0.5, 1.0, 2.0, 4.0 and 8.0 g *Bti.*

Trial 3b

Despite increasing the dosage rate of *Bti* in Trial 3b, there were no significant differences between treatments in the numbers of adult flies as was found in Trial 3a. The ANOVA of the numbers of closed pupae also found no significant difference between treatments (dosage rate). However, there was a clear "trend" that *suggests* increasing *Bti* doses results in increasing numbers of dead pupae. This supports the findings in Trial 3a. Although the two trials did not prove conclusively that increasing levels of *Bti* result in increased fly and larval death, in Trial 1 there was a dose-response relationship between *Bti* and pupal death. This is an important outcome because it confirms the positive hypothesis, that *Bti* can survive passage through the gut of horses.

Trial 3a and Trial 3b

There are a number of reasons why the expected decrease in fly and pupal survival was not seen as clearly in Trial 3b as in Trial 3a.

3.8.1 Inhibition of growth in the digestive tract

The *Bti* would have followed a course through the horse's digestive system from the mouth (post ingestion), through chemical digestion in the stomach, fermentation in the hindgut and out through the rectum in large, loosely joined "pellets" (Ellis *et al.*, 2005). This length of

exposure to anaerobic and aerobic digestion may reduce *Bti* survival. In addition, the pH of the duodenum, jejunum and ileum of the horse have been found to be 6.32, 7.10, 7.47, respectively, and 6.7 in the hindgut (Mackie *et al.*, 1988). This pH range is very different from the acid environment in the stomach. This diversity in pH may impact *Bti* survival. There is a dearth of information regarding favourable conditions for the growth and survival of *Bti* in its natural environment (Nicholson, 2002), let alone the survival of *Bti* in mammalian manure. However, in *in vitro* studies of *Bti* growth, numerous factors have been noted to affect *Bti* growth. Its growth is slow and takes place in its natural environment (such as soil aggregates) subject to extensive variations in temperature, humidity and nutrient conditions. Growth may be limited by competition for oxygen availability, and by competition with other micro- and macro-organisms (Fajardo-Cavazos *et al.*, 1993).

Horses are known to have a copious microbial population (Varloud *et al.*, 2007). Julliand *et al*. (2001) found small quantities of cellulolytic bacteria, but extensive populations of lactobacilli, lactate-utilizing bacteria and streptococci bacteria The different microbial composition of a horses diet will affect the microbial population of the horses gut (Julliand *et al.*, 2001), which may pose as competition to *Bt* .

However, the significant increase in the number of closed (dead) pupae in Trial 3a, and the culturing of *Bti* colonies from the manure of treated horses strongly suggest that *Bti* can survive passage through the gut of horses.

3.8.2 Trial Duration

The short duration of the two trials may have contributed to the lack of a significant dose-response in Trial 3b. Trial 3a was run for 6 weeks and generated significant results for the death of pupae as a function of *Bti* dose. Trial 3b was run for only 3 weeks and generated no significant results. In retrospect, it would have been advisable that both trials be run for at least 12 weeks. Horses need 4 – 5 days (Vander Noot *et al.*, 1967) to adjust to a new feeding regime. These particular horses were moved off veld pastures into a stable yard; were unfamiliar with a new management regime; and should have been given at least 3 weeks to

adjust to the change of management, temperature, environment and concentrate diet. Feeding passage should also have been tested before the trial using polystyrene balls, chromic oxide or dry ash in order to test the length of time *Bti* would remain in the horse's tract. Furthermore, a marker with the *Bti* treatments could have been included to determine when the *Bti* should have been emerging with the horse manure, if it survived passage through the horse's gut.

3.8.2 Statistical Design

These trials were essentially observational experiments and therefore unlikely to yield statistically significant results because of the limited data collected. The lack of replication of treatments means that the result found in Trial 3a, in which closed (dead) pupae were increased by increasing dosages of *Bti*, may have occurred by chance. Future research would need to increase the number of replicates, which is not easy in equine experiments; or a more complicated statistical design and much longer trial period could be employed to circumvent the problem of low animal numbers.

This trial illustrated clearly the amount of natural variation in day to day numbers of pupae in horse manure. This level of variation needs to be taken into account in the statistical design of future trials. If real differences between treatment means are to be found, this natural variation in fly and pupae numbers has to be accounted for in every way possible.

3.8.3 Dilution by Pasture Grass

The horses ate pasture grass during the day and this feed would have diluted the *Bti* fed to the horses in the mornings and the evenings. It may be necessary to feed higher concentrations of *Bti* than the doses used in this trial.

3.8.4 Coprophagy

The horse in the Control Stable regularly practiced coprophagy, consuming its own faecal material (Ellis *et al.*, 2005) and that of another, treated, horse. This behaviour has been linked to excessive energy in the feed (McGreevy *et al.*, 1995). The regular practice of coprophagy by the Control horse would have resulted in the death of house fly eggs laid in the consumed faeces, resulting in a lower adult fly count. In future trials of a similar nature, any horse practicing coprophagy should be removed from the trial.

3.8.5 Choice of Horses

Trial 3b availability of horses for this experimental work was very limited. The test group comprised a female foal, three mares, a stallion and a gelding. Ideally, it would be have been better to conduct the experiment using horses of the same sex and age; and similar weight. If both sexes were tested, there needed to be equal numbers of males and females in each treatment group.

