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Sheep Ectoparasite Resistance Update 2018-2020

NSW GOVERNMENT Department of Primary Industries

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Executive Summary

The impact of insecticide resistance on flystrike is usually a reduction in the protection period provided by treatments rather than complete control failure. The blowfly that initiates most flystrike in Australia is *Lucilia cuprina* which has developed widespread, high-level, stable resistance to the organophosphate insecticides. To provide up to date information on the presence/absence, levels and distribution of insecticide resistance across the sheep producing areas of Australia, this project investigated six insecticides registered for flystrike control and one of historical interest. These insecticides were 1) diazinon, which is representative of the Organophosphate group; 2) ivermectin a macrocyclic lactone (ML); 3) spinosad a spinosyns; 4) imidacloprid a neonicotinoid; 5) cyromazine a triazine 6) dicyclanil a pyrimidine derivative and 7) diflubenzuron, a benzoylphenyl urea, with the final three belonging to the IGR group of insecticides.

This study received 121 submissions of maggots removed from strikes, of these submissions 100 became viable strains that were tested. These were from WA (n=21), SA (n=12), Vic (n=11), Tas (n=1) and NSW (n=55). *In vitro* analysis of the toxicological response of these strains found a statistically significant difference in their susceptibility to diazinon, spinosad and imidacloprid dependent on the state of the strains' origin. In addition, resistance levels to diazinon have increased and the levels of susceptibility to spinosad and ivermectin were found to have decreased over time following comparison with historical data. Spinosad, ivermectin and imidacloprid are also used to control the sheep biting louse and incidental exposure of the blowfly can increase selection pressure and provide additional opportunity for the development of resistance. This is suggested by data collected on imidacloprid, for the first time, following its recent release for flystrike prevention (2019). The range of responses of field strains (n=100) to imidacloprid does not coincide with that of a laboratory susceptible strain (LS) suggesting a decade of use for lice control has applied selection pressure to the sheep blowfly. In addition, the response of dicyclanil/cyromazine resistant field strains (n=60) to imidacloprid was found to be significantly correlated with their level of susceptibility to dicyclanil, cyromazine, diazinon and Ivermectin, in descending order, but not with spinosad.

The percentage of cyromazine resistant strains has increased in the last 4-6 years from 62% (n=58) to 88% (n=100), with an increase in concurrent dicyclanil resistance from 13.8% to 73% and all submissions from NSW resistant to both (n=55). Cyromazine resistance was observed independent of dicyclanil resistance, however, dicyclanil resistance was always associated with cyromazine resistance. A correlation of 0.4303 ($p<0.0006$) was found between the responses of dicyclanil/cyromazine resistant field strains to these two insecticides. All submitted strains were characterized and pooled to form a number of reference strains which were a) dicyclanil and cyromazine susceptible (DSus), b) cyromazine resistant (CRes) or c) dicyclanil/cyromazine resistant (DRes). Levels of resistance to cyromazine and to dicyclanil were approximately the same in the CRes strain (4-fold), whereas, DRes was more susceptible to cyromazine (approximately 2-fold) but far more resistant to dicyclanil (approximately 49-fold). This indicates that dicyclanil resistance is not the result of "up-regulation" of cyromazine resistance but rather an additional resistance mechanism. In addition, there was a statistically significant increase in diazinon resistance and decrease in susceptibility to imidacloprid observed in the highly dicyclanil/cyromazine resistant reference strain which suggest the involvement of a general metabolic resistance mechanism such as the cytochrome P450 system which is associated with resistance and insect adaption. These reference strains were also used to ascertain the effect of dicyclanil resistance on the efficacy of currently marketed dressing products (*in vitro*) and several prophylactic products (*in vivo*). The *in vivo* study found dicyclanil/cyromazine resistance reduced the protection periods of three dicyclanil pour-on products by 69-78%, a cyromazine jetting fluid by 50% and an ivermectin jetting fluid by 33%. In contrast, on the same sheep, these treatments provided protection according to label claims against the dicyclanil and cyromazine susceptible strain.

Individual submitters have received the resistance profile for their property (n=100) and this project identified the need for, and informed on the development of, an integrated resistance management plan for flystrike control across Australia as non-insecticidal control measures, such as breeding sheep less prone to flystrike, will become increasingly important.

1. Introduction

This report provides information on the current insecticide resistance status of the Australian sheep blowfly, *Lucilia cuprina,* having determined the change in toxicological susceptibility and the extent of insecticide resistance in field populations to a number of different groups of insecticides. This report uses historical data to benchmark the current resistance status and demonstrates the benefits to the sheep and wool industry of periodic monitoring of insecticide resistance.

2. Literature Review

A History of Insecticide Use, Resistance Monitoring and Detection of Insecticide Resistance in the Australian Sheep Blowfly, *Lucilia cuprina*.

The Australian sheep blowfly. *Lucilia cuprina*, is one of many economically significant insects which has demonstrated its ability to develop resistance to insecticides. Despite this, there is a long history of insecticide use for the control of flystrike with insecticidal treatments remaining an essential component of flystrike management today.

Flystrike was a problem in Australia well before breech modification began[1], the first Joint Blowfly Committee was convened[2] and the first survey of *L. cuprina* [3] was conducted in the 1930's. Flystrike control relied upon the use of inorganic compounds, such as arsenic[4], well before the post war introduction of the Organochlorine (OC) group of insecticides. The OC insecticides applied to sheep included DDT, BHC, dieldrin and aldrin. However, resistance to dieldrin/aldrin was reported first in NSW[5,6] in 1958 and later in other states.[7-8] Despite the withdrawal of OC's from use in 1958[9], because of unacceptable meat residues, OC-resistance was investigated thoroughly[10-13].

The date organophosphates (OP's) were released for flystrike control has been reported as "from around 1950"[14] but is generally accepted as 1957[15]. With the withdrawal of OC's producers looked to the OP's to protect their flocks against flystrike. However, there were immediate reports of poor efficacy of OP treatments relative to OCs. The OC's were known for travelling around the animal, which made application technique unimportant, unlike the OP's which required thorough application. Despite these initial issues it wasn't until 1965[16,17] that routine monitoring detected resistance to the OP diazinon. OP-resistance was considered as non-specific and to have developed in two steps, firstly as low-level resistance in 1965 with an additional resistance mechanism in 1966.[18]. The latter is a genetically distinct form of OP-resistance which is malathion specific (R_{MAL})[19,20], conferring resistance to the OPs that contain a carboxyl ester group, of which none were ever registered to control flystrike. The non-specific esterase-based OP-resistance ($R_{op\text{-}1}$)[21-24] conferred resistance to a wide variety of OP-insecticides[25]. While the level of resistance to each OP differed, OP-resistance was described as low level in adult flies (2 to 10-fold) and moderate resistance in larvae (5 to 60-fold)[25]. Despite this, two resistance surveillance studies conducted in the 1966/67 and 1969/70 flystrike seasons, found OP resistance had increased quickly with an average OP-resistance frequency of 95% in the field populations tested. A survey conducted in 1985, found OP-resistance to be widespread, the resistance frequency estimated as 98%[24] and apparently stable, as the lack of a fitness disadvantage due to OP resistance had previously been established[26]. Despite the development of OP-resistance some fifty plus years ago, and the suspension of their registration as prophylactic treatments on Work Health and Safety grounds in 2007, OP's are still available but only to dress existing strikes and to control lice.

In 1966 a carbamate insecticide known as butacarb was released to control flystrike but was reported as only being sold in limited areas of NSW[27]. The same report stated that butacarb resistance developed within 6 months and as a consequence resistance to diazinon approximately doubled[27,28] which was shown to have negatively impacted protection period[29]. Later studies, in the 1980's, demonstrated that field populations had reverted to virtual susceptibility and butacarb was thought to be of only historical interest.[9] However, both a butacarb resistant and an OP/carbamate resistant reference strain were found to display low level cross resistance to the benzoylphenyl urea, diflubenzuron. Diflubenzuron belonged to the insect growth regulator (IGR) group of insecticides and was under consideration for use against a variety of insect pests. Following its later release for blowfly control, high level resistance developed, and it was removed from the market for flystrike protection. However, elevated mixed

function oxidases were shown to occur in diflubenzuron resistant flies[30] and an associated decrease in the efficacies of insecticides from other groups.

It wasn't until 1979 that a triazine Insect growth regulator (IGR), cyromazine, was released for flystrike prevention and for dressing existing strikes[31,32]. Due to its slow acting effects on existing strikes, cyromazine has predominantly been used as a prophylactic treatment. It was reported in the 1980's by this laboratory[33] that on occasion individual field strains were initially identified with elevated LC50 values in comparison to normal base line data. Bioassays conducted on subsequent generations failed to differentiate these strains from susceptible strains. This led to speculation[33] that these individuals had been selected by low residues of cyromazine on treated sheep, however, in the absence of selection pressure these genetic changes were at a fitness disadvantage and were lost. However, laboratory selection was able to produce a low level cyromazine resistant strain[34]. Producers became reliant on cyromazine and adapted their management practices around the use of this product with cyromazine resistance not confirmed in the field until 2011[35]. At that time cyromazine resistance was described as low-level and a subsequent survey found that 62% of the blowfly populations tested were cyromazine resistant with all submissions from NSW containing some cyromazine resistant larvae[36].

The synthetic pyrethroid (SP) group of insecticides has been used widely to control a variety of insect pests of agricultural significance. The SP deltamethrin was released to control sheep lice in 1981[37] and a cypermethrin /OP product to control *L. cuprina* was released in 1987, followed by a product which only contained alpha-cypermethrin. Unlike other flystrike prophylactic insecticides SP's paralyse the ovipositor of the female fly[38], preventing the laying of eggs or disrupting the pattern of oviposition, which results in the desiccation of scattered eggs[38, 39]. The selective capability of SP's applied as lice treatments on *L. cuprina* had been demonstrated *in vitro* along with the development of resistance to deltamethrin[40]. However, oviposition suppression was shown not to be affected by significantly correlated larval resistance to cypermethrin and the OP, diazinon[41]. Today, only alpha cypermethrin is available for lice and blowfly control and cypermethrin for lice control alone following the development of SP-resistance in lice in the late 1980's[42,43].

As previously discussed, there had been low level cross-resistance observed in both an OP resistant and an OP/carbamate resistant reference strain to the benzoylphenyl urea known as diflubenzuron. Also, laboratory selection with either butacarb or diflubenzuron was shown to increase the resistance level of the other[33]. However, *in vitro* and *in vivo* investigations of diflubenzuron in 1987 confirmed its effectiveness under fly wave simulation[44] and an apparent lack of resistance in field strains. Products with diflubenzuron as an active ingredient to prevent flystrike were released in 1993. A subsequent field survey conducted between 1996 and 1999 received 4 suspected field failures for investigation. Diflubenzuron resistance was confirmed in these and others while the correlation to OP-resistance was reconfirmed[45]. A field failure then yielded high level diflubenzuron resistance[46] and by 2002 the insecticide was withdrawn for use in flystrike control. An association with the general monooxygenases had been demonstrated[47] adding to the earlier speculation regarding OP and carbamate cross-resistance. In lice, resistance was reported to diflubenzuron in 2008[48] along with cross-resistance to triflumuron[49].

