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Informed Modelling of Sheep Blowfly Chemical Resistance

Department of Primary Industries and Regional Development

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Contents

1. Executive Summary

This study found that the levels of field evolved cyromazine and dicyclanil resistance in *Lucilia cuprina* (*L. cuprina*), and its occurrence across the sheep producing areas of Australia, have both increased since the previous study (2018-2020). For the first time we detected high-level cyromazine / high-level dicyclanil resistance in several submissions from southern NSW and Victoria, high-level dicyclanil / low-level cyromazine resistance in both Tasmania and Queensland, and low-level dicyclanil / low-level cyromazine resistance on Kangaroo Island. In total 130 field strains were viable and tested from the 150 submissions received.

The response of submitted strains to ivermectin and spinosad had previously been described as less susceptible than when these insecticides were released to control flystrike. We investigated any change in status since 2018-2020 and undertook the pairwise comparison of the five insecticides, ivermectin, spinosad, imidacloprid, dicyclanil and cyromazine. We found highly statistically significant correlations, of varying strengths, between all the insecticides, except for between dicyclanil and ivermectin and also between dicyclanil and spinosad. By calculating the Spearman's Correlation Coefficients, the most statistically significant correlation (R=.5486, n=128, p =2.0236E-11) was between dicyclanil and cyromazine which was of moderate strength. As with previous studies, we failed to find dicyclanil resistance where cyromazine resistance was absent, but the reverse does occur. Of great concern was a moderate strength correlation between dicyclanil and imidacloprid, which was also highly statistically significant (R = .5522, n=128, p = 1.4163E-11). These findings indicate partial imidacloprid cross-resistance conferred by one of the mechanisms responsible for dicyclanil resistance.

Despite cyromazine and dicyclanil being different chemical derivatives, they are both classed as insect growth regulators and are considered a single group in the flystrike chemical rotation strategy (FCRS). As imidacloprid is a neonicotinoid, which affects the insect's nervous system, it is a separate chemical group within the FCRS. The data we have gathered strongly indicates that the FCRS may be best served by imidacloprid being grouped with both cyromazine and dicyclanil, despite their different modes of action. As with the relationship between cyromazine and dicyclanil resistance, the relationship between imidacloprid and dicyclanil resistance is partial, indicating other mechanisms/genes are involved. In fact, while dicyclanil resistance has continued to spread and the levels increased over time, we found that the minimum, maximum and median resistance factors for imidacloprid decreased from the levels observed in 2018-20 (n=100) to those in 2022-24 (n=128). The commonality between the responses of strains to these insecticides, and resistance to them, requires further investigation.

Submitters were asked to supply details of insecticide usage for lice and blowfly control and their on-farm management practices. The "off-label" practices of applying double the recommended rate and using mixtures of products for prophylaxis and as dressings was reported. Through experimentation with dressing mixtures and concentrations other than the recommended rate, we found that neither practice increased efficacy and both practices increased selection pressure on a strain that had high-level dicyclanil resistance and one which only had low-level cyromazine resistance. In fact, addition of either the spinosad or diazinon based dressing products to the cyromazine or the ivermectin based dressings reduced their efficacy when assessed as the number of adult flies ultimately emerging following larval treatment. From a practical perspective, both practices increased the treatment cost per head without any increase in efficacy. There is a recommendation that producers should apply a dressing from one chemical group to existing strikes and overlay it with a different chemical group to achieve prophylaxis. Only preliminary results were obtained using imidacloprid as the prophylactic treatment overlaying the dressing products; however, they appear to support this as a sound strategy. It appeared that mixtures of imidacloprid with either ivermectin or spinosad, decreased the resistance factors observed to imidacloprid alone while the resistance factors to ivermectin and spinosad remained similar in the dicyclanil and cyromazine resistant strains. Due to time constraints, we were unable to confirm these preliminary results or conduct similar experiments with dicyclanil.

A collaboration between the University of Tasmania (UTAS), the University of Melbourne (UoM) and NSW Department of Primary Industries and Regional Development (NSW DPIRD) was successfully completed. To create a research tool, UTAS had previously incorporated insecticide resistance into the flystrike risk simulator model to consider the outcomes of insecticide rotation[1]. The UoM determined the number of genes, the dominance of the alleles, and the degree of fitness of the genotypes, of a dicyclanil and cyromazine resistant strain of *L. cuprina*. NSW DPIRD provided

UoM and UTAS with the resistance profile of the strain studied by UoM. In addition, NSW DPIRD provided UTAS with the toxicological profiles of 72 fly populations and their associated metadata on insecticide use and flystrike management. This included the resistance factors for imidacloprid, ivermectin, spinosad, dicyclanil and cyromazine and the percentage of survival of each strain at the dicyclanil and cyromazine susceptible discriminating concentrations. UTAS used this data to validate and calibrate the updated model. NSW DPIRD also supplied the toxicological and metadata for submissions from 2018-2020 which were resubmitted in 2022-2024. In strains that were formerly susceptible but now resistant, the modelled development of resistance was in total agreement with our findings given the treatment regimens utilised on farm. This was also the case when considering the increasing levels of pre-existing resistance observed. Subsequently, the validated model was used to "predict" the timing of resistance development and its effect on control strategies such as the rotation of two or more chemical groups or the use of mixtures. The model can answer a variety of questions regarding the development of resistance and the success of management strategies while providing guidelines for the successful pairing of insecticides in mixtures, if this is a resistance management strategy which is to be pursued in a similar way as it has been with drenches.

Armed with information on the presence and level of dicyclanil and cyromazine resistance, the genetics of this resistance and the fully validated flystrike resistance model, we should undertake practical, high-level, and predictive research. This would include determining the *in vivo* protection periods of registered chemicals and their various application methods (practical), undertaking a genetic study of common and specific genes (high-level), and using this information, model the outcomes of all available strategies (predictive). The final essential step is to make this information available to the industry and producers, enabling the individual tailoring of resistance management strategies.

2. Introduction

Resistance to cyromazine was confirmed in 2012 in the Australian Sheep Blowfly, *L. cuprina*, and the presence of dicyclanil resistance also noted.[2] Both were described as low-level, and cyromazine resistance was considered of greater importance because it had been used for two decades longer and provided a shorter protection period. The dicyclanil product (50 g/L) had a protection period of 18-24 weeks which was considered sufficient to maintain effective levels across an entire fly season. As a result, it was thought the opportunity to select dicyclanil resistant individuals in a population would be much less. This reasoning was reinforced by the release in 2017 of a higher dose product (65 g/L) with claims of 29 weeks protection. However, a lower dose product (12.5g/L) was also released claiming only 11 weeks protection, which is equivalent to the claim for the cyromazine spray-on product.[3] The use of the low dose product during the flystrike season increased the opportunity for further resistance development if it was not removed from the environment by shearing, crutching or slaughter. To provide updated information on cyromazine resistance, NSW DPIRD undertook a study between late 2017 and mid 2020 (n=100). Early submissions from NSW proved to be both cyromazine and dicyclanil resistant, which was supported by the farm histories of insecticide use. These histories outlined long term cyromazine use which was replaced by the exclusive, long-term use of dicyclanil for the prevention of flystrike. In response to these findings we shifted our attention to dicyclanil resistance, as the levels of resistance were much higher than those observed to cyromazine.[4] The practical implications of dicyclanil resistance to Australian sheep and wool producers was quantified by an *in vivo* study.[5] This study demonstrated that dicyclanil resistance produced marked reductions in the protection periods of dicyclanil based products and a cyromazine jetting fluid. The 2018-2020 study also determined the response of field submitted strains *in vitro* to five other insecticides, which included diazinon, diflubenzuron, imidacloprid, ivermectin and spinosad. Significant correlations were found between responses to dicyclanil and the insecticides cyromazine, diflubenzuron, diazinon and imidacloprid.[4] It is worth noting that an earlier study found a highly diflubenzuron resistant strain displayed cross-resistance to dicyclanil, cyromazine and ivermectin.[6] Both of these studies suggested the involvement of a general metabolic detoxification system in the observed resistances.

For research purposes, Dr Brian Horton, from the University of Tasmania (UTAS), had expanded the capability of his flystrike risk and treatment optimisation model[7] to include insecticide resistance. This model had the flexibility to include inputs for up to seven resistance genes.[1] AWI negotiated a collaboration with Dr Trent Perry, and subsequently Dr Simon Baxter, from the University of Melbourne (UoM), to determine the number and location of the genes responsible for dicyclanil and cyromazine resistance.[8] NSW DPIRD was included in the collaboration to supply toxicological and resistance profiles of field submissions and gather information on insecticide use, animal husbandry and farm management practices over the previous decade. The objective was for the UTAS model to incorporate this information, which would validate and calibrate the model, and then use the model to predict the outcomes of proposed resistance management strategies.

3. Literature Review

A history of insecticide use, resistance monitoring and detection of resistance in the Australian Sheep Blowfly, *L. cuprina* was detailed in the final report of Project ON-00491[4] which is referred to here as the 2018-2020 study .

Following the initial report of resistance to cyromazine[2] a field survey (n=58) found that 62% of submissions were resistant to cyromazine, of which 22% were also resistant to dicyclanil. In total, 14% of submissions were described as dicyclanil resistant, all of which were from NSW. At that time both cyromazine and dicyclanil resistances were described as low-level.[9] A subsequent *in vivo* investigation in 2013 found that cyromazine resistance reduced the protection periods provided by registered cyromazine and dicyclanil based products.[9] It was stated that this would only be of concern if conditions that favoured flystrike coincided with the periods after treatment when resistant larvae had a survival advantage over susceptible larvae.[10] A later *in-vivo* study (2018-2019), using higher level dicyclanil resistant larvae, demonstrated a further reduction in protection periods in all three dicyclanil based products, and a cyromazine based jetting fluid.[5] The 2018-2020 study confirmed that cyromazine resistance was present in individual populations on its own, however, dicyclanil resistance was only found in conjunction with cyromazine resistance (n=100).[4] In addition, the levels of resistance to cyromazine were low, with maximum resistance factors of (approx.) 4-fold, while resistance factors for dicyclanil were as high as 49-fold.[4] Based on this data, it was apparent that dicyclanil resistance was not the result of "up-regulation" of the cyromazine resistance but was rather a composite which included additional resistance mechanism/s that were either specifically against dicyclanil or were common but not effective against cyromazine.

New Zealand reports in 2012 of reduced protection from dicyclanil and the benzyl phenol urea (BPU) triflumuron against both *L. cuprina* and *L. sericata* were investigated.[11] For the two species, the maximum resistance factors to dicyclanil were RF= 5 and 28 respectively and to triflumuron RF= 16 and 7,460, respectively. Interestingly, BPUs are still used in New Zealand whereas in Australia the BPU diflubenzuron, was rapidly removed from the market following the determination of resistance levels up to RF= 791 in *L. cuprina*.[12] The development of resistance to diflubenzuron in Australia occurred in two steps with cross-resistance initially conferred by OP/carbamate resistance [13] and very high levels of resistance developing after its release.[12] Previous studies had found that a laboratory strain selected with diflubenzuron had RF= 617 and displayed a RF =10 to dicyclanil and a RF= 2 to cyromazine.[14] Conversely, a field strain selected with cyromazine had a RF= 20 to dicyclanil and a RF=362 to diflubenzuron.[14] The level of cyromazine resistance first reported from the field in Australian *L. cuprina* was RF= 2.3,[2] which increased to RF=8.1 following selection with cyromazine, while the level of resistance to dicyclanil in the same strain only increased from RF=1.3 to RF= 2.8. [15] These resistance factors are not high and in other insecticides might be considered part of the "normal" variation found amongst susceptible populations. However, even at these low levels of cyromazine resistance, the protection period provided by the spray-on cyromazine product was reduced to less than 4 weeks.[2] In New Zealand, there are commercially available mixtures of cyromazine plus ivermectin and cyromazine plus spinosad. Despite this, or possibly because of the use of mixtures, the main resistance problem in New Zealand are the BPUs, with no meaningful resistance reported to cyromazine or dicyclanil and no resistance reported to ivermectin (ML), imidacloprid (neonicotinoid) or spinosad (spinosyn) in either species.[16]

In Australia, the neonicotinoid, imidacloprid, was registered against sheep biting lice in 2009 and as a dual-purpose flystrike prophylactic product in 2016.[17] It is not unreasonable to assume that the use of imidacloprid for lice control pre-selected *L. cuprina* for imidacloprid resistance. As the toxicological response of *L. cuprina* populations to imidacloprid was studied for the first time in 2018-20,[4] a shift to less susceptibility or resistance was unable to be demonstrated without a base line. We did observe a greater than 13-fold range in response to imidacloprid across populations and the statistically significant groupings of these according to the state from which they were submitted (n=100). The frequency distribution of log (LC50) highlighted reduced susceptible when compared to the Laboratory Susceptible strain (LS). In addition, the highly dicyclanil/cyromazine resistant strains were found to display a statistically significant increase in the level of diazinon resistance and decreased susceptibility to imidacloprid. Based on these findings, the involvement of a general metabolic resistance mechanism, such as the cytochrome P450 system, was speculated.[4] This enzyme system had been suggested to also contribute to the resistances of diflubenzuron,[18] butacarb and deltamethrin[19,20] in *L. cuprina*. An earlier report of a field isolated strain displaying cyromazine and dicyclanil resistance, which was selected with cyromazine, but subsequently did not display any resistance to

ivermectin or spinosad, also suggested that cyromazine resistance was of lesser concern.[15] Despite this, a general decrease in the susceptibility of field populations to both ivermectin and spinosad was reported in 2018-2020.[4] Further analysis of the response of dicyclanil and cyromazine resistant strains (n=60) to imidacloprid showed significant correlations with their levels of susceptibility or resistance to dicyclanil, cyromazine, diazinon and Ivermectin, but not with spinosad. A subsequent study of a dicyclanil resistant strain and its response to imidacloprid and other insecticides reported the involvement of the cytochrome P450 enzyme system and increased Cyp12d1 transcription levels.[21] As dicyclanil resistance is now widespread in Australia, it was suggested that the protection period of imidacloprid should be investigated when failures occur and there is known to be resistance present. [22]

4. Project Objectives

Objective 1

- Determine the toxicological profile of sheep blowfly strains. The insecticides (chemical group) to be studied were dicyclanil and cyromazine (IGRs), imidacloprid (neonicotinoid), ivermectin (macrocyclic lactone), spinosad (spinosyn).
- The strains to include:
  - Strains that are the subject of genetic investigations by the UoM. Profiled at the commencement of the project. (Collaboration)
  - Submissions from specific areas identified as in close proximity to UoM successful trapping locations. (Collaboration)
  - Submissions from Tasmania, WA, SA (including Kangaroo Island) and Victoria, with particular emphasis on finding areas where dicyclanil and cyromazine resistance are not present. (Areas where there is the greatest gain)
  - Submissions from Queensland. (One of the "gaps" in current knowledge).

