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The efficacy of topically applied fluazuron and flumethrin in the control of sheep myiasis

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Thesis submitted in fulfilment of Master of Science degree in the field of Veterinary Science.

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Dedication

This thesis is dedicated to:

My children, Joshua and Rylan - may all your hopes, dreams and aspirations be realized;

My parents, Lorimer and Sandra, for your unending, selfless support and encouragement provided in the pursuit of my childhood dreams;

My coaches and mentors, in all aspects of my life – I will be forever indebted to you.

Declaration

I declare that the dissertation, which I hereby submit in fulfilment of the degree Master of Science in the field of Veterinary Science, at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at this or any other tertiary institution.

14 December 2016

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Dr Clinton Mark Austin   **Date**

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Abstract

Keywords: Sheep, parasites, blowflies, myiasis, fluazuron, flumethrin, wool, lanolin, pyrethroids, benzoylphenyl ureas, insect growth regulators.

Small stock farming and production accounted for approximately 8.4% of total animal product based agricultural output in the 2011 / 2012 season in South Africa. Large scale commercial farming aside, small stock farming also takes on an important role in poorer and developing rural areas of South Africa, where small stock are kept for a combination of economic and non-economic reasons including financial investment or security, food and resource production, as well as religious or traditional reasons.

Blowflies are Dipterids with complex life cycles and complete metamorphoses, causing damage to hides and frequent death in their ovine hosts, as a result of cutaneous myiasis caused by the larval stages. All economically important blowfly species causing veterinary myiasis belong to the superfamily Oestroidea, which contains the three major families Oestridae, Calliphoridae and Sarcophidae. The two most significant blowfly genera in South Africa, *Lucillia* and *Chrysomya*, both belong to the family Calliphoridae.

Chemical means of preventing and treating blowfly strike by topical application remains the most widely used method and appears to be indispensable at this stage. New molecules or formulations effective against blowfly strike are constantly being sought and form part of an active field of research. Bayer currently manufactures and markets Drastic Deadline Extreme®, a pour-on formulation containing flumethrin and fluazuron for the control of blue ticks (*Rhipicephalus decoloratus*) in cattle; its possible action against blowflies in sheep was investigated in an in-vitro model, subsequent to a pilot pharmacokinetic study evaluating the kinetics of fluazuron when applied topically to sheep in this particular combination.

The first objective of the project was to determine whether fluazuron has any effect at all on the development of blowfly larvae. An active ingredient from the same family of compounds,

namely triflumeron, has been successfully used for several years to control blowfly strike in sheep in South Africa (Zapp® Pour on – Bayer), but it was uncertain whether or not fluazuron would be effective. Raw fluazuron was applied to six pieces of beef according to a dose calculation based on the registered dose of the test product in cattle, while another six pieces were treated with saline in a similar fashion (n=6). Each piece of beef was placed in its own container along with six late instar larvae and placed in the incubator at 35°C for a further nine days. In this instance, the treated group demonstrated significant development defects with regard to pupation (uneclosed pupae) when analysed using the Mann-Whitney non-parametric t-test (p = 0.002).

The second phase of the project took the form of a pilot pharmacokinetic study, where 3 sheep (n=3) were treated with Drastic Deadline Extreme at a dose rate of 1ml/10kg applied to the skin and eleven blood sampling points from T0 to T+504 hrs were established. The serum was analysed for fluazuron concentrations by an approved laboratory using a previously validated analytical method for cattle. After no significant amounts of fluazuron were detected in the serum, a further 3 sheep were treated with the test product again at the same rate. This time, wool samples (three from each sheep) were collected from points varying distances below the application point on the dorsal thorax at weekly intervals, in order to establish fluazuron concentrations in the wool. It was concluded that virtually no fluazuron was absorbed trans-dermally into the bloodstream, instead remaining strongly dissolved in the wool fat or lanolin. The question then arose as to whether wool or lanolin bound fluazuron would be effective in preventing myiasis in sheep when considering the combined benefit of the active ingredient now being held in a depot while at the same time unlikely to be of a food safety concern

Phase three of the project comprised of two *in*-vitro studies which tried to determine if the combination product provided a zonal repellent effect to blowfly larvae, as well as whether or not the product could successfully interfere with larval development when applied to sheep pelts. Neither fluazuron nor flumethrin is known to have any significant repellent effect on 4adult flies, but the effect on larvae was uncertain. Two fresh sheep pelts were obtained from

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an abattoir and a 30cm x 30cm square section cut out of each pelt. One section was treated by applying the test product to the skin in the centre whilst the other was treated in a similar fashion with the same amount of saline. Both sections were left in a warm room for forty-eight hours to give the test product or saline time to spread through the lanolin, after which five sections (n=5) of 5cm x 5cm were cut from each section and placed in separate petri dishes. Five blowfly larvae, specially bred for this project on meat in an incubator, were placed in each petri dish and their behaviour monitored for forty-five minutes. When analysed using the Mann-Whitney non-parametric t-test, there was no significant difference in larval penetration of the pelt between the control and treated group (p = 0.4009)

The effect of the test product applied to sheep pelt on larval development and pupation was then evaluated. As in phase two, a similar procedure of pelt treatment was followed in order to obtain six pieces of pelt each (n=6), 5cm x 5cm, for the treated and control specimens. The pieces of pelt were each placed in their own container on top of moist paper towel along with six larvae, and then placed in an incubator for seven days. The contents of each container were then evaluated for the presence of adult flies or pupa, as well as macroscopic viability of each. Once again, after application of the Mann-Whitney non-parametric t-test, no significant difference was noted between the treated and control groups with regard to macroscopic larval development and pupation, as well as hatching and adult fly development (p=0.27)

The results of this entire study seem to indicate that although fluazuron would be effective at preventing blowfly development, it is not effective when applied cutaneously to sheep at the dose rate registered for use on cattle. To circumvent this, perhaps subsequent investigations can look at the effect of higher doses of the product, or compare its effect on shorn and fully woolled animals. It will also be important to ascertain if exposure to environmental factors would favour release of the bound fluazuron so that it may have an effect.

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List of abbreviations

ADI	Acceptable Daily Intake
APR	Acute Phase Response
BSA	Body Surface Area
BW	Body Weight
EMA	European Medicines Agency
IFNα	Interferon alpha
IGR	Insect Growth Regulator
JECFA	Joint Expert Committee on Food Additives
MRL	Maximum Residue Limit
NOEL	No Observed Effect Level
SIT	Sterile Insect Technique
TNFα	Tumour Necrosis Factor alpha
WHO	World Health Organisation
WHP	Withholding Period

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Chapter 1

1.1 Introduction

Small stock farming, in the form of sheep and goats production, is a significant part of South Africa's agricultural economy and accounted for a turnover of approximately 6.4 billion Rand in the 2011/2012 agricultural season. This equated to approximately 8.4% of the total animal product based output for the season (DAFF 2013). Productivity under southern African conditions is however not easy as parasites abundant in the environment induce major disease, which as a result of competition with the host animal for nutrients results in losses in animals products such as meat, milk, skins, wool and manure for the farmer (Perry and Randolph 1999). In the developed world an additional cost of veterinary treatment can be added to the equation.

Sheep in particular are affected by numerous parasites including mites, lice, blowflies, ticks and helminths (Bates 2004; Lehmann 1993; Taylor 2001). Blowflies are Dipterids with complex life cycles and complete metamorphoses (Bowman and Georgi 2009; Taylor et al. 2007). Fly strike or myiasis causes damage to hides and frequent death in their ovine hosts, as a result of cutaneous myiasis caused by the larval stages, resulting in considerable losses to the industry all over the world (French et al. 1995; Hall and Wall 1995; Tellam and Bowles 1997; Tourle et al. 2009). All economically important blowfly species causing veterinary myiasis belong to the superfamily Oestroidea (Taylor et al. 2007), which contains the three major families Oestridae, Calliphoridae and Sarcophidae. The two most significant blowfly genera in South Africa, Lucillia and Chrysomya, both belong to the family Calliphoridae.

The control of blowfly strike revolves around prevention and cure, with the former being by far the more effective method of control (Bath and De Wet 2000; Tellam and Bowles 1997). A number of husbandry procedures and management methods can aid in the prevention of blowfly strike (Bath and De Wet 2000; Bowman and Georgi 2009; Kahn and Line 2010; Tellam and Bowles 1997; Townend 1987), however chemical control in the form of topically

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applied insecticides remain the most widely used and appear to be indispensable at this stage (Bates 2004; Tellam and Bowles 1997).

Bayer currently markets Drastic Deadline Extreme®, a pour on formulation for use in cattle containing flumethrin and fluazuron, registered in particular for the control of resistant blue ticks (*Rhipicephalus decoloratus* and *R. microplus*). Within the formulation the pyrethroid flumethrin, a synthetic compound structurally similar to pyrethrins (Casida 1980), is highly effective against susceptible ticks while fluazuron, an insect growth regulator (IGR), inhibits the insect specific enzyme required for normal growth and development. In combination the pyrethroid acts as a contact poison and kills the susceptible parasites while the IGR induces moulting disturbances and death of the immature parasites (Siddall 1976). Despite the claim of efficacy against ticks, this specific product combination has not been evaluated for its effect against blowflies. For this study, we evaluate the potential efficacy of the formulation in preventing myiasis in sheep.

1.2 Hypothesis

The topical application of fluazuron and flumethrin on sheep pelts is an effective larvicide.

1.3 Aims and objectives

    i.   To determine the fate of topically applied fluazuron.

    ii.   To determine the efficacy in preventing blowfly larval development when applied to sheep pelts in combination with flumethrin.

1.4 Benefits arising from the study

    a.   Fulfilment of Master of Science degree in the field of Veterinary Science.

    b.   The possible development of a new treatment option for myiasis in sheep.

    c.   The possible development of new products to prevent and treat myiasis in sheep.

Chapter 2

2.1 Literature review

2.1.1 Introduction

Of the approximately 122.3 million hectares which make up South Africa, only 16.7 million hectares are potentially arable, which translates to just 13.7 % of available land being suitable for crop production (DAFF 2013). Large parts of the remaining land are either very mountainous or dry and arid and not suitable for sustaining dairy or beef cattle (Schoeman et al. 2010). Consequently, small stock production has been of current and historic importance to the South African agriculture industry and accounted for approximately R6.4 billion (8.4 %) of South Africa's total animal product output in the 2011/2012 season (DAFF 2013). Of the different species farmed in South Africa, sheep remain significant. As of August 2012, the breed composition of the South African commercial sheep flock was as follows: 11.25 million Merinos, 25 thousand Karakuls, 4.1 million wool sheep of other breeds and 6.0 million non-wool sheep (DAFF 2013), amounting to a total of 21.4 million animals.

Over the 2011/2012 season, 6.2 million sheep, lambs and goats were slaughtered at commercial South African abattoirs in South Africa yielding 136 200 tons of mutton, subsequently sold from abattoir to the meat trade at an approximate price of R46.24 per kg (DAFF 2013). The same season also yielded 43 600 tons of wool (calculated on a greasy basis), with a total value of auction sales calculated at 2.3 billion Rands (DAFF 2013). While it is not surprising that sheep form an important component of the commercial agricultural sector, small stock production takes on a new aspect in the poorer and developing rural areas of Southern Africa, where livestock are kept for a combination of economic and non-economic reasons (Chilonda et al. 1999), viz. physical investment, food and resource production (meat and wool) or religious purposes (slaughtered at ceremonies). Certain cultures in South Africa consider the ritual slaughter of livestock an important means of communicating with and honouring their ancestors (Mnguni 2006).

In the Eastern Cape region of South Africa, an area known for large scale, intensive sheep farming, interviews conducted with farmers identified many challenges associated with sheep farming in this area (Nel and Davies 1999). These challenges were grouped into:

- Economic factors including fluctuating prices of mutton and wool, mounting debt due to frequent drought and stock theft.
- Political factors which include violence against farmers in this area as well as marked uncertainty and fear amongst the farmers with regard to their future due to fears of land expropriation.
- Environmental factors of which drought remains the most important since the high frequency with which the region experiences this phenomenon has a severe effect on economic returns, employment and stock numbers. Internal and external parasites may be included in this category.

2.1.2 The effect of parasites

Figure 2.1 provides a schematic representation of how parasites may affect the productivity of a flock of sheep; as a result of their competition with the host animal for nutrients, they typically cause lower productive output of commodities such as meat, milk, skins, wool and manure (Perry and Randolph 1999). In the developed world, an additional cost of veterinary treatment also needs to be added to the equation.

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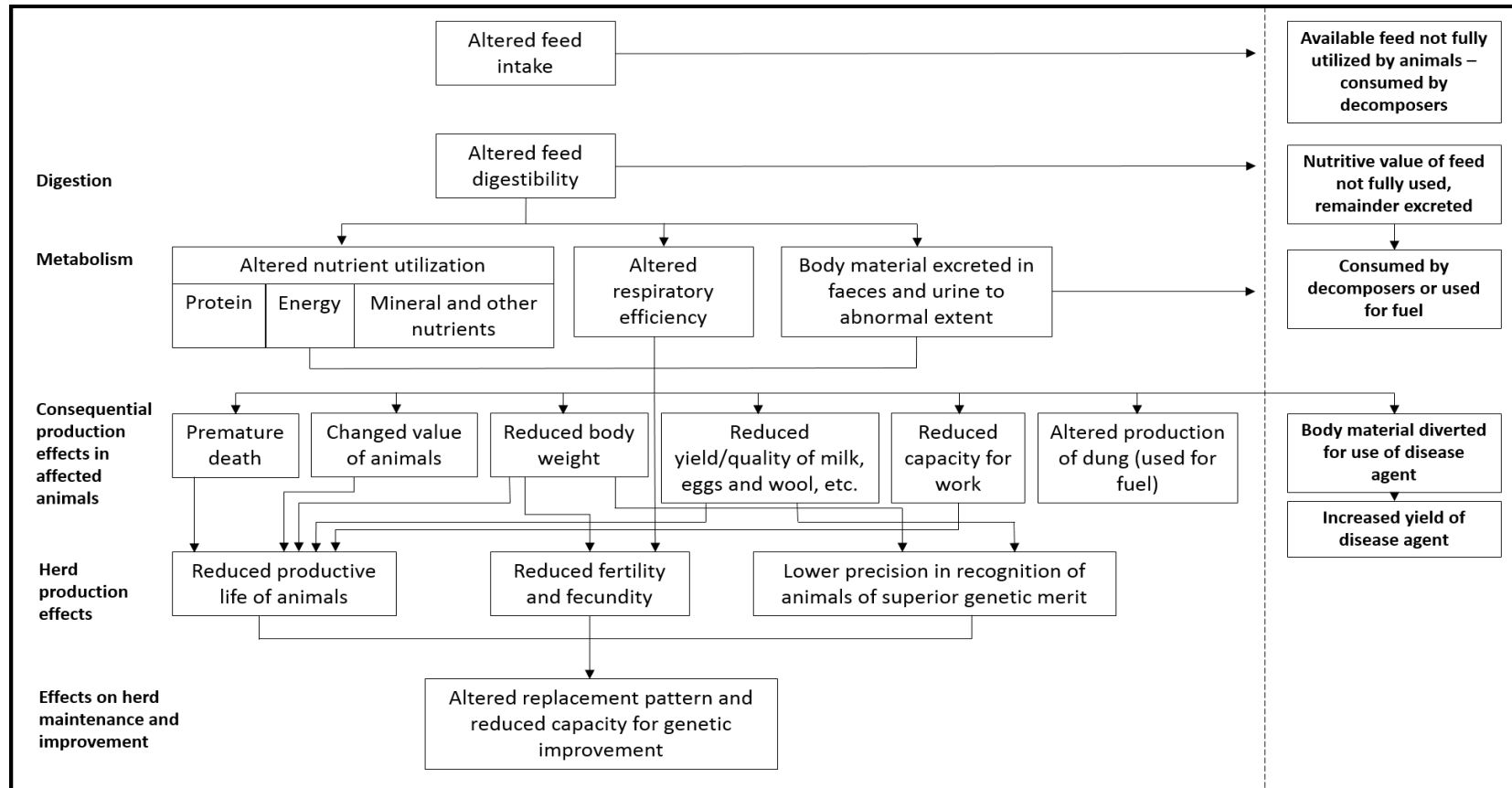
Figure 2.1: The pathways through which disease may affect herd or flock productivity leading to reduced productive output. The central area depicts the possible sequelae of parasite infestation while the column on the right gives a brief explanation for the effects seen. Redrawn from Morris and Marsh (1994).