In 1993 a macrocyclic lactone (ML) pesticide, known as ivermectin, was released for the prevention and treatment of fly strike. Currently, other ML's, such as moxidectin, are available as drenches while abamectin is available for the control of both internal parasites and lice. Following its release, it was anecdotally noted that producers used the ivermectin flystrike product to drench sheep as it was less expensive. This resulted in the blowfly product being removed from the market for reformulation to prevent this practice. Just prior to its re-release, base line data were collected on the toxicological response of fly populations to ivermectin[50] and a low-level cross resistance was observed in a highly diflubenzuron resistant field strain[46]. Despite this potential cross-resistance a field survey conducted in 2013 stated field populations were considered susceptible to ivermectin[51]. The findings of this project will provide additional information on the effect of dicyclanil/cyromazine resistance on the efficacy of an ivermectin based jetting fluid[52].

At approximately the same time as ivermectin was released a spinosyn, known as spinosad, was also registered to control flystrike. It was viewed favourably because spinosad degrades in light, has very low toxicity to mammals and low activity against some beneficial insects[53]. Spinosad was registered for the prevention and treatment of existing strikes as well as the control of sheep biting lice. Base line data collected on the toxicological response of blowfly

populations to spinosad reported a 16-fold difference in LC50 values in the field populations tested and a susceptible discriminating dose of 5mg/L was proposed[54]. An additional *in vitro* study of spinosad, used as a dressing, reported that highly diflubenzuron resistant larvae did not display a survival advantage, compared to susceptible larvae, despite not achieving 100% mortality[55].

In 1998 an amidine derivative known as dicyclanil, with very similar structure to cyromazine, was released for the prevention of flystrike only[56,57]. Dicyclanil based products are marketed as spray-on treatments and their ease of application and the long protection (up to 18-24 weeks) of the 50g/L product have assured its widespread adoption and ever-increasing market share. More recently a lower dose (12.5g/L) spray on product (up to 11 weeks protection) was released for use leading up to sale for slaughter, late in the season or prior to shearing or crutching. Also in 2018 a higher dose product was released (65g/L) with a protection claim of up to 29 weeks. Unfortunately, cyromazine resistance had already been confirmed in the field[35] along with 14% associated resistance to dicyclanil[36]. The occurrence of cyromazine resistance and concurrent dicyclanil resistance will be discussed as a major component of this project.

The most recent insecticide to be marketed for the control of flystrike is the neonicotinoid, imidacloprid, which was released for flystrike in 2019. The toxicological response of *L. cuprina* to imidacloprid has not previously been considered, however, it has been used extensively for the control of the sheep body louse, *B. ovis,* for more than 10 years. The findings reported here can be used as a benchmark for future studies and serve as a useful tool to identify future shifts in the susceptibility of *L. cuprina* to imidacloprid.

Given the limited availability of novel insecticides and the high cost of their development, there is an urgent need to extend the effective life of registered insecticides through their strategic use as part of integrated resistance management[58]. As the detection and monitoring of resistance have proved their worth in the past, the current findings on the resistance status of *L. cuprina* should aid the formulation and adoption of such a plan.

3. Project Objectives

3.1 Define the toxicological response and determine the resistance status of field populations of the sheep blowfly to spinosad, imidacloprid, ivermectin, diazinon, diflubenzuron, cyromazine and dicyclanil.

3.2 Specifically monitor field populations of sheep blowfly for resistance to cyromazine and dicyclanil by sampling from properties where cyromazine has been used consistently or if control failure is suspected.

3.3 Screen large numbers of larvae from each submitted population of sheep blowfly for cyromazine/ dicyclanil resistant individuals and quantify the level of resistance.

3.4 Preserve resistant and susceptible individuals as a resource for further research. Pooling populations to produce representative field strains for further studies on cross resistance and novel chemicals.

3.5 Implement a communications plan to promote sample submission, grower involvement in research, grower benefits of information flow back to farm, broader promotion of the AWI flystrike strategy.

3.6 To determine the effect of dicyclanil/cyromazine resistance on *in vitro* susceptibility to the three alternative insecticides spinosad, ivermectin and imidacloprid.

3.7 To determine the *in vitro* efficacy of currently marketed dressing products against dicyclanil/cyromazine resistant 3rd instar maggots.

3.8 To determine *in vivo,* the impact of dicyclanil/cyromazine resistance on the protection provided by dicyclanil, cyromazine and ivermectin based flystrike preventative products.

4. Success in Achieving Objectives

The study objectives 3.1-3.8 were achieved, despite widespread drought determining the locations of flystrike activity and hence blowfly submissions. This was one of the most comprehensive studies ever conducted by the NSW DPI Insecticide Resistance Laboratory, given the number of resistance profiles reported on here (100) and the number of insecticides involved (7).

The detection of dicyclanil resistance in the early stages of the project precipitated the formation of the AWI Sheep Blowfly Resistance Working Group which developed a producer targeted insecticide resistance management strategy (2 articles) for Beyond the Bale. These are now available on the AWI and FlyBoss websites as producer resources.

5. Methodology

Telephone or e-mail contact was made with existing networks in the sheep industry. Project outlines and information were distributed along with calls for contacts to distribute kits and for sample submissions. These networks included NSW Local Land Service District Veterinarians and Biosecurity Officers, SheepConnect Co-ordinators in Tasmania and South Australia, Leading Sheep Queensland, equivalent departments from other states, animal health companies and agricultural/rural retailers.

In total 455 maggot collection kits were distributed upon request and 121 submissions were received, whereupon, each strain of maggots was given a unique ID and placed into insecticide free laboratory culture. Neonate larvae of the second generation of each submission were used for *in vitro* testing of seven insecticides, which included 1) diazinon, representative of the Organophosphate group; 2) ivermectin a macrocyclic lactone (ML); 3) spinosad a spinosyn; 4) imidacloprid a neonicotinoid; 5) cyromazine a triazine derivative, 6) dicyclanil a pyrimidine derivative and 7) diflubenzuron a benzoylphenyl urea, with the final three belonging to the larger chemical group known as the Insect Growth Regulators (IGRs).

The laboratory assay used to measure each strain's level of larval susceptibility or resistance to diazinon, ivermectin, spinosad, imidacloprid and diflubenzuron was developed in this laboratory and has been used since the 1970's.[60] Briefly, PESTANAL®, analytical standard grade insecticides, were used and the sheep serum was fortified with 20 g L^{-1} yeast extract and 5 g L^{-1} potassium dihydrogen orthophosphate. Duplicate strips of chromatography paper were treated with acetonic solutions containing a serial dilution of insecticide to cover the 0–100% range of larval mortality. After placing the insecticide impregnated papers into glass phials, 1 mL of sheep serum and forty newly

hatched first-instar larvae were added to each. The assays were incubated under lights for 24 hrs, in the case of diazinon, and 48 hrs for the remaining insecticides, and then percentage mortality was determined. Solvent controls were used to determine the control mortality which was used to correct the dose mortality data using the Schneider-Orelli's formula[61] which is an adaption of Abbotts Correction[62]. Probit analysis[63] was performed using BioStatPro software[64] to calculate the concentration at which 50% of the maggots in the phial were killed (LC50), and also 95% mortality (LC95), along with the associated 95% fiducial or confidence limits. Each strain's level of susceptibility to the test insecticide was compared by calculating resistance factors (RF's) relative to the Laboratory Susceptible strain, LS, (LC50 field strain/LC50 LS strain).

The susceptibility of individual field strains to cyromazine and dicyclanil was determined using susceptible discriminating concentrations (SDC's) of 1 mg kg^{-1} for cyromazine and 0.1 mg kg^{-1} for dicyclanil incorporated into the larval food. In line with a previous study[36], an additional concentration of 8-fold the SDC for cyromazine was included. It was considered important to determine if field populations displayed higher-levels of resistance to dicyclanil and therefore 4-fold and 8-fold SDC concentrations were also included. Once strains were categorised, they were pooled according to their dicyclanil resistance levels to form reference strains of known resistance status. The same technique was used to determine the concentration of insecticide required to produce 50% mortality (LC50), and the associated 95% fiducial limits, for cyromazine and dicyclanil in these pooled reference strains. These were the dicyclanil/cyromazine resistant strains (DRes1 and DRes8), a pooled cyromazine resistant strain (CRes) and a strain susceptible to both of these insecticides (DSus). Results were expressed as resistance ratios (RR) relative to the pooled susceptible reference strain DSus, (LC50 resistant reference strain/LC50 DSus). These reference strains were used to determine:

a) The effect of dicyclanil resistance on the efficacy of the currently available dressing products against full gutted third instar larvae *in vitro*[59].

   Replicates of approximately 100 full gutted third instar larvae from the LS, DSus, DRes1 and DRes8 strains were exposed to the following dressings: a) 500g/L cyromazine (Vetrazin™); b) 16g/L ivermectin (Coopers Blowfly and Lice™); c) 200g/L diazinon (Coopers Diazinon™); d) 14g/L propetamphos (Young's Deadmag™); e) 15g/kg diazinon, 0.8g/kg piperonyl butoxide, and 1g/kg pyrethrins (WSD Fly Strike Powder); f) 25g/L spinosad (Extinosad Eliminator™); g) 2.8g/kg spinosad (Extinosad™). The dressings were diluted or used neat as directed by the manufacturer. The larvae were fully immersed for 5, 15, 30, 60 and 180 seconds. Controls were immersed for the same intervals in water. Larvae were placed on a small square of paper towel before being placed in a labelled pot. After 24 hours the total number of larvae were counted, vermiculite was added, and the larvae were left to pupate. Pupae and adult flies were counted when they emerged. The percentage mortality was determined by dividing the number of larvae exposed by the number of adults emerged multiplied by 100. Analysis was performed in Microsoft Excel. Odds Risk Ratios were performed according to the formula:

$$RR = \frac{D_E N_N}{D_N N_E} = \frac{D_E | D_N}{N_E | N_N} \qquad OR = \frac{D_E | H_E}{D_N | H_N}$$

   where N_E is the total number exposed out of which D_E died and H_E emerged as adults. N_N is the total number of the untreated controls, out of which D_N is controls that died and H_N the controls that emerged as adults.

b) The protection period provided by three dicyclanil spray on products, a cyromazine jetting fluid and an ivermectin based jetting fluid against strike establishment *in vivo* by neonate larvae[65,66,67].