Objective 2

- Using toxicological data, cross and multi-resistance data, and producer flock management histories, identify and build common strain "scenarios."
  - Use this data to validate the UTAS model.

Objective 3

- Provide individual strain or "scenario" information to UTAS for specific modelling and development of resistance management advice (Collaboration).

Objective 4

- Investigate the efficacy of mixtures and the recommended concurrent dressing and prophylactic treatment of flystrike and their effects on resistance.

5. Success in Achieving Objectives

Objective 1

- Toxicological profiling of 130 strains to five insecticides - *Achieved*.
  - From the sheep producing states of Australia, 147 field submissions were received of which 130 submissions produced viable cultures of *L. cuprina.* The toxicological profiles of these 130 strains to each of the five registered insecticides were determined. In addition, each submission was classified according to survival at three discriminating doses of dicyclanil and two of cyromazine.
- Toxicological profile of UoM strains - *Achieved.*
  - This collaboration was successfully completed. A full profile including LC50, LC95, associated fiducial limits and resistance factors, calculated using the NSW DPIRD Laboratory Susceptible strain (LS), were determined for the UoM laboratory susceptible (UoM-S) and a field derived dicyclanil resistant (UoM-R) strains. The data on dicyclanil and cyromazine was provided to both UoM and UTAS on the 25 July 2023 based on four replicates of testing, completed over time, for each strain with each chemical. The UoM-S strain was susceptible to both cyromazine and dicyclanil, as expected, while the UoM-R strain was classified as having low-level resistance to cyromazine and moderate-level resistance to dicyclanil.
- Submissions to mirror UoM trapping locations - *Not achieved.*
  - We were unable to pursue specific areas which were identified as close in proximity to UoMs trapping locations as we had to rely on producers to collect off struck sheep and were not in the field ourselves. However, the response from producers was such that we received submissions from most of the sheep producing areas of each state.
- Submissions from states other than NSW - *Achieved*
  - We intensively targeted states other than NSW for submissions and were successful. Due to an increased awareness in NSW of this type of project, the greatest number of submissions received was still from NSW (50). However, this was followed by submissions from Western Australia (40) with the lowest number of submissions being from Queensland and Tasmania (7 each).
- Submissions from Queensland - *Achieved*
  - A total of seven submissions were received from Queensland, the first submissions in decades from this state.

Objective 2

- Model validation- *Achieved.*
  - Resistance factors for each of the five insecticides and the percentage survival of each strain at the susceptible discriminating doses of dicyclanil and cyromazine were provided to UTAS throughout 2023 to validate the model. The data on these 72 strains also included the GPS location of the submission and the management and insecticide usage data provided by producers.

Objective 3

- Individual strain information to UTAS for specific modelling – *Achieved.*
  - Full details and data sets for over 75 individual strains and twenty resubmitted strains were provided to UTAS.

Objective 4

- Efficacy of mixtures – *Achieved.*
  - The efficacy of dressing mixtures was investigated.
  - Additionally, in response to information provided by submitters, we investigated the efficacy of double strength and half strength individual dressings and mixtures of dressings.
- Efficacy of dressing with one chemical group overlayed by a prophylactic treatment from another – *Commenced but not completed.*
  - Investigations of the efficacy of imidacloprid and dicyclanil mixtures with dressing actives were commenced but were not completed due to the project ending. These experiments need to be completed and the initial findings for imidacloprid confirmed.

6. Methodology

Submissions were canvassed through existing networks in the sheep industry. These included NSW Local Land Service, the Nutrien Ag Solutions network, the Elders network, AgnVet, and privately owned Agricultural companies such as Brennan Agriculture, Dept of Agriculture and Fisheries Queensland, producer bodies such as AgForce Queensland and Monaro Farming Systems, private agricultural consulting companies such as Aggregate Consulting, industry resource providers such as the SheepConnect Co-ordinators in Tasmania and South Australia, Leading Sheep Queensland, other state government departments dealing with primary industries, agricultural teaching establishments and individual producers. Project outlines and information were distributed along with requests to distribute kits, promote sample submissions in their newsletters and to refer enquiries they received to the project.

Maggot collection kits were distributed upon request and submission received by mail. On arrival, the number of maggots in the sample were counted, the strain 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 five insecticides, which included 1) ivermectin, a macrocyclic lactone (ML); 2) spinosad, a spinosyn; 3) imidacloprid, a neonicotinoid; 4) cyromazine, a triazine derivative, and 5) dicyclanil, a pyrimidine derivative, with the final two belonging to the larger chemical group known as the Insect Growth Regulators (IGRs).

A modified version of an assay developed in this laboratory was used to measure each strain's level of larval susceptibility or resistance to imidacloprid, ivermectin, and spinosad.[23] Briefly, PESTANAL®, analytical standard grade insecticides, were used and sheep serum 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 48 hrs, and the percentage mortality was determined. Solvent only controls determined the control mortality which was used to correct the dose mortality data using the Schneider-Orelli's formula[24] which is an adaption of Abbotts Correction.[25] Probit analysis[26] was performed using BioStatPro software[27] to calculate the concentration at which 50% of the maggots in the duplicate phials were killed (LC50), and 95% mortality (LC95), along with the associated 95% fiducial or confidence limits. Each strain's level of susceptibility to the test insecticide was determined by calculating resistance factors (RFs) relative to the Laboratory Susceptible strain, LS, (LC50 field strain/LC50 LS strain). The frequency distribution of Log LC50 data were plotted and the normality of the data for each insecticide was calculated using Shapiro-Wilk analysis.[28] Spearman's correlation coefficient[29] calculations were undertaken as it is a nonparametric measure of rank correlation. The statistical significance or insignificance was demonstrated by the p value which was set at the 99% confidence limit i.e. $p<0.01$. The strength of an association between insecticides was described based on the interpretation of Rho.[30]

A technique which incorporates a serially diluted range of dicyclanil or cyromazine concentrations into a larval food source was utilised to determine mortality.[14] The percentage mortality was defined as the number of flies which did not eclose divided by the number of neonate larvae exposed, multiplied by 100. This dose mortality data was corrected for control mortality and subject to Probit analysis as outlined above. The individual field strains were classified according to their survival at the susceptible discriminating concentrations (SDC's) of 1 mg kg^{-1} for cyromazine[31] and 0.1 mg kg^{-1} for dicyclanil and were defined as low-level resistant if there were survivors. An additional concentration of 4-fold the SDC for cyromazine was included to define higher level resistance. In addition, it was considered important to determine if field populations displayed higher-levels of resistance to dicyclanil and therefore 4-fold (moderate-level) and 8-fold (higher level) the SDC concentration were also included. Once strains were categorised, they were pooled according to their resistance levels to form reference strains.

When the insecticide resistance profile of a submitted strain was complete, the results were conveyed to the submitter via email, and the opportunity was taken to provide details of additional AWI information regarding insecticide resistance management. The format of the result sheet and the emailed letter were developed with the assistance of Schuster' Consulting Group and approved by AWI. Results were expressed in terms of the submissions classification of cyromazine and dicyclanil resistance and the response of the strain to each insecticide relative to the national response and relative to the Laboratory Susceptible strain.

Individual property profiles are confidential and are not provided as part of this report; instead, aggregated data are presented. In addition, 67 strains that were submitted in 2018-2020 were identified as good candidates for resubmission in 2022-24 because of their resistance profiles. The owners of the properties were contacted by email or telephone, and collection kits were sent to the proportion who responded positively. Resubmissions were received, cultured, resistance profiled and the toxicological data from both time points, and the information provided on management and insecticide usage, were forwarded to the UTAS for modelling.

To determine dressing efficacy, a commercially available product was purchased to represent each of the four insecticides registered as wound dressings at the time of this study. These were ivermectin, spinosad, cyromazine and diazinon. Composite strains of those with survivors at 8-fold the dicyclanil SDC (DResH) and another which contained survivors of the cyromazine SDC only (CResL) had been in the laboratory unselected by dicyclanil or cyromazine for >3 generations and were not considered pure breeding strains. Using LS as a negative control strain, full gutted 3rd instar larvae of each strain were exposed for 180 seconds to the commercially available dressing products and mortality assessed at fly eclosion. The mortality of water treated controls of each strain were used to correct for control mortality.[24] The dressing products were used at twice the registered rate, the registered rate and half the registered rate. The half-registered rate was included as producers who make two products to the recommended rate and then combine them halve the rate of each active ingredient in the mixture. Also, each of these products were used in combination with one another at the three rates to determine the efficacy of mixtures and the effect of mixtures on cyromazine and dicyclanil resistance.

The technique used on neonate larvae, outlined above,[23] was modified by increasing the yeast component of the fortified sera by 10% and the temperature of incubation by 4 degrees Celsius. This was used to examine the response of the three reference strains LS, CResL and DResH to dicyclanil, imidacloprid, ivermectin and spinosad individually and as mixtures. A standard range of concentrations was determined for each insecticide for each strain. Then acetonic dilutions were prepared in a 1:1 ratio (halving the concentration of each insecticide) and 1st instar larvae were exposed as previously detailed. Following correction for control mortality, Log Probit analysis was undertaken to determine the LC50 and LC95s of each component at the relative concentration as previously outlined.

7. Results

7.1 Producer Submissions and Information

Approximately 710 maggot collection kits were distributed across Australia, of which 147 field and three reference strain submission were received. **Figure 1** shows the distribution of submissions from across Australia over the two spring and autumn fly seasons. The marker pins were located according to the GPS co-ordinates of the sheep yards on the submitting property.

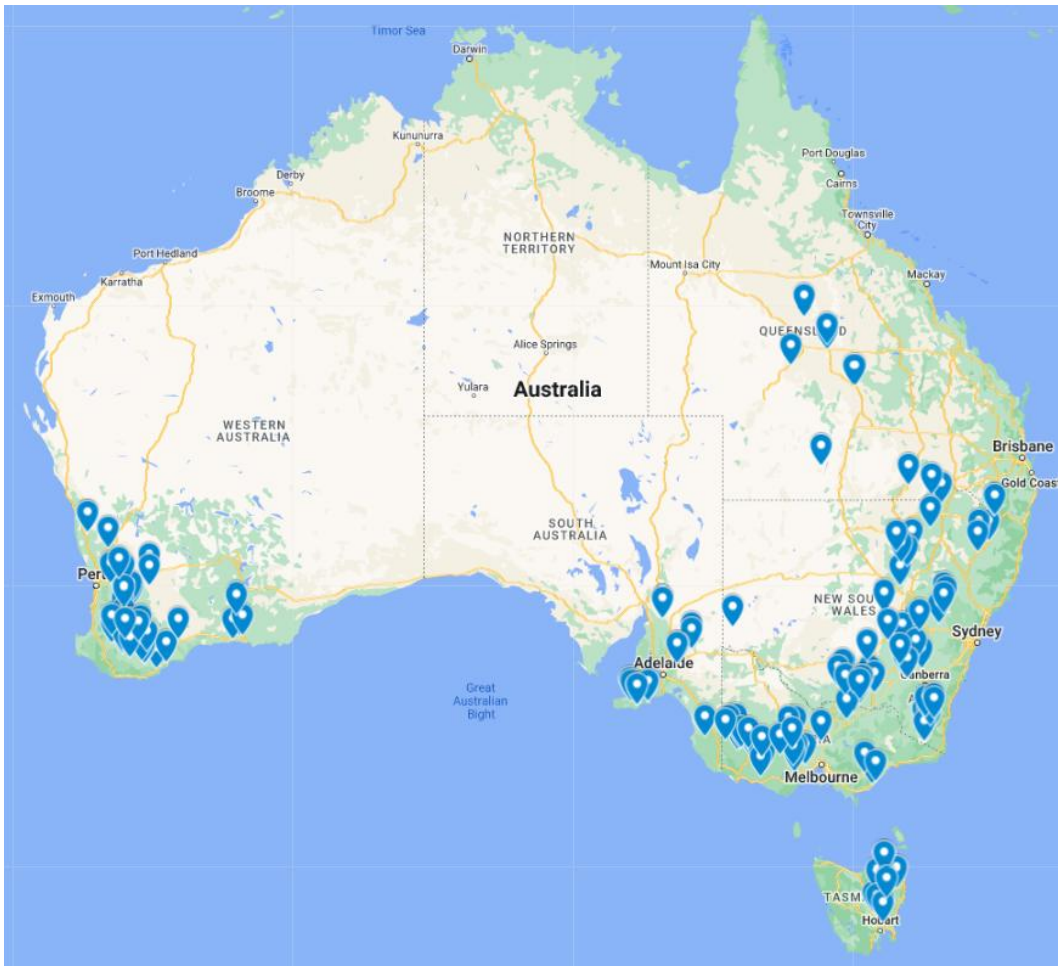


Figure 1. The geographical location of submissions which were received between spring 2022 and winter 2024. Producers collected maggots off struck sheep and submitted them for insecticide resistance profiling. (n=147).