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Sheep in particular are affected by numerous parasites (Bates 2004; Lehmann 1993; Taylor 2001):

- Mites which live in the skin and cause intense pruritus, leading to self-trauma by the sheep in the form of rubbing and biting and damage to the wool in the form of wool breaks.
- Lice, which also cause severe self-trauma as a result of pruritus
- Blowflies which result in damage to hides and frequent death
- Ticks which can transmit numerous diseases e.g. Heartwater
- Helminths which may lead to decreased production, clinical disease and sometimes death

2.1.3 Blowflies and their relationship with sheep

2.1.3.1 Phylogeny

Blowflies are phylogenetically classified as follows: Phylum Arthropoda, Class Insect, Order Diptera, Superfamily: Oestroidea, Family Caliphoridae (Bowman and Georgi 2009; Taylor et al. 2007). All species in the order Diptera have complex life cycles with a complete metamorphosis, implying that the larvae are completely different in structure and behaviour to the adults. Dipterous flies can be ectoparasites as adults or larvae but are rarely parasites in both life cycle stages.

Blowfly larvae cause a cutaneous myiasis (blowfly strike) in sheep which results in considerable losses in the wool industry all over the world including South Africa (French et al. 1995; Hall and Wall 1995; Tellam and Bowles 1997; Tourle et al. 2009). While the term myiasis originally referred to the disease in humans caused by dipterous larvae (Hope 1840), the definition has been expanded to include the same disease that these larvae induce in their living vertebrate host as they feed on dead or living tissue, liquid body substances or ingested food (Zumpt 1965).

At present, there are two main systems for classifying myiasis (Bowman and Georgi 2009; Hall and Wall 1995): Anatomically which refers to the location of the infestation (nasal myiasis for example) or biologically (entomologically), which relates to the parasitic relationship and the level of dependence on the host. In the latter case the parasite can be further classified as obligatory or facultative. In obligatory myiasis, the fly larvae are completely dependent on the host to complete their life cycle, while in facultative myiasis the fly larvae, which are usually free living, can under certain circumstances become parasitic (Kahn and Line 2010).

Facultative blowflies may also be further subdivided into primary parasites which parasitize only living hosts, and secondary parasites which may feed on decaying flesh or become invaders in weak, debilitated, soiled, immobilized or wounded animals (Bowman and Georgi 2009; Hall and Wall 1995).

All economically important blowfly species causing veterinary myiasis belong to the superfamily Oestroidea (Taylor et al. 2007). Within this superfamily, the three major families of myiasis producing flies are Oestridae, Calliphoridae and Sarcophidae. The most significant genera producing fly strike in sheep in South Africa are Lucilia and Chrysomya (Bath and De Wet 2000; Howell et al. 1978), both belonging to the family Calliphoridae. *Lucilia cuprina* is a primary blowfly and is the main species associated with myiasis in wooled sheep (Bowman and Georgi 2009; Leipoldt 1996; Soulsby and Mönnig 1982). Lucilia blowflies measure up to 10 mm in length and have a characteristic green to bronze sheen as evident in figure 2.3. The adults are recognized by the presence of a bare stem vein, bare squamae and 3 pairs of post-sutural, dorso-central bristles on the thorax. Males and females may be distinguished by the distance between the eyes which are very close together, almost touching anteriorly, in the males but separated in the females (Taylor et al. 2007). Figure 2.2 shows an example of a *Lucilia* spp. larva, which is smooth and segmented, typically measuring 10 to 14 mm in length. A pair of oral hooks are present at the anterior extremity and peritremes bearing spiracles can be found at the posterior end (Taylor et al. 2007; Urquhart 1996).

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Figure 2.2: Late instar larva of a *Lucilia* species with the head at bottom left of the image (Anderson and Kaufman 2011). Photograph by Richard Major © Australian Museum.

Figure 2.3: Adult sheep blow fly, *Lucilia cuprina* showing typical colouring and morphology (Anderson and Kaufman 2011). Photograph by Lesley Ingram, Bugwood.org.

Chrysomya albiceps is a secondary blowfly and strikes only where primary blowflies have already set up an infection (Bath and De Wet 2000). The adult *Chrysomya* flies are blue-green in colour and have orange-brown eyes. The thorax has longitudinal stripes and the hind margins of the abdominal segments have black stripes. The anterior spiracle is white or pale

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yellow (Soulsby and Mönnig 1982; Taylor et al. 2007). Larvae of the genus *Chrysomya* may reach 18 mm in length in their third stage of development. The larvae have a number of fleshy projections resembling thorns on most of the body segments which gives rise to their common name of "the hairy maggot blowfly" (Taylor et al. 2007). These rough looking maggots eat the smoother looking maggots of the primary blowfly infection (Bath and De Wet 2000).

2.1.3.2 Predisposing factors

A number of factors predispose sheep to myiasis by attracting and enticing blow flies to lay their eggs (Bruère and West 1993; Raadsma et al. 1988; Tellam and Bowles 1997; Wardhaugh and Morton 1990), with faecal and urine staining around the crutch region, especially in lambs, being the most important factor. Fleece rot caused by *Pseudomonas aeruginosa* and infection of the wool with *Dermatophilus congolensis* can also produce odours which attract blowflies. Likewise, foot rot can also result in fly strike at the lesions themselves or on the flanks where exudate from the lesions has rubbed off. Wethers with balanoposthitis may be struck on the ventral abdomen or prepuce and rams may be struck on the head if injured through fighting; this is known as poll strike.

Merino breed sheep are particularly susceptible to fly strike (Raadsma et al. 1988; Tellam and Bowles 1997; Townend 1987), as their increased skin folds provide warm and moist, sheltered environments conducive to blowfly strike, especially around the crutch region. Their longer and thicker fleece that takes longer to dry after rain tends to provide the perfect environment for bacterial growth and secondary fly strike (Tellam and Bowles 1997).

2.1.3.3 Lifecycle

Blowflies usually occur in the warm summer months. Adult flies are attracted to moist wounds, skin lesions or soiled wool such as the area around the breech or perineum. The female flies feeds at the target area and lay approximately 300 eggs, which hatch within a day. The hatched larvae (maggots) move about the skin or wound surface, feeding on dead

cells, exudate, secretions and debris. Initially the larvae do not ingest live tissue (Kahn and Line 2010). With time, these larvae irritate and injure successive layers of skin and stimulate further production of exudates. The epidermis becomes thin and this allows larvae to burrow into the subcutis producing tissue cavities in the skin that can measure a few centimetres in diameter, which can ultimately progress to haemorrhage, infection, dehydration, anaphylactic shock, toxaemia and death (Kahn and Line 2010; Taylor et al. 2007). Once feeding has commenced, the larvae moult for the first time after 12 to 18 hours and again approximately 30 hours later. They will feed for 3 to 4 days before dropping to the soil to pupate for anything from 7 days to several weeks depending on the temperature. Figure 2.4 shows a simple representation of the blowfly life cycle.
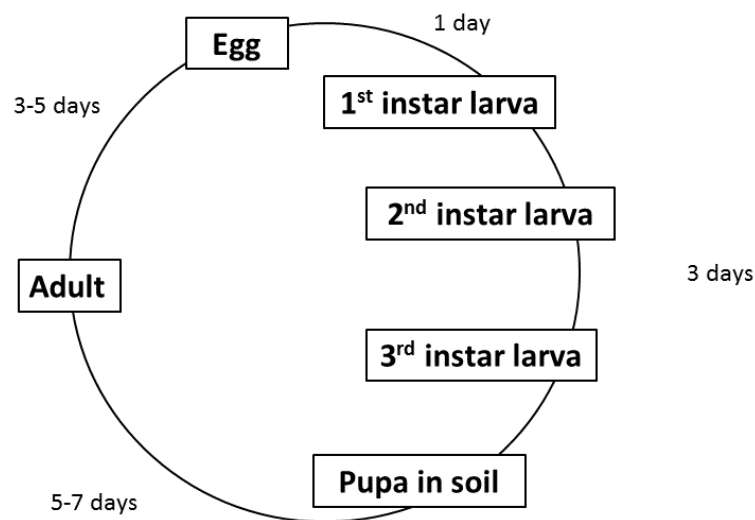
Figure 2.4: A simple representation of the various stages of the blowfly lifecycle redrawn from Bruère and West (1993)

In all insects, including flies, chitin plays a vital role in the lifecycle, making up one of the major components of the cuticle or exoskeleton. This exoskeleton is a mostly rigid structure which has very limited ability to expand and allow for body growth (Merzendorfer and

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Zimoch 2003), with the resulting need for insects to periodically shed the old cuticle and replace it with a larger and looser one during moulting. The importance of this fact will become clear further along in this discussion when chemicals which interfere with chitin synthesis are discussed.

2.1.3.4 Pathophysiology

Clinical signs in sheep infested with fly larvae may include irritation, pruritus, discomfort, dullness, lethargy and varying degrees of anorexia accompanied by weight loss as a result of the feeding larvae. Affected animals are often separate from the herd and closer inspection will usually reveal wounds emitting foul smelling odours (Bath and De Wet 2000; Kahn and Line 2010; Taylor et al. 2007).

Reduced feed intake and external symptoms are however not the only contributing factor to production losses in sheep affected by myiasis. The host's immune system appears to play an important role in inducing metabolic changes in target tissues (Colditz 2002; Colditz 2003), due to the activation of the acute phase response (APR) following bacterial proliferation on the skin (Colditz et al. 2001). The pro-inflammatory cytokines such as Interleukin 1α and β, Interleukin 6, Tumour Necrosis Factor alpha (TNFα) and Interferon (IFN) α and β produced by leukocytes, as well as the epithelial cells, are responsible for initiating the APR.

The effects of the APR in the body are widespread (Colditz 2003) and include fever, inappetance, lethargy, skeletal muscle catabolism from decreased amino acid accretion and increased proteolysis. Lipolysis and increased leptin production in adipose tissues, increased hepatic gluconeogenesis and a decrease in albumin production are also involved. The APR also targets tissues of the gastrointestinal tract, causing them to express acute phase proteins such as serum amyloid A and lipopolysaccharide binding protein (Vreugdenhil et al. 1999), with resultant decreased nutrient uptake from ingested matter. The pro-inflammatory cytokines have also been postulated to affect skeletal growth via their direct effects on osteoblast and osteoclast activity and their indirect effects on micronutrients (Stephensen 1999).

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2.1.4 The control of blowflies and myiasis

The control of blowfly strike revolves around prevention and cure, with the former being by far the more effective method of control (Bath and De Wet 2000; Tellam and Bowles 1997). A number of procedures and management methods can aid in the prevention of blowfly strike (Bath and De Wet 2000; Bowman and Georgi 2009; Kahn and Line 2010; Tellam and Bowles 1997; Townend 1987), including:

- Avoiding surgical procedures during the most active periods of blowfly activity, as the surgical wound could attract female flies.
- Culling of sheep with excessive skin pleats, sheep that suffer from repeated strike as well as sheep that have excessive dagging (matting of faeces in perineal wool), as these are the perfect sites for bacterial growth as discussed above.
- Keeping the flock free of helminth infestations with effective anthelminthics, as helminth infestations can cause diarrhoea and soiling of the wool around the anus, attracting blowflies to the area.
- Proper burial or disposal of carcasses, since carcasses serve as a perfect breeding environment.
- Changing the shearing times so that sheep have short wool during peak blowfly season, in order to reduce predisposing factors.
- Crutching, which refers to the shearing of wool around the perineal area, to again reduce predisposing factors.
- Docking of tails to the correct length to prevent soiling with urine and faeces.
- Dehorning of animals to reduce the number of wounds caused by fighting.
- Mulesing (surgical removal of excessive skin pleats) will deprive the blowflies of attractive breeding sites by removing the skin pleats which create a warm, moist and sheltered environment in which to lay eggs.
- Vaccination against fly strike is currently an active field of research and shows some promise in the future of blowfly control (East and Eisemann 1993; Sandeman 1990). Vaccination of sheep with antigens, including the secretory proteases of larvae as well as different extracts from the gut or peritrophic membrane, has been demonstrated to retard larval growth to varying degrees and in some cases cause larval mortality.

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- Fly population control: Another direction of research also has potential in the future control of blowfly: this involves the release of genetically modified, sterile, male flies into the population (Schetelig and Wimmer 2011; Scott et al. 2004), otherwise known as the Sterile Insect Technique or SIT. The basic principles of the sterile insect technique involve breeding large numbers of the target species and then causing sexual sterility within the group by exposing them to gamma radiation. These irradiated flies are then released into the target population; the sterile males mate with the wild females and in so doing prevent them from reproducing (Klassen and Curtis 2005).