   Yearling merino wethers were treated 6 weeks post shearing on the backline and breech according to the manufacturers' instructions for their individual body weight. The treatment groups were: (a) 12.5 g/L dicyclanil, (CLiKZiN Spray-On™); (b) 50 g/L dicyclanil (CLiK™ Spray-On); (c) 65 g/L dicyclanil (CLikExtra™ Spray-On); (d) 500 g/L cyromazine (Vetrazin™ Liquid) applied by hand jetting; (e) 16.0 g/L ivermectin (Coopers® Blowfly and Lice Jetting Fluid) applied by hand jetting; and (f) Untreated controls. Six sheep from each group were challenged at fortnightly intervals with newly hatched larvae from a pooled dicyclanil and cyromazine susceptible strain and on the opposite side of the midline with a pooled dicyclanil/cyromazine resistant field strain. These neonate

larval implants were created directly under the treatment and were checked at 24, 48 and 72 hours. Larvae were removed from the sheep, after 24 hours on the untreated controls and 48 and/or 72 hours, dependant on their size, from the treated sheep. Once removed the larvae were allowed to continue development through pupation and the number of flies which emerged were counted. An implant was considered positive if flies successfully emerged, while a break in the protection period was declared if 3 out of the 6 challenged sheep had a positive implant for that strain. This was confirmed by the subsequent implant. The percentage reduction observed in the protection period was calculated by dividing the number of weeks post treatment at which the break in protection occurred in the treatment group divided by the number of weeks protection claimed by the product multiplied by 100.

The parameters of this *in vivo* study were specifically selected to avoid issues highlighted by a similar study using a cyromazine resistant strain which was reported on 2014[68]. Some of these parameters and the differences between these two studies are listed in the **Table 1**. below.

Table 1. Comparison of some trial design parameters of the 2013-14 and 2018-19 *in vivo* studies.

Trial Parameter	**2014 Study – Cyromazine Resistance**	**2019 Study – Dicyclanil Resistance**
Trial Conducted	Autumn, Winter 2013	Spring, Summer 2018/19
Sheep Age	Adult (not described)	Yearlings (Flystrike naïve)
Wool length at treatment	7 months	6 weeks off shears
Previous treatment with insecticide	Yes, post shearing but none in 6 months prior	Nil
Cyromazine Treatment Used	Spray-on and Jetting Fluid	Jetting Fluid
Possibility of contamination between treatment groups	Yes. Sheep run as a single mob	No. Sheep run as discreet groups
Total Number of Treatment Groups (Total Number Sheep)	4 (28)	6 (72)
Number untreated control sheep (Max Number implants/sheep)	7 (7)	15 (4)
Implants commenced	Mid-Autumn	Last month of Spring
Number of weeks between implants	3	2
Number of sheep implanted per treatment group	5	6
Number of implants positive for a break in protection	2	3
Implants assessed as	Positive=live larvae on strike at 48 or 72 hours Negative = All larvae on strike dead.	Positive = larvae removed at 48 or 72 hours, pupated and adults emerged. Negative = larvae on strike dead or removed at 48 or 72 hours but did not develop through to adult flies.

c) Finally, these dicyclanil/cyromazine susceptible and resistant reference strains were used to investigate the change in *in vitro* susceptibility to ivermectin, spinosad and imidacloprid resulting from dicyclanil and/or cyromazine resistance using the laboratory assays outlined above.

6. Results

6.1 Producer Submissions

Approximately 455 maggot collection kits were distributed across Australia and 121 samples were submitted (27%) despite widespread drought conditions in many areas. Figure 1 shows the distribution of submissions from across the southern areas of Australia. In total submissions were received from 24 of the natural resource management regions identified on the MLA map of National sheep numbers (June 2016).

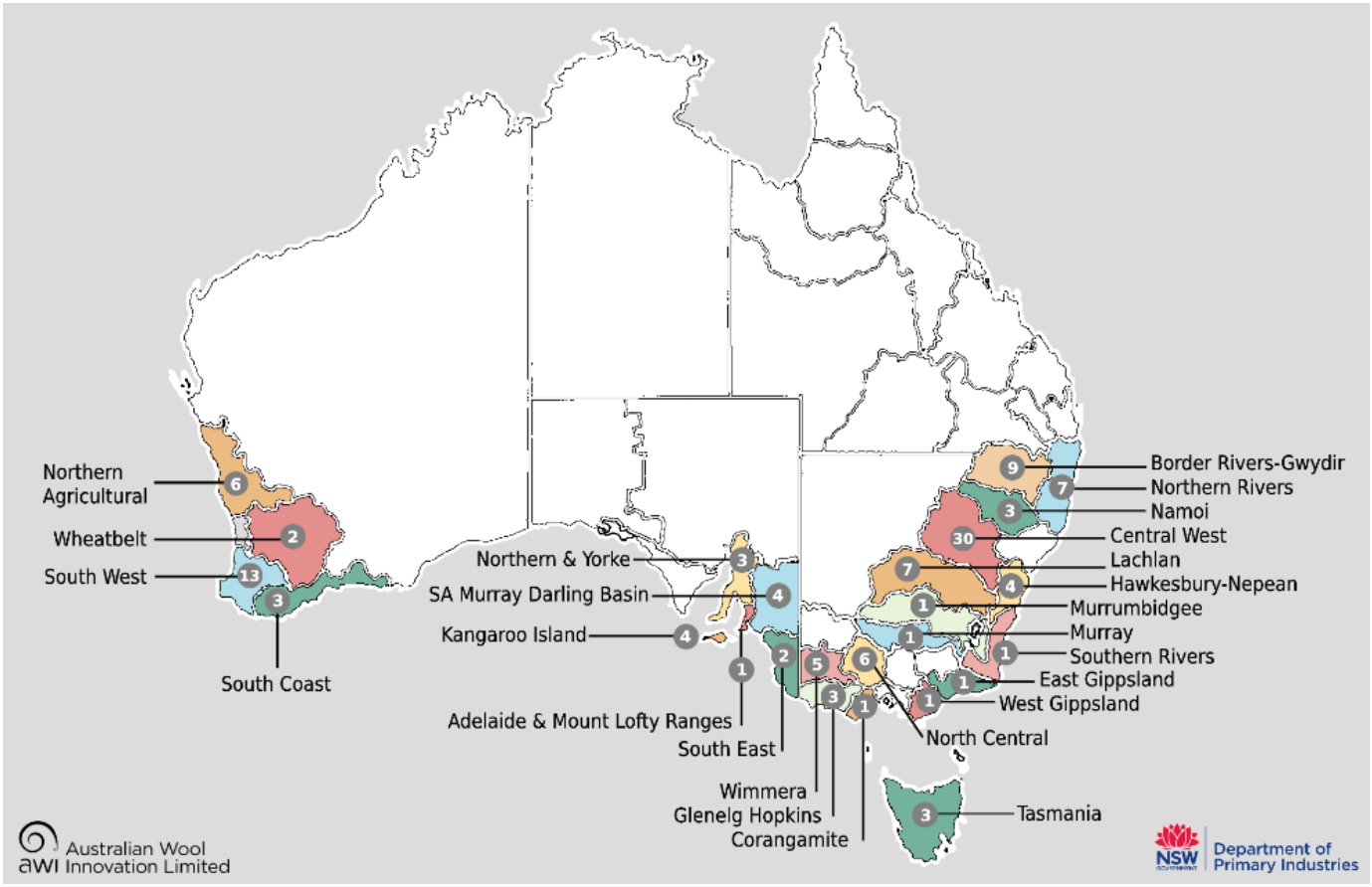


Figure 1. The number of submissions of maggots, collected from struck sheep, which were received from each region identified on the MLA national sheep numbers map (2016) (n=121).

Submissions from NSW made up 52% of all submissions while those from other states were low with 19.8% from WA, 11.6% from SA, 14.1% from Vic, and 2.5% from Tasmania (n=121). During the project we did not receive any submissions from Queensland despite contacting and then sending kits to key members of the Leading Sheep program, Department of Agriculture and Fisheries and directly to producers following their requests. Of the 121 submissions received, 21 (17.4%) were not viable, being either the incorrect species, dead on arrival or containing too few individuals for successful culture (**Figure 2**).

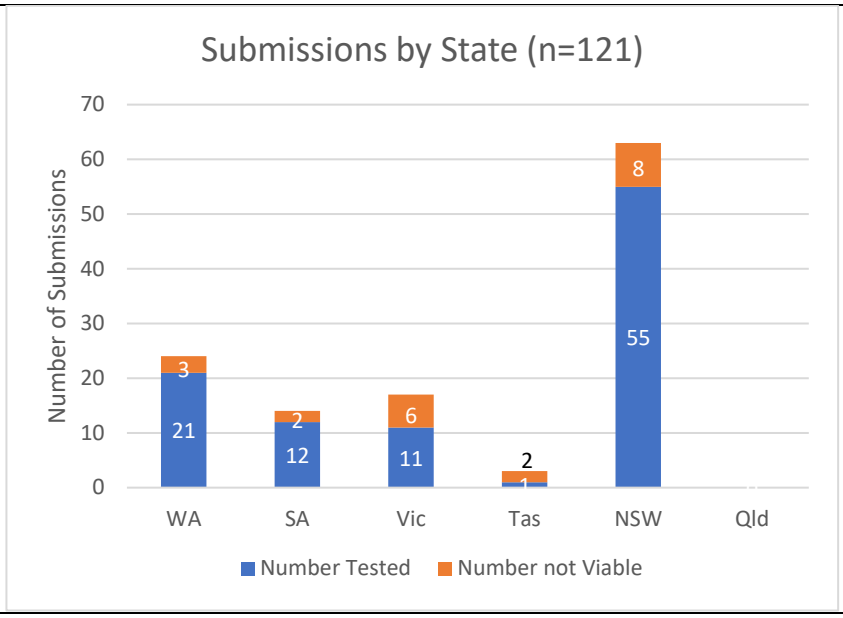


Figure 2. The number of maggot submissions received from each state that were successfully cultured and then tested or which were not because the submission was the incorrect species, died in transit or was too few to successfully culture (n=121).

The maggot collection kits contained a strike record sheet for submitters to complete and return. From this information we determined if the maggot sample had been removed from insecticide treated sheep and the product used following their last shearing. Untreated sheep made up 38% of submissions, leaving 62% of submissions taken from treated sheep (**Figure 3**). Of the total submissions received dicyclanil had been the most frequently used insecticide (34.7%). As diflubenzuron-based products are no longer marketed for the control of flystrike, we are assuming that a louse treatment was reported here despite there also being a separate question on louse treatments.

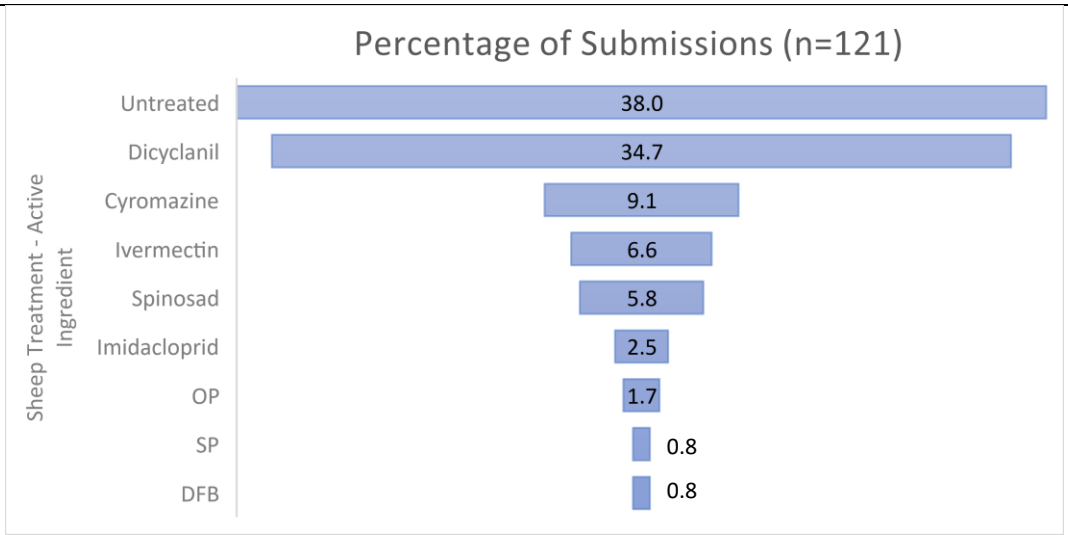


Figure 3. The percentage of maggot submissions which were removed from untreated or treated sheep and the active ingredients they had been treated with (OP- Organophosphate, SP – Synthetic Pyrethroid and DFB – Diflubenzuron).