Out of the 147 strains submitted by producers, 20 (13.3%) were not viable. These strains may not have been *L. cuprina*, contained less than four pupae, or the adults may have emerged and were dead on arrival due to postage delays. The state-by-state viability of submissions is detailed in **Figure 2** below.

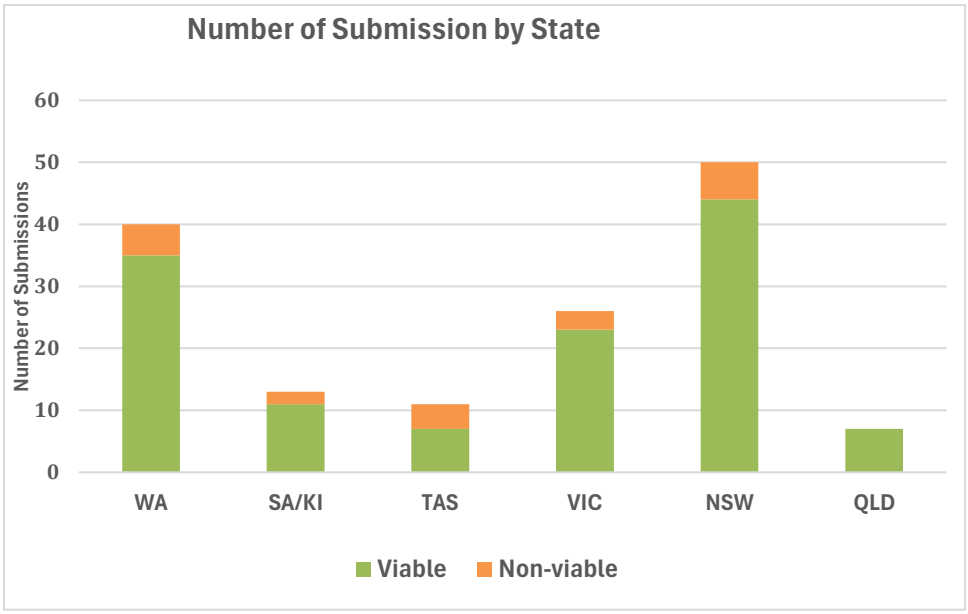


Figure 2. The number of submissions of maggots received from each state that were successfully cultured and resistance profiled (viable) or which were considered non-viable due to incorrect species, dead on arrival, or too few to successfully culture. (n=150). This also includes three reference strains.

The maggot collection kits contained a strike record sheet for submitters to complete and return. This submission sheet was developed in consultation with Dr Brian Horton (UTAS) and was modified after six months to include the dates of crutching and shearing which were required for the UTAS model. From this information we determined if the maggots had been removed from insecticide treated or untreated sheep (n=147) (**Figure 3**).

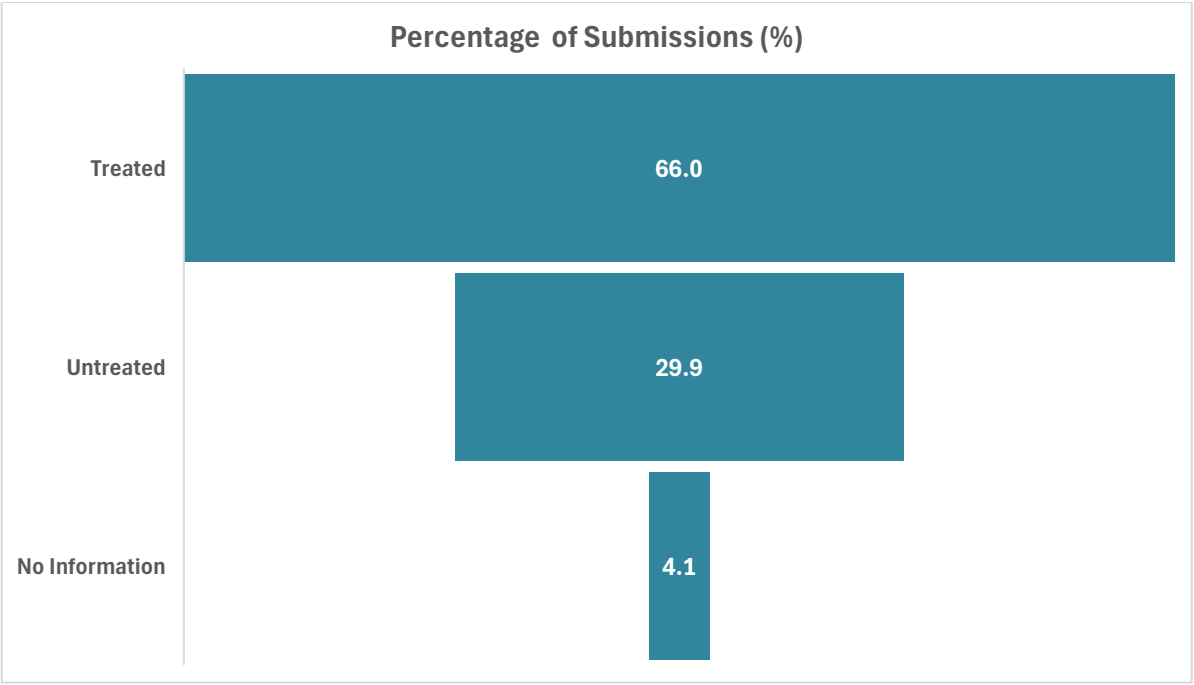


Figure 3. The percentage of submissions (maggots) which were collected off untreated or treated sheep.

The strike record sheet also provided data on the site of the flystrike on the sheep from which the submitted maggots were removed. This information can be seen in **Figure 4**

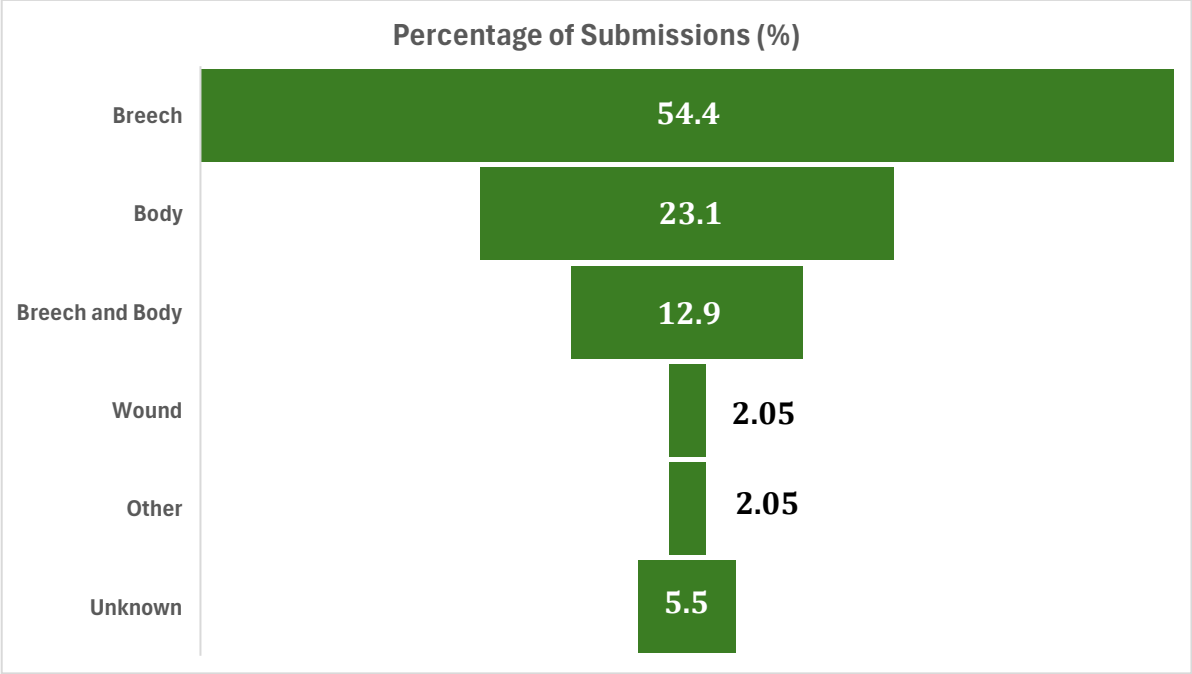


Figure 4. Flystrike location on sheep from which the submitted maggots were collected, expressed as the percentage of total submissions. (n=147).

7.2 Insecticide Use – Active Ingredients

The submission sheet requested information about the on-farm use of insecticides over the previous decade for both lice and flystrike prevention and control. Of those listed, dicyclanil was reported as the most applied active ingredient followed closely by ivermectin. It is important to note that dicyclanil is registered only to prevent flystrike while ivermectin is registered for use against both lice and flystrike including prevention and treatment. In addition, there were reports of 'off-label' practices, such as the mixing of products together, which is a strategy that producers are familiar with because of drenches. **Figure 5** shows the active ingredients used for the 12 months preceding submission. Unfortunately, approximately 34% of submissions failed to provide this information, while 7.5% reported using mixtures.

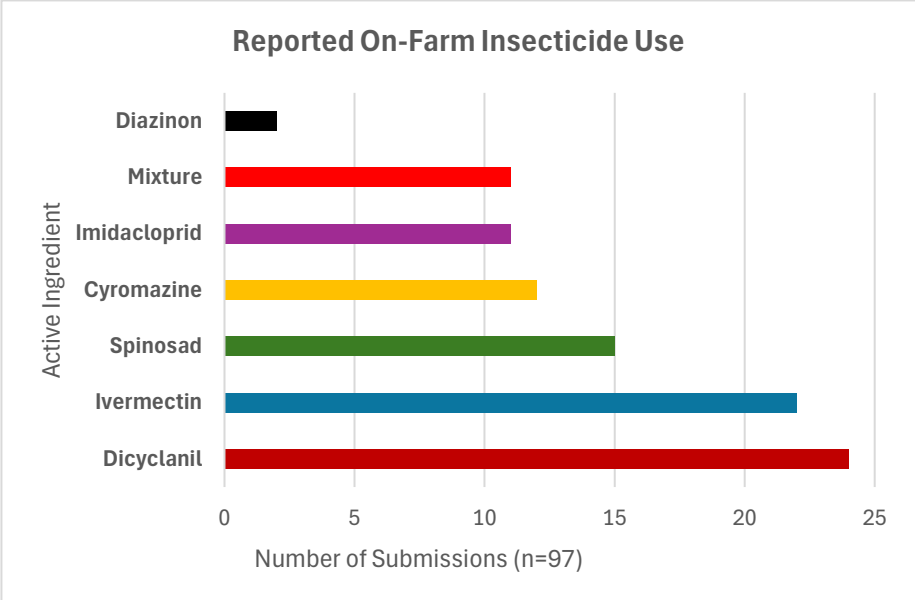
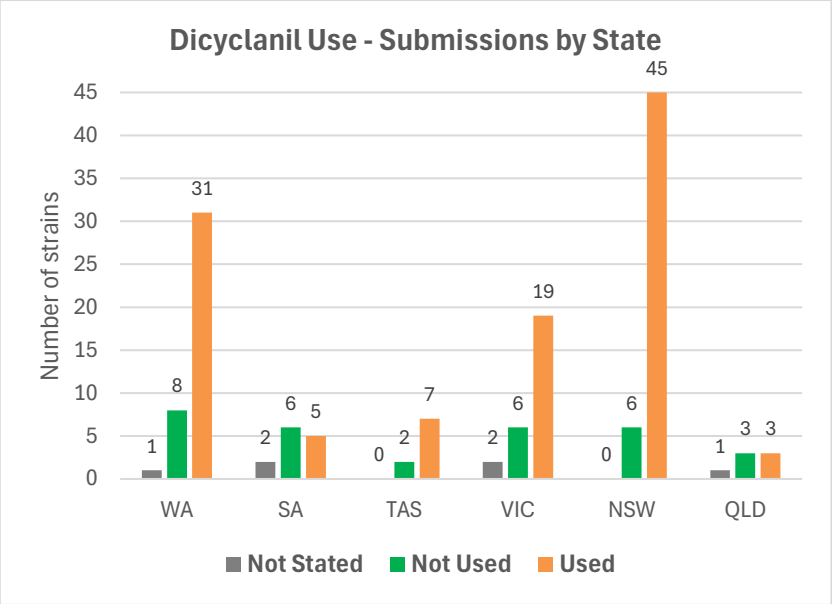


Figure 5. The active ingredients of the products reported to have been used on-farm over the preceding 12 months for flystrike and lice prevention or control.