- Chemical means of treating blowfly strike are numerous and varied but topically applied insecticides remain the most widely used method and appear to be indispensable at this stage (Bates 2004; Tellam and Bowles 1997). The earliest treatments for blowfly strike used dressing containing arsenic, copper sulphate, sulphur and cresylic acid (Levot 1995) and later the organochlorines, organophosphors and pyrethroids. Of these, the latter two are still in use while the organochlorines were withdrawn from use in 1958 as a result of the environmental persistence of these compounds, the long time to depletion from adipose tissue and the development of resistance (Hughes and McKenzie 1987; Shanahan 1958).

Bayer currently markets Drastic Deadline Extreme®, a pour on formulation for use in cattle containing flumethrin and fluazuron, registered in particular for the control of resistant blue ticks (*Rhipicephalus decoloratus*). The potential effects, if any, on blowflies, larvae and subsequent myiasis had not been investigated.

2.1.5 Pyrethroids

Pyrethrins are naturally occurring, insecticidal chemicals derived from the Chrysanthemum plant (Riviere and Papich 2008). As shown in Figure 2.5, natural pyrethrins can occur as six different compounds namely pyrethrin I, pyrethrin II, jasmolin I, jasmolin II, cinerin I and cinerin II (Spurlock and Lee 2008), which differ from each other in the terminal substituents in the side chains of the acid and alcohol components (Casida 1980).

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In recent years the pyrethrins have slowly been replaced by pyrethroids, synthetic compounds which are structurally similar to pyrethrins but have been modified by the addition of various substituent groups such as chlorine, bromine or cyanide onto the basic molecule in order to render them more photostable, increasing the persistency of the pyrethroids (Ballantyne et al. 1993). Additional advantageous effects of the additions include increased potency and broader spectrum of activity (Casida 1980).

The pyrethroids exert their insecticidal effect via their action on voltage gated sodium channels in nerve cells. By binding to these channels the pyrethroids cause them to remain open, which results in the nerve cell entering a stable, hyper-excitable state. This repetitive membrane depolarization that results subsequently induces complete paralysis and the ultimate death of insects (Casida and Quistad 2004; Pérez-Fernández et al. 2010; Riviere and Papich 2008).

In general, the pyrethroids are considered safe compounds for humans and animals as they are extremely selective for insect nerve cells when compared to mammalian nerve cells, due to several factors (Warmke et al. 1997):
- insectoid sodium channels are a hundred-fold more sensitive to the pyrethroid effect;
- the pyrethroid insecticides are more effective at the low body temperature of insects (15 – 20° C) than humans (37.5° C);
- the selective uptake of pyrethroids by the nerve lipoid sheath and the poor detoxification mechanisms present within the insect neural network.

Pyrethroids are widely used in agriculture for crop, wood and livestock protection because of their low mammalian toxicity, high insecticidal potency and lack of persistence in the environment. Their low persistency is due to the fact that they are easily hydrolysed (Ballantyne et al. 1993; Coats 1990). They are, however, extremely toxic to aquatic organisms (Everts et al. 1983; Mauck et al. 1976; Weston et al. 2005).
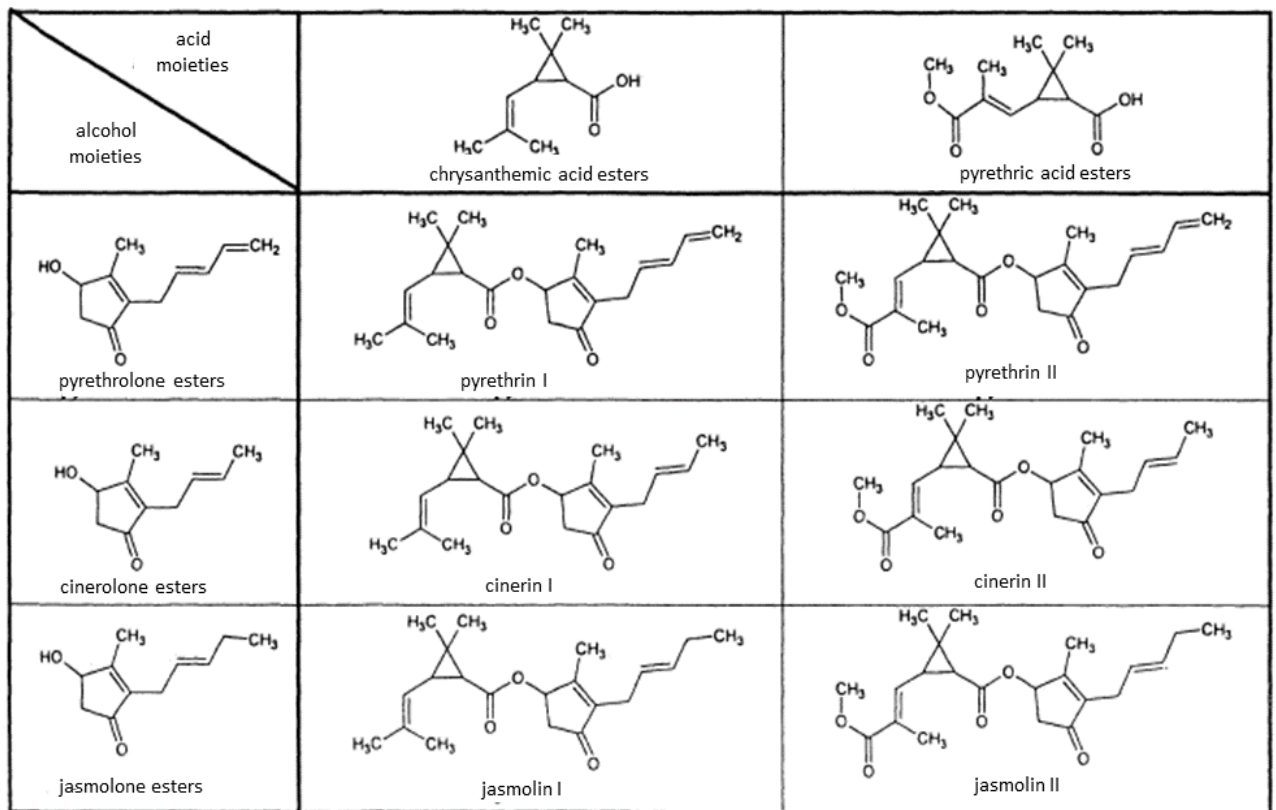
Figure 2.5: The chemical structures of the different pyrethrin esters (Spurlock and Lee 2008)

Flumethrin is a fourth generation pyrethroid formed by the reaction of 4-fluoro-3-phenoxybenzaldehyde and *trans*-(*E*)-3-[2-chloro-2-(4-chlorophenyl)vinyl-2,2-dimethylcyclopropanecarboxylic acid chloride in the presence of cyanide (World Health Organisation 1997). The chemical name of flumethrin is cyano(4-fluoro-3-phenoxyphenyl)methyl-3-[2-chloro-2-(4-chlorophenyl)ethenyl]-2,2-dimethylcyclopropanecarboxylate with a molecular formula of (World Health Organisation 1997); the structural formula is shown in Figure 2.6. Although flumethrin is used extensively in veterinary products to control many different ectoparasites, the chief application remains the control of ticks (Anadon et al. 1995), and no literature could be found documenting the use of flumethrin against blowfly larvae.
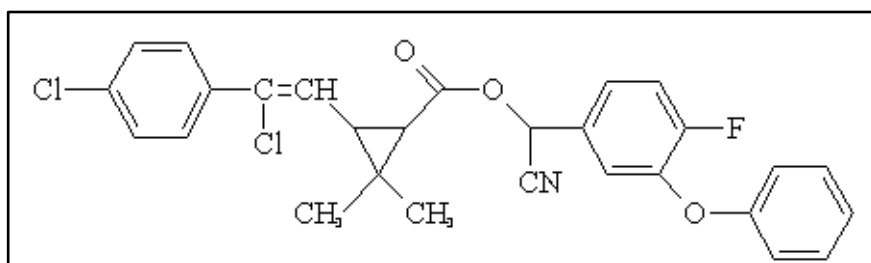
Figure 2.6: The structural formula of flumethrin (World Health Organisation 1997).

2.1.6 Insect growth regulators

Insect Growth Regulators (IGRs) are a more recent development in the field of ectoparasite control (Bates 2004; Tunaz and Uygun 2004). The IGRs function through the inhibition of insect specific enzyme systems. One of the most important characteristics of insect growth regulators is that they do not necessarily kill target pests directly but interfere in some way with the growth and development processes, causing moulting disturbances, which results in the death of the immature parasites and ultimately impairs insect survival (Siddall 1976).

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The IGRs are currently classified into 3 categories (Graf 1993):

- **Juvenile hormone analogues:** They prevent the metamorphosis of insects into adults by mimicking the action of naturally occurring juvenile hormones (Taylor 2001). Under normal circumstances, esterase enzymes within the insect's haemolymph catalyze the destruction of endogenous juvenile hormone once the larva is fully developed, thereby allowing the larva to metamorphose into the adult stage. The juvenile hormone analogues, which are more stable and resistant to esterase enzymes, are able to bind permanently to juvenile hormone receptor sites, consequently inhibiting the further development to adult (Dhadialla et al. 1998). Methoprene is an example of a juvenile hormone analogue (Graf 1993).

- **Chitin inhibitors:** These substances appear to interfere with the deposition of chitin in the insect cuticle, rather than interfere with its synthesis (Friedel et al. 1988). Two different groups of chitin inhibitors can be identified, namely triazine and pyrimidine derivatives and the two compounds are closely related with similar modes of action. Cyromazine is a well-known and widely used chitin inhibitor, and is an example of a triazine derivative.

- **Chitin synthesis inhibitors:** While their mechanism of action is not completely understood, it is believed that they interfere with the assembly of chitin chains into microfibrils (Cohen 1993). Benzoylphenyl ureas such as triflumeron, lufenuron and fluazuron are classified as chitin synthesis inhibitors and immature insects exposed to these compounds are not able to complete ecdysis, subsequently dying during the process of moulting. Benzoylphenyl ureas also appear to demonstrate a transovarial effect in that they are incorporated into the egg nutrients when the female lays eggs, where they interfere with hatching (Taylor 2001).

Fluazuron is commonly used for the control of the blue ticks (*Rhipicephalus decoloratus* and *R. microplus*) in cattle (Kryger et al. 2005), which stands out in stark contrast to the rest of the benzoylphenyl ureas, which have notoriously poor efficacy against ticks (Graf 1993).

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Despite an extensive literature review, no information could be found indicating efficacy of fluazuron against blowflies. However, with fluazuron being a chitin synthesis inhibitor and blowflies, like all insects, have chitin exoskeletons, it stands to reason that fluazuron may well be effective at disrupting blowfly larval or adult development. Figure 2.5 shows the structural formula of fluazuron - its chemical name is N-(3-(3-chloro-5-trifluoromethyl-2-pyridinyloxy)-4-chlorophenyl)-1-2,6-difluorobenzoyl)-urea with a molecular formula of $C_{20}H_{10}C_{12}F_5N_3O_3$ (JECFA 1998).
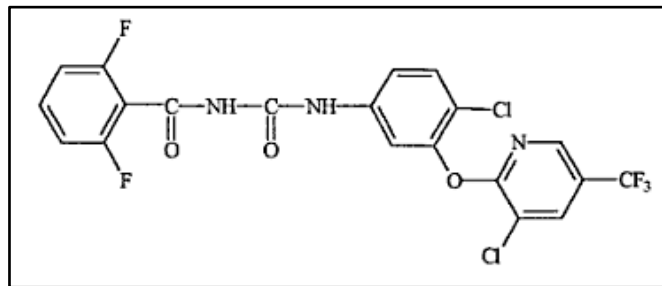
Figure 2.7: The structural formula of fluazuron (JECFA 1998).

2.1.7 Resistance

The efficacy of topically applied insecticides is heavily dependent on exposure of the parasite to toxic concentrations of the pesticide for as long a period as possible (Hennessy 1994). One of the problems associated with topically applied pesticides is the area of the concentration-time curve known as the "tail", where pesticide concentration fall below lethal levels for a varying degree of time, usually fairly extended in the case of topically applied products, with the result that there is an extended period of exposure of parasite population to sub-lethal drug concentrations with subsequent survival and propagation of organisms carrying alleles containing resistance determinants. This "tail" phenomenon is practically unavoidable when it comes to the application of topical insecticides as the concentration of active ingredient bound to the skin, hair coat or wool gradually decreases with time. It then stands to reason that this effect will be exacerbated in oil soluble or lipophilic active ingredients which dissolve into or bind strongly to the lanolin in wool, dramatically prolonging the presence of the active ingredient on the sheep.

The simple decision to use a specific chemical against a specific pest is already the greatest risk for increasing the likelihood of resistance development (Heath and Levot 2015); the risk is further increased by a number of factors including the frequency with which the pest is exposed to the chemical, method and effectiveness of application as well as persistency of the chemical on the host. These factors act as artificial selection mechanisms, resulting in the removal of susceptible individuals from the population and perpetuating the genetic transmission of resistant traits to subsequent generations.

In Australia and New Zealand, resistance development to blowfly remedies, including organophosphates and benzoylphenyl ureas, has been rapid and widespread (Heath and Levot 2015; Levot 1995), with a link between resistance to the different chemical groups being discovered in the 1990s. It was determined that resistance to organophosphates in blowflies was influenced by microsomal oxygenase enzymes, which in turn showed a strong correlation to diflubenzuron susceptibility (Kotze et al. 1997; Kotze and Sales 2001). This phenomenon of cross-resistance between different chemical classes will also serve to accelerate the development of resistance within blowfly populations.

A similar potential fate could be looming within a South African context if product usage and artificial selection trends follow the same path, which is likely as resistance to arsenic and Diazinon was demonstrated in this country as far back as 1975 (Blackman and Bakker 1975). In the author's experience working in the veterinary pharmaceutical industry, new active ingredients with unique modes of action are not brought onto the market very frequently, with the risk that the rate of resistance development may well outstrip the rate at which effective formulations can be developed and commercialized. Developing and commercializing new formulations is a complex, extended and costly affair (Geary and Thompson 2003); for this reason, extending the registration of an existing product registered for use on a different species to sheep could cut down significantly on costs and time required.

2.1.8 Conclusion

Fly strike can be a source of major losses for sheep producers. With resistance amongst the current products being a potential problem, the aim of this study is to investigate the potential preventative efficacy of a fluazuron against blowfly larvae in sheep within a commercial fluazuron/Flumethrin combination pour-on formulation already marketed in South Africa. The combination has the potential to control fly strike in sheep through the disruption of moulting caused by fluazuron however the behaviour of the formulation on sheep skin and wool is unknown.

