Research Article

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Resistance to dicyclanil and imidacloprid in the sheep blowfly, *Lucilia cuprina*, in Australia

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Abstract

Background: The sheep blowfly, *Lucila cuprina*, is a myiasis-causing parasite responsible for significant production losses and welfare issues for the Australian sheep industry. Control relies largely on the use of insecticides. The pyrimidine compound, dicyclanil, is the predominant control chemical, although other insecticides also are used, including imidacloprid, ivermectin, cyromazine and spinosad. We investigated *in vitro* resistance patterns and mechanisms in field-collected blowfly strains.

Results: The Walgett 2019 strain showed significant levels of resistance to both dicyclanil and imidacloprid, with resistance factors at the IC_{50} of 26- and 17-fold, respectively, in *in vitro* bioassays. Co-treatment with the cytochrome P450 inhibitor, aminobenzotriazole, resulted in significant levels of synergism for dicyclanil and imidacloprid (synergism ratios of 7.2- and 6.1-fold, respectively), implicating cytochrome P450 in resistance to both insecticides. *Cyp12d1* transcription levels were increased up to 40-fold throughout the larval life stages in the resistant strain compared to a reference susceptible strain, whereas transcription levels of some other *cyp* genes (*6g1*, *4d1*, *28d1*) did not differ between the strains. Similar resistance levels also were observed in flies collected from the same property in two subsequent years.

Conclusion: This study indicates that *in vitro* resistance to both dicyclanil and imidacloprid in this field-collected blowfly strain is likely mediated by cytochrome P450, with Cyp12d1 implicated as the enzyme responsible; however, it remains possible that another P450 also may be involved. A common resistance mechanism for the two drugs has important implications for drug rotation strategies designed to prolong the useful life of flystrike control chemicals.

Supporting information may be found in the online version of this article.

Keywords: *Lucilia cuprina*; blowfly; flystrike; resistance; dicyclanil; imidacloprid

1 INTRODUCTION

The sheep blowfly, *Lucilia cuprina*, is the primary cause of flystrike on sheep in Australia. Flystrike is a serious financial and animal welfare issue for the Australian sheep industry, costing up to AU $175 million per annum due to production losses (i.e. reduced wool growth and bodyweight gain, and animal death) and costs associated with treatment and prevention.[1] Flystrike control relies largely on the use of insecticides.[2] These chemicals are generally applied as prophylactic treatments given in advance of fly waves, although some also are used as dressing treatments on existing strikes. The insecticides currently used for flystrike control belong to various chemical classes: pyrimidine (dicyclanil), triazine (cyromazine), macrocyclic lactone (ivermectin), neonicotinoid (imidacloprid), synthetic pyrethroid (alpha-cypermethrin), spinosyn (spinosad) and organophosphates (diazinon, chlorfenvinphos). Dicyclanil dominates the market for flystrike control chemicals,[3] as it provides a much longer period of protection than the other chemical groups (up to 29 weeks for the highest concentration dicyclanil product compared to up to10–14 weeks).

The sheep blowfly has shown an ability to develop resistance to some insecticides.[4,5] Resistance to the organophosphate class resulted in inadequate protection against flystrike when applied prophylactically, with their use continuing only as dressing treatments for existing strikes. Low-level resistance to the benzoyl phenyl urea compound, diflubenzuron, emerged in the field soon after its introduction for flystrike control in the early 1990s. Within several more years, a high level of resistance had emerged and became widespread in field strains,[6] leading to the withdrawal of claims for flystrike control for compounds in this chemical class in the mid-2000s. Levot[7] first reported resistance to cyromazine in *in vitro* assays, with a low level of resistance to dicyclanil also detected. By 2014, this low level of *in vitro* resistance to cyromazine was described as being 'quite common'.[8] Sales *et al.*[5] recently described a number of strains of blowfly collected from regions within the state of New South Wales (NSW) that showed survivors