7.3 Dicyclanil and Cyromazine Use

Of particular interest were the numbers of submitters from each state who reported that they had not used dicyclanil (**Figure 6A**) or had not used cyromazine (**Figure 7A**) over the preceding 10 years. For ease of comparison, these were also expressed proportionally (%) (**Figures 6B** and **7B**). This data shows that over the past decade NSW, followed by WA, has the greatest proportional use of both insecticides.

6A



6B

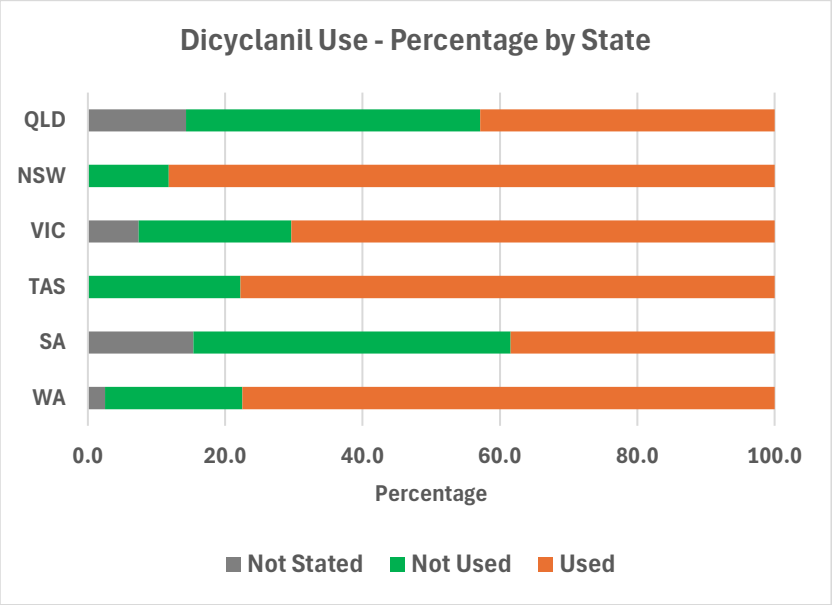
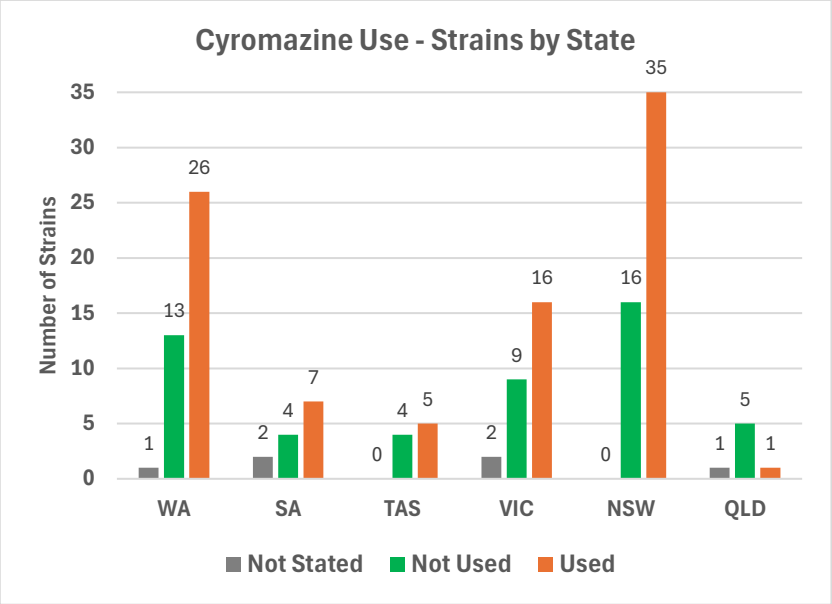


Figure 6. Submitter information on dicyclanil use on-farm over the past 10 years, attributed to the state from which the strains were submitted. (n=147).
Figure 6A Expressed as the number of submissions by state.
Figure 6B Expressed as the percentage of submitted strains for the state.

7A



7B

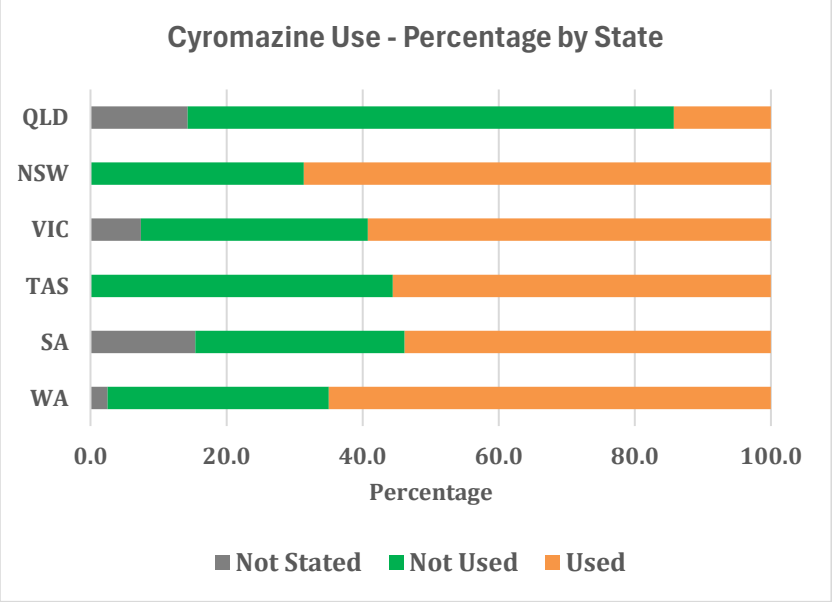


Figure 7. Submitters information on cyromazine use on-farm over the past 10 years, attributed to the state from which the strains were submitted. (n=147).
Figure 7A Expressed as the number of submissions by state.
Figure 7B Expressed as the percentage of submitted strains for the state.

The number of submissions where the producer suspected the presence of insecticide resistance in the fly population on the property (or not) was categorised according to use of dicyclanil **(Figure 8**) and cyromazine (**Figure 9**) in the previous 12 months. This indicates that more people are not using cyromazine if they suspect resistance than those that are avoiding the use of dicyclanil. The reason for this can only be speculated; however, the use of dicyclanil at marking and wound protection may be contributing factors.

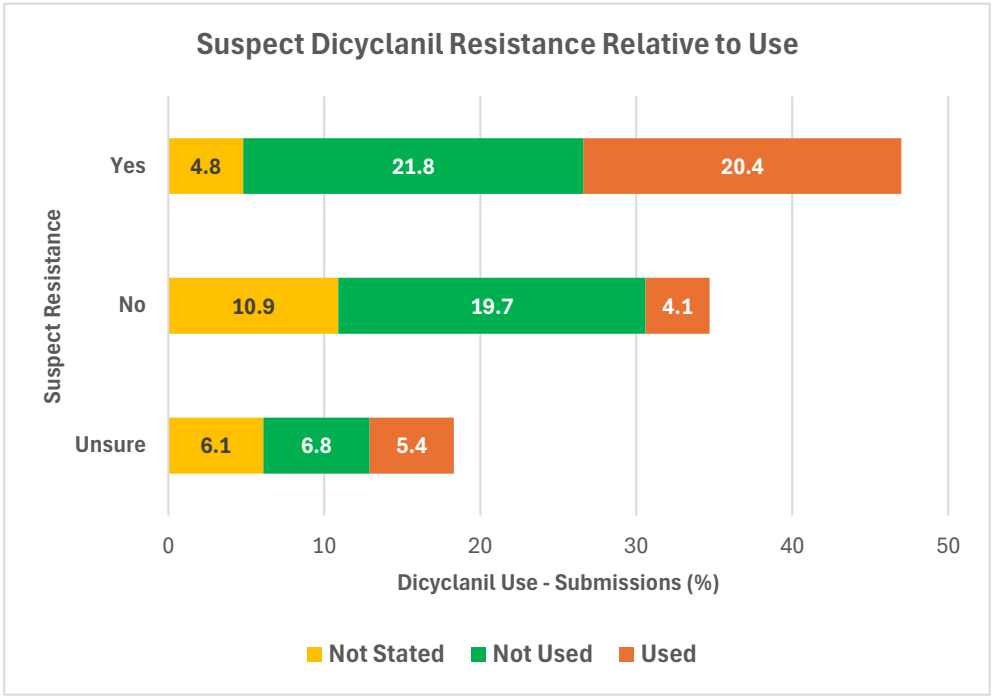


Figure 8. Submitters do or do not suspect insecticide resistance on their property, compared to dicyclanil use in the previous 12 months.

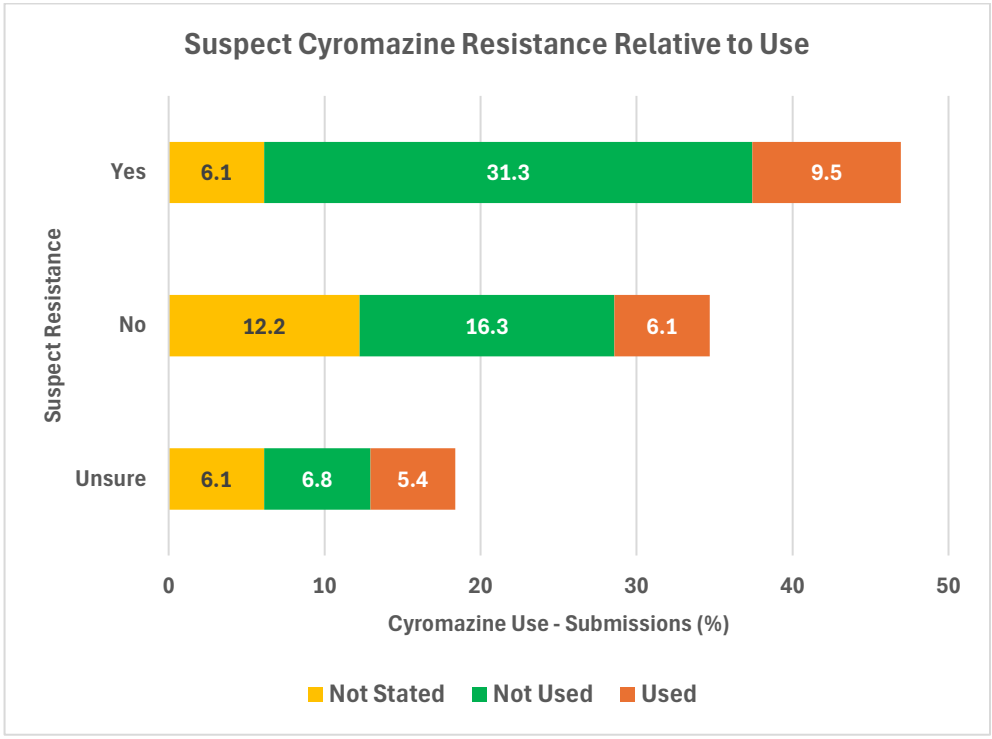
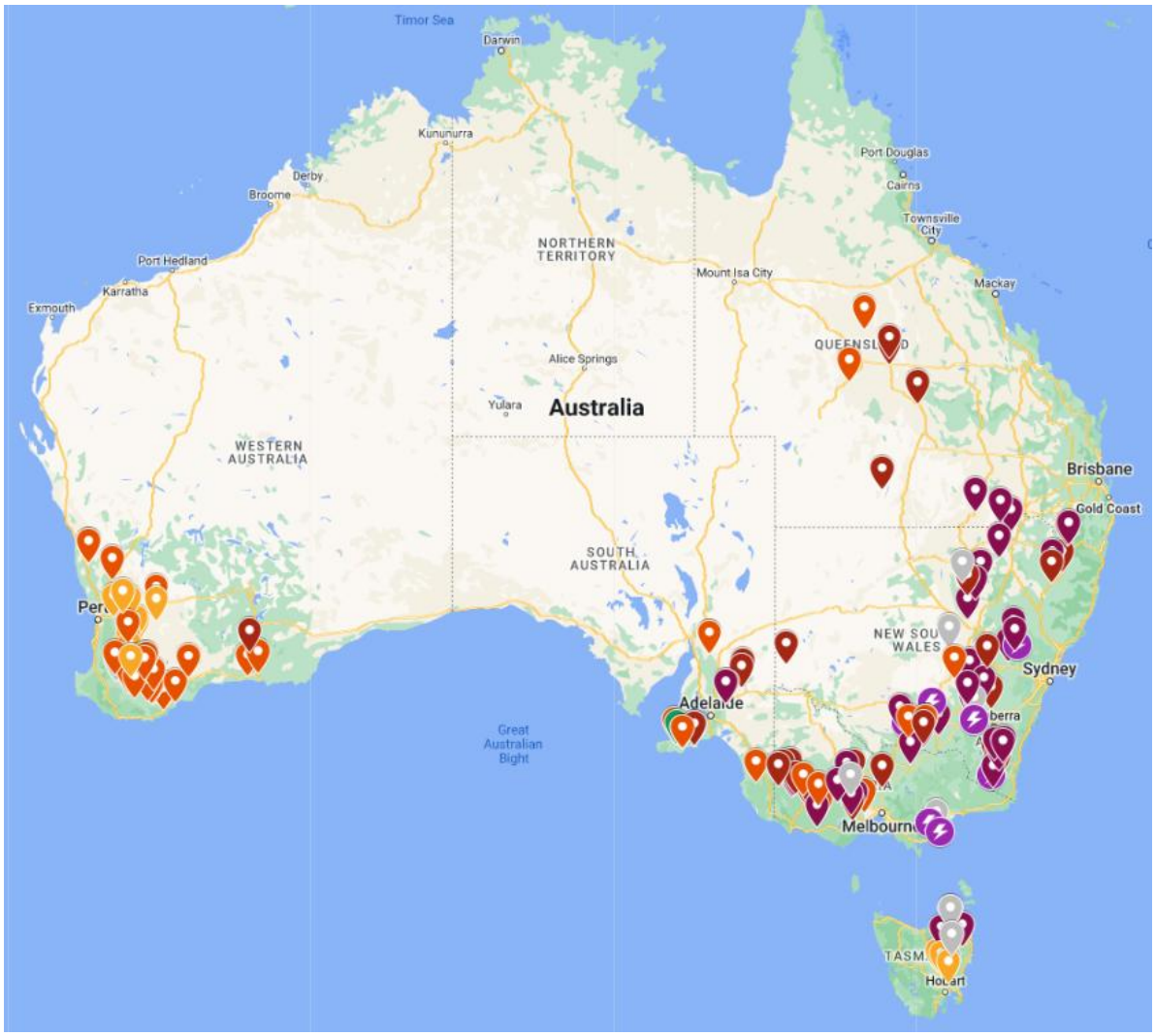