Chapter 3

The pharmacokinetics and *in-vitro* efficacy of a topically applied, low dose flumethrin and fluazuron combination in a sheep myiasis model.

By CM Austin, V Naidoo

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The pharmacokinetics and *in-vitro* efficacy of a topically applied, low dose flumethrin and fluazuron combination in a sheep myiasis model.

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Abstract

Small stock farming plays an important role in the South African livestock industry and whether farmed for commercial, subsistence or other reasons, parasites remain a significant hindrance to productivity. Blowflies in the form of *Lucillia* spp and *Chryomya* spp feature prominently as a leading cause of myiasis and subsequent production losses, possibly even death in sheep. Chemical means of preventing and treating blowfly strike by topical application remains the most widely used method and appears to be indispensable at this stage; efficacious new molecules or formulations effective against blowfly strike are constantly sought. For this study we evaluate the potential of fluazuron to be one of these effective molecules through the use of pharmacokinetics and in vitro efficacy. When pure fluazuron was applied to raw meat at the recommended dose per bodyweight for cattle, significant effects on adult fly development were observed ($p=0.002$). A pilot pharmacokinetic study confirmed virtually no systemic absorption, from wool binding. More importantly, when applied topically to post-mortem collected sheep pelts at the same dose rate, no significant effects were observed on larval repellence ($p=0.74$) or larval development ($p=0.27$) in comparison to the control group, with fly development progressing as expected. The results of this study while showing that fluazuron is effective against blowfly larvae affecting sheep, the wool binding of the molecule may preclude its use.

Keywords: Blowflies, myiasis, sheep, flumethrin, fluazuron, chromatography

3.1 Introduction

Small stock production, particularly in the form of sheep and goat farming, plays a significant role in South African agriculture with an approximate 0.5 billion dollar (US) contribution to the 2011/2012 agricultural season representing circa 8.4% of the total animal product based output that season (DAFF 2013). Despite the value of sheep to the South African economy, sheep farming in the area is under a number of constraints, of which cutaneous myiasis (blowfly strike) results in considerable losses to the wool and mutton production (French et al. 1995; Hall and Wall 1995; Tellam and Bowles 1997; Tourle et al. 2009). Myiasis as a disease is characterised by larvae that feed of the dead or living tissue of a live host (Zumpt 1965).

Blowflies are Dipterids with complex life cycles and complete metamorphoses (Bowman and Georgi 2009; Taylor et al. 2007). Dipterous flies can be ectoparasites as adults or larvae but are rarely parasites in both life cycle stages. All economically important blowfly species causing veterinary myiasis belong to the superfamily Oestroidea (Taylor et al. 2007), which contains the three major families Oestridae, Calliphoridae and Sarcophidae. The two most significant blowfly genera in South Africa, *Lucilia* and *Chrysomya*, both belong to the family Calliphoridae, with the former being a primary blowfly associated mainly with wooled sheep (Bowman and Georgi 2009; Leipoldt 1996; Soulsby and Mönnig 1982), while *Chrysomya albiceps* is a secondary blowfly and strikes only where primary blowflies have already set up an infection (Bath and De Wet 2000).

Blowflies usually occur in the warm summer months. Adult flies are attracted to moist wounds, skin lesions or soiled wool such as the area around the breech or perineum. The female flies feeds at the target area and lay approximately 300 eggs, which hatch within a day. The hatched larvae (maggots) move about the skin or wound surface, feeding on dead cells, exudate, secretions and debris. Initially the larvae do not ingest live tissue (Kahn and Line 2010). With time, these larvae irritate and injure successive layers of skin and stimulate further production of exudates. The epidermis becomes thin and this allows larvae to burrow

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into the subcutis producing tissue cavities in the skin that can measure a few centimetres in diameter, which can ultimately progress to haemorrhage, infection, dehydration, anaphylactic shock, toxaemia and death (Kahn and Line 2010; Taylor et al. 2007). Once feeding has commenced, the larvae moult for the first time after 12 to 18 hours and again approximately 30 hours later. They will feed for 3 to 4 days before dropping to the soil to pupate for anything from 7 days to several weeks depending on the temperature, before the adult fly emerges to begin the life cycle again.

A number of factors predispose sheep to myiasis by attracting and enticing blow flies to lay their eggs (Bruère and West 1993; Raadsma et al. 1988; Tellam and Bowles 1997; Wardhaugh and Morton 1990), with faecal and urine staining around the crutch region, especially in lambs, being the most important factor. Fleece rot caused by *Pseudomonas aeroginosa* and infection of the wool with *Dermatophilus congolensis* can also produce odours which attract blowflies. Likewise, foot rot can also result in fly strike at the lesions themselves or on the flanks where exudate from the lesions has rubbed off. Wethers with balanoposthitis may be struck on the ventral abdomen or prepuce and rams may be struck on the head if injured through fighting - this is known as poll strike. Merino breed sheep are particularly susceptible to fly strike (Raadsma et al. 1988; Tellam and Bowles 1997; Townend 1987) as their increased skin folds provide warm and moist, sheltered environments conducive to blowfly strike, especially around the crutch region. Their longer and thicker fleece that takes longer to dry after rain tends to provide the perfect environment for bacterial growth and secondary fly strike (Tellam and Bowles 1997).

The control of blowfly strike revolves around prevention and cure, with the former being by far the more effective method of control (Bath and De Wet 2000; Tellam and Bowles 1997). A number of husbandry procedures and management methods can aid in the prevention of blowfly strike (Bath and De Wet 2000; Bowman and Georgi 2009; Kahn and Line 2010; Tellam and Bowles 1997; Townend 1987), including culling of sheep with excessive skin pleats or those that suffer from repeated strike or matting of faeces in perineal wool, keeping the flock free of helminth infestations with effective anthelminthics, crutching and changing

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shearing times, docking tails, dehorning, mulesing and environmental fly population control. Chemical means of treating blowfly strike are numerous and varied but topically applied insecticides remain the most widely used method and appear to be indispensable at this stage (Bates 2004; Tellam and Bowles 1997).

The insect growth regulators (IGRs) are a more recent development in the field of ectoparasite control (Bates 2004; Graf 1993; Tunaz and Uygun 2004). The IGRs function through the inhibition of insect specific enzyme systems and can be classified into 3 categories (Graf 1993): The juvenile hormone analogues prevent the metamorphosis of insects into adults by mimicking the action of naturally occurring juvenile hormones (Taylor 2001); chitin inhibitors which appear to interfere with the deposition of chitin in the insect cuticle, rather than interfere with its synthesis (Friedel et al. 1988); and the chitin synthesis inhibitors that interfere with the assembly of chitin chains into microfibrils (Cohen 1993). Benzoylphenyl ureas such as triflumeron, lufenuron and fluazuron are classified as chitin synthesis inhibitors and immature insects exposed to these compounds are not able to complete ecdysis, subsequently dying during the process of molting (Taylor 2001) .

With chitin playing a vital role in the lifecycle all insects, including blowflies, the IGRs have already demonstrated their effectiveness as a means of blow-fly control. The aim of this study was to investigate potential value of an existing pour-on combination of 1% flumethrin and 2.5% fluazuron (Drastic Deadline eXtreme® - Bayer) against blowfly larvae when applied to sheep pelts. The product is registered for the control of blue ticks on cattle but its use in sheep against myiasis has never been investigated.

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3.2 Materials and methods

Lucillia spp. larvae were artificially bred for these studies using a similar method to that described by Mukandiwa (L. et al. 2012; Mukandiwa et al. 2012). Blowflies were collected from carcasses at a vulture restaurant in Hartebeespoort, South Africa and successive generations of larvae were bred on commercial beef cuts in an incubator kept constant at 35°C under constant humidity. As reported by Webber, the laboratory bred larvae became smaller with successive generations (Webber 1955), most likely from nutritional reasons. Nonetheless the colony was able to provide viable flies that were able to fly and reproduce in all the tested generations.

3.2.1 In vitro studies
3.2.1.1 The efficacy of pure fluazuron applied to meat

Twelve pieces of fresh beef were treated with either pure fluazuron dissolved in acetone (0.25mg/1ml) to provide a final fluazuron dose of 2.5 mg/kg of meat, or acetone alone as the control. The administered dose was applied to either side of the meat in 2 doses 40 minutes apart, allowing the acetone to vaporise for 40 minutes after each application. The doses were administered by spraying the liquid onto the surface of the meat using a graduated 1ml syringe and a 25 gauge needle.

The pieces of meat were then placed into plastic jars with six late instar *Lucillia cuprina* larvae each, with the openings covered with a porous cloth secured with an elastic band, in order to allow for airflow but prevent the escape of larvae or adult flies. The jars containing meat and larvae were placed in an incubator for 9 days before being removed and the contents examined for the presence of larvae, pupae or adult flies. With the results not showing a normal distribution, differences between groups were ascertained with a 2-tailed Mann-Whitney test.

Figure 3.1: Treatment group jars containing treated meat and larvae.

3.2.1.2 The efficacy of fluazuron and flumethrin on wool penetration

The following study made use of a fully woollen pelt freshly collected from a local abattoir, from an animal slaughtered for commercial purposes that was independent of the study. The animal had no history of prior exposure to fluazuron. Two sections of pelt were cut (30 cm by 30 cm) and treated with Drastic Deadline Extreme or saline at 0.26 ml per section respectively. This dose was calculated based on the registered dose for cattle, the known absence of absorption of the product, and the assumption of uniform distribution of the product into the wool of the animals. The dose was based on the body weight to skin surface area of a 25 kg dog, similar in size to a large lamb, which would have a body surface area of 0.85 m2. With exposure being 1 ml/10kg, the topical exposure of fluazuron would be 2.94 ml per m2, and thus 0.26 ml for the square of woollen skin with a surface area of 0.09 m2.

The saline or drug was applied onto the skin by parting the wool via pipette. The treated skins pieces were allowed to stand at 28°C for 24 hours to allow the test product to diffuse over the surface of the pelt. After the 24 hour period, 5 cm was trimmed off a single edge of each pelt to provide a fresh edge. Five swatches of pelt measuring 5 cm x 5 cm were then cut from the fresh edge of each pelt and put in separate, labelled petri dishes. The petri dishes were placed underneath a desk lamp at a distance of 50 cm to provide some additional warmth and light for photography. Five incubator bred larvae of similar size were placed into each petri dish alongside the swatch and their behaviour observed for 45 minutes (Figures 3.3). For this

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experiment, assuming that diffusion of the product over the skin was evenly distributed, larvae were exposed to an estimated fluazuron concentration of 73.5 mg/m^2 of skin. Difference in larval penetration was ascertained by a Mann-Whitney non-parametric t-test as the results were not normally distributed.
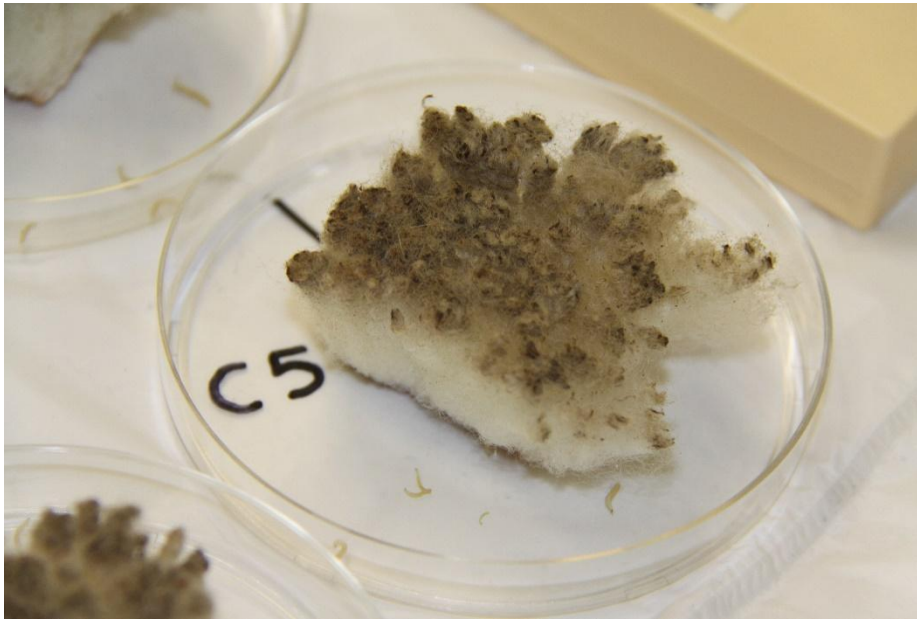
Figure 3.2: Petri dish from the control group containing wool swatch and larvae.

3.2.1.3 The effect fluazuron and flumethrin on larval development when applied to wool

Sheep pelts were prepared as above. Six swatches of wool were cut in the same manner and placed in separate jars. Six incubator bred, late instar larvae of similar size were placed directly onto each pelt and the opening of the jar closed with cloth secured with an elastic band to allow for air flow and not larval escape. Jars were subsequently incubated at 35°C and monitored for 10 days. After 10 days, each jar was examined separately for the presence of adult flies and their macroscopic morphology. The normal pupation and emergence of a macroscopically normal adult fly was considered a development success. Any non-pupated larvae, macroscopically abnormal pupae, unhatched pupae or macroscopically abnormal adult

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flies were considered a development failure. Difference in the number of normal adult flies, abnormal flies and uneclosed larvae were compared between the treatments by a Mann Whitney non-parametric t-test as the results were not normally distributed.

3.2.2 *In vivo* studies

3.2.2.1 Animal Phase

The following study was approved by the Animal Use and Care Committee of the University of Pretoria, in accordance with the National Standard for the use of care of animals in research (SANS10386). The approval letter is contained in Appendix 1.

3.2.2.1.1 Pharmacokinetic Study 1

Three adult Merino sheep were treated topically with commercially available Drastic Deadline Extreme (Bayer South Africa) at a dose of 1 ml/10kg of body mass (the recommended cattle dose), applied to 4 parts to bare skin in the axillae and inguinal areas. Drastic Deadline extreme consists of fluazuron at 2.5 % m/v and flumethrin at 1% m/v. Sheep were placed the seated position for application of the product. Sheep were kept in the seated position and limbs adducted for a period to prevent run off once. Blood samples were collected into serum tubes were collected from all three sheep before treatment, and at 8, 16, 24, 36, 48, 72, 96, 168, 336 and 504 h after treatment. Samples were frozen and delivered to FDA Laboratories in Pretoria, South Africa for analysis.