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when cultured on food sources containing a 'susceptible discriminating concentration' of dicyclanil. This study also reported on an *in vivo* larval implant trial using a blowfly strain showing high levels of *in vitro* resistance that showed significantly reduced flystrike protection periods following administration of dicyclanil- and cyromazine-based products.[5]

Given the dominant position of dicyclanil for flystrike control, resistance to this compound would have a significant impact on the sheep industry.[2] The ongoing usefulness of the alternative chemicals available presently for flystrike control will depend to some extent on whether cross-resistance between dicyclanil and the alternative compounds is present in field blowfly populations. As the structures of dicyclanil and cyromazine are very similar, it is not surprising that cross-resistance between these two chemicals has been reported previously,[9,10] however no other cross-resistances have been reported for the sheep blowfly among the other chemicals currently used for flystrike control. The present study therefore aimed to investigate resistance patterns in blowflies recovered from the field in NSW by Elanco Animal Health as part of their on-farm product support and pharmacovigilance processes. We also aimed to measure the effect of metabolic enzyme inhibitors on the toxicity of insecticides to blowfly larvae with a view to revealing resistance mechanisms and assessing the potential for the use of synergists to restore sensitivity to insecticides in resistant blowflies. As these synergism experiments revealed a likely involvement of cytochrome P450 enzymes in the observed resistances, we measured expression patterns over the blowfly life cycle for two P450 genes implicated in metabolism of dicyclanil and imidacloprid in other insects: *cyp6g1* and *cyp12d1*.[11–13] In addition, expression levels of two P450 genes from families associated with detoxification of xenobiotics, including some insecticides, but with no known action on dicyclanil or imidacloprid (*cyp28d1*, *cyp4d1*) also were measured as probable negative controls.[14,15]

2 MATERIALS AND METHODS

2.1 Insects

The field-collected blowfly strains used in this study were maintained in culture at the Elanco Animal Health facility at Kemps Creek, NSW. At various times, pupae were sent by courier to the CSIRO laboratory in Brisbane, QLD. The pupae were used to establish cages of adult flies in order to supply larvae for subsequent insecticide bioassay experiments. The number of generations that the flies had been maintained in culture before their use in assays was defined using a 'G' number. Adult flies were maintained at 28 °C and 80% relative humidity with a photoperiod of 16 h:8 h, light:dark, and fed a diet of sugar and water. Protein meals (ovine liver) were provided on Day (D)4 and D7 after adult eclosion in order to stimulate gonad maturation for subsequent egg-laying.

2.1.1 *Laboratory susceptible (LS)*

The laboratory reference drug-susceptible strain, derived from collections made in the Australian Capital Territory (Canberra, Australia) over 45 years ago. This strain has been maintained in a laboratory since that time (in Canberra for 30 years, and then at CSIRO in Brisbane for the last 15 years), with no history of exposure to insecticides.

2.1.2 *Walgett 2019*

Collected in November 2019 on a property in north-west NSW. On 28 November 2019, Elanco were notified of a breakdown in protection from flystrike in sheep that had been treated with CLiK Extra™ (65 g L^{-1} dicyclanil). Approximately 6000 mixed-age merino ewes had been treated with CLiK Extra on 7 September, before lambing. Joining had been delayed as a result of dry seasonal conditions. Flystrike was evident in 20–30% of the sheep in some mobs following lambing, with the majority of strikes 10–20 cm in size and predominantly on the tail and rump area. Wool samples analyzed for dicyclanil levels indicated that the majority of sheep had levels of the compound that should have prevented flystrike. However, a small number of samples did show levels that were quite low, indicative of variable application on at least some sheep within the flock. Third-stage larvae were collected from active strikes and submitted to the laboratory for resistance testing.

CLiK™ (50 g L^{-1} dicyclanil) had been used regularly on this property, usually once per year. The product was typically applied after crutching in June/July, before lambing, but was delayed as a result of seasonal conditions in 2019. Vetrazin™ (cyromazine) had been used before the availability of CLiK.