The strike record sheet also provided data on location of the strike from which the submitted maggots were removed. (**Figure 4**). The sheet also asked if the maggot sample had been removed from a single sheep (46.3%), from multiple sheep (28.9%) with the remaining 24.8% of submissions undeclared.

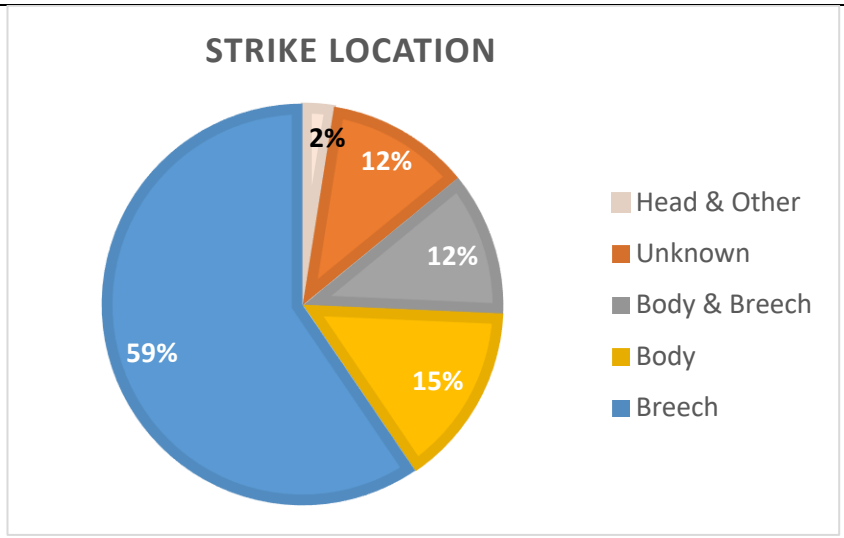


Figure 4. The locations from which the submitted maggots were collected off the sheep expressed as the percentage of total submissions (n=121).

When the insecticide resistance profile of a strain was completed the results were conveyed to their submitter and the opportunity was taken, when possible, to gather additional information regarding the management practices and insecticide usage on the property.

The individual property profiles supplied to submitters are confidential and are not provided as part of this report. Instead results are presented as **a)** a comparison between States of the range of susceptibility or resistance observed to an insecticide; **b)** all of the submissions considered as a single population to assess the normality of the distribution of the toxicological response to an insecticide; and **c)** the change in susceptibility to the insecticide over time by comparison with data generated in this laboratory during previous studies.

6.2 Resistance Levels of Field Submissions to the Organophosphate, Diazinon

6.2.1 Diazinon -State Comparison

As expected, regardless of the state of origin, all submissions were resistant to the OP diazinon. Resistance levels of field strain to diazinon ranged from a Tasmanian strain with the minimum RF of 8-fold through to the maximum RF of 63.5-fold in a strain from NSW (n=100). There were two submissions which appeared as outliers for their state, one from NSW (RF= 63.5) which was received late in autumn 2020 and the other from South Australia (RF=50.4). However, the outlier from SA fell within the NSW range. There was a statistically significant difference in resistance levels between the strains from WA and NSW ($p<0.05$) but SA and Vic were not different to either.

6.2.2 Diazinon – Frequency Distribution of Diazinon Resistance in the Australian *L. cuprina* Population

A "normal" distribution of response can still be observed in a resistant population if the resistance has stabilised in the population and selection pressure remains constant. Comparison with a hypothetical, normally distributed population, with the same mean and standard deviation, indicated the response to diazinon in 2018-20 was not normally distributed. It was negatively skewed indicating a tendency towards higher levels of resistance and leptokurtic with a congregation around the mean Log (LC50). The Shapiro-Wilk test for normality also rejected the null hypothesis that the population was normally distributed.

6.2.3 Diazinon - Comparison over Time

In this study, resistance factors (RF's) calculated to diazinon were higher, maximum= 63.5-fold and minimum= 8 (n=100), than those reported in 1988.[69] These were a maximum RF of 25-fold and a minimum Rf of 8-fold (n=33). In 1988 OP's were being used, resistance gene frequency was stabilised at 98%, however, these RF's were not considered high. The level of resistance was found to be higher in a subsequent study in 1994, with RF's ranging from 2.2 to 42.1 with the mean RF of 19.6 (n=125), however this is still lower than observed in the current study.

6.3 Susceptibility of Field Submissions to the ML, Ivermectin

6.3.1 Ivermectin - State Comparison

A significant difference in the susceptibility of field strains from different states to ivermectin was not observed. The RF's ranged from 1.0-fold in a strain submitted from Western Australia to 7.1-fold in a strain submitted from NSW, with three outliers observed, one each in WA, Vic and NSW.

6.3.2 Ivermectin – Frequency Distribution of Ivermectin Susceptibility in the Australian *L. cuprina* Population

The populations susceptibility to ivermectin is normally distributed, compared to a "normal" hypothetical population, with the LS strains Log (LC50) value being lower than the field populations mean Log (LC50) but still falling within the field response. The frequency distribution is not skewed but the LC50 values of the strains are concentrated about the mean Log (LC50) i.e. there is leptokurtosis. The Shapiro-Wilk normality test accepted the null hypothesis and confirmed the distribution was normal.

6.3.3 Ivermectin – Comparison over Time

Previous studies of ivermectin, with smaller numbers of field strains, were conducted in 2013/14 (n=58) and 1998/99 (n=74)[36]. Statistical analysis of these data shows an increase in ivermectin RF's in the 20 years between 1998 and 2018-20 ($p<0.05$). However, the maximum RF only increased 2.5-fold in that period. As the majority of submissions to all three studies were from NSW, a comparison between studies was undertaken using only NSW data. The maximum RF's did not change (i.e. 2.5-fold increase) and the minimum RF only increased 2-fold. The 2018-20 resistance factors were statistically significantly higher ($p<0.001$) than in both previous studies with more than half of the RF values for 2018-20 being higher than the maximum Rf observed in 1998-99.

6.4 Susceptibility of Field Submissions to the Spinosyn, Spinosad

6.4.1 Spinosad – State Comparison

ANOVA performed on RF values determined to spinosad in field submissions indicated that there was a significant difference between the strains from WA to those from SA ($p<0.05$). There was no significant difference between the remaining states to either WA or SA. The RF's ranged from approximately 0.4-fold in a strain submitted from New South Wales to 5.0-fold, in a strain submitted from Western Australia.

6.4.2 Spinosad- Frequency Distribution of Spinosad Susceptibility in the Australian *L. cuprina* Population

The normal distribution of the 2018-20 populations response to spinosad was confirmed by the Shapiro-Wilk test. Once again, the distribution was not skewed but it was concentrated around the mean of the population (leptokurtic) with the Log (LC50) of the LS strains falling within the distribution but lower than the mean of the field populations.

6.4.3 Spinosad – Comparison over Time

The minimum RF of 0.16 in a 1998-99 study (n=41) increased by 2.5-fold to 0.41 in the 2018-20 study (n=100) with an approximate doubling of the maximum RF from 2.61 (1998-99) to 4.93 (2018-20). Despite these apparently low increases in RF values there was a statistically significant decrease in susceptibility ($p<0.00001$) over the intervening 20 years.

6.5 Susceptibility of Field Submissions to the Neonicotinoid, Imidacloprid

6.5.1 Imidacloprid – State Comparison

In the absence of base line data on imidacloprid, this study reports a large range of susceptibility from a minimum RF of 3.2-fold from Tasmania to a maximum RF of 42.5-fold from Victoria which appeared as an outlier for that state but not compared to NSW. However, the susceptibility of field strains from WA, Vic and NSW to imidacloprid were statistically significantly different to each other ($p<0.05$) whilst those of SA and Tas were similar to both WA and Vic.

6.5.2 Imidacloprid – Frequency Distribution of Imidacloprid Susceptibility in the Australian *L. cuprina* Population
The frequency distribution of field strain susceptibility to imidacloprid was not normally distributed. In addition, the response of the LS strain to imidacloprid fell outside the very broad range observed in the field strains, indicating a shift towards resistance

Whilst skewness was negligible, the Log (LC50) values were more spread out from the mean than in a normally distributed population (platykurtic). This was supported by the Shapiro -Wilk test which rejected the null hypothesis of normality.

6.5.3 Imidacloprid – Comparison over Time
An evaluation of the change in susceptibility over time was not able to be conducted as previous data was not available on imidacloprid for comparison. The data collected during this study will enable comparisons in the future.

6.6 The Toxicological Susceptibility of Field Strains to the Benzoylphenyl Urea, Diflubenzuron

From previous studies regarding the nature of the widespread and high-level resistance found to diflubenzuron (DFB) the highest concentration, based on solubility, was used to screen the field strains. The percentage mortality produced at 512.0 mgL^{-1} was determined for each field submission which is 3,657-fold the LC50 value of LS. The percentage survival at this extreme dose ranged from 6.25% through to 93.5% survival, both being strains from NSW. Overall, the highest average percentage survival (69.5%) was amongst submissions from South Australia.

6.7 Susceptibility of Field Submissions to the Triazine IGR, Cyromazine

Low level resistance to cyromazine (without dicyclanil resistance) was defined as those field submissions which could survive the susceptible discriminating concentration (SDC) of 1mg $Kg.^{-1}$ Cyromazine resistance was not detected in Tasmania (n=1) nor on its own in NSW as the strains were also resistant to dicyclanil (n=55) (**Figure 5**). The percentage of cyromazine resistant maggots was assessed *in vitro* for each submission at the second generation and ranged from “present but below 1%” to 73%. The offspring of the strains which survived the cyromazine SDC, but not the dicyclanil SDC, were pooled to form the CRes reference strain.

6.8 Susceptibility of Field Submissions to the Amidine Derivative IGR, Dicyclanil

Concurrent dicyclanil and cyromazine resistance was found in 100% of submissions from NSW (n=55) which was not the case for other states **(Figure 5)**. The frequency of dicyclanil resistant individuals ranged from 2% to 93% in the dicyclanil/cyromazine resistant submissions. Offspring of those strains that survived the dicyclanil SDC were pooled to form DRes1 and those that survived 8-fold the dicyclanil SDC were pooled to form DRes8.