The objectives of this trial were to observe whether *Bti* is able to survive passage through the digestive tract of the horse, and to find an approximate dietary concentration of the bacterium that would act as a "starting-point" in future, replicated trials, designed to confirm the optimum dosage rate. Despite the statistical limitations of this research, the two feeding trials did provide two significant results: *Bti* survived passage through the equine gastrointestinal tract; and there was a dose-response in the killing of house fly pupae by *Bti* administered in the feed. Further trials are therefore called for, in order to confirm these preliminary findings. A more sophisticated trial design, with more homogenous test horses, a greater number of horses, and longer trial periods, are needed to provide confirmation of the results in Trial 3a.

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Appendix 1: Ethics letter from UKZN Ethics Committee 2010

UNIVERSITY OF
KWAZULU-NATAL

RESEARCH OFFICE
WESTVILLE CAMPUS
E-Mail: moodleyv@ukzn.ac.za
Tel.: 27-31-260 2273 Fax: 27-31-260 2384

30 November 2009

Reference: 042/10/Animal

Ms C Martins
MSc Agric Student
School of Agricultural Sciences and Agribusiness
University of KwaZulu-Natal
PIETERMARITZBURG

Dear Ms Martins

Ethical Approval of Research Project using Animals

I have pleasure in informing you that on recommendation of the review panel, the Animal Ethics Sub-committee of the University Ethics Committee has granted ethical approval for 2010 on the following project:

"Biological control of the common housefly *(Musca domestica)* a vector of disease in horses".

Yours sincerely

Professor Theresa HT Coetzer
Chairperson: Animal Ethics Sub-committee

Cc Registrar
Research Office
Head of School

Appendix 2: Veterinary clearance for Trial 3a and Trial 3b

DR D.E. MULLINS

BSc. (Rhodes) BVSc. (Pret)

Equine Veterinary Services

Tel: 033 263 1608
Cell: 082 822 6049
Fax: 033 263 1751
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Lakeview
PO Box 468
Mooi River
3300

Vet Report

Patient: 6 x Miniature Horses

Farm: University of Natal

Owner: University of Natal

Date: 21/04/2010

Procedure: Confirm that the horses are fit and healthy at the end of a biological trial.

Results: The 2 mares, one gelding, one filly and 1 stallion were part of a biological trial that has just been completed. These horses are in very good condition after the trial. There skin condition is much improved. They have gained weight and have shiny, healthy coats. There are still a few ticks on some of the horses but they are due for dipping this week. They are also to be dewormed this week again.

Comments: In my opinion these horses have been very well cared for during the trial. And have responded very well to the trial.

Name: Dr J.A. Lawrence **Signature:**

CHAPTER 4: EFFECT OF SPRAYING *BEAUVERIA BASSIANA* AND *BACILLUS THURINGIENSIS* VAR. *ISRAELENSIS* ONTO HORSE FAECES FOR THE CONTROL OF HOUSE FLIES (*MUSCA DOMESTICA* L.*)*

Abstract

House flies have evolved resistance against many pesticides, causing horse owners to look for alternative, affordable and environmentally friendly products to control them. The aim of this trial was to test the effect of *Beauveria bassiana* (*Bb*) and *Bacillus thuringiensis* var. *israelensis* (*Bti*) sprayed on to horse faeces collected from equine establishments in the Ashburton area of Pietermaritzburg, KwaZulu-Natal. The objective was to determine a suitable dose of two biological control agents that would provide effective control of the house fly. Samples of 500 g of faeces were sprayed with increasing concentrations of *Bb* and *Bti* (at doses of 0, 1, 2, 4 g/250ml water in Trial 4a, and 0, 4, 8 and 12 g/250 ml water in Trial 4b) for six weeks. The statistical design used in this trial was inadequate to cope with the high variation about treatment means for hatched flies and closed pupae, and no significant differences were found between the means. However, the trend illustrated in the treatment means suggest that *Bb* and *Bti* do have an effect on house fly survival. A simplified statistical model was then used that compared the number of hatched house flies on untreated manure to the number hatching from manure treated with any level of *Bb* (1 to 4g/ 250 ml water). A significant reduction was found in the number of hatched flies on treated manure. No significant results were found for a corresponding reduction in the number of closed (dead) pupae, which suggests that *Bb* acts before the larva pupates. Future trials, designed with higher levels of replication, are needed to definitively determine the effects of these two biocontrol agents on the numbers of hatched flies and closed pupae in horse manure.

4.1 Introduction

Female house flies utilize accumulations of horse manure next to horse stables as breeding sites (Hafez, 1948). Spraying pesticides has been a common method for controlling house

flies in agricultural environments (Levine *et al.*, 1991). The cost of pesticides used to control the house fly population is high (Axtell, 1986) and house flies have developed resistance to various common pesticides such as pyrethroids, insect growth regulators (e.g. fipronil (Dryden *et al.*, 2000)) and organophosphates (e.g. tetrachlovinphos, (Scott *et al.*, 2000)).
Beauveria bassiana (Bb), a fungus, and *Bacillus thuringiensis* var. *israelensis* (*Bti*), a bacterium, are two biological control agents that can kill house flies (Schnepf *et al.*, 1998; Scott *et al.*, 2000). *Beauveria bassiana (Bb)* is an omnipresent fungus found naturally in many areas of the world (Feng *et al.*, 1994). It is known to attack lepidopteran species such as the *Bombyx mori* L. and has been exploited in greenhouses and on agricultural crops to control agricultural pests such as weevils, aphids and whiteflies. *Beauveria bassiana* is pathogenic to house flies in poultry houses (Kaufman *et al.*, 2005; Mwamburi *et al.*, 2009).