2.1.3 *Walgett 2020*

Collected from the same property in 2020. Some mobs of sheep on this property were treated again with CLiK Extra between late June and the middle of July 2020, as per product label, ahead of the 2020/2021 fly season. On 6 November 2020, Elanco were notified of a breakdown in protection from flystrike in these mobs; 3–4% of sheep were struck, predominantly on the body. Third-stage larvae again were collected from active strikes and submitted to the laboratory for resistance testing.

2.1.4 *Walgett 2021*

Collected from the same property in 2021. CLiK Extra was applied to the sheep on the property between 14 June and 12 July 2020, with dose volumes and application method as per product label. Elanco was subsequently notified of a low level (0.4%) of breakdown in protection from breech flystrike on 15 October. Third-stage larvae were again collected from active strikes and submitted to the laboratory for resistance testing.

2.2 Chemicals

Dicyclanil, imidacloprid, spinosad, ivermectin, piperonyl butoxide (PBO), tranylcypromine, MGK264, aminobenzotriazole, ketoconazole, diethyl maleate and SKF525 A (proadifen) were purchased from Sigma-Aldrich (St Louis, MO 63103, USA). Cyromazine was purchased from Chem Service (West Chester, PA 19381, USA). Trichlorophenylpropynyl ether (TCPPE) was supplied by Elanco Animal Health (Greenfield, IN 46140, USA). All chemicals were prepared at 10 mg mL^{-1} in ethanol and stored at −20 °C. Various dilutions subsequently were prepared by serial dilution in ethanol.

2.3 Bioassays

The effects of insecticides and insecticide/synergist combinations on the growth of blowfly larvae were assessed using a bioassay system in which larvae developed from the 1st instar to the pupal stage on cotton wool impregnated with the compound of interest at various concentrations (modified from Kotze *et al.*).[16] Sheep liver was placed into cages of gravid flies for a period of 2 h (10:00 h until 12:00 h) to stimulate oviposition. In order to minimize the risk of bacterial contamination of subsequent bioassays, egg clumps were collected from the liver and then dispersed using a detergent solution and surface-sterilized in a mild bleach

solution. Egg clumps were mixed gently in a 1% (v/v) solution of Tween 80 for 10 min, collected onto a tea strainer, washed with water, and then mixed for 10 min in 42 mg L^{-1} sodium hypochlorite. The eggs then were collected onto a 100-μm filter, washed with water, and dispensed onto a disc of filter paper (Whatman grade 1) that previously had been soaked in a larval nutrient medium consisting of sheep serum (Life Technologies/Thermo Fisher Scientific, Adelaide, SA, Aukland 1061, NZ), containing 80 g L^{-1} yeast extract (Millipore, Macquarie Park, NSW, 64271 Darmstadt, Germany), 35 mM KH_2PO_4, 250 U mL^{-1} penicillin, 250 μg mL^{-1} streptomycin and 1.25 μg mL^{-1} amphotericin B. The filter paper was placed in the dark at room temperature.

On the same day as the flies were stimulated to oviposit, 4 mL amounts of insecticide or insecticide/synergist combination solutions were dispensed into 70-mL plastic pots containing a filter paper disc and 0.2 g shredded cotton wool. Control containers were prepared by the addition of 4 mL ethanol alone to the cotton wool. The solvent was allowed to evaporate in a fume cabinet overnight.

At 10:00 the next morning (D0), 3 mL larval nutrient medium (described above) was added to the cotton wool in each bioassay pot, and mixed into the wool using a pipette tip until no free liquid remained visible. The newly-hatched larvae on the filter disc prepared the previous day were transferred into a dish containing PBS, and groups of 60 larvae were collected using a pipette and dispensed into each bioassay pot. The assay pots were placed at 28 °C. After 24 h (D1) 1 mL nutrient medium was added to each pot, followed by 2 mL on each of D2 and D3. Late on D4, the containers were placed into larger pots with a layer of sand at the base to serve as a medium for pupation, and returned to the incubator. Pupae were recovered from the sand using a sieve on D9 and counted. The effect of the insecticides or insecticide/synergist combinations on larval development was described by calculating the pupation rate relative to control assays. For assays with insecticides alone, the number of pupae in insecticide treatment assays was expressed as a percentage of the mean number of pupae in assays prepared using ethanol alone. For assays examining insecticide/synergist combinations, the number of pupae in assays with both chemicals was expressed as a percentage of the mean number of pupae in assays with synergist alone.