Figure 9. Submitters do or do not suspect insecticide resistance on their property, compared to cyromazine use in the previous 12 months.

7.4 Dicyclanil and Cyromazine Resistance – Classification of Submissions

Each strain was screened at discriminating concentrations of dicyclanil (SDC, 4-fold and 8-fold) and cyromazine (SDC and 4-fold) and classified according to the presence of any survivors regardless of the number. Only one strain, originating from Kangaroo Island, was identified as susceptible to both insecticides and can be seen in **Figure 10.**



Pin	Legend
	Not Viable
	Susceptible – dicyclanil and cyromazine
	Low level cyromazine resistance only
	Low level dicyclanil resistance*
	Medium level dicyclanil resistance*
	Higher level dicyclanil resistance*
	Higher level cyromazine resistance**
*	And **low level cyromazine** resistance
**	And **higher level dicyclanil** resistance

Figure 10. The location of submissions which were received from spring 2022 until winter 2024, classified according to dicyclanil and cyromazine resistance and designated by pin colour. (n=147).

When this information is presented as the proportional contribution by state to each of resistance classification, it shows that Western Australia did not have strains with high dicyclanil resistance, that only NSW and Victoria had strains with high cyromazine and high dicyclanil resistance and strains with cyromazine only resistance originated from either Western Australia or Tasmania (**Figure 11).**

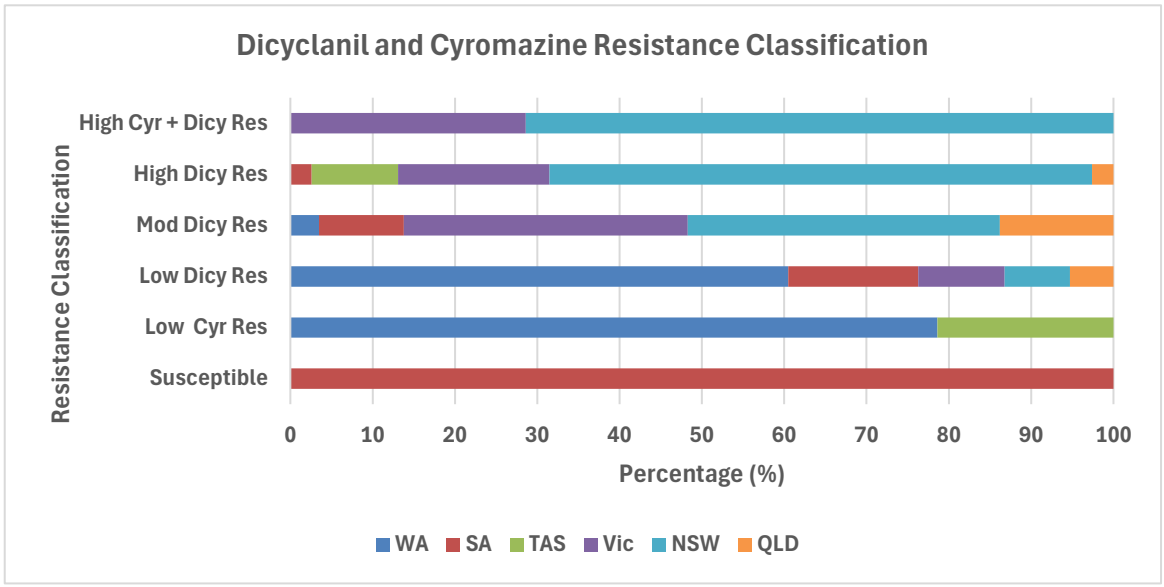


Figure 11. The composition of the dicyclanil and cyromazine resistance classifications by state. (n= 127).

7.5 Neonicotinoid Use.

The submission forms were interrogated to determine the extent to which neonicotinoids had been used in the previous 10 years for either lice and/or flystrike prevention. The percentage of submissions falling into each category can be seen in **Figure 12.** This indicates that approximately equal proportions of submitters either used (48.7%) or had not used (48.6%) a neonicotinoid in the past 10 years.

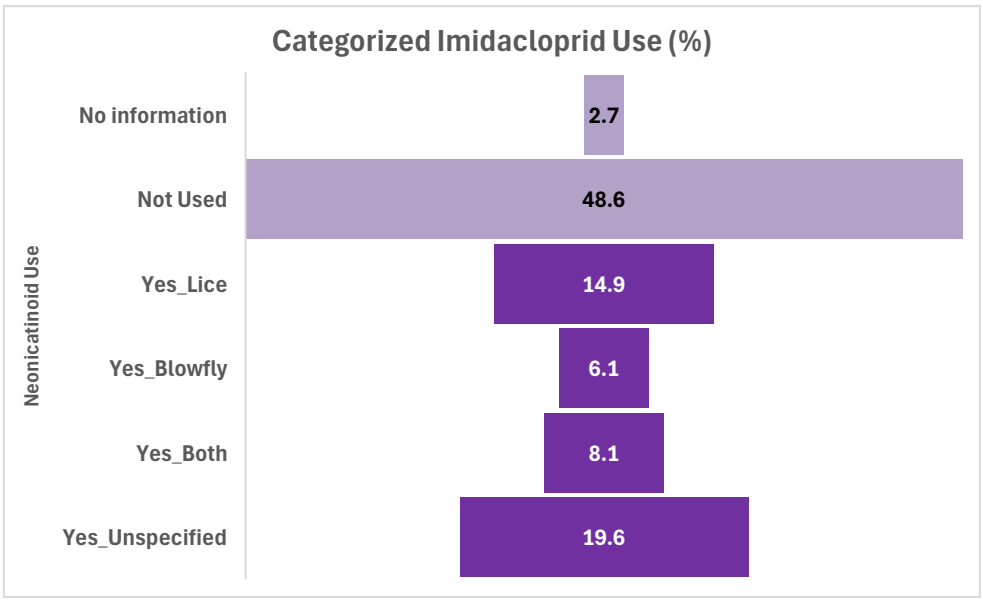


Figure 12. The use of neonicotinoid-based products on farm over the last 10 years expressed as a percentage of the submissions received and classified according to use type. (n=147).

7.6 State by State Response of Strains to Insecticides.

The dose mortality data collected on each strain against each insecticide, expressed as LC50s, were attributed to the state from which they were submitted. This data is presented as box plots to show the distribution of the data points, the outliers, and the median LC50 for each state. This information is displayed for each insecticide in **Figures 13** through to **Figure 17.**

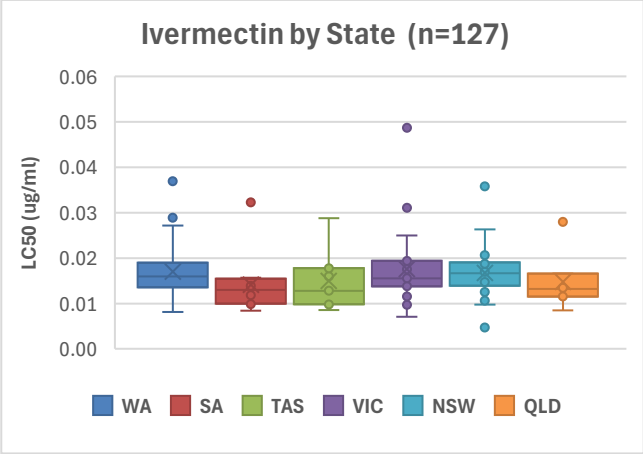


Figure 13. The distribution of response to ivermectin, at the LC50, of submitted strains according to the state of their origin.

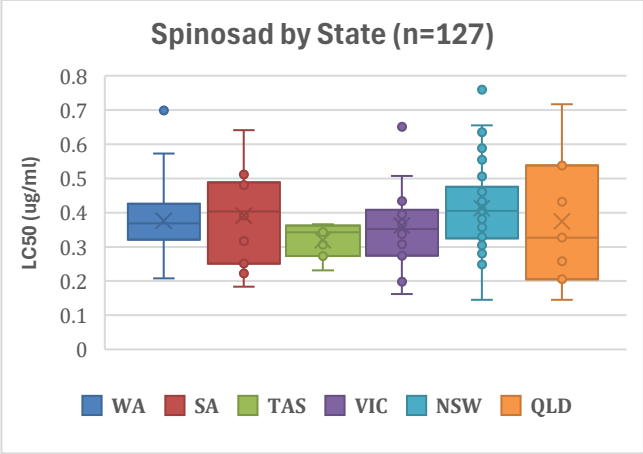


Figure 14. The distribution of response to spinosad, at the LC50, of submitted strains according to the state of their origin.

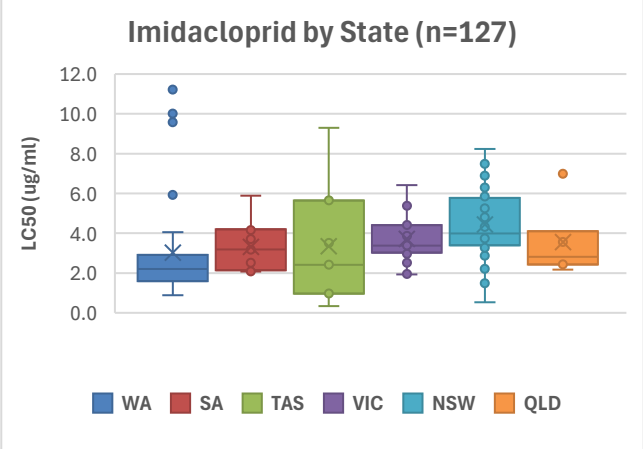


Figure 15. The distribution of response to imidacloprid, at the LC50, of submitted strains according to the state of their origin.

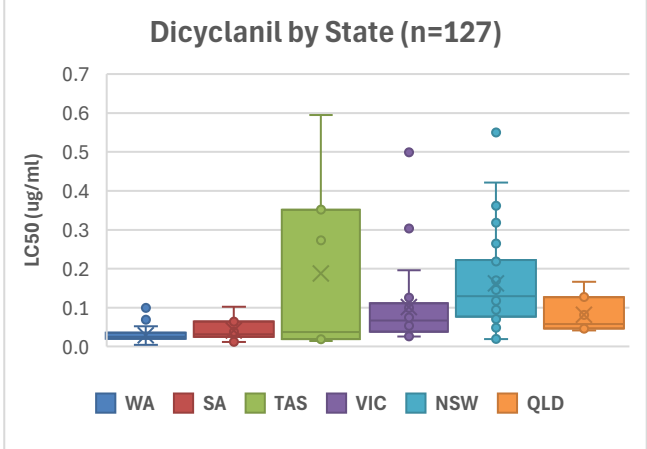
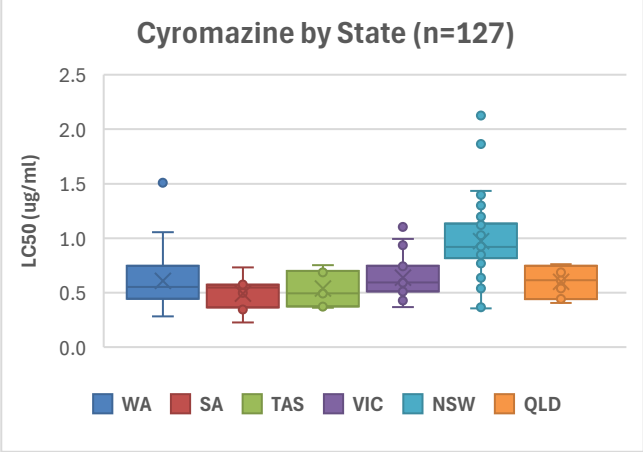


Figure 16. The distribution of response to dicyclanil, at the LC50, of submitted strains according to the state of their origin.