Bacillus thuringiensis is a gram-positive spore forming bacterium, and is a well-established as a "biorational pesticide". Classified by its ability to form a parasporal body containing crystals, these protein crystals (controlled by *Cry* genes) contain specific insecticidal crystals (δ-endotoxins) that selectively bind to the gut of target insects. The specificity of the toxins to binding sites in hosts is largely responsible for the bacterium's ability to be species specific (Schnepf *et al.*, 1998). Over 100 *Cry* genes have been discovered (Hofte *et al.*, 1989). Some of the *Bt* isolates can kill the house fly (Indrasith *et al.*, 1992; Schnepf *et al.*, 1998; Mwamburi *et al.*, 2009). *Bti* strains possess a range of acaricidal, nematicidal and insecticidal toxins and a thuringiensin (β-exotoxin) nucleotide. *Bacillus thuringiensis* var. *israelensis* is toxic to members of the family Diptera. Pathogenesis is caused by lysis of the midgut epithelial cells, resulting in gut paralysis and death by starvation (Schnepf *et al.*, 1998). Alternatively, death may be caused by binding of the active protein toxins to the midgut surface receptor cells, where influx of ions and water occurs and death caused by gut lysis within two days (Gill *et al.*, 1992).

Schnepf *et al.* (1998) noted that *Bt* can last for several years after spraying application. Mwamburi *et al*. (2009) reported that feeding as well as spraying had lethal effects on house fly adults and pupae. There have been numerous studies of *Bb* and *Bti* (Table 4.1).

Table 4.1: Previous studies of *Beauveria bassiana* (*Bb*) and *Bacillus thuringiensis* var. *israelensis* (*Bti*)

Biological agent sprayed:	**Insect:**	**Dosage sprayed/Method**	**Outcome:**	**Reference:**
Bti	(Chickens) *Musca domestica*	1g and 2g of *Bti* powder/l of water	No significant difference between 1g and 2g *Bti*. House fly larvae reduced by 43%,	(Mwamburi *et al.*, 2009)
Bt – in the form of Dipel (a biological insecticide with *Bacillus* as a base)	Maize Crops *Crysoperla carnea* (spider mites)	1g/l of water – plants sprayed with +- 0.4 ml per plant) – recommended dose	Increased mortality, Spider mites had a lower intrinsic rate of natural increase, *Bt* increased larval development	(Dutton *et al.*, 2003)
Bt – Dipel	Maize crop - *Spodoptera littoralis*	1g/l of water – 0.4 ml per plant	Higher mortality, slower developmental phase - It was also found that transgenic (genetically modified) maize had a higher effect on the insect than sprayed maize	(Dutton *et al.*, 2005)
Bb - balEnce	House fly *Musca domestica*	10g of 5 x 10^{11} conidia of Bb suspended in 15ml oil sol^{n} with an emulsifying agent	Post-spraying: high adult house fly mortality. Number of house fly larvae was lower than in	(Kaufman et al., 2005)

			pyrethroid treated samples.	
Bb – two field isolates NC2 and NC3	Lesser mealworm - *Alphitobius diaperinus*	2.37 x 10^{11} conidia per square meter	Higher susceptibility in larvae. 68% mortality in adults. Repetition caused a further 33% mortality.	(Crawford *et al.*, 1998)
Bb – *in vitro* and field examinations	Diamond back moth – *Plutella xylostella*	*Bb* suspended in oil and in water	Reduced larval population compared to control. Two applications of *Bb* spray reduced larval populations more than one application.	(Vandenberg *et al.*, 1998)

There is a dearth of information regarding the biological control of house flies using *Bb* and *Bti* in the equine environment. The objective of the current experiment was to determine the most effective spraying concentration of the biological control agents to provide horse owners with an alternative, eco-friendly method to control the house fly in the environment of horse stables.

The null hypothesis tested was that spraying of *Bb* and *Bti* at a range of concentrations on to horse faeces would not provide any control over the number of flies. The positive hypotheses were that some control would be provided by the application of these two biological control agents, and that the level of control would be dose related.

4.2 Materials and methods

4.2.1 Drip test

Before the experiment was conducted, a "drip test" was performed to determine the optimal amount of water needed to penetrate the samples of horse faeces, without saturating the containers and potentially downing the maggots. Samples of 500 g of race-horse manure were placed in transparent plastic open-top containers with six holes burnt through the bottom with a heated glass rod. A hand held spray bottle was used to spray known quantities of distilled water on to the samples, to test the amount of water needed to penetrate through 500 g of horse manure. The volumes tested were 50, 100, 150, 200, 250 and 300 ml of distilled water sprayed over six trays of 500 g of race horse manure. The optimum amount of water sprayed was found to be 250 ml, which resulted in 5 – 6 droplets on the newspaper placed under the tray.

4.2.2 Collecting the race horse manure

The Ashburton Training Centre is situated close to Pietermaritzburg, South Africa. It has large manure dumps which attract prolific numbers of house flies to breed there. The trainers of the yard agreed to collection of manure for the experiment, and 20 kg was collected every Thursday and Friday through January, February, March and April 2010.