Each insecticide was examined at five to eight (depending on the slope of the dose response) 1.41-fold serially diluted concentrations. For assays with Walgett 2019 G9–10, G18–21 and G27–29, three separate experiments were performed for each insecticide or insecticide/synergist combination, using larvae from across the specified generations. For assays with Walgett 2020 G3 and Walgett 2021 G3, three assays were conducted at each drug concentration within the single generation.

The pupation rate dose–response data were analyzed with PRISM® software (GraphPad, San Diego, CA, USA) using nonlinear regression, with the 'variable slope' option selected, in order to calculate the inhibitory concentration (IC) values (with 95% confidence intervals) representing the concentration of inhibitor required to reduce the pupation rate by 50% (IC_{50}) or 95% (IC_{95}) compared to control treatments.

2.4 Gene transcription

Blowflies were collected at various stages through the life cycle in order to examine transcription patterns of selected P450 genes (*cyp6g1*, *cyp12d1*, *cyp28d1*, *cyp4d1)*. Gravid female flies from the LS and Walgett 2019 G22 strains were stimulated to lay eggs as described above. Samples of eggs (≈120 mg) were collected into 2-mL screw top vials containing a mixture of 0.1, 1.0 and 2 mm zirconia/silica beads (Biospec Products, Bartlesville, OK, USA), snap-frozen immediately in liquid nitrogen, and stored at −80 °C. Remaining eggs were collected and treated with Tween 80 and sodium hypochlorite and placed into 70-mL bioassay pots as described above for control (no drug) bioassays. Larvae were sampled after 24, 48, 72 and 96 h, with varying numbers taken at each time point as the larvae increased in size: ≈50 mg at 24 h, 20 larvae at 48 h, ten larvae at 72 h, and five larvae at 96 h. Pupae were collected after a further 6 days (five per sample), and adult female and male flies were collected after a further 7 days (five per sample). At each time point, the samples were added to 2-mL vials containing beads, and snap-frozen as described above for eggs. Four replicate samples were taken for each life stage at each time point. An examination of the posterior spiracle openings (as described by O'Flynn and Moorhouse)[17] in larvae sampled at each time point showed that the larvae were 1st instar at the 24 h time point, 2nd instar at 48 h, and 3rd instar at 72 and 96 h.

We also examined the effect of exposure to dicyclanil on the transcription of each of the target genes in Walgett 2019 G26 larvae. Newly-hatched larvae were placed into bioassay pots containing 0 (control), 0.5 or 2 μg dicyclanil. Larvae were collected

Table 1. IC_{50} and IC_{95} values and resistance factors for the LS and Walgett 2019 G9-10 strains

	Blowfly strain									
	LS				Walgett					
Insecticide	IC_{50} (μg/assay)	95% CI[†]	IC_{95} (μg/assay)	95% CI[†]	IC_{50} (μg/assay)	95% CI[†]	Resistance factor[‡]	IC_{95} (μg/assay)	95% CI[†]	Resistance factor[‡]
Dicyclanil	0.081	0.076–0.088	0.12	0.10–0.14	2.08	1.73–2.49	26	6.35	3.77–10.7	53
Imidacloprid	0.84	0.77–0.92	1.65	1.27–2.15	14.0	12.1–16.3	17	25.2	16.6–38.5	15
Cyromazine	1.9	1.3–2.9	2.22	0.69–7.20	9.4	8.4–10.4	4.9	18.9	14.2–25.1	8.5
Ivermectin	0.012	0.009–0.017	0.033	0.015–0.076	0.032	0.028–0.037	2.6	0.080	0.053–0.119	—
Spinosad	0.22	0.17–0.29	0.59	0.28–1.24	0.48	0.42–0.55	2.1	0.91	0.63–1.34	—

[†] 95% CI = 95% confidence interval.
[‡] Resistance factor = IC_{50} (or IC_{95}) resistant strain/IC_{50} (or IC_{95}) susceptible strain; Resistance factors only shown if the IC_{50} or IC_{95} values of the susceptible and resistant strains were significantly different, as judged by nonoverlap of 95% CI.

and frozen at 24 h and 48 h as described above. Four replicate samples were taken at each concentration for each time point. Preliminary dose–response data indicated that the pupation rate would be unaffected by 0.5 µg and reduced to ≈60% by 2 µg dicyclanil.