For the pharmacokinetic phase, the intention was to analyse the data by non-compartmental analysis, using the following method: The maximum plasma concentration (Cmax) and the time to the maximum concentration (Tmax) would be read directly off the concentration versus time plasma profile. The area under curve to the last quantifiable time point (AUC_{last}) is determined using the linear trapezoidal rule $AUC_{last} = \sum_{i=1}^{n} 0,5 * ((C_i + C_{i+1}) * \Delta t)$; the total area under curve (extrapolated to infinity) (AUC_{inf}) is calculated as: $AUC_{inf} = AUC_{last} + AUC_{extra} = AUC_{last} + C_{Last}/Lz$ with C_{last} being the computed last measured concentration; the

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area under the moment curve from the time point zero to the last measured time pojnt (AUCM$_{last}$) was calculated as $AUMC_{last} = \sum_{i=1}^{n} 0{,}5 * (t_i * C_i + t_{i+1} * C_{i+1}) * \Delta t$. The elimination half-life (t$_{1/2}$), clearance (Cl) and volume of distribution during terminal phase (V$_z$) and volume of distribution at steady state (V$_{ss}$) are determined as t$_{1/2}$= ln(2)/Lz; V$_z$ = Cl/Lz = Dose/(AUC*Lz); V$_{ss}$ = (Dose*MRT)/AUC and Cl = dose/AUC$_{tot}$

3.2.2.1.2 Pharmacokinetic Study 2

For this study 3 sheep were treated with the test product applied to the dorsal midline, at the same dose registered for cattle. Wool from treated animals was sampled at 7, 14 and 21 days after treatment using a commercial wool clipper by removing a square section of 100 cm^2 from both lateral sides of the thorax at similar positions, sampling successively lower on the thorax with each sample. The two bilateral wool samples from each sampling were subsequently combined to form one analytical sample. The first sample was collected one third of the height of the thorax ventral to the dorsal midline. The second sample was taken from a position halfway between the dorsal and ventral midlines and the third sample taken one third of the height of the thorax dorsal to the ventral midline. The wool samples were analysed for their fluazuron concentrations by FDA Laboratories, a commercial analytical laboratory in South Africa. Due to the commercial nature of the laboratory, the full method was not made available for reporting.

3.2.2.2 Laboratory Methodology

3.2.2.2.1 LC-MS conditions and methods

A Liquid Chromatography tandem Mass Spectrometry (LC-MS/MS) method was employed to accurately determine the concentration of fluazuron in the serum. The mass spectrometer employed electrospray ionization within the positive ion mode. Sample preparation involved the additional of a buffer for improved solubilisation followed by extraction of the analyte

from the matrix by means of acetonitrile as the organic solvent. Subsequent to this extraction the sample was further cleaned up by means of reverse phase solid phase extraction. Following trapping of the analyte, and removal of interferences by means of solvent washes, the analyte was selectively eluted. This eluent was evaporated and reconstituted in LC reconstitution solvent.   Injection for LCMS/MS analysis was automated for best precision by means of an auto-sampler. The Lower Limit of Quantification (LLOQ) and Limit of Detection (LOD) were determined at 50 µg/kg.

A double extraction technique was used to obtain the analyte from the matrix in a high yield, with the second step as to clean this extract for a high sensitivity detection with the minimum of interference in detector signal. The very modern technique of liquid chromatography mass spectrometry was used for the detection. The sensitivity of this technique generally provides a high detection sensitivity and excellent reproducibility, which was also shown valid when applied to this quantification study of fluazuron.

For both the serum and wool fluazuron quantification studies calibration standards prepared from reference standard material were prepared within the particular matrix for the best representation of the effect of the matrix. For the serum study the calibrator range was 0.5 to 100 µl/L. The curve presented a calibration correlation coefficient of 0.9998 and an RSQ (Pearson Product-Moment Correlation Coefficient) of 0.9995. For the wool study the calibrator range was 500 to 4000 ppb (µg/kg). The curve presented a calibration correlation coefficient of 0.9911 and an RSQ (Pearson Product-Moment Correlation Coefficient) of 0.9823. This shows the high performance of the serum method and an excellent method for the matrix of wool, which is more demanding. This high linearity of the methodology is evident by the good correlation of the other validation parameters including a high signal to noise ratio of the analyte signal and a high accuracy of the theoretical quality control spiking to the actual measured value.

At the quality control spiking level the ratio of the analyte peak to blank response was at least a factor of several thousand for both methods, indicating a high Lower Limit of

Quantification (LLOQ) and Limit of Detection (LOD) for this analyte in these matrixes. Accuracy and precision was for both methods determined as two spiking level of the analyte in the matrix (serum or wool). This study, which investigate how close a spiked standard result is calculated compared to the theoretical amount added, is a measure of the certainty that results are accurate and close to the actual when measured. The advancement of the LC/MS/MS methodology was demonstrated within this demanding application. For the serum validation study the uncertainly was 0.9 at the very low level of 1 μl/L and 35.5 at the higher level of 75 μl/L. For the wool validation study the uncertainly was 320 at the level of 1000 ppb and 813 at the level of 3000 ppb. This makes this methodology highly suitable for the quantification application of these particular experiments/studies.

3.3 Results

3.3.1 The effect of fluazuron on larval development when applied to meat

For the meat treated group, of the 36 larvae placed on meat in the negative control groups, a total of 35 were recovered of which only 2 showed incomplete hatching, while the rest developed into morphologically normal flies. In contrast to this, of the 36 larvae placed on meat treated with the test product in the treatment groups, 28 were recovered of which none showed normal maturation. Twenty-five failed to emerge from their pupae, whilst the 3 that did manage to emerge showed grossly abnormal morphology. Table 3.1 provides a summary of the results on adult fly development, while Figure 3.3 shows the specimens collected from the control and treatment groups respectively. Statistical analysis showed the difference to be significant at p =0.002.

Table 3.1 Normal and abnormal adult flies, as well as uneclosed pupae recovered from treated and control groups

Group	Untreated			Fluazuron		
	Normal adults	Abnormal adult	**Uneclosed pupae**	Normal adults	Abnormal adult	**Uneclosed pupae**
1	5	0	0	0	0	3
2	6	0	0	0	0	6
3	6	0	0	0	0	5
4	6	0	0	0	1	4
5	6	0	0	0	0	5
6	6	0	0	0	2	2
Average	**5.83**	**0.00**	**0.00**	**0.00**	**0.50**	**4.17**
SD	**0.41**	**0.00**	**0.00**	**0.00**	**0.84**	**1.47**

Normal adults: Adult flies which show macroscopically normal development; Abnormal adults: Adult flies which show macroscopically abnormal development; Uneclosed pupae: Pupae from which adult flies have failed to emerge

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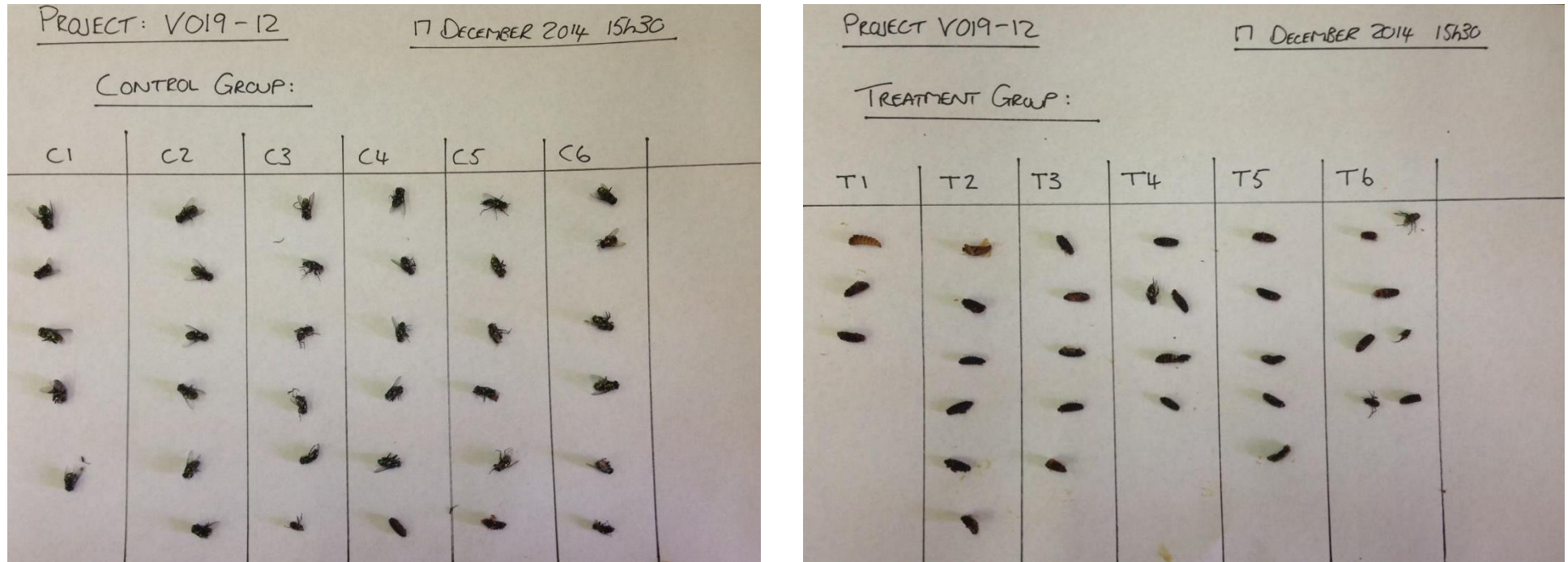
Figure 3.3: Specimens collected from the six replicates for the control groups (Left) and the treatment groups (Right)
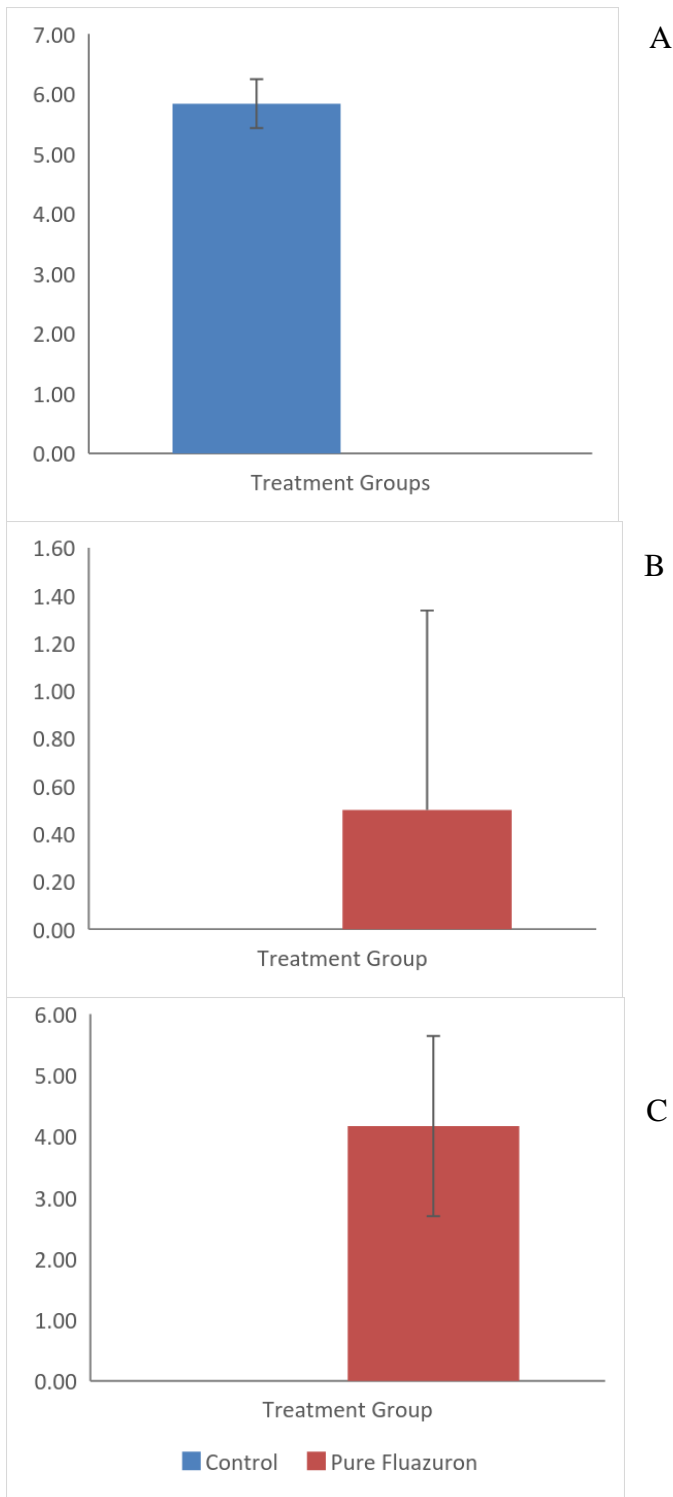
Figure 3.4: Average number of healthy adult flies (A) from the control group, as well as abnormal flies (B) and non-eclosed pupae (C) from the fluazuron treated group obtained

following exposure to acetone or fluazuron in acetone treated meat. In each case thirty six healthy larvae in 6 replicates were exposed.

3.3.2 The effect on larval wool penetration

Within 15 minutes of beginning the experiment, all of the larvae in control groups 1, 4 and 5 had penetrated into or underneath the skin swatch. By 45 minutes, 3 of the larvae in group 3 had penetrated the skin swatch whereas none of the larvae in group 2 made any attempt to penetrate the wool or skin tissue. On repeating the experiment with the treated pelt, all of the larvae in treatment group 4 penetrated the skin swatch within 15 minutes. By 45 minutes, 3 of the larvae in treatment groups 1 and 2 had penetrated the skin swatch and 2 of the larvae in group 5. None of the larvae in group 3 made any attempt to penetrate the skin swatch. The results of the experiment are summarized in table 3.2 and figure 3.7. No significant difference was present for larval penetration ($p = 0.74$).