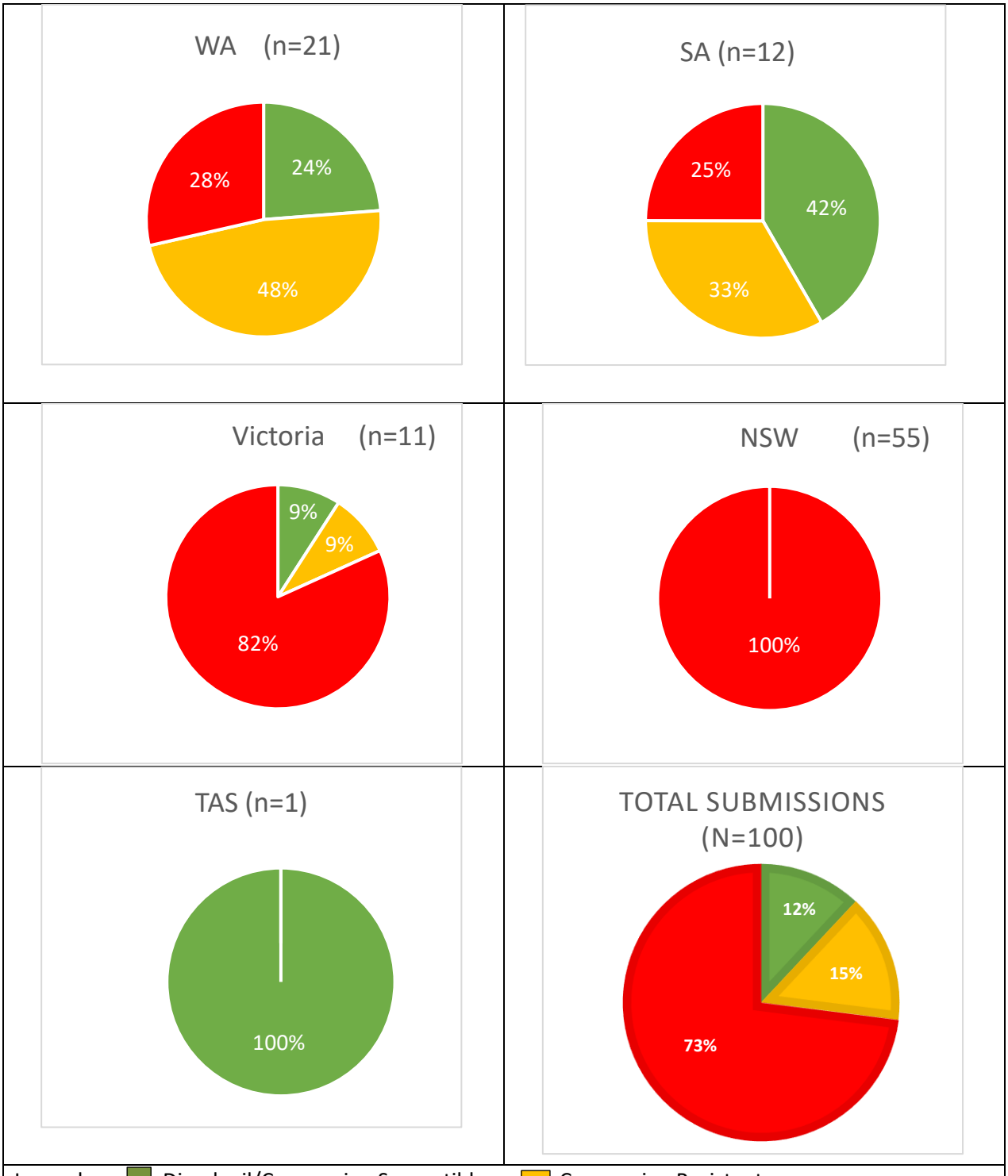


Figure 5. The percentage of field submissions from each state which were susceptible to both cyromazine and dicyclanil, resistant to cyromazine or displayed concurrent dicyclanil and cyromazine resistance as determined by the susceptible discriminating doses (SDCs).

6.9 Strains with Concurrent Dicyclanil and Cyromazine Resistance

Strains which were found to be dicyclanil resistant were also found to be cyromazine resistant whilst in states other than NSW cyromazine resistance was found to occur independently.

6.9.1 The Relationship Between Resistance to Dicyclanil and Cyromazine

The percentage survival, at the SDC's of dicyclanil and cyromazine, were used to determine associations in dicyclanil/cyromazine resistant field strains (n=60). A Pearson's correlation coefficients of 0.4303 indicated a statistically significant ($p<0.0006$) relationship between the strain's response to both insecticides. The inclusion of strains which were resistant to cyromazine but not to dicyclanil increased the Pearson's Correlation to $r = 0.5325$ ($p< 7.3937E^{-7}$) which is highly statistically significant. This reinforces that cyromazine resistance is required for dicyclanil resistance to occur, however, the observed levels of dicyclanil resistance far exceeded those of cyromazine resistance.

6.9.2 Field Strains - *In vitro* Susceptibility to Other Insecticides

Following screening with the SDC's individual submissions were classified as susceptible to dicyclanil and cyromazine, cyromazine resistant, lower dicyclanil/cyromazine resistance or higher level dicyclanil/cyromazine resistance. A statistically significant ($p<0.001$) association of increasing RF's to diazinon with increasing resistance to dicyclanil/cyromazine was observed. This association was also observed with imidacloprid but not with ivermectin nor spinosad.

6.9.3 Pooled Reference Strains - *In vitro* susceptibility to other insecticides

Following characterization of each submitted field strain according to survival of cyromazine at 1-fold the SDC and dicyclanil at 8-fold the SDC, the strains were pooled accordingly. This process resulted in 3 pooled reference strains which included a strain susceptible to both insecticides (DSus), a strain resistant to cyromazine only (CRes) and a strain with higher level resistance to dicyclanil/cyromazine (Dres8). Following their formation, the CRes and the DRes8 strains were selected with the appropriate SDC level to eliminate susceptible types from the strain but not to increase the level of resistance in the resistant individuals. Over the course of the study field strains were added to the appropriate pooled strain. Comparison of RF values found a statistically significant decrease in susceptibility to diazinon, ivermectin and imidacloprid with CRes less susceptible than DSus and DRes less susceptible than CRes. Susceptibility to spinosad was greater in DSus than in the other two strains. Interestingly Dres8 had a lower RR (2.2) against cyromazine than CRes (3.8) while CRes's RR to dicyclanil was similar (3.6) a much higher level of resistance (RR= 48.7) occurred in the DRes8 strain.

6.10 Associations Between Insecticides in the Dicyclanil/Cyromazine Resistant Field Strains

Pearson's Correlation Coefficients, based on the method of covariance, were calculated to determine the association between the susceptibility of the dicyclanil/cyromazine resistant field strains to two insecticides. It gives information about the magnitude of the association as well as the direction of the relationship. The data examined for cyromazine and dicyclanil was the % survival at their SDC, for diflubenzuron the % survival at $512mgL^{-1}$ and for diazinon, ivermectin, spinosad and imidacloprid their resistance factor.

The correlation between diazinon and diflubenzuron, which had been reported on in the 1990's, was not observed in this study, nor was a correlation between ivermectin and dicyclanil despite evidence of a relationship in the *in vivo* study. Conversely, there was a weak correlation between cyromazine and ivermectin with the largest and most significant associations being between dicyclanil and imidacloprid followed by dicyclanil and diflubenzuron. An association also occurred between cyromazine and imidacloprid which was only slightly more significant than the association between dicyclanil and cyromazine.

6.11 In vivo Trial

6.11.1 The Effect of Dicyclanil Resistance on the *In vivo* Protection Periods Provided by Registered Preventative Products

A challenge study on sheep treated 6 weeks post shearing, with newly hatched 1st instar larvae of a pooled dicyclanil/cyromazine resistant strain was conducted. We found protection from a $12.5gL^{-1}$ dicyclanil spray-on product was reduced from 11 weeks down to <3 weeks given all 6 sheep were positive at the first implant. Assuming

3 weeks protection. this constitutes a 73% reduction in protection period. Protection was also reduced from 18 to 4 weeks, (78% reduction) and from 29 to 9 weeks (69% reduction) following challenge of a 50mgL^{-1} and a 65gL^{-1} dicyclanil based spray-on product respectively. Jetting fluids with cyromazine and ivermectin as actives also had protection periods reduced from 14 to 7 weeks, (50% reduction) and from 12 to 8 weeks (33% reduction) respectively. When challenged with maggots which were dicyclanil and cyromazine susceptible all products protected for the periods listed on the product labels. **(Figure 6)**

6.11.2 Comparison Between *In vivo* Results in 2013 and 2018-19

Unfortunately, during the 2013-14 *in vivo* study the challenge process failed on 11 occasions on the untreated control sheep (**Figure 20e**), which was not the case in the 2018-19 study (**Figure 7f**). There were a number of parameters which were modified in the 2018-19 *in vivo* study protocol, detailed in **Table 1.,**, The most important of these were wool length at treatment which was changed from 7 months wool to 6 weeks post-shearing and the use of only a cyromazine jetting fluid rather than both a jetting fluid and a pour-on formulation. Despite these differences the cyromazine jetting fluid (500gL^{-1}) delivered approximately the same protection against the cyromazine resistant strain in the 2013-14 study (8 weeks) (**Figure 7a**) as it did against the dicyclanil/cyromazine resistant strain DRes (7 weeks) in the 2018-19 study(**Figure 7b**). However the 50gL^{-1} dicyclanil based pour on treatment provided 11 weeks protection against the cyromazine resistant strain in 2013-14 (**Figure 7c**) but less than 4 weeks protection against the dicyclanil/cyromazine resistant strain (**Figure 7d**) when challenged. These results reflect the findings of the *in vitro* testing where much higher levels of resistance were observed to dicyclanil than to cyromazine in the field. Both *in vivo* studies attained the protection periods claimed on the labels of products against susceptible strains with the exception of the 50gL^{-1} dicyclanil spray-on product in the 2013-14 study where the cut-off was reached 17 weeks post treatment against the cyromazine susceptible strain **(Figure 7c)**.