RNA was extracted using an RNeasy kit (Qiagen, Clayton, VIC 40724 Hilden, Germany), as per the manufacturer's instructions, with initial homogenization by shaking on a Powerlyzer 24 (Mo Bio Laboratories Inc./Qiagen, Carlsbad, CA 92010, US,A). Following extraction, the samples were quantified using a Nanodrop and treated with TurboDnase (Ambion/Thermo Fisher Scientific, SC 29842, USA) to remove any genomic DNA. RNA quality was assessed using an Agilent Bioanalyser. cDNA synthesis was performed on extracted RNA using SuperScript III Reverse Transcriptase (Life Technologies/Thermo Fisher Scientific), according to the manufacturer's instructions.

Quantitative PCR primers were designed for each of the blowfly genes using PRIMER 3 software (Supporting Information, Table S1).[18,19] Three housekeeper genes (*18S*, *28S* and *RPLPO*) were used as reference genes for data normalization.[20] A ViiA7

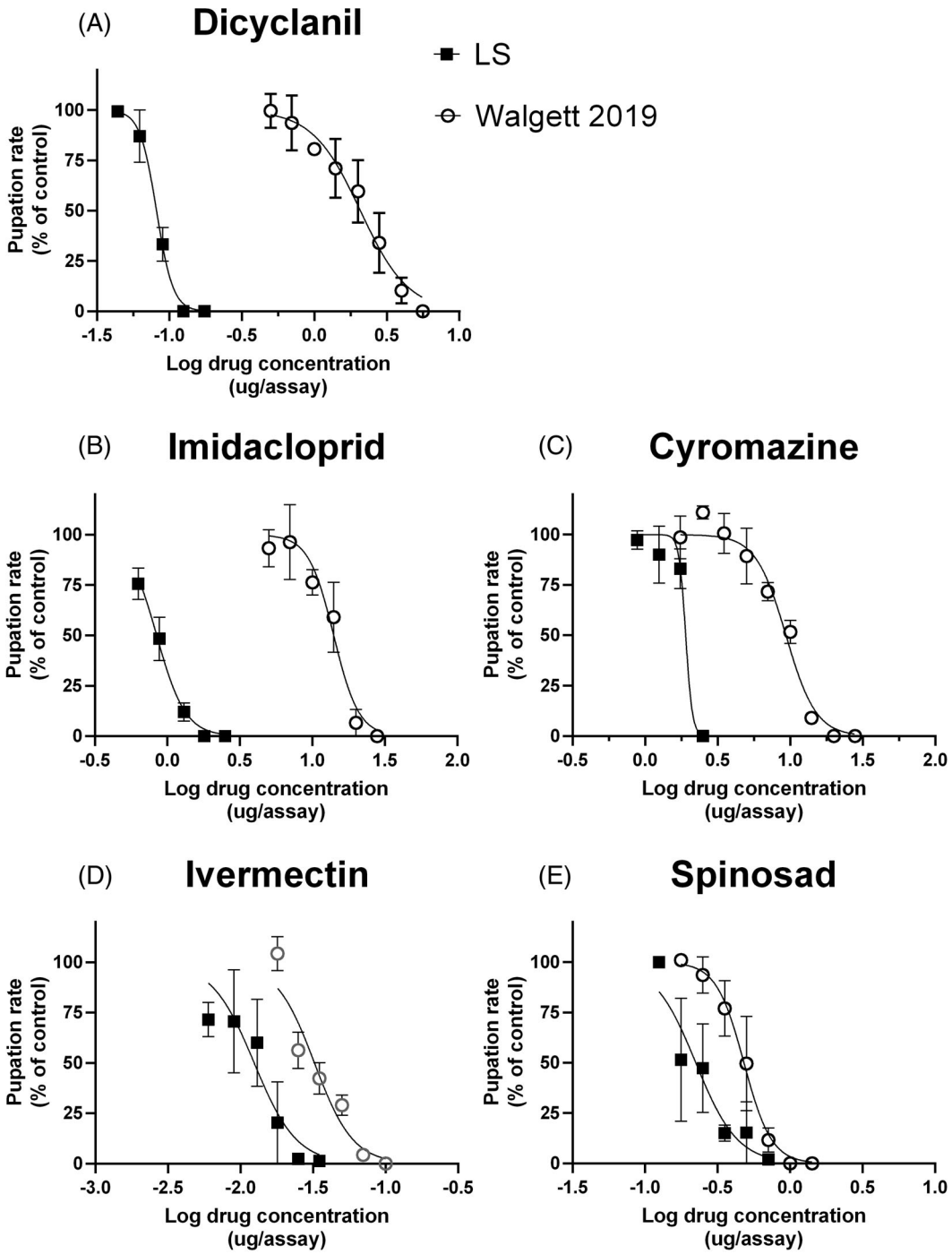

Figure 1. Dose responses of LS and Walgett 2019 G9–10 larvae to dicyclanil (A), imidacloprid (B), cyromazine (C), ivermectin (D) and spinosad (E). Each data point represents mean ± SE, $n = 3$ separate experiments, each with single assays at each insecticide concentration.

Table 2. Synergism of insecticides by piperonyl butoxide (PBO) against Walgett 2019 G9–10 and LS larvae

		Insecticide alone or plus PBO							