Table 3.2: Larvae which penetrated skin swatch within 45 minutes following placement of 5 larvae

Group	Control	Test
1	5	3
2	0	3
3	3	0
4	5	5
5	5	2
Mean	3.60	2.60
SD	2.19	1.82

Control: Wool swatches treated with saline; Test: Wool swatches treated with test product
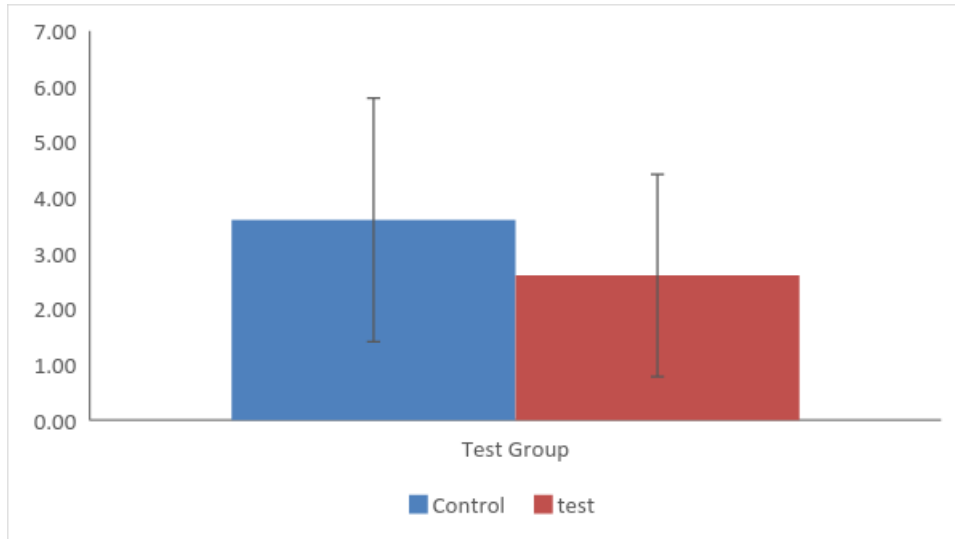
Figure 3.6: Comparison of the number of larval penetration between treated and non-treated woollen skin swatches.

3.3.3 The effect on larval development when applied to wool

After 10 days, a total 18 of the larvae out of 26 organisms recovered from the 6 control groups had developed normally through pupation and hatching into macroscopically normal adult flies, when considered morphologically. Seven pupae failed to eclose and 1 fly in group 1 was macroscopically abnormal. Of the remaining 10 larvae that were placed out of the original 36, no trace could be found in the jars. It is assumed that the larvae may have died at an early stage and been consumed by other larvae.

The groups exposed to the treated skin swatches demonstrated 21 normal developments, 5 pupation failures and 4 morphologically abnormal adult flies; a better recovery rate of 30 organisms out of the original 36 placed was achieved. No significant difference was present between the control and treated groups ($p = 0.27$). Interesting to note was the discovery of 8 relatively small and lighter coloured pupae in group 2 of the treated groups. Identification of these pupae proved impossible and we assume contamination of the sample with another species. Table 3.3 summarizes the results of this study.

Table 3.2: Development of larvae (n=6) when exposed to fluazuron treated wool

Group	Untreated			Fluazuron Group		
	Normal Flies	Abnormal Flies	Uneclosed pupae	Normal Flies	Abnormal Flies	Uneclosed pupae
1	2	1	2	5	0	0
2	5	0	1	4	0	1
3	3	0	2	3	1	2
4	3	0	1	2	1	1
5	4	0	0	4	2	0
6	1	0	1	3	1	0
Average	**3.00**	**0.17**	**1.17**	**3.50**	**0.83**	**0.67**
SD	1.41	0.41	0.75	1.05	0.75	0.82

Normal adults: Adult flies which show macroscopically normal development; Abnormal adults: Adult flies which show macroscopically abnormal development; Uneclosed pupae: Pupae from which adult flies have failed to emerge

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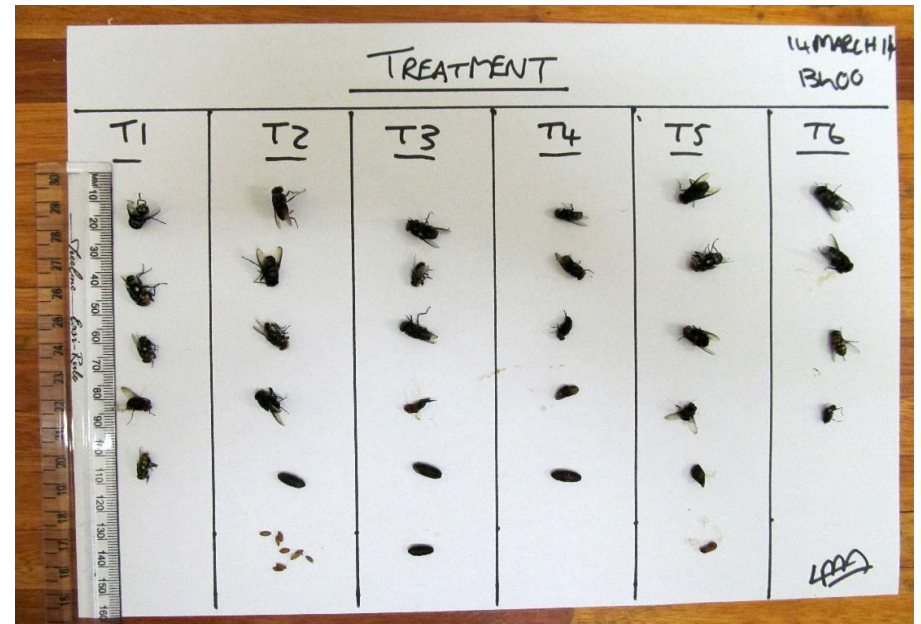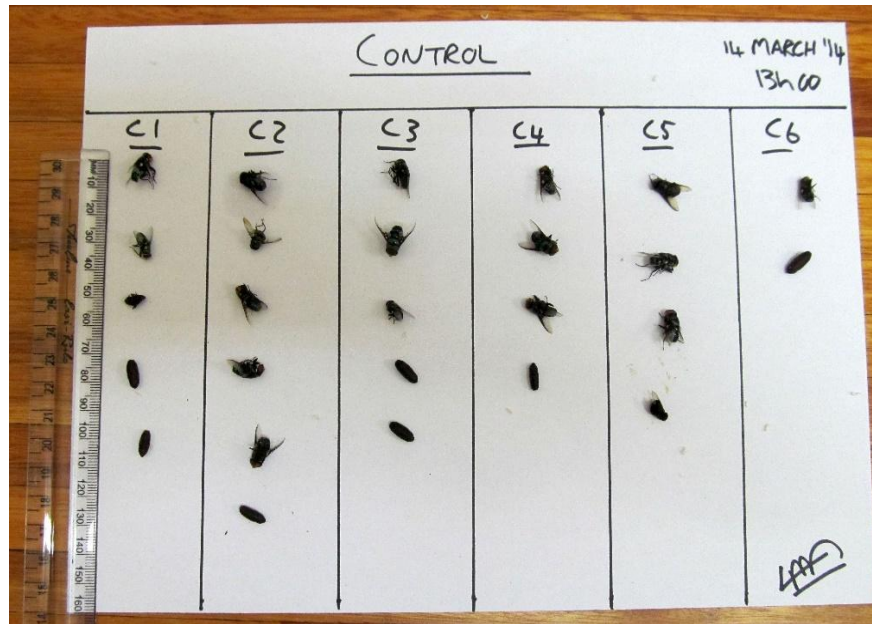
Figure 3.7: Adult flies and non-eclosed pupae recovered from the control (left) and treatment groups (right)

Normal adults: Adult flies which show macroscopically normal development; Abnormal adults: Adult flies which show macroscopically abnormal development; Uneclosed pupae: Pupae from which adult flies have failed to emerge

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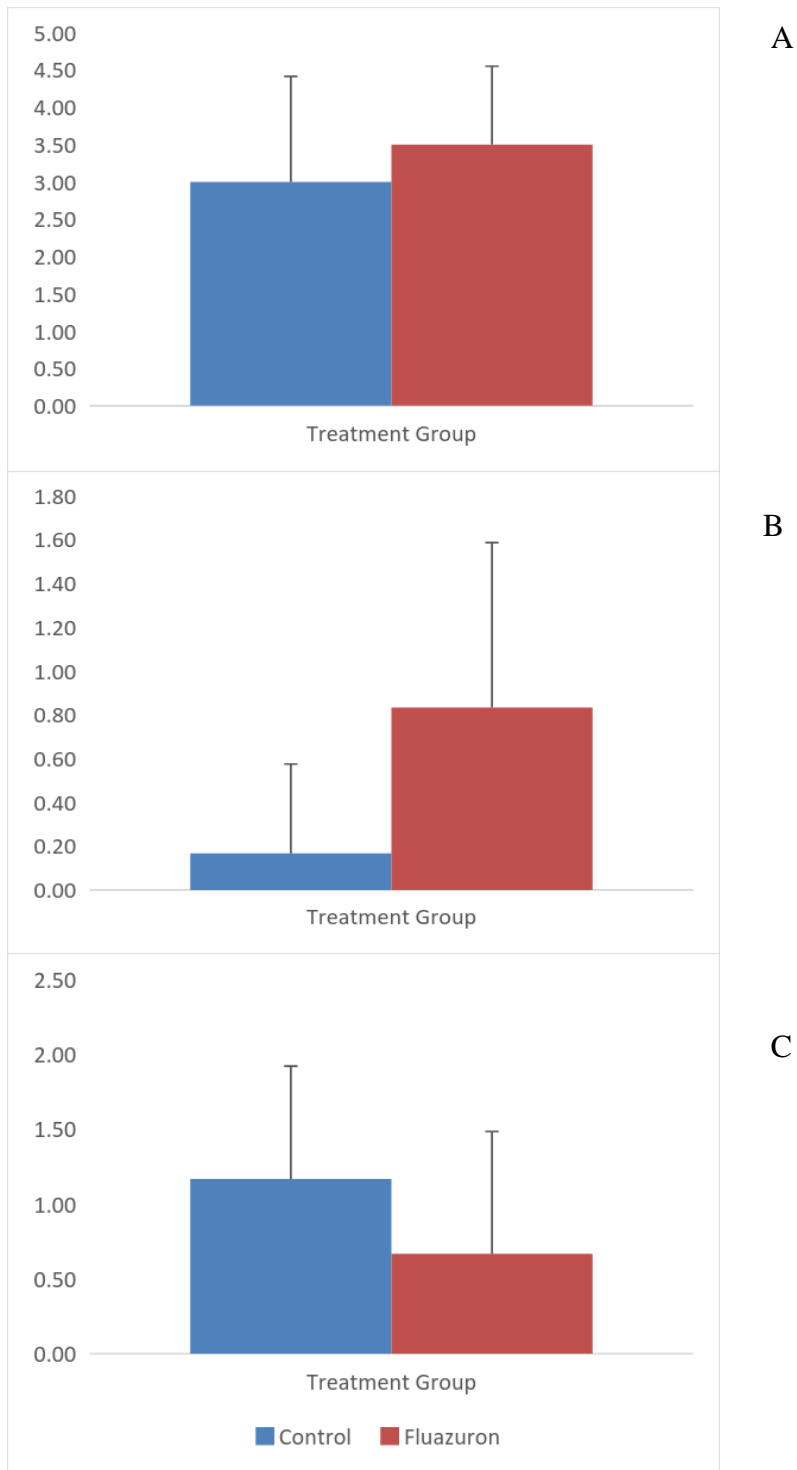
Figure 3.8: Average healthy adult flies (A), abnormal flies (B) and non-eclosed pupae (C) obtained following exposure of woollen skin to saline or fluazuron in its commercial formulation. In each case thirty-six healthy larvae were exposed.

3.3.4 Serum and wool kinetic study

For the first pharmacokinetic study, all plasma samples evaluated were below the limit of quantification of 50 ppb. For the second study while once again no drug was detectable in the plasma, substantial drug was present within the wool samples (Fig 3.5). The concentration measured between the three sheep was highly variable at 1453.8 ± 1154.78, 608.33 ± 803.13 and 1152.23 ± 1333.92 mg/kg at 168, 336 and 504 hours respectively. Due to the absence of detectable plasma concentrations, the calculation of the applicable pharmacokinetic parameters were not possible. The raw data is presented in Appendices 2 and 3 respectively.
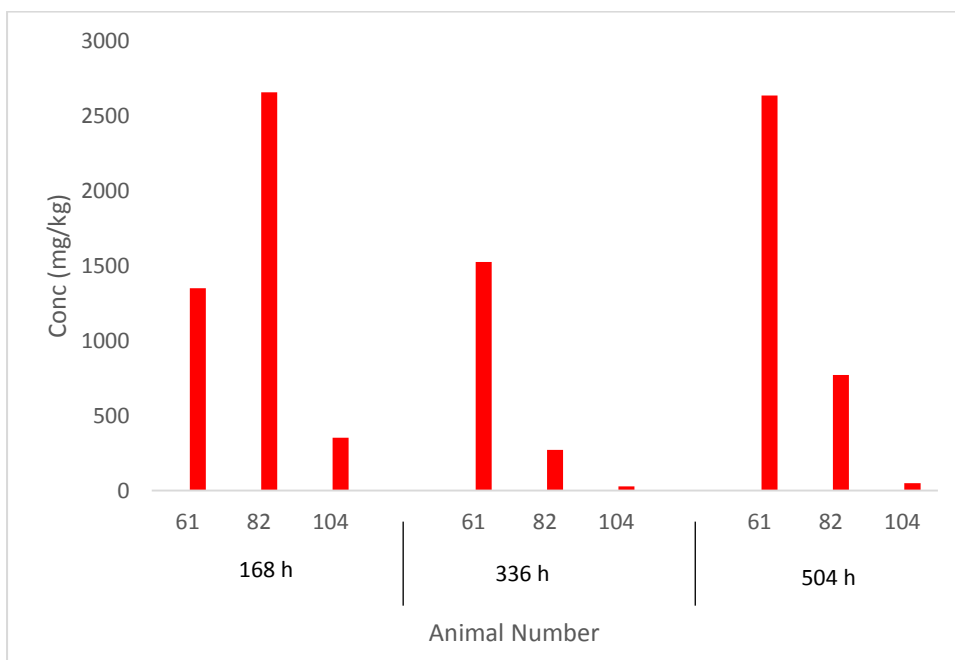
Figure 3.5: Wool concentrations of fluazuron at 168, 336 and 503 hours, after topical application to three sheep (animals 61, 82 and 104)

3.4 Discussion

The mere decision to use a chemical against a specific pest is the greatest risk factor in the development of insecticide resistance (Heath and Levot 2015), as its use places artificial selection pressure on the target population with the inevitable development of resistance. When considering this in combination with the speed of blowfly resistance development, it is fairly obvious that new chemicals effective against blowflies need to be constantly developed in order to stay ahead in the race. Of these newer chemical compounds in use, the benzoylphenyl ureas have been demonstrated to be effective against susceptible blowflies when applied to sheep, with the newest addition triflumeron even being used to control blowflies both off and on sheep (Plant and Lewis 2011; Smith and Wall 1998). As a class, the benzoylphenyl ureas are chitin synthesis inhibitors which prevent the immature insects exposed to these compounds from being able to completely ecdysis, subsequently dying during the process of moulting. Benzoylphenyl ureas also appear to demonstrate a transovarial effect in that they are incorporated into the egg nutrients when the female lays eggs, where they interfere with hatching (Taylor 2001).