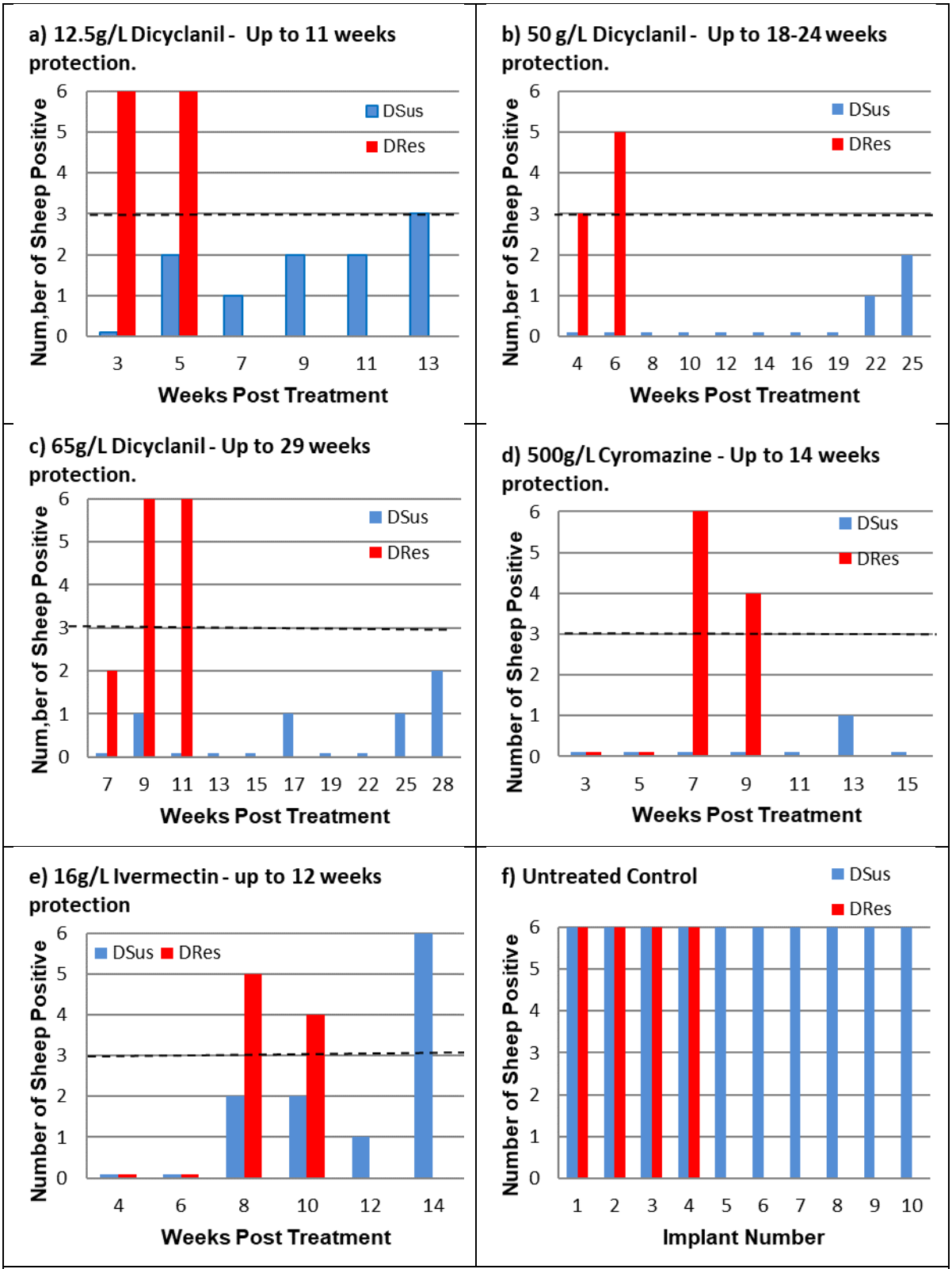


Figure 6 The number of sheep declared positive, according to treatment group, when challenged by dicyclanil susceptible (DSus) and dicyclanil/cyromazine resistant (DRes) strains based on any surviving larvae removed from sheep developing successfully through to fly emergence.
--------- 3/6 Cut-off of positive sheep defining a break in protection period.

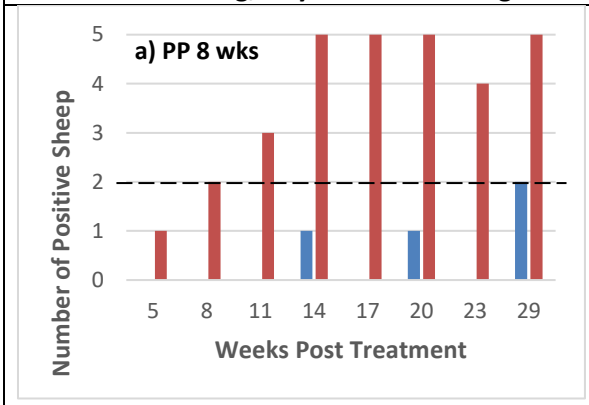
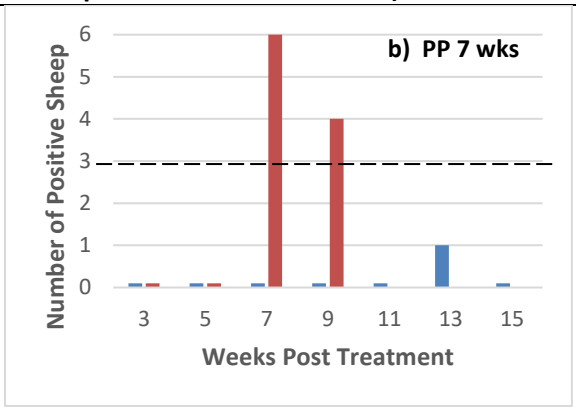
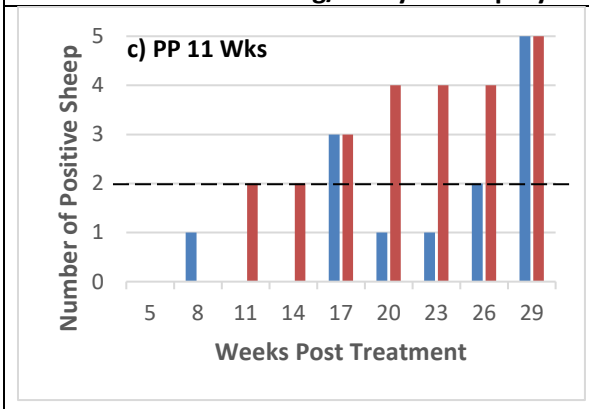
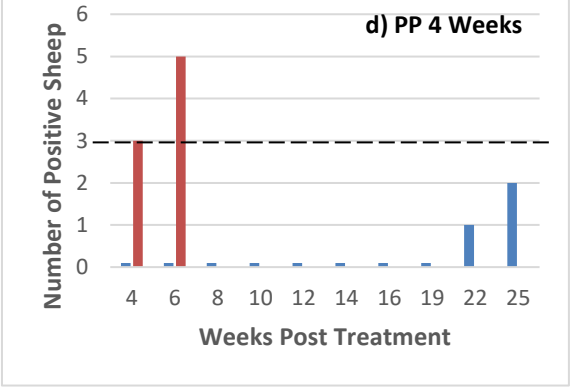
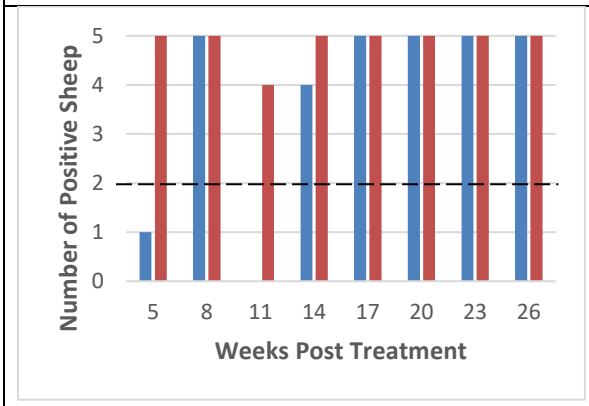
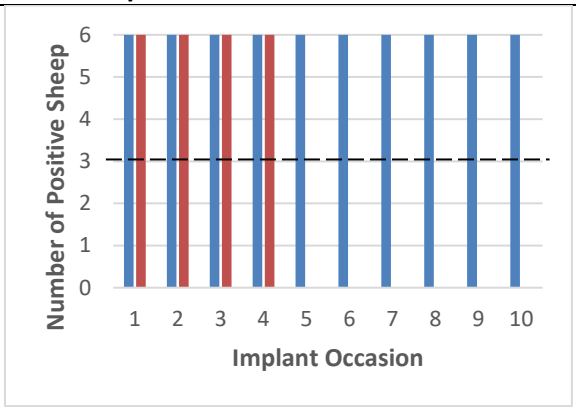
2013 Cyromazine Trial (7 months wool)	2018-19 Dicyclanil Trial (6 weeks wool)
500g/L Cyromazine Jetting Fluid (Claim: up to 14 Weeks Protection)	
	
50g/L Dicyclanil Spray-On (Claim: up to 18-24weeks)	
	
Untreated Control Sheep	
	
Cyromazine **Susceptible** & **Resistant** Strains	Dicyc/Cyrom **Susceptible** & **Resistant** Strains

Figure 7 a), c) and **e) 2013**. Protection provided by two treatments when challenged by cyromazine susceptible and resistant strains based on larvae assessed as alive or dead. A cut-off of 2 out of 5 implanted sheep declared positive defined a break in protection

Figure 7 b), d) and **f) 2018-19**. Protection provided by two treatments when challenged by dicyclanil/cyromazine susceptible and resistant strains assessed as fly development from larvae removed from the sheep. A cut-off of 3 out of 6 implanted sheep declared positive defined a break in protection period.

---------- Cut-off of positive sheep defining a break in protection period.

PP – Protection Period achieved against the resistant strain.

6.12 Dressing Efficacy

Full gutted 3rd instar larvae from the Laboratory Susceptible strain (LS), the cyromazine and dicyclanil susceptible pooled field strain (DSus) and the dicyclanil/cyromazine resistant composite strains (DRes8 and DRes1) were exposed to commercially available dressing products ranging from 5 seconds to 180 seconds. Mortality was determined by counting the number of adults that emerged and subtracting them from the number of larvae exposed. This figure was divided by the total number of larvae exposed multiplied by 100. Products were ranked from most effective to least effective based on the average mortality calculated across the time course, as follows:

> DSus strain: Flystrike Powder > Spinosad aerosol > ivermectin jetting fluid > cyromazine jetting fluid > Diazinon > Propetamphos > Spinosad jetting fluid.
>
> DRes: Spinosad aerosol > Ivermectin jetting fluid > Cyromazine jetting fluid > Flystrike powder > Spinosad jetting fluid > Propetamphos > Diazinon.
>
> Calculation of the relative risk of mortality from exposure for 180 seconds to one dressing product compared to another can be seen in **Table 2** for each strain.

Table 2. The relative effectiveness of flystrike dressing products (identified by numbers 1-7). following 180 seconds exposure of various *Lucilia cuprina* strains. Risk ratios were calculated from the % mortality which determined by fly emergence. Risk ratios are an estimation of risk to individuals exposed to one treatment (rows) relative to another (columns) i.e. if a value <1 the dressing listed in the row is less effective than the dressing listed in the column and if the value is >1 the dressing listed in the row is more effective than the one in the column.