State	(n=)
WA	35
SA	11
Tas	7
Vic	23
NSW	44
QLD	7
Total	127

Legend

Figure 17. The distribution of response to cyromazine, at the LC50, of submitted strains according to the state of their origin.

7.7 Frequency Distribution of the LogLC50 of Strains to Insecticides.

Frequency distributions were developed using Log (LC50) data for submitted strains (n=127) to each insecticide studied. The Schapiro Wilk Test[28] in BioStat[27] was used to determine and describe the deviation from normality observed in the frequency distributions. The Mean, Median and Standard Deviation are detailed in **Table 1** along with the Schapiro Wilk Index (W), and assessments of skewedness and kurtosis. This analysis determined that only the response to cyromazine was normally distributed i.e. the null hypothesis (H_0) that the population is normally distributed was rejected for all except cyromazine.

Table 1. Shapiro-Wilk analysis of normality on the LogLC50 frequency distribution data for the response of strains to five registered active ingredients. (n=127).

Insecticide	Median	Mean	±SD	Fishers Skewedness	Skew	Kurtosis	Kurtosis Type	S-W Index (W)	*P value*	H_O
Dicyclanil	-1.2717	-1.2128	0.4327	0.2838	No	2.3135	Platykurtic	0.9699	6.28E-03	Reject
Imidacloprid	0.5256	0.5057	0.2485	-0.6823	Left	4.4859	Leptokurtic	0.9717	9.14E-03	Reject
Ivermectin	-1.8038	-1.8070	0.1464	0.1202	No	4.6362	Leptokurtic	0.9728	1.16E-02	Reject
Spinosad	-0.4279	-0.4294	0.1662	1.4654	Right	3.4184	Leptokurtic	0.8945	5.85E-08	Reject
Cyromazine	-0.1639	-0.1751	0.1771	0.0517	No	2.8583	Mesokurtic/ Platykurtic	0.9955	5.85E-08	Accept

7.8 Correlation Analysis Between the Responses of Submitted Strains to the Insecticides

As the frequency distributions of the insecticides, except for cyromazine, were not normally distributed, Spearman's correlation coefficients were calculated to determine the relationships between the responses of the strains to insecticide pairings. The LC50 values of 128 strains were analysed in a pairwise fashion for the five insecticides of interest. A Rho value (R) of zero (0) denotes no linear correlation and a positive value denotes a positive linear correlation with the strength of the correlation defined by cut-off values and the statistical significance at the 99% level ($p<0.01$). A p-value assesses the statistical significance of an observed correlation, indicating the probability of obtaining a correlation by random chance if no real relationship existed (the null hypothesis). However, if the Rho value designates the correlation as very weak, we can assume there is little relevance of the correlation.

Positive monotonic correlations of various strengths occurred between all insecticide pairings apart from dicyclanil and ivermectin (R = .144, n = 128, p = 0.1040 i.e. not significant) and dicyclanil and spinosad (R = .1756, n = 128, p = 0.0475) was also classified as a very weak correlation but was marginally significant at the 95% level (P<0.05) but not significant at the 99% level (P<0.01). The moderate strength correlations of note were between dicyclanil and cyromazine, as expected, and dicyclanil and imidacloprid. As seen in **Table 2,** both were highly significant as was the weak correlation between cyromazine and imidacloprid and the moderate strength correlation between ivermectin and spinosad.

Table 2. The Spearman Correlation Coefficient (*p<0.01)* calculated by pairwise examination of the response to insecticides at the LC50 of submitted strains. (n=128). Statistical significance defined at the 1% level (p<0.01).

Insecticide	Spearman's Correlation Coefficient (Rho) H1 $p \neq 0$ (Rho Figure – Defined Strength of the Correlation if Significant) (** Significant at $p<0.01$ or not significant)			
	Imidacloprid	Ivermectin	Spinosad	Cyromazine
Dicyclanil	0.5522 (Moderate) (1.4163E-11)**	0.1444 (Very Weak) (0.1040)	0.1756 (Very Weak) (0.0475)	0.5486 (Moderate) (2.0236E-11)**
Imidacloprid		0.2519 (Weak) (0.0041) **	0.3152 (Weak) (0.0003) **	0.3152 (Weak) (1.1575E-6) **
Ivermectin			0.4058 (Moderate) (2.0097E-6)**	0.2660 (Weak) (0.0024) **
Spinosad				0.2552 (Weak) (0.0036) **
**** Correlation is significant at the 0.01 level (2-tailed)**				

7.9 Change in Resistance over Time

In total, 25 resubmissions were received in this study. Two properties submitted two kits in the same project i.e. three submissions in total. Unfortunately, three resubmissions were dead on arrival, but the remainder were retested. A summary of the change in the strain's resistance classification to dicyclanil and cyromazine can be seen in **Table 3**. As previously stated, the strains with dicyclanil resistance also have low-level cyromazine resistance except for those listed as having high-level cyromazine resistance and high-level dicyclanil resistance. Only six of the twenty-five resubmissions (24%) remained at the same resistance classification.

Table 3. Changes in the classification of dicyclanil and cyromazine resistance status of submissions from the same property between 2018-20 and 2022-24.

Individual Strain Resistance Classification		Number of Strains
2018-2020	**2022-24**	
Susceptible	**Low Cyromazine Resistance**	1
Susceptible	**Low Dicyclanil Resistance**	3
Susceptible	**High Dicyclanil Resistance**	1
Low Cyromazine Resistance	**Low Dicyclanil Resistance**	3
Low Dicyclanil Resistance	**Low Dicyclanil Resistance**	4
Low Dicyclanil Resistance	**Mod Dicyclanil Resistance**	4
Mod Dicyclanil Resistance	**Mod Dicyclanil Resistance**	2
Mod Dicyclanil Resistance	**High Dicyclanil Resistance**	1
Low Dicyclanil Resistance	**High Cyromazine Resistance**	2
Susceptible	**No Result**	1
Low Cyromazine Resistance	**No Result**	1
Low Dicyclanil Resistance	**No Result**	1
No Result	**Low Dicyclanil Resistance**	1
	Total Number	**25**

A comparison of the minimum, maximum and median resistance factors observed in field strains over multiple studies indicate that resistance factors have increased to spinosad and ivermectin (**Table 4**). Conversely, in the most recent study, all three statistical measures of the RF values for imidacloprid were lower than those determined in the 2018-2020 study. This may be due to the overall proportion of submissions from NSW being less in the 2022-2024 study than in the 2018-2020 study. An increasing trend in the maximum resistance factors of both spinosad and ivermectin was observed. Both insecticides are worthy of continued surveillance given increasing reliance, especially on ivermectin.

Table 4, Comparison of the minimum, maximum and median resistance factors determined for field submissions to registered active ingredients of flystrike products over time.

Insecticide	Resistance Factor of Field Strains			Study Year	Number of Strains (n=)
	Minimum	Maximum	Median		
Spinosad	0.16	2.61	1.10	2002	41
	0.42	4.94	1.64	2018-2020	100
	0.97	17.4	2.48	2022-2024	127
Ivermectin	0.63	2.71	1.31	2002	74
	0.54	2.29	1.02	2012-2013	58
	0.98	5.84	2.86	2018-2020	100
	0.67	6.96	2.24	2022-2024	127
Imidacloprid	3.22	42.46	15.51	2018-2020	100
	1.15	37.79	11.29	2022-2024	127
Cyromazine	1.23	10.52	3.34	2022-2024	127
Dicyclanil	0.53	65.66	5.91	2022-2024	127

7.10 Dressing Efficacy at the Recommended Rate and Off-Label Rates

The presence of cyromazine only, or cyromazine and dicyclanil, resistance on the efficacy of registered dressing products can be seen in **Table 5** for maggots exposed to the registered rate, twice the registered rate and half the registered rate.

The most efficacious active at the recommended rate (RR) was ivermectin when assessed by the number of adult flies which hatched following successful pupation of the treated larvae. In practical terms, surviving larvae which successfully pupate, and from which flies eclose, provide the next generation in a fly population. There was no observed increase in efficacy by doubling the RR of ivermectin as it was 100% efficacious at the recommended rate against all three strains. However, cyromazine efficacy did increase to 100% against CresL and DResH when the rate was doubled. At all three rates diazinon, spinosad and cyromazine based dressings, were less efficacious against the resistant strains, Cres and DRes8 compared to LS. In fact, the spinosad dressing product failed to achieve 100% mortality, even against the laboratory susceptible strain. As expected, the diazinon based dressing only achieved 100% mortality against the LS strain but it did so at all concentrations. (**Table 5**).

Table 5. The corrected mortality (%), measured at fly eclosion, following 180 seconds of exposure of 3rd instar larvae to the stated active ingredient in a representative dressing product. The dressings products were diluted to produce twice, the recommended and half, the recommended rates.

Dressing Active	Dressing Rate	% Corrected Mortality (fly eclosion)		
		LS	CResL	DResH
Ivermectin	2X	100	100	100
	1X	100	100	100
	0.5X	100	100	97.1
Spinosad	2X	75.2	60.7	74.4
	1X	64.5	58.9	51.1
	0.5X	54.9	48.9	6.7
Diazinon	2X	100	72.6	23.2
	1X	100	56.1	32.9
	0.5X	100	21	6.9
Cyromazine	2X	100	100	100
	1X	100	94.6	96.4
	0.5X	100	65.6	76.2

7.11 Mixtures of Products.

The most efficacious mixture was that of ivermectin and cyromazine, however, against the CResL and DResH strains, this mixture was less effective than ivermectin on its own which achieved 100% efficacy. The mixing of diazinon with any of the other three dressing products, while increasing the efficacy of the diazinon component, decreased the efficacy of the other dressing in the mixture. This was regardless of doubling the rate against the CResL and DResH strains. A similar reduction in efficacy was observed when spinosad was mixed with the ivermectin or cyromazine dressing products.

As both imidacloprid and dicyclanil based prophylactic products are applied as spray-on formulations, their use against neonate larvae of the same three strains was to be investigated. Due to the large numbers of submissions we received, only unreplicated preliminary data was obtained for imidacloprid but not for dicyclanil. The resistance factors for imidacloprid were reduced when the dressing actives ivermectin or spinosad were included in the assay compared to the resistance factors determined for imidacloprid alone. These preliminary results for imidacloprid need to be confirmed and the mixing of dicyclanil when used as a prophylactic with a dressing product also needs to be studied to determine the effect of this recommendation on the development of resistance.

8. Discussion

Submissions were received from every sheep and wool producing state in Australia, covering a greater area than has previously been achieved. This included submissions from Queensland for the first time in three decades (seven strains), and from Tasmania (eleven strains). As NSW producers are familiar with the concepts of submitting and testing for resistance, direct canvassing was only undertaken in areas of NSW which have previously been under-represented, such as the Riverina. Despite this limited approach in NSW, the greatest number of submissions were still received from NSW (n=50), followed by Western Australia (n=40). Unfortunately, five WA submissions were dead on arrival due to flooding on the Nullarbor Plain and the limitations of the postal system over the December and January periods. Mail delivery times also negatively impacted other states, with one submission taking six weeks to arrive from Tasmania, and another four weeks from Victoria.

NSW submitters were also the most diligent in completing the submission sheets that requested information on insecticide use, animal husbandry and management practices. It was unfortunate that some submission sheets were incomplete as this information was required by UTAS to validate and calibrate the flystrike resistance model. These necessitated producers being contacted directly, as we attempted to fill the information gaps.

The submissions we received were of maggots of varying sizes, removed from struck sheep. It could be assumed that submissions are only off treated sheep and are not representative of the on-farm fly population. However, in this study, approximately 30% of submissions were removed from untreated sheep which included unshorn lambs. Sampling bias would also occur if the only strains submitted were collected by producers who thought they had an insecticide resistance problem. In this study, less than half (47%) of the submitters thought there was resistance to dicyclanil or cyromazine on their property. Both factors indicate a lack of bias in the data presented here, given the submission process used.

Many producers identify breech and wound strike as their major flystrike concern and often treat the breech exclusively. In this study, wound and breech strike accounted for 56% of submissions. One positive outcome of only treating the breech is a reduction in the overall amount of insecticide applied to sheep, thus, reducing selection pressure on the blowfly population. In fact, the UTAS model demonstrated that only treating the breech delays the initial development of resistance and the rate at which resistance will increase if resistance is already present. Body strike made up 23% of the submissions, a testament to the periods of steady rainfall and/or storm events that have occurred over the past two years. Rather than treating the entire sheep or every category of sheep on a property, the targeted, timely use of insecticides on the breech to protect lambs at marking and mulesing, yearlings, ewes, and particularly lambing ewes, can be considered as an element of an integrated pest management (IPM) approach, reducing the use of insecticides. If a product from another chemical group is used as a dressing or prophylactically on other classes of sheep, then this can also be considered a resistance management strategy.