For both trials, 500 g of horse manure was weighed and placed into labeled rectangular transparent plastic containers. Twice a week for 4 weeks in Trial 4a, two samples of 500 g of faeces were each sprayed with either 0, 1 g, 2 g, and 4 g of *Bb* in 250 ml of water or 0, 1 g, 2 g, and 4 g of *Bti* in 250 ml of water. Twice a week for 2 weeks in Trial 4b, two samples of 500 g of faeces were sprayed with either 0 g, 4 g, 8 g and 12 g of *Bb* or 0 g, 4 g, 8 g and 12 g of *Bti* in 250 ml of water. Initially, hand sprayers were used to spray the trays. However, these were replaced with three hydraulic sprayers, which proved to be more efficient, accurate, and less time-consuming. To prevent cross-contamination, each hydraulic sprayer was labelled "Control", "*Bb"* or "*Bti"*. Trays were sprayed inside a fume cupboard.

The treated trays were sealed with white nylon netting ("tutu" material). The gauze of the nylon netting was porous enough to allow sunlight and an occasional light mist spray of water through to feed the growing house flies. However, it was fine enough to prevent flies or maggots from escaping. The nylon was stapled to the trays, and sealed with glue.

Once all 14 trays had been sprayed and sealed, they were moved to a glass-house for 21 days for incubation and fly growth. Glasshouse conditions were set to be for optimal for the growth of house flies. The number of adult house flies and dead pupae were counted on each tray at the end of 21 days.

4.4 Statistical analysis

In Trial 4a, the data were analyzed using the Fit Model procedure in JMP 8.0 (SAS). There were two treatments: (*Bb* and *Bti*), each sprayed on to faeces at 4 levels: 0, 1, 2 and 4 g biocontrol agent per 250 ml water. With the exception of the 0 (Control) level, there were 2 replicate samples taken twice a week for 4 weeks, giving a total of 16 observations for both hatched house flies and closed pupae numbers per treatment level. For the 0 (Control) levels for both *Bb* and *Bti,* there was a single sample taken twice a week for 4 weeks, giving a total of 8 observations for the two zero treatment levels. Data sets for the number of house flies found on horse faeces after spraying with *Bb* and *Bti* were combined to allow comparison of the two biocontrol treatments (total number of observations in combined data set = 112).

Variation about treatment means in the combined data set was high, for both hatched house fly and closed pupae numbers. For example, in the Control group (n=8) for *Bb* sprayed manure samples, the number of hatched house flies varied from 2 to 644 hatched flies. In contrast, in samples sprayed with 2 g *Bti* per 250 ml water, the number of hatched flies ranged from 0 to 199 per tray. Transformation of the response variate (y) was performed in an attempt to equalize these variances. Natural log (Ln) and $\log_{10}$ (Lg_{10}) transformations were tested on both the hatched fly and closed pupae data sets.

The combined data set was also separated into two subsets: *Bb* and *Bti*, allowing the full 16 observations for the 0 g /250 ml level to be included in each of the subsets. The number of observations in each data set was thus 64.

In Trial 4b, the data were analyzed using the Fit Model procedure in JMP 8.0 (SAS). There were two treatments: (*Bb* and *Bti*), each sprayed on to faeces at 4 levels: 0, 4, 8 and 12 g biocontrol agent per 250 ml water. With the exception of the 0 (Control) level, there were 2 replicate samples taken twice a week for 2 weeks, giving a total of 8 observations for both hatched house flies and closed pupae numbers per treatment level. For the 0 (Control) levels for both *Bb* and *Bti*, there was a single sample taken twice a week for 2 weeks, giving a total of 4 observations for the two zero treatment levels. Data sets for the number of house flies found on horse faeces after spraying with *Bb* and *Bti* were combined to allow comparison of the two biocontrol treatments (total number of observations in combined data set = 56).

Variation about treatment means in the combined data set was again very high, for both hatched house fly and closed pupae numbers. For example, in the 8 g/250 ml group (n=8) for *Bb* sprayed manure samples, the number of hatched house flies varied from 13 to 610 hatched flies. In contrast, in the Control group for *Bti* sprayed manure samples, the number of hatched flies ranged from 3 to 150 per tray. Transformation of the response variate (y) was performed in an attempt to equalize these variances. Natural log and $\log_{10}$ transformations were tested on both the hatched fly and closed pupae data sets.

The combined data set was also separated into two subsets: *Bb* and *Bti*, allowing the full 8 observations for the 0 g /250 ml level to be included in each of the subsets. The number of observations in each data set was thus 32.

4.5 Results

Treatment means for the number of house flies hatched and closed pupae counted on horse manure treated with increasing levels of either Bb or Bti in Trial 4a are given in Tables 4.2 to 4.4. In Table 4.2, the analysis of the combined dataset (n=112) for Bb and Bti treated manure

is presented. In Tables 4.3 and 4.4, the sub-datasets (n=64) for *Bb* and *Bti* are given. Separating the two datasets in this way allowed the full 16 Control (0 g biocontrol agent/250 ml water) to be included in the analysis for the two biocontrol agents.