Although it would be logical to assume that IGR's by virtue of their ability to essentially render all in-contact individuals infertile, and thus reduce the overall population size, would make them less at risk for the development of resistance this has not been the case. Resistance to benzoylphenyl ureas is a well-known phenomenon in Australia and New Zealand (Heath and Levot 2015; Levot 1995), with a link between resistance to organophosphates and diflubenzuron being discovered in the 1990s. It was determined that resistance to organophosphates in blowflies was influenced by microsomal oxygenase enzymes, which in turn showed a strong correlation to diflubenzuron susceptibility (Kotze et al. 1997; Kotze and Sales 2001). This makes a strong case for investment into the development of new active ingredients as well as the responsible management of our existing arsenal in order to maintain effectiveness for as long a period as possible. As fluazuron is a benzoylphenyl urea, chemically distinct from other members of this class of compounds, we postulated that it could also be effective in controlling blowflies, possibly even where other benzoylphenyl ureas are failing due to resistance development. When combined with a

contact insecticide like flumethrin, such as in the test formulation, a synergistic action may well be of benefit.

From this study we were able to demonstrate that pure fluazuron applied to raw meat at a concentration of 2.5 mg/kg has the ability to interfere with blowfly larval development into adult flies, indicating likely interference with chitin synthesis as anticipated. More interesting, the two pharmacokinetic studies clearly demonstrated that the fluazuron lacked systemic bioavailability when administered topically, which is markedly different to cattle. In one study, van Schalkwyk was able to demonstrate that fluazuron was readily absorbed percutaneously in cattle at a dose of 2.5 mg/kg, to reach a mean peak serum concentration of 26.2 µg/kg of fluazuron at 14 days after application (van Schalkwyk 2010). While the absence of systemic absorption could be taken as a negative result, this could also be of potential benefit as firstly it could allow for a shorter food withholding time to be set while secondly the wool could prove to be a depot for the drug, with resultant slow release of the drug over a longer period of time. Also important to note was that efficacy in the *in vitro* study was achieved at the exposure dose of 2.5 mg/kg, while at 504 hours after topical treatment will concentrations in the wool were over 400 fold higher at 1152.23 ± 1333.92 mg/kg.

To ascertain if wool bound fluazuron could have benefit in the management of sheep myiasis, a simulated *ex vivo study* was undertaken whereby two pelts from freshly slaughtered animals were treated with Drastic Deadline Extreme and subsequently used to ascertain efficacy. In all cases, the fluazuron appeared to have loss its efficacy in comparison to when it was applied to only meat. This leads to the conclusion that the fluazuron is too tightly bound to the wool fats to be effective. If shortcomings were to be identified for this study, it would the *ex vivo* testing of efficacy used for this last phase. The reason for this comes from the static nature of the test, which does not take into consideration animal movement and environmental conditions. It is quite possible that under daylight conditions, direct exposure to the sun could promote the release of the bound fluazuron, making it effective. It may even

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be possible, that the wool fats are saturatable and that administration at higher doses may overcome the binding effect, thereby rendering the molecule effective.

3.5 Conclusion

While we were able to demonstrate that fluazuron possesses the same larvae effect as the other benzoylphenyl ureas, this chemical appears to be inhibited by the presence of wool fats. This tends to suggest that fluazuron may not be an ideal agent for use in the management of sheep myiasis.

Chapter 4

4.1 General Discussion and Conclusion

The control of parasites on and in livestock is an ongoing battle, with the offending organisms constantly evolving mechanisms to survive chemical means of control, while pharmaceutical companies and other institutions continue the efforts to stay ahead of the evolutionary curve by development of new products, whether they be new chemical entities or new combinations of existing molecules. In reality, the threat of resistance developing to existing drugs is the main stimulus driving the discovery of new anti-parasitic drugs (Geary and Thompson 2003).

The traditional synthetic neurotoxins, such as the organophosphates and pyrethroids, have been used extremely effectively for decades in the control of ectoparasites from a combination of their potency, ease of application and relatively low cost. Unfortunately these very factors driving their use also led to global problems of resistance, concerns about environmental contamination and human health largely from a degree of complacency in the way these products were used (Wall 2007). A more recent concern has been the decrease in the screening for new anti-parasitic drugs in the animal health industry (Geary and Thompson 2003). It therefore stands to reason that more sophisticated methods of parasite control, chemical and otherwise, need to be utilized in order to preserve the efficacy of existing active ingredients. Chemical class rotation is essential for the preservation of insecticide susceptibility and the development of the benzoylphenyl ureas for ectoparasite control has significant implications for resistance management (Levot 1995).

The efficacy of topically applied insecticides is heavily dependent on exposure of the parasite to toxic concentrations of the pesticide for as long a period as possible (Hennessy 1994). One of the problems associated with topically applied pesticides is the area of the concentration-time curve known as the "tail", where pesticide concentration fall below lethal levels for a varying degree of time, usually fairly extended in the case of topically applied products, with

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the result that there is an extended period of exposure of parasite population to sub-lethal drug concentrations with subsequent survival and propagation of organisms carrying alleles containing resistance determinants. This "tail" phenomenon is practically unavoidable when it comes to the application of topical insecticides as the concentration of active ingredient bound to the skin, hair coat or wool gradually decreases with time. It then stands to reason that this effect will be exacerbated in oil soluble or lipophilic active ingredients which dissolve into or bind strongly to the lanolin in wool, dramatically prolonging the presence of the active ingredient on the sheep.

The development of resistance in parasites is also not limited to the neurotoxic drugs. Reports from Australia raise concerns of widespread resistance to benzoylphenyl ureas in blowflies, most likely from the long term use of molecules such as diflubenzuron and triflumuron against sheep lice infestations (Levot and Sales 2004), as well as the cross resistance demonstrated between the historically over-used organophosphates and benzoylphenyl ureas (Kotze et al. 1997; Kotze and Sales 2001). This phenomenon subsequently led to the removal of flystrike prevention claims from benzoylphenyl urea-based products in that country (Waghorn et al. 2013). However, with no cross resistance being reported between the various classes of insect growth regulators, for this study we ascertained the potential of the fluazuron to be effective in blowfly control. At present, the compound is predominantly used for tick control on cattle, with no use in sheep.

This study clearly demonstrated the ability of fluazuron to prevent the development of blowfly larvae when pure fluazuron was applied directly to meat. It also demonstrated that fluazuron is not systemically absorbed in sheep, instead remaining strongly bound to the lanolin in the wool, a potential benefit when considering toxicological safety as well as food safety and residue concerns. However, when applied to sheep pelts at the same dose rate as that intended for tick control on cattle the test product was not effective at preventing larval development, and further studies would be needed to determine the minimum effective dose rate for topical application to sheep. Should a higher dose rate be effective, the effect of the additional flumethrin in the test formulation would need to also be quantified, as a possible

synergism would render the product more effective and less susceptible to the development of resistance. Based on the outcome of this study and the dose rate of the test product used, the study hypothesis is rejected and further studies are warranted.

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Appendix 1: Animal Use and Care Committee Approval

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
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ANIMAL USE AND CARE COMMITTEE
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Ref: V019-12

24 April 2012

Prof V Naidoo
UPBRC
Faculty of Veterinary Science
(vinny.naidoo@up.ac.za)

Dear Prof Naidoo

V019-12 : The efficacy and safety of a topically applied flumethrin and fluazuron combination in a sheep myiasis model *(C Austin)*

The application for ethical approval, dated 28 March 2012 was approved by the Animal Use and Committee at its meeting held on 23 April 2012.

Kind regards

Elmarie Mostert

AUCC Coordinator

Copy   Dr C Austin

Appendix 2: Analytical report for pharmacokinetic study 1

Food & Drug Assurance Laboratories (Pty) Ltd
Reg No.2007/010792/07
Cnr Charles and Alexander Street,
Brooklyn, Pretoria, South Africa
PO Box 2302, Brooklyn Square, Pretoria 0075
Tel: + 27 12 346 8569 - Fax 086 656 7771

FDA
LABORATORIES

Bayer (Pty) Ltd
PO Box 143
Isando
1600

Tel Number: 011 921 5747
Fax Number: 011 921 5745

Batch Number:	2012-C-00589
Client Ref Number:	**V019-12**
Report Number:	BAY001-0055
Enquiries:	Azel Swemmer
Date Samples Received:	18 Jun 2012
Date Samples Analysed:	4 Jul 2012
Date of Report:	4 Jul 2012

TEST CERTIFICATE

No	Sample Reference Number	Matrix	Lab Number	Fluazuron µl/L Method Number LC-MS/MS 045
1	Pre-Bleed 81910	Serum	2358	0.9
2	Pre-Bleed R001	Serum	2359	<0.5
3	Pre-Bleed 1010	Serum	2360	<0.5
4	0h 81910	Serum	2361	<0.5
5	0h R001	Serum	2362	<0.5
6	0h 1010	Serum	2363	<0.5
7	8h 81910	Serum	2364	<0.5
8	8h R001	Serum	2365	<0.5
9	8h 1010	Serum	2366	<0.5
10	24h 81910	Serum	2367	<0.5
11	24h R001	Serum	2368	<0.5
12	24h 1010	Serum	2369	0.5
13	48h 81910	Serum	2370	0.5
14	48h R001	Serum	2371	<0.5
15	48h 1010	Serum	2372	0.5
16	72h 81910	Serum	2373	0.6
17	72h R001	Serum	2374	<0.5
18	72h 1010	Serum	2375	0.5
19	96h 81910	Serum	2376	0.6
20	96h R001	Serum	2377	<0.5
21	96h 1010	Serum	2378	0.6
22	168h 81910	Serum	2379	0.6
23	168h R001	Serum	2380	<0.5
24	168h 1010	Serum	2381	0.7
25	336h 81910	Serum	2382	0.8
26	336h R001	Serum	2383	0.5
27	336h 1010	Serum	2384	0.6
28	504h 81910	Serum	2385	<0.5
29	504h R001	Serum	2386	<0.5
30	504h 1010	Serum	2387	<0.5

The result obtained is only relevant to the laboratory sample as received by the laboratory. Opinions and interpretation of test results fall outside the scope of the laboratory. Stability of the analyte in the matrix between the sampling date and the date of analysis falls outside the scope of the laboratory.

The Test Report shall not be reproduced except in full, without the written approval of the laboratory. Electronic signatures are available for verification.
2012-C-00589 / BAY001-0055
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A Swemmer
Laboratory Manager

The result obtained is only relevant to the laboratory sample as received by the laboratory. Opinions and interpretation of test results fall outside the scope of the laboratory. Stability of the analyte in the matrix between the sampling date and the date of analysis falls outside the scope of the laboratory.

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Electronic signatures are available for verification.
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2012-C-589
Serum

Fluazuron MRM 1

Concentration (µl/L)	Peak area Fluazuron	Peak area IS	Analyte ratio-blank ratio	Response factor
Blank-1	0			
Blank-2	0			
Blank-3	0			
Average	0			
0.5	1190		1190	2380.00
1	2480		2480	2480.00
10	18400		18400	1840.00
25	57100		57100	2284.00
100	220000		220000	2200.00
Correl			0.9998	2236.8000
RSQ			0.9995	
Slope			2.21E+03	
Y-Intercept			-4.12E+02	

Fluazuron calibration curve
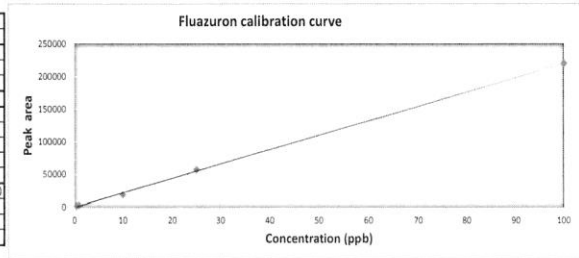
Controls

Analyte	Fluazuron	Peak area IS
Peak Area	2400	
PA(-)Blank	2400	
Conc.(ppb)	1.07	
Spiking level	1	
Uncertainty	0.90	
Upper limit	1.90	
Lower limit	0.10	
Status	**PASS**	TRUE

Analyte	Fluazuron	Peak area IS
Peak Area	110000	
PA(-)Blank	110000.00	
Conc.(ppb)	50.02	
Spiking level	75	
Uncertainty	35.43	
Upper limit	110.43	
Lower limit	39.57	
Status	**PASS**	TRUE

2012-C-589

Sample name	Lab number	Peak area Analyte	An ratio - Blank ratio	conc (µl/L) Fluazuron	Fluazuron conc (µl/L)	conc (µl/L) RF (lvl2)	Reported Fluazuron conc (µl/L)
Pre-bleed 81910	2358	2290	2290.000	1.24	1.2	0.92	0.9
Pre-bleed R001	2359	437	437.000	0.40	<0.5	0.18	<0.5
Pre-bleed 1010	2360	402	402.000	0.38	<0.5	0.16	<0.5
0h 81910	2361	668	668.000	0.50	0.5	0.27	<0.5
0h R001	2362	462	462.000	0.41	<0.5	0.19	<0.5
0h 1010	2363	546	546.000	0.45	<0.5	0.22	<0.5
8h 91910	2364	365	365.000	0.37	<0.5	0.15	<0.5
8h R001	2365	546	546.000	0.45	<0.5	0.22	<0.5
8h 1010	2366	836	836.000	0.58	0.6	0.34	<0.5
24h 81910	2367	664	664.000	0.50	0.5	0.27	<0.5
24h R001	2368	594	594.000	0.47	0.5	0.24	<0.5
24h 1010	2369	1150	1150.000	0.72	0.7	0.46	0.5
48h 81910	2370	1250	1250.000	0.77	0.8	0.50	0.5
48h R001	2371	162	162.000	0.27	<0.5	0.07	<0.5
48h 1010	2372	1320	1320.000	0.80	0.8	0.53	0.5
72h 81910	2373	1420	1420.000	0.84	0.8	0.57	0.6
72h R001	2374	1090	1090.000	0.69	0.7	0.44	<0.5
72h 1010	2375	1320	1320.000	0.80	0.8	0.53	0.5
96h 81910	2376	1490	1490.000	0.87	0.9	0.60	0.6
96h R001	2377	877	877.000	0.60	0.6	0.35	<0.5
96h 1010	2378	1510	1510.000	0.88	0.9	0.61	0.6
168h 81910	2379	1570	1570.000	0.91	0.9	0.63	0.6
168h R001	2380	1020	1020.000	0.66	0.7	0.41	<0.5
168h 1010	2381	1720	1720.000	0.98	1.0	0.69	0.7
336h 81910	2382	1940	1940.000	1.08	1.1	0.78	0.8
336h R001	2383	1310	1310.000	0.79	0.8	0.53	0.5
336h 1010	2384	1470	1470.000	0.87	0.9	0.59	0.6
504h 81910	2385	481	481.000	0.42	<0.5	0.19	<0.5
504h R001	2386	876	876.000	0.60	0.6	0.35	<0.5
504h 1010	2387	1040	1040.000	0.67	0.7	0.42	<0.5