The contribution of lice treatments to the selection of resistant individuals in sheep blowfly populations has been demonstrated.[33,34] As several of the chemical groups are registered for use against lice and flystrike, we asked submitters for details of insecticide use against both parasites on their property over the previous 12 months. The use of dicyclanil was the most frequently reported (25%), despite it being only for the prevention of flystrike. The use of ivermectin based products, reported by 23% of submitters, is not surprising given it is marketed for flystrike prevention, lice control, internal parasite control and as a flystrike dressing. The 4-week period of flystrike protection provided by spinosad reduces its use as a flystrike preventative treatment to prior to sale, slaughter or shearing and late in the season. This suggests that much of its third-place ranking can be attributed to lice control and use as a dressing. The most concerning information supplied by producers was the use of “off-label” mixtures for flystrike prevention and as dressings. The number of reports regarding the use of mixtures approximated the reported use of imidacloprid for blowfly and lice control. The most popular mixture used by submitters was cyromazine and ivermectin. Interestingly, UTAS modelling found that a mixture is effective in slowing down the development of resistance only if the two components of the mixture have equivalent protection periods (PP). By having the same PP

each chemical protects the other. However, if they do not then the product with the shortest PP reduces to ineffective levels leaving the second product, the one with the longer PP, unprotected and at levels that can select resistant individuals in fly populations. Anecdotally, some NSW producers have returned to using cyromazine, however, the proportion of them using it for short term protection, or as part of a mixture, has not been determined. Despite resistance reducing the protection period provided by cyromazine, especially in NSW, there may be merit in using cyromazine as a dressing in a rotation strategy. We have demonstrated that cyromazine is more effective than either diazinon (use not permitted after 10 September 2024) or spinosad at preventing treated larvae from pupating and producing flies for the next generation, despite being slow acting against larvae.

The current study focused on dicyclanil and cyromazine resistance, which were both first detected in NSW. Therefore, it was no surprise that NSW submitters had the greatest proportion of dicyclanil and cyromazine use over the preceding decade followed by Western Australia. Despite producers suspecting that resistance was present on their property, a greater percentage of producers continued to use dicyclanil in the preceding 12 months than cyromazine. This may be explained by some producers continuing to use dicyclanil predominantly for the protection of lambs post marking/mulesing and the breeches of lambing ewes. In fact, some submitters maintained they had little choice in registered products to protect these categories of sheep.

Often studies of resistance rely upon changes observed in the concentration which produces 50% mortality in a population, i.e. the LC or LD50. While this data is useful to describe the response of a population to an insecticide, it does not indicate the presence of resistant individuals when they are at very low frequencies in a population. To detect emerging resistance, it is necessary to screen large numbers of individuals from each population at doses capable of discriminating between susceptible and resistant individuals. This concentration is known as the susceptible discriminating dose (SDD). The SDDs were established for both cyromazine and dicyclanil prior to their commercial release. We used these discriminating doses to develop a resistance classification for each viable submitted strain. The six resistance classifications we report here are 1) susceptible, 2) low cyromazine, 3) low dicyclanil (and low cyromazine), 4) moderate dicyclanil (and low cyromazine), 5) higher dicyclanil (and low cyromazine) and 6) higher resistance to both cyromazine and dicyclanil. The value of screening large numbers of a population was highlighted by three strains which appeared to be susceptible to cyromazine, and another six susceptible to dicyclanil, but which screening showed were not susceptible as they contained resistant individuals at frequencies below 10^4. Given our levels of detection, we had only categorised one strain as susceptible from the 127 tested. The classification of strains has proved to be particularly useful to track the onset of resistance, the increase in resistance level, its spread from east to west across the country, its appearance on islands and its increasing level over time i.e. between 2018-2020 and 2022-2024.

In the 2-to-6-year period between resubmissions, we have seen changes in the range and levels of dicyclanil and cyromazine resistance. Previously, submissions from Tasmania were shown to be more susceptible to insecticides compared to submissions from the mainland. This was even the case for the organophosphate resistance which had been present for decades. In contrast to the susceptibility of the 2018-2020 Tasmanian submissions, three strains submitted between 2022-24 displayed low-level cyromazine only resistance but four strains had also developed high-level dicyclanil resistance. Generally, we would expect the long protection period provided by the 50mg/L dicyclanil product (18 to 24 weeks) to successfully cover the much shorter Tasmanian flystrike periods. We also found that submissions received from Tasmania prior to well into December are *Lucilia sericata* not *L. cuprina.* The very late appearance of *L. cuprina* should easily ensure low to zero selection pressure from dicyclanil, with effective high levels of dicyclanil present from summer through to early autumn. If we can assume that resistance has not been imported into Tasmania, the shortened protection period required from dicyclanil, and the longer colder winters appear not to have been sufficient to prevent the development of cyromazine and dicyclanil resistance in Tasmanian *L. cuprina*.

In the 2018-2020 study susceptible strains were found in Victoria, Tasmania, on Kangaroo Island, mainland SA, and WA. Despite categorising approximately 22% more submissions in this study, only one susceptible strain was identified, which was from Kangaroo Island, SA. In addition, the proportion of strains classified as cyromazine only

resistant had decreased along with a commensurate increase in higher level dicyclanil resistant strains. Alarmingly, for the first time we described seven strains from NSW (5) and Victoria (2) with both high-level dicyclanil and high-level cyromazine resistance. This indicates that with continued selection pressure the resistance levels and frequencies of both insecticides will continue to increase.

Frequency distributions of the Log (LC50) responses of the 2022-2024 submissions (n=127) indicated there was little difference in the range of these distributions from those determined in 2018-2020 (n=100) for spinosad, ivermectin, and imidacloprid. The two exceptions were an additional resistant outlier in spinosad and two susceptible outliers to imidacloprid. However, in contrast to the previous study, this data was not normally distributed except for cyromazine. This lack of normality is an indicator of the selective forces at work on Australian *L. cuprina* populations. Statistically significant linear relationships of varying strengths were calculated, using Pearsons Correlation Coefficients, in 2018-2020 between dicyclanil/cyromazine, dicyclanil/imidacloprid, dicyclanil/diflubenzuron, dicyclanil/diazinon, diazinon/Imidacloprid, cyromazine/ivermectin, cyromazine/imidacloprid and ivermectin/spinosad. Diflubenzuron was studied despite only being registered for lice control as resistance had previously rendered it ineffective for flystrike prevention. Historically, true cross resistance was reported between diflubenzuron, the OP's and the carbamates[35] even before diflubenzuron was registered for use. Highly diflubenzuron resistant strains displayed elevated mixed function oxidase levels,[36] more precisely cytochrome P450,[37] which conferred 2-fold resistance to cyromazine and 10-fold resistance to dicyclanil.[14] As the 2022-2024 data was not normally distributed, the pairwise correlations between dicyclanil, cyromazine, imidacloprid, ivermectin and spinosad were determined using the Spearman's correlation coefficient calculation. Highly statistically significant correlations ($p < 0.01$) of various strength were calculated between all combinations of the five insecticides with the exceptions of dicyclanil/ ivermectin and to a lesser extent dicyclanil/spinosad. In addition to the three significant correlations of moderate strength there were six "weak" but significant correlations. This suggested the involvement of shared resistance genes, which we now know to be the case from the work by UoM,[8], and/or the utilization of existing general detoxification mechanisms.

Monooxygenase-mediated metabolism is a common resistance mechanism found in many types of insects and can detoxify a variety of insecticides. Cytochrome P450s, are a large group of heme containing monooxygenases that are found in plants, animals, fungi, and some microorganisms, capable of degrading chemicals foreign to the organism or are part of the normal metabolic degradation processes. Historically, P450s have been found to be the cause of resistance in insects to the pyrethroid, neonicotinoid, organophosphate, and organochlorine chemical groups and are responsible for cross-resistance between the chemical groups.[38] Elevated P450 levels have been implicated or proven to cause resistance in *L. cuprina* to the four chemical groups listed above, with the addition of the carbamate, butacarb; the benzoylphenyl urea, diflubenzuron; the pyrimidinone, dicyclanil and the amino triazine, cyromazine. In particular, the relationship between dicyclanil and imidacloprid resistance has been investigated in vitro and the cytochrome P450 system implicated with transcription of the Cyp12d1 gene reported to be increased 40-fold.[16] As a result, the role of imidacloprid in any flystrike chemical rotation strategy needs to be considered carefully. Given imidacloprid does not have a dressing claim, its greatest contribution to the rotation strategy is for use against lice and to reduce selection pressure by replacement of dicyclanil when shorter periods of protection (up to 14 weeks) are required.

As observed in the previous study, all strains classified as having dicyclanil resistance also had low-level cyromazine resistance (n=227), however cyromazine resistance is found on its own. This was noted in our previous report[4] where it was suggested that dicyclanil resistance was the result of additional gene/s added to the existing cyromazine resistance. As previously stated, the University of Melbourne (UoM) has verified this by identifying multiple genes, their degree of dominance and chromosomal locations.[8] It found one gene is exclusive to cyromazine resistance, two are exclusive to dicyclanil resistance and a number confer resistance against both insecticides. Using this information, the UTAS model showed that 20 years of widespread exclusive use of cyromazine was sufficient to initiate resistance, and following the release of dicyclanil, that resistance to both insecticides developed at the same rate. This modelling supports producer reports prior to 2001 of reduced protection periods provided by cyromazine.[39] However, it took a further 11 years before cyromazine resistance was confirmed in the field and the laboratory[2]. Prior to this confirmation, populations had last been screened for resistance to cyromazine in 1998 when survivors to the

cyromazine SDC were often detected. However, these individuals did not survive in the absence of the insecticide, and the populations soon reverted to susceptibility in the laboratory. At that time strains were selected in the laboratory but, because the resulting resistance factors were low, the threat of resistance was considered minimal in comparison to the high-level diflubenzuron resistance which was detected at approximately the same time.[12] The previous field findings[15] of a lack of fitness disadvantage in overwintering cyromazine resistant individuals was confirmed by the UoM and shown to include dicyclanil resistance.[8] In practical terms this means that if selecting insecticides are present on the sheep throughout the autumn fly season, these will overwinter and the emerging overwintered flies will include the most resistant individuals of the population. Where this is the case, and depending on the level or resistance present, the strategy of applying dicyclanil to sheep prior to fly emergence will not completely prevent a fly wave and because of the reduced efficacy may only delay visible flystrike.

To protect against flystrike in the presence of resistance to cyromazine and dicyclanil, the rotation of chemical groups has been advocated and includes treatments for lice control. This is a commonly used strategy in IPM which has seen varying degrees of success dependent upon the level of adherence achieved. Interestingly, anthelmintics followed a rotation strategy to counter resistance but now rely on multivalent drenches i.e. mixtures. The UTAS flystrike model has been employed to determine the efficacy of rotation as a strategy. The model found that while rotation cannot prevent the development of resistance it can increase the useful life of the currently available products and should continue to be encouraged because of the level of protection it provides sheep throughout that time. The amount of time that is gained by rotating chemicals will be dependent upon the degree to which resistance already exists in the fly population, the products used and the strictness of adherence to rotation. The UTAS model has also determined the contribution that mixtures could play in flystrike prevention. The model indicates that there is one requirement for success i.e. each chemical in the mixture must have a similar protection period. Despite the model indicating that mixtures may be a positive strategy, it is acknowledged that the formulation of such products may be problematic, and the economics of manufacturers and/or use may not be favourable. Certainly, for dressings we have shown that combining dressing products or increasing the concentration of dressing products does not increase efficacy and in fact decreases the efficacy, particularly of ivermectin. We demonstrated that ivermectin can prevent 100% of larvae continuing their life cycle and forming the next generation of flies. The only outcome of mixing dressing products or doubling their concentrations is an increase in the cost of treatment per head. In addition, if producers combine two products already mixed at the recommended rate, they halve the concentration of each product, drastically reduce the efficacy, increase selection pressure and will drive the development of resistance.

There is a current recommendation to dress an existing strike with one chemical group and if prophylaxis is required to overlay that treatment with a product from another chemical group. Preliminary in vitro results suggest that this is a sound strategy with respect to dressing with ivermectin or spinosad followed by imidacloprid as a prophylactic. Resistance factors were considerably reduced to imidacloprid in the cyromazine only and higher level dicyclanil resistant strains. The resistance factors of ivermectin and spinosad were also reduced but less noticeably. This work needs to be completed for imidacloprid and dicyclanil and the information provided to UTAS for modelling and predictions regarding simultaneous control and prevention options.