There were no significant differences in the number of hatched house flies or closed pupae found on the manure treated with *Bb* or *Bti* (Table 4.2). The percentage variance accounted for in these full models, with interaction effect (biocontrol agent x concentration level), was very low: 5.4 and 5.8, respectively, for hatched house flies and closed pupae. Although the means presented in Table 4.2 seem to suggest that increasing the dosage of either *Bb* or *Bti* used to spray the manure samples decreases the number of hatching flies and increases the number of closed pupae, the variation about these treatment means is so large that no significant differences between treatment means could be found. The SE mean for each treatment mean is presented alongside each mean in Tables 4.2 to 4.4 and gives a clear indication of the size of this variation about these means. It is not justified to delete outliers in this study, since the counts were genuine observations. However, it is obvious that 16 observations per treatment are not adequate when there is such extreme biological variation in the data. Natural and $\log_{10}$ transformations were attempted in order to equalize the variation about the means, but these transformations did not improve the models and are thus not presented.

Separating the data into two subsets (*Bb* and *Bti* treated manure samples) did not significantly improve the percentage variance accounted for (Tables 4.3 and 4.4). Table 4.3 and 4.4 do suggest, at the 16% level and 19% significance level, respectively, that increasing the amount of *Bb* or *Bti* in the spray decreases the number of hatched house flies *Bb* or increases the number of closed pupae, *Bti*. These trends are not statistically significant, at the 5% level, but do encourage further research into this product, with greater numbers of observations at each treatment level.

Table 4.2 Trial 4a: Table of means of hatched house flies and closed pupae on treated horse manure: for main effect (biocontrol agent: *Bb* or *Bti*) and interaction effect (biocontrol agent and concentration)

	Concentration (g/250 ml)	Hatched flies†	Closed pupae†
Interaction effect: Biocontrol agent x concentration			
Bb	0	104.0 (77.6)	44.6 (11.8)
	1	26.1 (12.8)	44.6 (7.2)
	2	19.6 (4.2)	57.2 (13.0)
	4	45.3 (21.5)	65.9 (28.0)
Bti	0	109.9 (81.7)	55.6 (19.7)
	1	103.4 (58.6)	45.8 (9.0)
	2	44.9 (14.2)	69.2 (19.5)
	4	52.8 (34.2)	100.2 (29.9)
Main effect: Biocontrol agent			
Bb		48.7 (13.2)	53.1 (9.07)
Bti		77.8 (22.6)	67.7 (11.0)
P-values:			
Main effect: biocontrol agent		0.29	0.33
Interaction effect		0.74	0.85
Var. accounted for ($r^{2,}$ %)		5.4	5.8
N observations		112	112

† SE Mean in parentheses

Table 4.3 Trial 4a: Table of means of hatched house flies and closed pupae on horse manure treated with increasing concentrations of *Bb* (number of Control observations = 16)

	Concentration (g/250 ml)	Hatched flies†	Closed pupae†
Treatment: Bb concentration			
Bb	0 (Control)	106.9 (54.4)	50.1 (11.2)
	1	26.1 (12.8)	44.6 (7.2)
	2	19.6 (4.2)	57.2 (13.0)
	4	45.3 (21.5)	65.9 (28.0)
P-value:			
Effect: *Bb* concentration level		0.16	0.83
Var. accounted for ($r^{2,}$ %)		8.1	1.5
N observations		64	64

† SE Mean in parentheses

Table 4.4 Trial 4a: Table of means of hatched house flies and closed pupae on horse manure treated with increasing concentrations of *Bti*

	Concentration (g/250 ml)	Hatched flies†	Closed pupae†
Treatment: Bti concentration			
Bti	0 (Control)	106.9 (81.7)	50.1 (19.7)
	1	103.4 (58.6)	45.8 (9.0)
	2	44.9 (14.2)	69.2 (19.5)
	4	52.8 (34.2)	100.2 (29.9)
P-value:			
Effect: *Bti* concentration level		0.65	0.19
Var. accounted for ($r^{2,}$ %)		2.7	7.6
N observations		64	64

† SE Mean in parentheses

If the datasets are simplified further in Trial 4a, so that they separate observations into Control (Untreated) observations (not sprayed with *Bb* or *Bti;* n = 16) and Treated

Observations (disregarding the level of *Bb* or *Bti* in the spray; n = 16*3 = 48), the *Bb* model gives further evidence that spraying with *Bb*, at any dosage level, will significantly reduce the number of house flies hatching from treated manure (Table 4.5). There was not a significant corresponding reduction in the number of closed pupae, which suggests that *Bb* acts before the maggot pupates. The number of maggots in the manure was not counted in this trial. Simplifying the datasets in this way did not improve the variation accounted for in the *Bti* model (Table 4.5).

Table 4.5 Trial 4a: Table of means of hatched house flies and closed pupae on horse manure treated (number of treated observations = 48) or untreated (number of Control observations = 16) with *Bb*

	Concentration (g/250 ml)	Hatched flies	Closed pupae
Treatment: Bb concentration			
Bb	Control (untreated)	106.9[a]	55.9
	Treated (dosage ignored)	30.3[b]	50.1
P-value:			
Effect: *Bb* concentration level		0.03	0.76
Var. accounted for ($r^{2,}$ %)		7.4	0.1
N observations		64	64

Trial 4b: Trial 4b was a repeat of Trial 4a but employed higher concentrations of *Bb* or *Bti* in the sprays applied to the manure samples (4, 8 and 12 g/250 ml water in Trial 4b, compared to 1, 2 and 4 g/250 ml water in Trial 4a). Unfortunately, this trial only ran for 2 weeks because of the onset of winter and a decrease in fly populations at the racetrack. In light of the extreme biological variation in the number of flies hatching and in the number of closed pupae in the manure samples, it is now obvious that this collection period was too short, allowing for collection of only 8 observations per treatment level. Treatment means for the number of house flies hatched and closed pupae counted on horse manure treated with increasing levels of either *Bb* or *Bti* in Trial 4b are given in Tables 4.6 to 4.7. In Table 4.6,

the analysis of the combined dataset (n=64) for *Bb* and *Bti* treated manure is presented for Trial 4b. In Tables 4.7 and 4.8, the sub-datasets (n=32) for *Bb* and *Bti* are given. Separating the two datasets in this way allowed the full 8 Control (0 g biocontrol agent/250 ml water) to be included in the analysis for the two biocontrol agents.