		Insecticide alone		Insecticide plus PBO at 250 µg/assay			Insecticide plus PBO at 500 µg/assay		
Insecticide	Strain	IC_{50} (µg/assay)	RF[†]	IC_{50}[‡] (µg/assay)	RF[†]	SR[δ]	IC_{50}[‡,¶] (µg/assay)	RF[†]	SR[δ]
Dicyclanil	Walgett	2.08	26	1.28*	16	1.6	0.83*	10	2.5
	LS	0.081	—	0.088	—	—	nt	nt	nt
Imidacloprid	Walgett	14.0	17	5.0*	5.9	2.8	4.0*	4.7	3.5
	LS	0.84	—	0.40*	—	2.1	nt	nt	nt
Cyromazine	Walgett	9.35	4.9	9.32	4.9	—	5.53*	2.9	1.7
Ivermectin	Walgett	0.032	2.6	0.025	2.0	—	0.013*	—	2.5
Spinosad	Walgett	0.48	2.1	0.31*	—	1.5	0.26*	—	1.8

[†] RF, resistance factor = IC_{50} resistant strain (with or without PBO)/IC_{50} susceptible strain in absence of PBO; RF values only shown if the difference between the two IC_{50} values was significant, as judged by nonoverlap of 95% CI. IC_{50} values for LS with cyromazine, ivermectin and spinosad, in the absence of PBO, are shown in Table 1.
[‡] * symbol denotes that the IC_{50} value in the presence of PBO was significantly different to the IC_{50} for that strain treated with insecticide alone, as judged by nonoverlap of 95% confidence intervals.
[δ] SR = Synergism ratio = IC_{50} in absence of PBO / IC_{50} in presence of PBO in the same isolate; SR values only shown if the difference between the two IC_{50} values was significant, as judged by nonoverlap of 95% confidence intervals.
[¶] nt = not tested (synergism effects assessed only at 250 µg/assay PBO for LS).