LS @180 Seconds	**1**	**2**	**3**	**4**	**5**	**6**	**7**
1 *Cyromazine*[a]	1.00	1.00	1.00	5.00	1.00	3.00	1.01
2 *Ivermectin*[b]	1.00	1.00	1.00	5.00	1.00	3.00	1.01
3 *Diazinon*[c]	1.00	1.00	1.00	5.00	1.00	3.00	1.01
4 *Propetamphos*[d]	0.20	0.20	0.20	1.00	0.20	0.60	0.20
5 *Diazinon Powder*[e]	1.00	1.00	1.00	5.00	1.00	3.00	1.01
6 *Spinosad*[f]	0.33	0.33	0.33	1.67	0.33	1.00	0.34
7 *Spinosad Aerosol*[g]	0.99	0.99	0.99	4.94	0.99	2.97	1.00

DSus @ 180 seconds	**1**	**2**	**3**	**4**	**5**	**6**	**7**
1 *Cyromazine*[a]	1.00	0.94	1.94	1.38	0.92	2.58	0.92
2 *Ivermectin*[b]	1.06	1.00	2.06	1.47	0.97	2.74	0.98
3 *Diazinon*[c]	0.52	0.49	1.00	0.71	0.47	1.33	0.48
4 *Propetamphos*[d]	0.72	0.68	1.40	1.00	0.66	1.86	0.67
5 *Diazinon Powder*[e]	1.09	1.03	2.12	1.51	1.00	2.82	1.01
6 *Spinosad*[f]	0.39	0.36	0.75	0.54	0.36	1.00	0.36
7 *Spinosad Aerosol*[g]	1.08	1.02	2.10	1.50	0.99	2.79	1.00

DRes1 @ 180 seconds	**1**	**2**	**3**	**4**	**5**	**6**	**7**
1 *Cyromazine*[a]	1.00	1.04	8.47	1.67	2.32	2.55	1.00
2 *Ivermectin*[b]	0.96	1.00	8.11	1.60	2.22	2.44	0.96
3 *Diazinon*[c]	0.12	0.12	1.00	0.20	0.27	0.30	0.12
4 *Propetamphos*[d]	0.60	0.63	5.08	1.00	1.39	1.53	0.60
5 *Diazinon Powder*[e]	0.43	0.45	3.65	0.72	1.00	1.10	0.43
6 *Spinosad*[f]	0.39	0.41	3.32	0.65	0.91	1.00	0.39
7 *Spinosad Aerosol*[g]	1.00	1.04	8.46	1.67	2.32	2.55	1.00

DRes8 @ 180 seconds	**1**	**2**	**3**	**4**	**5**	**6**	**7**
1 *Cyromazine*[a]	1.00	1.06	7.88	8.09	1.56	2.12	0.98
2 *Ivermectin*[b]	0.94	1.00	7.43	7.63	1.47	2.00	0.92
3 *Diazinon*[c]	0.13	0.13	1.00	1.03	0.20	0.27	0.12
4 *Propetamphos*[d]	0.12	0.13	0.97	1.00	0.19	0.26	0.12
5 *Diazinon Powder*[e]	0.64	0.68	5.04	5.18	1.00	1.35	0.63
6 *Spinosad*[f]	0.47	0.50	3.72	3.83	0.74	1.00	0.46
7 *Spinosad Aerosol*[g]	1.02	1.09	8.06	8.28	1.60	2.17	1.00

a) 500g/L cyromazine (Vetrazin); b) 16g/L ivermectin (Coopers Blowfly and Lice); c) 200g/L diazinon (Coopers Diazinon); d) 14g/L propetamphos (Young's Deadmag); e) 15g/kg diazinon, 0.8g/kg piperonyl butoxide, and 1g/kg pyrethrins (WSD Fly Strike Powder); f) 25g/L spinosad (Extinosad Eliminator); g) 2.8g/kg spinosad (Extinosad)

7. Discussion

Flystrike on sheep is the end result of a multifactorial process involving sheep, blowfly and environmental variables which have all recently been reviewed.[70] The toxicological effect of insecticides on the sheep blowfly, *Lucilia cuprina*, are studied *in vitro* to remove, negate or standardize a large number of these factors and variables. Such assays consider the insecticide concentration and the resulting mortality that exposure or ingestion produces, in this case, in blowfly larvae (maggots). By quantifying this response across a range of concentrations of the insecticide, the effects of intoxication can be compared between strains of blowflies. In this way it is also possible to detect insecticide resistance, if it is present at high enough frequencies, and classify strains accordingly. If available, reference strains which are insecticide susceptible, are widely used to benchmark strains collected from the field. Strains which have a previously defined resistant or susceptible status to one or more insecticides are also used for benchmarking. Undertaking *in vitro* resistance investigations in the laboratory removes any elements of chance which have been mentioned in association with sheep becoming flystruck.[71]

While this study is unable to provide much information on the Tasmania blowfly population (n=1) or any information on the Queensland population (n=0), 24 of the natural resource regions across the southern areas of Australia are represented. These natural resource regions were utilized by the Meat and Livestock Association to map the national sheep numbers in 2016 and this project provides an overlay for insecticide resistance in the sheep blowfly. Submissions from these areas were examined using *in vitro* assays which determined their individual susceptibility to seven different insecticides. Six of these insecticides represent the main insecticide groups which are currently marketed for flystrike control, while the 7th, diflubenzuron (DFB), is no longer marketed. DFB was included as true cross resistance was reported between it, the OP's and the carbamates prior to its introduction and very high levels of resistance developed following its release. Highly DFB resistant strains were also found to be cross resistant to other insecticides like cyromazine.

By comparing the range of resistance factors calculated for diazinon we found strains from the eastern state of NSW were statistically different in their susceptibility to those from the agriculturally isolated states of Western Australia and Tasmania. However, the strains from the two States linking east and west, SA and Vic, were not distinct from either. Despite reduction in use of OP's over the last decades, this study identified an elevation in resistance levels, compared to studies conducted in the late 80's and mid 90's. As OP-resistance has long been stabilised in the Australian *L. cuprina* population this apparent increase in resistance level suggests ongoing selection pressure. This is also supported by the negative skewness and lack of normality observed in the frequency distribution plot to diazinon.

In general, strains from NSW displayed a wider range in susceptibility and a higher level, expressed as RF's, to the non-IGR insecticides than the other submitting states. However, the difference in resistance levels was only statistically significant for diazinon and imidacloprid. This broader range of susceptibility may have been exaggerated by the larger proportion of submissions which were received from NSW relative to the other States (n=55). When considering the submitted samples as a single population it is apparent that the levels of resistance has increased to diazinon and the levels of susceptibility to ivermectin and spinosad have decreased significantly from those reported 20 or 30 years ago. We do not have previous data for imidacloprid to allow a similar comparison as imidacloprid was only registered for flystrike prevention in 2019.

It is generally accepted that resistance genes arise by chance mutations, and, that when they occur, they often place the mutated individual at a fitness disadvantage. This disadvantage is obviously reduced when the individual is in contact with the insecticide. The contribution of lice treatments to this selection process in the sheep blowfly was demonstrated with the organochlorines[72,73] where prior exposure to lice treatments was considered responsible for the rapid development of resistance to them in flies. The very wide range of the imidacloprid frequency distribution , the lack of a normal distribution and the fact that the laboratory susceptible strain's LC50 does not fall within the field range, are all indicators that over the last decade imidacloprid lice treatments have applied selection pressure to the blowfly population. In addition, statistically significant associations were confirmed between the response of dicyclanil/cyromazine resistant strains to imidacloprid and to four of the other insecticides, these being diazinon, ivermectin, dicyclanil and cyromazine. Reports of cytochrome P450 mediated resistance to imidacloprid have been recorded in a number of insect pests.[74,75] Cytochrome P450 is also responsible for the metabolism of DDT and a

range of OP's including diazinon. This may explain the association we observed between the level of susceptibility to imidacloprid and diazinon in the dicyclanil/cyromazine resistant strain. While cytochrome P450 monooxygenase levels were previously found to be increased in diflubenzuron resistant *L. cuprina,*[76] an association between imidacloprid and DFB was not observed in this study. Interestingly, the only other insecticide that did not have a statistically significant association with imidacloprid was spinosad.

As ivermectin and spinosad based products are also marketed for both lice and flystrike control it should be noted that the 2018-20 populations were considered susceptible to both. However, widespread and regular exposure may provide the opportunity for a small number of individuals to tolerate quite high doses of the insecticide. In practice, exposure to residues would naturally change the response of the population to a less susceptible level which should revert within a few generations back to susceptibility in the temporary absence of treatments. This process may account for some of the higher outlying RF values observed in the field populations. to diazinon, ivermectin, spinosad and imidacloprid. These outlying strains may enable the rapid increase of resistance if the insecticide is overused. This reversion to susceptibility following the removal of selection pressure is the rationale behind the long running recommendation of rotating between insecticide groups.

Of the 100 strains examined 15 were resistant to cyromazine while 73 were resistant to both dicyclanil and cyromazine. This has confirmed the findings of six years ago (2012-14)[36] when 36/58 (62%) of the investigated strains were cyromazine resistant, however, at that time only 8/58 (13.8%) were also dicyclanil resistant. In the former study not all of the cyromazine resistant strains were from NSW, however, dicyclanil resistance was confined to NSW[36]. This is no longer the case with concurrent dicyclanil/cyromazine resistant submissions coming from all of the mainland states (n= 73) which included every strain from NSW (n=55). It is interesting to note that cyromazine resistance occurs without dicyclanil resistance, but the converse has not been found. Also, a pooled cyromazine resistant strain displayed resistance ratios (RRs) of 3.8-fold to cyromazine and approximately the same to dicyclanil (3.6-fold). However, a pooled dicyclanil/cyromazine resistant strain, with a relatively high level of resistance to dicyclanil of approximately 49-fold resistance did not display a commensurately high level of resistance to cyromazine (2-fold). As expected, regression analysis of the NSW strains found a statistically significant correlation between a strains' resistance to dicyclanil and cyromazine of 0.4303 ($p<0.0007$). Also, the correlation increased to 0.5325 ($p< 7.3937E-7$) when the strains which were only cyromazine resistant were included. These and earlier findings[35] suggest that the development of dicyclanil resistance requires the presence of genetic variation provided by cyromazine resistance but cannot be attributed to up-regulation of the cyromazine resistance mechanism. It appears to be an additional mechanism which is dicyclanil specific, or given the association with imidacloprid, possibly cytochrome P450 mediated while excluding cyromazine. In addition, cyromazine resistance was shown by bioassay to be incompletely dominant in the sheep blowfly[35] as was the dicyclanil resistance reported here (Sales unpublished data).

Cyromazine-resistant blowflies were shown to over-winter successfully[68] and as the maggots that leave a treated strike and survive constitute the next generation, it was important to determine the efficacy of dressing products against the dicyclanil/cyromazine resistant strain. The odds risk ratio showed that cyromazine remains an effective dressing despite the resistance discussed here. Most dressings were less effective against the dicyclanil/cyromazine resistant strains than against the cyromazine and dicyclanil susceptible or LS strains ($p < 0.05$). The spinosad jetting fluid was the exception as it did not perform well regardless of the strain, in contrast to the spinosad aerosol which performed well. This was likely due to the significant difference in concentration between spinosad aerosol and the spinosad jetting fluid.

As 100% of NSW strains were dicyclanil/cyromazine resistant, it is important to note that the majority of NSW submitters (70%) declared use of dicyclanil exclusively, or dicyclanil and cyromazine, while 22% failed to provide information on insecticide usage. To avoid an increase in the level and frequency of dicyclanil/cyromazine resistance, the widespread and exclusive use of dicyclanil should be avoided where possible in all States. In this way it may be possible to maintain the use of the full range of registered products to control flystrike.