There were no significant differences in the number of hatched house flies or closed pupae found on the manure treated with *Bb* or *Bti* (Table 4.6). The percentage variance accounted for in these full models, with interaction effect (biocontrol agent x concentration level), was, as in Trial 4a, very low: 7.9% and 11.3%, respectively, for hatched house flies and closed pupae. The variation about these treatment means is so large that no significant differences between treatment means could be found. With only eight observations per treatment level, Trial 4b was less likely than Trial 4a to yield significant differences between treatments.

Separating the data into two subsets (*Bb* and *Bti* treated manure samples) did not significantly improve the percentage variance accounted for (Tables 4.7 and 4.8). Simplifying the data into Treated and Untreated, as shown in Table 4.5 for Trial 4a, did also not improve the variation accounted for in Trial 4b.

Table 4.6 Trial 4b: Table of means of hatched house flies and closed pupae on treated horse manure: for main effect (biocontrol agent: *Bb* or *Bti*) and interaction effect (biocontrol agent and concentration)

	Concentration (g/250 ml)	Hatched flies†	Closed pupae†
Interaction effect: Biocontrol agent x concentration			
Bb	0	62.8 (25.7)	29.0 (16.7)
	4	84.6 (14.7)	38.4 (9.8)
	8	131.3 (69.5)	28.6 (7.2)
	12	126.0 (47.9)	102.4 (64.6)
Bti	0	95.5 (33.6)	14.3 (4.5)
	4	79.1 (24.9)	38.3 (14.0)
	8	65.3 (15.1)	53.9 (17.9)
	12	56.0 (14.3)	35.3 (8.9)
Main effect: Biocontrol agent			
Bb		101.2 (24.1)	49.6 (19.0)
Bti		73.9 (10.1)	35.4 (7.1)
P-values:			
Main effect: biocontrol agent		0.34	0.51
Interaction effect		0.56	0.38
Var. accounted for ($r^{2,}$ %)		7.9	11.2
N observations		56	56

† SE Mean in parentheses

Table 4.7 Table of means of hatched house flies and closed pupae on horse manure treated with increasing concentrations of *Bb* (number of Control observations = 16)

	Concentration (g/250 ml)	Hatched flies†	Closed pupae†
Treatment: Bb concentration			
Bb	0 (Control)	79.1 (20.5)	21.6 (8.5)
	4	84.6 (14.7)	38.4 (9.8)
	8	131.3 (69.5)	28.6 (7.2)
	12	126.0 (47.9)	102.4 (64.6)
P-value:			
Effect: *Bb* concentration level		0.77	0.31
Var. accounted for ($r^{2,}$ %)		3.9	11.8
N observations		32	32

† SE Mean in parentheses

Table 4.8 Table of means of hatched house flies and closed pupae on horse manure treated with increasing concentrations of *Bti*

	Concentration (g/250 ml)	Hatched flies†	Closed pupae†
Treatment: Bti concentration			
Bti	0 (Control)	79.1 (20.5)	21.6 (8.5)
	4	79.1 (24.9)	38.3 (14.0)
	8	65.3 (15.1)	53.9 (17.9)
	12	56.0 (14.3)	35.3 (8.9)
P-values:			
Effect: *Bti* concentration level		0.79	0.39
Var. accounted for ($r^{2,}$ %)		3.6	10.1
N observations		32	32

† SE Mean in parentheses

4.6 Discussion

At the onset of the spraying trial the author was unaware of the high level of natural biological variation present in the number of eggs, pupae and maggots in the horse manure samples before spraying. Therefore, the number of replicates for each treatment was inadequate for this trial. This can be seen by the huge within-treatment variation in the number of house flies (Table 4.2).

At the onset of the trial, the concentrations of *Bb* and *Bti* to be sprayed could only be guessed at. The two biocontrol agents had not been previously tested in an equine environment, so the concentrations of the biological agents used were based on those used in the trial run by Mwamburi *et al*. (2009), in which 1g and 2g of *Bti* powder per litre of water were sprayed in poultry houses. Treatment means in Trial 4a *suggest* that all levels of *Bb* and *Bti* reduced the number of hatched flies and increased the number of closed pupae. However, the variation between means is too large for statistical significance to be shown. Future trials, run over a longer period of time, with more replications, would seem to be worthwhile and would be necessary to determine the optimum dose of the biological control agent. At the levels used in this trial, *Bb* seems to be more effective than *Bti* in reducing the number of hatched house flies.

There was some evidence that *Bb* and *Bti* work at different points in the life cycle of the fly. Table 4.2 suggests that higher concentrations of *Bti* are more successful in increasing the numbers of closed pupae in the faeces than the same concentrations of *Bb*. Given that the *Bb* treatment is more successful at reducing the number of hatched house flies, this would seem to suggest that its action must be at the maggot (larval) stage, preventing the maggots pupating and being counted as closed pupae, but also reducing the number of flies ultimately hatching. In contrast, *Bti* would appear to increase the number of closed pupae, thus reducing the number of hatched flies.