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AB SCIEX

Analyte: Fluazuron MRM 1 (507.955/158.100 Da)

Data File	2012-C-589 27 June 2012.wiff	Result Table	2012-C-589 27 June 2012 (1).rdb
Acquisition Date	6/28/2012 10:44:49 AM	Algorithm Used	Analyst Classic
Acquisition Method	Fluazuron LCMSMS2.dam	Instrument Name	4000 Q TRAP
Project	Pesticides\Fluazuron		

RT

RT (Exp. RT):	8.38 (8.36) min	
Area:	3040.	
Sample Type:	(Unknown)	

Blank 1

RT (Exp. RT):	0.00 (8.36) min	
Area:	0.00	
Sample Type:	(Unknown)	

Blank 2

RT (Exp. RT):	0.00 (8.36) min	
Area:	0.00	
Sample Type:	(Unknown)	

Blank 3

RT (Exp. RT):	0.00 (8.36) min	
Area:	0.00	
Sample Type:	(Unknown)	

Level 1

RT (Exp. RT):	8.36 (8.36) min	
Area:	1190.	
Sample Type:	(Standard)	

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Level 2		
RT (Exp. RT):	8.36 (8.36) min	
Area:	2480.	
Sample Type:	(Standard)	

Level 3		
RT (Exp. RT):	8.35 (8.36) min	
Area:	18400.	
Sample Type:	(Standard)	

Level 4		
RT (Exp. RT):	8.36 (8.36) min	
Area:	57100.	
Sample Type:	(Standard)	

Level 7		
RT (Exp. RT):	8.36 (8.36) min	
Area:	220000.	
Sample Type:	(Standard)	

2358		
RT (Exp. RT):	8.37 (8.36) min	
Area:	2290.	
Sample Type:	(Unknown)	

2359		
RT (Exp. RT):	8.37 (8.36) min	
Area:	437.	
Sample Type:	(Unknown)	

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2360		
RT (Exp. RT):	8.35 (8.36) min	
Area:	402.	
Sample Type:	(Unknown)	

2361		
RT (Exp. RT):	8.36 (8.36) min	
Area:	668.	
Sample Type:	(Unknown)	

2362		
RT (Exp. RT):	8.37 (8.36) min	
Area:	462.	
Sample Type:	(Unknown)	

2363		
RT (Exp. RT):	8.40 (8.36) min	
Area:	546.	
Sample Type:	(Unknown)	

2364		
RT (Exp. RT):	8.37 (8.36) min	
Area:	365.	
Sample Type:	(Unknown)	

2365		
RT (Exp. RT):	8.35 (8.36) min	
Calculated Conc:	0.44009 ng/mL	
Area:	546.	
Sample Type:	(Unknown)	

2366		
RT (Exp. RT):	8.38 (8.36) min	
Area:	836.	
Sample Type:	(Unknown)	

2367		
RT (Exp. RT):	8.38 (8.36) min	
Area:	664.	
Sample Type:	(Unknown)	

2368		
RT (Exp. RT):	8.37 (8.36) min	
Area:	594.	
Sample Type:	(Unknown)	

2369		
RT (Exp. RT):	8.37 (8.36) min	
Area:	1150.	
Sample Type:	(Unknown)	

2370		
RT (Exp. RT):	8.36 (8.36) min	
Area:	1250.	
Sample Type:	(Unknown)	

2371		
RT (Exp. RT):	8.43 (8.36) min	
Area:	162.	
Sample Type:	(Unknown)	

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2372		
RT (Exp. RT):	8.37 (8.36) min	
Area:	1320.	
Sample Type:	(Unknown)	

2373		
RT (Exp. RT):	8.36 (8.36) min	
Area:	1420.	
Sample Type:	(Unknown)	

2374		
RT (Exp. RT):	8.38 (8.36) min	
Area:	1090.	
Sample Type:	(Unknown)	

2375		
RT (Exp. RT):	8.37 (8.36) min	
Area:	1320.	
Sample Type:	(Unknown)	

2376		
RT (Exp. RT):	8.37 (8.36) min	
Area:	1490.	
Sample Type:	(Unknown)	

2377		
RT (Exp. RT):	8.36 (8.36) min	
Area:	877.	
Sample Type:	(Unknown)	

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AB SCIEX

2384		
RT (Exp. RT):	8.39 (8.36) min	
Area:	1470.	
Sample Type:	(Unknown)	

2385		
RT (Exp. RT):	8.41 (8.36) min	
Area:	481.	
Sample Type:	(Unknown)	

2386		
RT (Exp. RT):	8.39 (8.36) min	
Area:	876.	
Sample Type:	(Unknown)	

2387		
RT (Exp. RT):	8.35 (8.36) min	
Area:	1040.	
Sample Type:	(Unknown)	

C Low		
RT (Exp. RT):	8.34 (8.36) min	
Area:	2400.	
Sample Type:	(Unknown)	

C High		
RT (Exp. RT):	8.33 (8.36) min	
Area:	110000.	
Sample Type:	(Unknown)	

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2378		
RT (Exp. RT):	8.38 (8.36) min	
Area:	1510.	
Sample Type:	(Unknown)	

2379		
RT (Exp. RT):	8.37 (8.36) min	
Area:	1570.	
Sample Type:	(Unknown)	

2380		
RT (Exp. RT):	8.37 (8.36) min	
Area:	1020.	
Sample Type:	(Unknown)	

2381		
RT (Exp. RT):	8.38 (8.36) min	
Area:	1720.	
Sample Type:	(Unknown)	

2382		
RT (Exp. RT):	8.38 (8.36) min	
Area:	1940.	
Sample Type:	(Unknown)	

2383		
RT (Exp. RT):	8.39 (8.36) min	
Area:	1310.	
Sample Type:	(Unknown)	

Appendix 3: Analytical report for pharmacokinetic study 2

Food & Drug Assurance Laboratories (Pty) Ltd
Reg No. 2007/010792/07
Cnr Charles and Alexander Street,
Brooklyn, Pretoria, South Africa
PO Box 2302, Brooklyn Square, Pretoria 0075
Tel: + 27 12 346 8569 · Fax: 086 656 7771

FDA
LABORATORIES
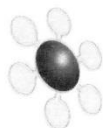
Bayer (Pty) Ltd
PO Box 143
Isando
1600

Tel Number: 011 921 5747
Fax Number: 011 921 5745

Batch Number:	2013-C-00103
Client Ref Number:	PO4500520824
Report Number:	BAY001-0071
Enquiries:	Azel Swemmer
Date Samples Received:	20 Mar 2013
Date Samples Analysed:	18 Jul 2013
Date of Report:	18 Jul 2013

TEST CERTIFICATE

No	Sample Reference Number	Matrix	Lab Number	Detection limit	Fluazuron (mg/kg) Method Number In House
1	Wool Ani.61 168h	Wool	530	50ppb	1351.1
2	Wool Ani.82 168h	Wool	531	50ppb	2656.5
3	Wool Ani.104 168h	Wool	532	50ppb	353.8
4	Wool Ani.61 336h	Wool	533	50ppb	1525.0
5	Wool Ani.82 336h	Wool	534	50ppb	271.7
6	Wool Ani.104 336h	Wool	535	50ppb	28.3
7	Wool Ani.61 504h	Wool	536	50ppb	2635.1
8	Wool Ani.82 504h	Wool	537	50ppb	771.6
9	Wool Ani.104 504h	Wool	538	50ppb	50.0

The concentration of Fluazuron can be taken as an estimate value due to the high concentrations present in the samples.

A Swemmer
Laboratory Manager

The result obtained is only relevant to the laboratory sample as received by the laboratory. Opinions and interpretation of test results fall outside the scope of the laboratory. Stability of the analyte in the matrix between the sampling date and the date of analysis falls outside the scope of laboratory.

The Test Report shall not be reproduced except in full, without the written approval of the laboratory. Electronic signatures are available for verification.
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2013-C-103 15 July 2013
Matrix: Wool

Fluazuron MRM1
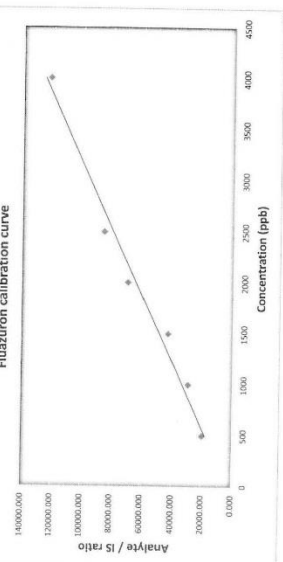
Concentration (ppb)	Peak area	IS Peak area	Analyte / IS ratio	Ratio - Blank	Response factor
Blank 1	0				
Blank 2	0				
Blank 3	0				
Average	0				
500	19900			19900	39.8
1000	29200			29200	29.2
1500	42700			42700	28.5
2000	69200			69200	34.6
2500	85400			85400	34.2
4000	122000			122000	30.5
					32.8
Correl			0.9911		
RSQ			0.9823		
Slope			3.07E+01		
Y-Intercept			2.47E+03		

Sequence controls

Low	
Analyte Peak area	31300
Ratio - blank	31300
Conc (ppb) using Avg Rf	954.6
Spiking level	1000
Uncertainty	319.9337
Upper limit	1319.93
Lower limit	680.07
Status	PASS

High	
Analyte Peak area	100000
Ratio - blank	100000
Conc (ppb)	3149.9
Spiking level	3000
Uncertainty	813.53
Upper limit	3813.53
Lower limit	2186.47
Status	PASS

Fluazuron calibration curve

Batch 2013-C-103	Peak area	IS Peak area	Analyte / IS ratio	Area - Blank	Conc (ppb or µg/kg) with Avg Rf	Dilution factor	Corrected conc (ppb)	Reported Conc (µg/kg)	Concentration PPM
530	443000			443000	13511.1	100.0	1351113.2	1351113.2	1351.1
531	871000			871000	26664.8	100.0	2656477.7	2656477.7	2656.5
532	116000			116000	3537.9	100.0	353790.4	353790.4	353.8
533	500000			500000	15249.6	100.0	1524958.5	1524958.5	1525.0
534	891000			891000	27174.8	10.0	271747.6	271747.6	271.7
535	92700			92700	2827.3	10.0	28272.7	28272.7	28.3
536	864000			864000	26351.3	100.0	2635128.3	2635128.3	2635.1
537	253000			253000	7716.3	100.0	771629.0	771629.0	771.6
538	164000			164000	5001.9	10.0	50018.6	50018.6	50.0

AB SCIEX

Analyte: Fluazuron MRM 1 (507.955/158.100 Da)

Data File	2013-C-103 Repeat 15 July 2013.wiff	Result Table	2013-C-103 Repeat 15 July 2013 (1).rdb
Acquisition Date	7/16/2013 8:41:00 AM	Algorithm Used	Analyst Classic
Acquisition Method	Fluazuron LCMSMS2.dam	Instrument Name	4000 Q TRAP
Project	Pesticides\Fluazuron		

RT		
RT (Exp. RT):	8.40 (8.36) min	
Area:	22600.	
Sample Type:	(Unknown)	

Blank1		
RT (Exp. RT):	8.08 (8.36) min	
Area:	0.00	
Sample Type:	(Unknown)	

Blank2		
RT (Exp. RT):	8.01 (8.36) min	
Area:	0.00	
Sample Type:	(Unknown)	

Blank3		
RT (Exp. RT):	0.00 (8.36) min	
Area:	0.00	
Sample Type:	(Unknown)	

Level 1		
RT (Exp. RT):	8.33 (8.36) min	
Area:	19900.	
Sample Type:	(Standard)	

Level 2		
RT (Exp. RT):	8.33 (8.36) min	
Area:	29200.	
Sample Type:	(Standard)	

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AB SCIEX

Level 3		
RT (Exp. RT):	8.34 (8.36) min	
Area:	42700.	
Sample Type:	(Standard)	

Level 4		
RT (Exp. RT):	8.34 (8.36) min	
Calculated Conc:	2170. ng/mL	
Area:	69200.	
Sample Type:	(Standard)	

Level 5		
RT (Exp. RT):	8.35 (8.36) min	
Area:	85400.	
Sample Type:	(Standard)	

Level 7		
RT (Exp. RT):	8.35 (8.36) min	
Area:	122000.	
Sample Type:	(Standard)	

SB		
RT (Exp. RT):	0.00 (8.36) min	
Area:	0.00	
Sample Type:	(Unknown)	

530		
RT (Exp. RT):	8.35 (8.36) min	
Area:	443000.	
Sample Type:	(Unknown)	

531		
RT (Exp. RT):	8.36 (8.36) min	
Area:	871000.	
Sample Type:	(Unknown)	

532		
RT (Exp. RT):	8.35 (8.36) min	
Area:	116000.	
Sample Type:	(Unknown)	

533		
RT (Exp. RT):	8.36 (8.36) min	
Area:	500000.	
Sample Type:	(Unknown)	

534		
RT (Exp. RT):	8.37 (8.36) min	
Area:	891000.	
Sample Type:	(Unknown)	

535		
RT (Exp. RT):	8.36 (8.36) min	
Area:	92700.	
Sample Type:	(Unknown)	

536		
RT (Exp. RT):	8.37 (8.36) min	
Area:	864000.	
Sample Type:	(Unknown)	

537		
RT (Exp. RT):	8.38 (8.36) min	
Area:	253000.	
Sample Type:	(Unknown)	

538		
RT (Exp. RT):	8.37 (8.36) min	
Area:	164000.	
Sample Type:	(Unknown)	

SB		
RT (Exp. RT):	0.00 (8.36) min	
Area:	0.00	
Sample Type:	(Unknown)	

C Low		
RT (Exp. RT):	8.35 (8.36) min	
Area:	31300.	
Sample Type:	(Unknown)	

AB SCIEX

C High	
RT (Exp. RT):	8.35 (8.36) min
Area:	100000.
Sample Type:	(Unknown)

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