Providing effective prevention and control of flystrike in the presence of resistance is much easier to address through rotations and mixtures if resistance is not conferred across chemical groups. In the previous study[4] we determined that the largest and most significant correlations occurred between the response of strains to dicyclanil and to imidacloprid followed by dicyclanil and diflubenzuron. A correlation existed between cyromazine and imidacloprid which was only slightly more significant than that between dicyclanil and cyromazine. As the intervening 2-6 years has seen an increase in both the presence and levels of resistance to dicyclanil and cyromazine it appears that resistance confers survival advantage, to varying degrees, between several insecticides. The exception to this is the ivermectin/dicyclanil combination. A very weak correlation for the spinosad/dicyclanil combination is not significant at the 1% level ($p<0.01$) however it is at the 5% level ($p<0.05$) which is an often-used standard cutoff for significance. Despite the statistical significance this correlation is of little relevance compared to the stronger correlations outlined above. Formerly, the correlation between ivermectin/spinosad was speculated to be one of shared susceptibility.

Despite this, we found a move away from susceptibility in both insecticides since their introduction, which should be monitored. In fact, each of the insecticides involved in the treatment and prevention of flystrike should continue to be scrutinised for changes in efficacy particularly in conjunction with the increasing levels of dicyclanil and cyromazine resistance. It is very clear that the exclusive use of a single insecticide to prevent or control flystrike will ultimately result in the development of resistance, regardless of the advantages gained from ease of application or long protection periods. If a new insecticide became available soon, this should be added to the chemical rotation plan and exclusive use should be avoided at all costs. In addition, any flystrike control that can be achieved through non-chemical means should also be exploited, but as part of an integrated plan.

9. Impact on Wool Industry – Now & in 5 years' time

The Australian wool producer faces many challenges and certainly one of them is the successful management of flystrike prevention and treatment. The development of resistance to cyromazine and dicyclanil is the result of the almost exclusive, sequential use of these two insecticides for decades, especially in states like NSW. However, this has been contributed to by spray-on (pour-on) formulations that deliver high concentrations of the chemical in a confined area and then moves in the fleece, creating a concentration gradient over the animal. As the chemical degrades, there will be a multitude of concentrations over the surface of the sheep, some of which allow the survival of resistant individuals. This type of selection pressure contrasts with the selection pressure from a thorough jetting treatment, where there is a single concentration applied over a large area of the sheep. This should mean that there is not a chemical gradient on the jetted sheep and any gradient that is produced by degradation is minimised. In this way the entire application should be effective until the chemical has degraded to a non-effective level and selection pressure is reduced.

In the absence of resistance, the application of a higher dose dicyclanil treatment prior to the commencement of the fly season was recommended as the concentration of dicyclanil would remain above the minimum effective concentration throughout the entire fly season until flies are no longer active. However, if this strategy was correct, high level dicyclanil resistance would not have developed in colder areas, such as Tasmania, where the start of the season is late, and the end of the season is early. In fact, the only time that a chemical does not apply selection pressure to the *L. cuprina* population is when the selecting chemical is not present on sheep. In areas where resistance now exists, the effective concentration will vary among individual flies and depend overall on the level of resistance in the most resistant individuals in the population. Flystrike prevention and control strategies will need to be modified accordingly, and it was for this reason that a rotation system was advocated, not just for a wool-growing year but across multiple years.

While the development of dicyclanil resistance was expected, it has still had a major effect on farm practices. The conversion to spray-on or pour-on products had almost caused the demise of wet jetting or dipping and often the infrastructure and/or labour no longer exist on farm to use these techniques. Within the flystrike chemical rotation strategy, the preference for spray-on formulations reduces choice to three preventative chemical groups i.e., the IGR's, dicyclanil/cyromazine, the neonicotinoid, imidacloprid or the synthetic pyrethroid, alpha-cypermethrin. Unfortunately, alpha-cypermethrin is only registered for use on the body, although it is understood that an either breech or body claim is being considered. The options in a rotation strategy are reduced further if a lice treatment is required that year. As such there has been an increase in the use of ivermectin and a resurgence in the use of jetting or spray-races. Automatic jetting races are seen by producers as being a more accurate means to quickly deliver the correct dose to the right area on the sheep and allows the use of more economical products. These are key factors as many producers are governed by the economic realities of the single person operation, time constraints and the schedules imposed by mixed farming.

When producers were asked about the use of dicyclanil on their property, a number noted that they thought there was little alternative at marking, mulesing and/or on ewes' breaches prior to lambing. Following a limited check of commercially available products, it appears that the only alternative to dicyclanil is a spinosad based product. Unfortunately, producers may consider the aerosol product as too expensive for widescale use, despite its good wound coverage, and the water-based product as counter intuitive to the drying process required for healing mulesing and marking wounds. Wound dressing alternatives, particularly those that are insecticide free but promote rapid healing, would be beneficial as many producers also stated that this was the only category of sheep which received any insecticidal treatments on their property.

Cross-resistance is a major threat to the success of the flystrike chemical rotation strategy. Of current concern is cross-resistance conferred by the cytochrome P450-mediated component of the dicyclanil/cyromazine resistance to the other chemical groups in the rotation. Cytochrome P450's detoxifying foreign compounds such as insecticides also play a key role in insect metabolic synthesis and degradation pathways, such as production of pheromones and juvenile hormone[40]. Historically, synergist studies of *L. cuprina* have determined that resistance to organochlorines, organophosphates, a carbamate, benzyl phenol ureas and synthetic pyrethroids can be at least partly attributed to cytochrome P450 detoxification. More recently a dicyclanil resistant strain with 40 times the susceptible level of a cytochrome P450 enzyme, Cyp12d1, was also found to confer resistance to the neonicotinoid imidacloprid[22]. This finding supports the correlations reported in this and the previous study between dicyclanil/imidacloprid. It is important to note that susceptibility to cyromazine had statistically significant correlations with all the studied insecticides, as for dicyclanil, except for ivermectin. While the exception of ivermectin is good news for producers, there should still be strict adherence to rotation rather than switching dependency from dicyclanil to ivermectin. As there have also been partial resistances demonstrated between each of these insecticide groups in other insects and at other times in *L. cuprina*, the rationale for use of the rotation strategy remains constant.

Cytochrome P450 based metabolic resistance to spinosad has been found in several agriculturally important insects including the housefly. This was attributed to a sex specific spinosad resistance gene, CYP4G2, unlike the CYP12d1 gene found in *L. cuprina*. It could be argued that *L. cuprina* is less likely to develop resistance to spinosad on its own given its short protection period resulting from its rapid break down by UV light and hydrolysis. In other insects spinosad resistance has resulted from repeated treatments that maintained high selection pressure over extended periods. The rapid break-down of spinosad in the *L. cuprina* environment will be aided by adherence to the flystrike chemical rotation strategy. This adherence increases in importance when considering the dressing efficacy studies, we conducted in which the aqueous spinosad dressing consistently failed to kill a proportion of 3rd instars even at double the recommended concentration. The inability of the spinosad dressing to produce 100% mortality, in even the laboratory susceptible strain, would aid the rapid selection of resistance.

Unfortunately, there is no single product, procedure or strategy that can quickly and effectively overcome the effect of dicyclanil resistance, given the heavy reliance on it to date. To reduce selection pressure with cyromazine and dicyclanil, their use should be restricted to within a multiyear rotation strategy and only one at a time should be used. It has been suggested here that imidacloprid should be included in the same chemical rotation group as dicyclanil and cyromazine for the prevention of flystrike or be reserved for lice treatments. The information provided by the UTAS model on such strategies will prove to be invaluable to producers when additional information is available on these other registered active ingredients.

The long-term goal must be to reduce reliance on chemicals to control flystrike. This includes the breeding of sheep which are not susceptible to flystrike. This is not a quick or easy undertaking; however, many producers have made positive gains by including previous flystrike in their culling criteria. Another measure is on-farm fly population control. This includes the disposal of carcasses, which provide a protein source for female *L. cuprina* flies that is required before being able to produce eggs. Carcasses also provide a breeding ground for other fly species, particularly *Chrysomya rufifacies*, the hairy maggot blowfly that can strike existing wounds. By eliminating reservoirs of these other species, wound strikes, such as cracked horns, shearing cuts, and cancers will be reduced. An incredibly effective but often-overlooked method of controlling the fly population is to remove and kill every maggot off every struck sheep. This directly impacts the number of flies available for the next fly wave. There are other non-chemical control measures which target the fly population, such as the on-farm release of infertile male flies and the employment of effective fly traps, both of which have been studied in the past and, given the current resistance problems, are being investigated again. Technological advancements are supplying other options such as RNA interference (RNAi)[41], which relies on the insertion of double stranded RNA into the insect to block the production of proteins vital to an insect's survival.

The management of insecticide resistance, prevention and control of flystrike in Australia is reliant upon the implementation of well-planned integrated approaches. Realistically, while the reliance on insecticides might be reduced, in many areas their use will always be required. As a result, new chemistries, the possible use of mixtures and the development of additional non-chemical alternatives are required to add to the flystrike toolbox. Presently, without new insecticide products imminent, the strict adherence to the strategic rotation of current insecticides, reducing the susceptibility of sheep to flystrike and reducing, manipulating or eliminating the fly population are becoming a necessity.

10. Conclusions and Recommendations

This study shows that dicyclanil and cyromazine resistance has developed across the country, increasing in frequency and level, with some producers applying a rotation strategy where practicable. Of major concern is the apparent generality of the dicyclanil resistance mechanism/s and the effect this has on the efficacy of the other chemical groups registered for flystrike, particularly imidacloprid. The imidacloprid based product is a spray-on providing up to 14 weeks protection and we suggest that it be considered in the same resistance group as dicyclanil and cyromazine despite their different modes of action. Imidacloprid is useful as a lice treatment in a rotation or as an alternative to dicyclanil later in the season or in areas where the flystrike season is shorter than three months. While the use of dicyclanil and imidacloprid should be mutually exclusive, the correlation between imidacloprid and cyromazine was weaker and the use of these two may provide the flexibility that is often required within the rotation strategy. However, where dicyclanil resistance exists, the protection period of dicyclanil, cyromazine and imidacloprid is expected to be reduced.

The UoM study determined the number and location of the genes that are involved in dicyclanil and cyromazine resistance and confirmed that resistant individuals are as fit as susceptible ones. This means that resistance does not cause detrimental or lethal effects on individuals during overwintering or in the absence of the insecticide. UTAS has modelled the development of resistance to cyromazine and showed that resistance would have been present after 20 years of use and slowly increased in frequency. The model showed that, once dicyclanil was released, the rate of resistance development to both chemicals was identical due to the presence of these fitness modifiers. In the field, this accounts for the seemingly rapid development and spread of dicyclanil and cyromazine resistance. Armed with this information UTAS has modelled the rotation of chemical groups and proved that this strategy provides protection against flystrike over time despite ultimately still ending up with resistance to both rotated chemical groups. The model demonstrates that, despite the same end point, that rotation should be continued as the sheep welfare outcomes are much better over the period. The UTAS modelling of mixtures has been beneficial as it provided a formula for successful combinations, proving it is only effective if those with similar protection periods are combined.

Producers want to know a) if there is resistance on their property and b) how resistance will affect the protection period of the products they use to control flystrike. An on-sheep (*in vivo*) investigation is required to provide these practical answers to producers and should include various levels of resistance. This fundamental study should include each delivery method registered for a chemical group i.e. spray on, hand jet, dip, and jetting race, to determine the protection achieved when applied according to the manufacturers' instructions. These treatments should be challenged with resistant types and life stages e.g. gravid females in the case of alpha cypermethrin, eggs/neonate larvae for preventative treatments, and 2^{nd}-3^{rd} instars for dressings. This will give an accurate and detailed picture of the protection that producers can currently expect and efficacy in the short to medium term (2- 5 years). UTAS can use this information to model the likelihood of field failure and the options available. Conversely, if the data on protection periods proves there is limited scope for the chemical prevention of flystrike, it will provide the hard data and hopefully the impetus for the broadscale move towards non-chemical practices.

One non-chemical means of flystrike prevention is the release of sterile males which has a contraceptive effect on the fly population as females produce unfertilised eggs. This has been trialled in NSW on a summer shearing farm with 100% success. In addition, on this NSW DPIRD research station, where recruitment from adjacent properties is low and a closed flock is managed, it has continued to provided protection over three flystrike seasons. AWI supports the Kangaroo Island project which is looking to eradicate *L. cuprina* long term. On-farm and/or control at a local level could be available following field validation. AWI is also supporting the development of a fly trap specific for *L. cuprina* which, used in conjunction with on-farm modelling, would inform on shearing and crutching dates. The adoption of these approaches, breeding sheep more resistant to flystrike, and the development of a practical annual flystrike action plan for each individual property would reduce reliance on chemicals to prevent flystrike.

In conclusion, the collaboration between NSW DPIRD, UTAS and UoM has been very productive and has provided unbiased findings. These include the real-world data on resistance prevalence and levels across Australia, actual patterns of insecticide use and associated resistance development and the required genetic information on number and locations of the resistance genes. This information enabled validation of the flystrike model that predicted the likelihood of success, the outcomes of chemical rotation, and the use of mixtures against flystrike. There is still more fundamental research required including high-level gene studies, a comprehensive study of registered products to determine protection periods against the classifications of dicyclanil and cyromazine resistance described here and modelling for best practice flystrike prevention. This information would also contribute to the aspirational goals of providing continually updated information on insecticide resistance and effective flystrike prevention, increasing the scope of non-chemical flystrike prevention measures and the development of practical on-farm management "packages" to prevent flystrike.

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