A positive result for this experiment would have been to find fewer adult house flies, or alternatively, to have found greater numbers of closed pupae as the biological control agents

increased in dose. Although the treatment means presented in Table 4.2 suggest that any dose of *Bb* (1 to 4 g/250 ml) decreases the number of hatched flies and that dosages of *Bti* above 2 g/250 ml both decreases the number of hatched flies and increases the number of closed pupae, statistically significant results could not be reported. An important finding of this trial was the level of biological variation in the number of eggs, larvae and pupae found in samples of horse manure. Clearly, the number of replications used in this trial was inadequate in light of this variation and any future trials would need to include a much higher level of treatment replication.

It could be argued that it would have been better to treat manure containing very similar levels of eggs, larvae and pupae. It might have been possible to isolate house fly eggs (Mwamburi *et al.*, 2009), raise them in an insectary and inoculate fresh horse manure already treated with the biological control agents with a given number of eggs. However, this trial attempted to mimic stable yard conditions, where the number of eggs, larvae and pupae on the manure pile will vary from day to day and, perhaps, from stable to stable. If a biocontrol spray is to be used, it needs to be administered at a level which will offer effective control against manure infested at different levels. Samples of manure were thus taken over an extended period of time and, on any given sampling date manure was mixed thoroughly to reduce between-sample variation for that day. Between-day variation in infestation was not controlled. It was hoped that the number of samples taken would be sufficient to reduce the variation about treatment means to a level that would allow significant differences between treatment means to be found. This was not the case. To be useful in the commercial environment, biocontrol sprays must be able to reduce house flies whether manure is lightly or heavily infested. Future trials should not attempt to control the level of infestation in the treated samples but should increase the level of replication to account for the variation witnessed in this trial, or perhaps use other data transformations to equalize the variation about means.

In conclusion, although high levels of biological variation resulted in a lack of significant differences between treatment means, there is some evidence that spraying horse manure with any dose of *Bb* above 1 g /250 ml water reduces the number of house flies hatching, possibly

by preventing eggs hatching or larvae pupating. In addition, there is weaker evidence that spraying horse manure with *Bti* at over 2 g/250 ml water may reduce both the number of house flies hatching and increase the number of closed pupae found on the manure. Future trials should test higher dosages of *Bti*, and take steps to control or manage the biological variation in egg, larvae and pupae numbers between samples of manure through sampling technique, replication or statistical design and analysis.

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5. THESIS OVERVIEW

House flies have been a nuisance pest since the beginning of biocenosis and therefore the control of their population has been pivotal to maintaining livestock health (Axtell, 1986). There is a demand for alternate means of house fly control in agriculture, in place of pesticides (Scott *et al.*, 2000). Despite huge investments in insecticide research, house flies have developed resistance against most insecticide groups (Scott *et al.*, 2000). These potent chemicals are sometimes misused and can cause damage to the environment. Use of pesticides also requires skilled labour, increasing the costs of using pesticides (Bennett *et al.*, 2003).

Two entomopathogenic agents known to be effective as biological control agents against house flies are a fungus, *Beauveria bassiana (Bb),* and a bacterium, *Bacillus thuringiensis* var. *israelensis* (*Bti*).

Beauveria bassiana is an ubiquitous fungus which occurs naturally in numerous areas of the world (Roberts *et al.*, 1994). The existence of the fungus was first reported by Agostino Bassi in 1834, when the fungus was isolated from the silkworm *Bombyx mori* L. (Lepidopteran Bombycidae) (Feng *et al.*, 1994). Currently, *Bb* is an extensively studied entomopathogen and is being used commercially to control arthropod pests in agriculture (Hajek *et al.*, 1994).

The discovery that *Bt* has insecticidal activity was made over a hundred years ago by Ishiwata (1901) (Martin & Travers, 1989). The insecticidal protein from *Bt* has been used in genetically modified plants (Bennett *et al.*, 2003). However, the use of *Bti* in controlling house flies in the manure of livestock and poultry projects by spraying and feeding (Mwamburi *et al.*, 2009) may be considered as a new research field.

5.1 Conclusions

The goal of this project was to assess the impact of house flies in the equine environment and to begin the development of an alternative method of house fly control for horse owners.

The survey conducted in Chapter 2 concluded that house flies are considered one of the worst pests in the horse industry in KwaZulu–Natal. Although owners considered control of other pests, such as the *Culicoides* midges (the vectors of the *African horse sickness virus*) to be sufficient, they felt their methods of house fly control were not. Most horse owners (97%) were eager to try out alternative, environmentally-friendly biological control products on their horses and in their stable yards. The survey also revealed that horse owners use many different methods to control house flies. The majority of horse owners use a mixture of physical and chemical methods. However, this was only due to the lack of biological control alternatives. The high level of interest displayed by horse owners in biological control options proved that there is a market for commercial biocontrol products. The two avenues available to apply biological control agents are either in the animal feed or by spraying the agents directly on to manure. Over 80% of owners would prefer to administer the control agent in the feed. Both approaches were tested in trial work reported in Chapters 3 and Chapter 4.