An *in vivo* technique known as the implant technique, has been used extensively for decades to initiate flystrike for a variety of purposes. It has been used to determine and stimulate immunological responses of sheep to flystrike[77,78,79], provide flystrike challenge to vaccinated sheep[80] , determine the fitness of resistant genotypes of *L.*

cuprina[72,73,81], to test the efficacy of controlled release drug delivery systems[82], to determine the insecticidal properties of novel toxins[83], to elucidate the biological requirements for larval development on the sheep[84] and to determine the physiological effects of flystrike on the sheep.[85] It is also used routinely to determine the persistence of insecticides on sheep[86] and to determine the effect of resistance on the period of protection provided by insecticidal treatments[45]. As with *in vitro* studies, every attempt is made to eliminate or standardize variables. In this way an *in vivo* study considers the concentration of insecticide present on the sheep and the mortality it produces in exposed maggots. This can be conducted over a time course to allow the insecticide to decay in the way it does on sheep in a paddock. The protection period is the number of weeks post-treatment for which the treatment prevents the initiation of a strike when challenged. Insecticide resistance is investigated by comparison of simultaneous challenge using a resistant and a susceptible strain with all other variables maintained the same. The success of an implant on a sheep indicates that some sheep in a flock would be at risk of strike if flies are active and other conditions are suitable for flystrike to occur. Here the success of an implant on three sheep out of the six in a treatment group was considered evidence of a break in the protection period. Such an *in vivo* investigation was undertaken using a pooled cyromazine and dicyclanil susceptible strain and pooled dicyclanil/cyromazine resistant strains. The protection period achieved by three dicyclanil based spray-on treatments, a cyromazine jetting fluid and an ivermectin based jetting fluid were determined. Technical literature for the dicyclanil based products[87] states that dicyclanil prevents larval development between the 1st and 2nd larval stages, thus preventing the development of a strike. We used neonate larvae but assessed success or failure based on the number of adult flies which developed from larvae removed from the sheep after 48-72 hours exposure. The products provided protection for the claimed period against larvae from the susceptible strain, however, they did not against the dicyclanil/cyromazine resistant strain. A 69-78% reduction in protection period was obtained from the dicyclanil based spray-on products and a 50% and a 33% reduction in protection from the cyromazine and the ivermectin jetting fluids respectively. Protection from the cyromazine based jetting fluid was reduced in this study to a similar period as that obtained in an earlier study involving challenge from a cyromazine resistant strain[68]. In comparison, against the dicyclanil/cyromazine resistant strain, the dicyclanil based spray-on products provided far less protection than in the former study which also showed reduced protection (11 weeks for $50gL^{-1}$)[68]. This also supports the concept of an additional dicyclanil specific component to the cyromazine resistance mechanism.

Insecticide resistance is a cumulative legacy and once resistance genes have become established at detectable frequencies, reversion to the mutation rate is highly unlikely. Consequently, once initially detected the resistance needs to be managed to avoid a rapid further increase in the frequency of resistance. Unfortunately, this study provides evidence that this rapid increase has occurred for dicyclanil/cyromazine resistance since cyromazine resistance was first reported[35]. In addition, historical precedents in the development of resistance in *L. cuprina* would suggest that when the same chemicals are used on sheep for both lice and blowfly control this can result in a more rapid development of insecticide resistance than if two unrelated groups of insecticide are used against the two ectoparasites. Ivermectin, spinosad and imidacloprid, the three alternatives to dicyclanil and cyromazine for blowfly control, are also used for lice control. Therefore, lice treatments should be counted in any rotation of insecticide groups. Unquestionably, the best practice is to reduce the chance of resistance developing in the first place. However, in the current situation implementation of a comprehensive and extensive integrated resistance management plan for both blowfly and lice control is imperative to preserve these remaining insecticides for flystrike control.

8. Impact of Wool Industry – Now & in 5 Years' Time

In 1982 a situation report on myiasis of livestock in Australia was provided to the OIE[88]. It outlined three areas of research to control sheep flystrike which were underway during that and the previous decade. These included genetic manipulation by selective breeding and surgical modification of the Merino, identification of pathogens for biological control of blowflies and the transfer of genetically manipulated genes into the *L. cuprina* population to prevent reproduction or to cause mortality. Since that time there has been a technological explosion and these three areas of research have been discounted, delivered or are close to completion. However, just as in 1982 sheep producers will need to rely on insecticide treatments to prevent flystrike for the immediate future. Therefore, it is timely that this study of insecticide resistance in *L. cuprina* was undertaken and the findings should provide the impetus for the widespread promotion and adoption of targeted and strategic insecticide usage.

Due to the apparent exclusive and widespread use of dicyclanil in NSW, and the subsequent selection of resistance, integrated resistance management measures must be adopted[89,90]. By adopting these measures producers may prevent flystrike and at the same time halt the stabilisation of dicyclanil resistance in the NSW blowfly population. Adoption of an integrated resistance management plan in all States is advisable to slow or halt the continued selection of cyromazine and dicyclanil resistance. By doing this the full arsenal of insecticidal products registered to control flystrike should remain available.

Anecdotally there may have been a shift to the use of flystrike preventative treatments independent of imminent flystrike risk. It could be argued that this, and the widespread use of the dicyclanil based products capable of providing protracted periods of protection, have resulted in a temporal and spatial continuum of selection pressure on the sheep blowfly. This study also suggests that the use of the same chemicals for prevention or control of *Bovicola ovis*, the sheep body louse, has imposed selection pressure on the sheep blowfly despite flies not being the target. Unfortunately, insecticide resistance is often a cumulative legacy and a shift to the warranted and strategic use of insecticides is required to control flystrike and manage insecticide resistance. This should be part of an integrated resistance management plan designed to maximize the benefits gained from judicious insecticide usage and the adoption of a wide range of non-insecticidal fly strike preventative measures. Such a program will minimize the negative production and welfare impacts usually associated with flystrike insecticide resistance.

9. Recommendations

The following recommendations are made to support and build on the findings of this project which has identified the critical need for the widespread adoption of an effective resistance management plan.

- Submissions from both Tasmania and Queensland are needed to determine the presence and level of cyromazine and dicyclanil resistance. The shorter warmer winters in Queensland provide the opportunity for high frequencies of cyromazine and dicyclanil resistance to develop, dependent on the extent and degree of selection pressure applied by their use across the state. Because of the colder winters, an apparent reduced reliance on these insecticides and also the practice of good animal biosecurity, it is postulated that Tasmania may be free of dicyclanil and cyromazine resistance. However, this needs to be confirmed. The toxicological susceptibility of the strain from Tasmania will be of interest to this state given the presence of resistance, to varying degrees, in the remaining states.
- Additional monitoring of WA, SA and Victoria blowfly populations are required to elaborate on the current findings.
- An investigation on the stability of dicyclanil and cyromazine resistance in the NSW populations is required to predict and quantify the benefits of measures such as insecticide rotation.
- Close monitoring of the alternative insecticides, particularly imidacloprid, is warranted given the findings of this study and the resistance situation in other insects.
- Continued investigation of the resistant reference strains will be undertaken by this laboratory.

Recommendations General

- Resistance monitoring of blowfly populations should be conducted periodically to monitor changes in the toxicological susceptibility of *L. cuprina* to insecticides, especially dicyclanil, cyromazine, imidacloprid and possibly ivermectin.
- Recommendations made by the AWI Sheep Blowfly Resistance Working Group should be followed with the two documents available on the Flyboss website.
- Non-insecticidal flystrike management strategies should be supported and form a major part of an integrated resistance management plan which is widely adopted across Australia.
- Selection pressure from cyromazine and dicyclanil should be reduced by use of an alternative chemical group when practical circumstances allow.
- Where possible two treatments in the same growing cycle should be avoided but if required the second treatment should be from a different insecticide group. This also includes treatments for lice control.
- When dressing active strikes use an insecticide from a different group to the one used for flystrike prevention.
- Novel molecules under development should be considered in the context of the current resistance status by screening with resistant reference strains and current field strains.
- Baseline data should be collected on soon to be or recently released insecticides to aid the ongoing management of insecticides and resistance.

10. Conclusions

In 1968 G.H.S. Hooper, president of the Entomological Society of Queensland, made two points regarding insecticide resistance in Australia[91]. Firstly, “There is an urgent need for extended work on the documentation of resistance in Australia, particularly with respect to laboratory confirmation.” Secondly, “Resistance knows no geographical or political barriers and dissemination of information is a prime responsibility of all workers in this field.” These points were recognised as still valid by the ParaBoss Technical Committee when they kindly endorsed this project.

In the history of our laboratory this has been one of the most comprehensive insecticide resistance studies undertaken to date. While more field strains have been involved in past studies none have investigated such a large number (n=100) with so many insecticides (n=7). At the same time, an *in vivo* resistance study (of 35 weeks) and an *in vitro* efficacy study on marketed dressing products (n=7) were also conducted.

This study has confirmed concurrent dicyclanil and cyromazine resistance *in vitro* and *in viv*o and provides up to date information on its increased levels, wider distribution and the effect it has on the efficacy of alternate insecticides. We have also determined that a reduction in the level of susceptibility in field strains to ivermectin and spinosad has occurred over the last 20 years and shown that imidacloprid, despite it only recently being marketed for blowfly control, has already applied selection pressure on *L. cuprina* through lice treatments. Overall, this project has highlighted the benefits of periodic monitoring for insecticide resistance to inform on effective flystrike control, responsible insecticide usage and insecticide resistance management.

At this time, the greatest tools available to producers for the control of flystrike are information and a willingness to employ it. The information provided by this study should be another driver for the widespread adoption of integrated resistance management which is the presently available answer to the long-term problem of flystrike.

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12. List of Abbreviations and/or Glossary

a. ANOVA: Analysis of Variance. A statistical method to determine if there is a significant difference between sets of data.
b. Frequency: Number of strains with the same LC50 value or resistance factor.
c. *In vivo:* An assay performed on sheep.
d. *In vitro:* An assay performed in the laboratory in a tube or pot.
e. Laboratory Susceptible strain (LS) a strain of sheep blowflies which have been in the Laboratory for over 60 years and are naïve to insecticide exposure.
f. LC50: The concentration capable of killing 50% of the maggots of a tested population or strain.
g. 95% Fiducial limits: The lower limit and upper limit that you can be 95% confident that the true mean or proportion of a population lies between.
h. *Lucilia cuprina*: commonly known as the Australian sheep blowfly which is the species which predominantly initiates flystrike in Australia.
i. Resistance Factor (RF): LC50 of the selected strain divided by the LC50 of the Laboratory Susceptible Reference strain (LS).
j. Resistance Ratio (RR): LC50 of the selected strain divided by the LC50 of a characterized strain such as the dicyclanil susceptible strain DSus.
k. Submission: Maggots collected off struck sheep and sent to our laboratory.
l. Strain: The submitted maggots which are then bred for testing.

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