In the observational trials reported in Chapter 3, there is encouraging evidence that the feeding of *Bti* to miniature horses controls the pupal phase of house flies. There was a significant increase in the number of closed (dead) pupae when feeding increasing doses of *Bti*. The regression equation suggests that there will be 3.1 times as many closed pupae in the faeces when horses are fed 1 g of *Bti* in their feed, than when horses are fed no *Bti*. Higher doses of *Bti* in the feed were tested in Trial 3b, but this trial was very short in duration and the results were disappointing. It is suggested that, in any future trials, the lowest dosage of the bacterium in the feed should be 1 g *Bti* per meal.
Laboratory isolation tests in Chapter 3 confirmed that *Bti* survived passage through the tract of the horse and could therefore be administered in the feed as a potential biological control agent in horses.

Chapter 4 looked at the effect of *Bb* and *Bti* on adult house flies and pupae by spraying the agents, at differing concentrations, on to 500 g samples of race horse manure. Every race yard has piles of horse manure (dung skips) which were collected from the stables each morning and afternoon. This trial highlighted the high levels of biological variation in egg, larvae and pupae numbers that will be found in samples of horse manure, taken from the same skip two

days apart. The statistical design of the two trials reported in this chapter was inadequate to cope with the high level of variation about treatment means for fly and larval counts. However, despite the lack of significant differences between treatment means, the trend in the means suggests that *Bb* and *Bti* did have an effect on house fly survival. A simplified statistical model, which compared the number of hatched house flies on untreated manure, with the number on manure treated with any level of *Bb* (1 to 4 g /250 ml water), found a significant reduction in the number of hatched flies on treated manure. There was no significant corresponding reduction in the number of closed pupae, which suggests that *Bb* acts before the larva pupates. A parallel analysis of the effect of *Bti* on treated and untreated manure did not reveal a significant difference.

The optimal dose of *Bb* or *Bti* to be sprayed on to manure could not be determined because of the high variation about treatment means. This experiment could be used as a basis for future trials; using similar dosages for *Bb* but perhaps higher dosages for *Bti* (starting at 2 g/250 ml water). Trial periods should be extended and/or the number of samples (replicates) increased, perhaps as much as four-fold, to reduce variation about treatment means. Transformation of data before analysis may also be necessary to equalize variation about treatment means.

5.2 Recommendations

At present, only agrochemical pesticides are registered for the control of house flies in the equine environment. As far as can be determined, this is the first research project in which the biological control agents *Bb*, a fungus, and *Bti*, a bacterium were applied, either in the feed or directly on to horse manure, in an attempt to control house flies. Given the paucity of research into the use of biological control of house flies in horses or other livestock, this study provides a basis for future research.

Horse owners in KwaZulu-Natal are almost all receptive to the idea of using biocontrol agents in the stable yards, and, as most of the owners stable their horses, there is a large market for commercial biocontrol products. The majority would prefer to administer the agents in the horse feed and future research should focus on the development of feed

supplements, unless spraying the biocontrol agents on to the manure directly is shown to be more effective.

There is sufficient evidence in this research that *Bti*, supplied in the horse feed increases the number of closed pupae in a dose-dependent manner. Since no plateau in response was found, any future research into using Bti as a dietary biocontrol agent should use dosage rates at least as high as those tested in Trial 3b (0.5 to 8 g/meal).

Further research into the effect of changes in pH on *Bti* might be useful to improve survival rates of the bacterium as it passes through the digestive tract of the horse. It might be necessary to protect the bacterium in some way, say, through the acidic stomach environment. It was, however, encouraging that *Bti* could be isolated in manure after passage through the equine digestive tract.

The diluting effect of pasture consumed on the dosage rate of the bacterium and the practice of coprophagy both need to be considered and managed in future trials.

Although the trials reported in Chapter 3 in this thesis were essentially observational experiments and therefore unlikely to yield statistically significant results from the limited data collected, future trials will need to make use of more horses, with greater attention to gender, size and age profiles. If sufficient horses are not available, then more complicated statistical designs will have to be used.

The trials reported in Chapter 4 did not provide a clear dose response as to the optimal dose of *Bb* or *Bti* to be sprayed on to manure. Variation about treatment means was extremely high because of high levels of natural variation in egg, larvae, pupae and fly numbers on the manure samples, and inadequate replication in the statistical design. This experiment could be used as a basis for future trials; using similar dosages for *Bb* but perhaps higher dosages for *Bti* (starting at 2 g/250 ml water). Trial periods should be extended and/or the number of samples (replicates) increased, perhaps as much as four-fold, to reduce variation about treatment means. Transformation of data before analysis may also be necessary to equalize

variation about treatment means. It is important that future research in this field recognizes and manages the inherent natural variation in the different fly life-stages in manure samples

In theory, both *Bb* and *Bti* and should kill the *Culicoides* midges, which are the lethal vectors for the *African horse sickness virus*. This attractive option could be tested alongside control of house and stable flies in the horse environment.

Research into using *Bb* and *Bti* to control house flies in the environments of other livestock species, such as dairy cattle and pigs, could help commercial and subsistence farmers economically by introducing a new, more environmentally friendly, forms of house fly control, without the problem of resistance developing, and toxic side-effects to non-target organisms.

Finally, there is a dearth of objective research into the effect of flies on livestock and financial consequences of not controlling these pests on farms and in stable yards. Although horse owners clearly *perceive* flies as a huge nuisance factor in their facilities, there is little or no published research to quantify the effect that flies have on the growth, development, health and behaviour of horses. There is scope for future research in this area, and in the development of objective ways of measuring the irritation and distress created by flies interacting with horses.

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