thermocycler (Applied Biosystems/Thermo Fisher Scientific, Foster City, CA 94404, USA) was used for quantitative polymerase chain reaction (qPCR) reactions using the Sensifast™ SYBR® Lo-Rox qPCR system (Meridian Bioscience, Cincinatti, OH 45244, USA), with the following PCR cycling conditions: 95 °C for 2 min, followed by 40 cycles at 95 °C for 5 s, 60 °C for 30 s. PCRs were run in quadruplicate. Reaction efficiencies were determined by performing PCRs with seven 5-fold serial cDNA dilutions (Table S1). Melting curve analysis of each primer pair identified the qPCR products to be homogenous. The identity of the *cyp* qPCR products was confirmed by cloning of amplicons into PgemT Easy cells (Promega, Madison, WI 53711, USA), isolation of plasmids, and sequencing using M13 forward and reverse primers (Australian Genome Research Facility, Brisbane, QLD 4072, Australia). BLAST searches confirmed that the qPCR products were the correct target *cyp* gene.

Analysis of the qPCR data was performed by normalizing target gene transcription to the three reference genes.[21] For the life stage data, transcription for each target gene in Walgett larvae was compared separately to the data for LS larvae at the same time point. For assessing the effects of dicyclanil exposure on transcription, the data for each target gene in drug-exposed larvae was compared separately to the data for that gene in control (no drug exposure) larvae. This analysis generated data describing the normalized expression level for each target gene between Walgett and LS larvae over time, or between Walgett larvae exposed or not exposed to dicyclanil. The data were analyzed using ANOVA following $\log_{10}$ transformation. Significant differences between transcription levels of the target genes were identified using Tukey's multiple comparison test at $\alpha = 0.05$.

3 RESULTS

The average pupation rates for control assays with the LS and Walgett 2019 strains were 88% and 80%, respectively, across the course of the study. The pupation rates for control assays in the single experiments conducted with the Walgett 2020 and Walgett 2021 strains were 85% and 88%, respectively.

Walgett 2019 G9–10 larvae showed reduced sensitivity to each of the insecticides compared to the reference susceptible LS strain, with resistance factors at the IC_{50} being greatest towards dicyclanil (26-fold) and imidacloprid (17-fold) (Table 1, Fig. 1). Resistance factors were <5-fold for cyromazine, ivermectin and spinosad. Dicyclanil and cyromazine resistance factors based on IC_{95} values were ≈2-fold higher than the resistance factors based on IC_{50}s (53 *versus* 26 for dicyclanil, 8.5 *versus* 4.9 for cyromazine), reflecting the less steep dose–response curves for both strains compared to LS [Fig. 1(a), (c)]. For imidacloprid, the separate resistance factors based on IC_{50} or IC_{95} values were similar (15 *versus* 17).

We investigated the effect of PBO on the toxicity of the various insecticides to Walgett 2019 larvae, and also examined the effect of the synergist on toxicity of dicyclanil and imidacloprid to LS larvae. Preliminary experiments showed that pupation rates in Walgett larvae were maintained at over 75% at PBO levels of 250 µg and 500 µg/assay (Fig. S1), and hence these two concentrations were used in synergist / insecticide combination experiments to determine if synergism occurred and whether it was dependent on the PBO concentration. These preliminary experiments also showed that LS larvae were more sensitive than Walgett to the presence of PBO alone (Fig. S1), and hence only the 250 µg/assay level was used in combination assays with this strain. The presence of PBO at 500 µg/assay resulted in significant levels of synergism with Walgett larvae for each of the insecticides tested, with synergism ratios of 3.5 for imidacloprid, and 2.5 for dicyclanil and ivermectin (Table 2). However, for dicyclanil, imidacloprid and cyromazine, this synergism by PBO did not reduce the sensitivity of the larvae to levels measured in un-synergized LS strain larvae (Table 2; Fig. 2). Hence, the Walgett larvae retained significant levels of resistance to these three insecticides in the presence of PBO at 500 µg/assay (resistance factors of 10-, 4.7- and 2.9-fold, respectively). On the other hand, Walgett larvae treated with 500 µg/assay PBO were equally sensitive as un-synergized LS larvae to ivermectin and spinosad (resistance factors not significant) (Table 2). A comparison of the datasets for 250 and 500 µg/assay PBO indicated that synergism ratios were

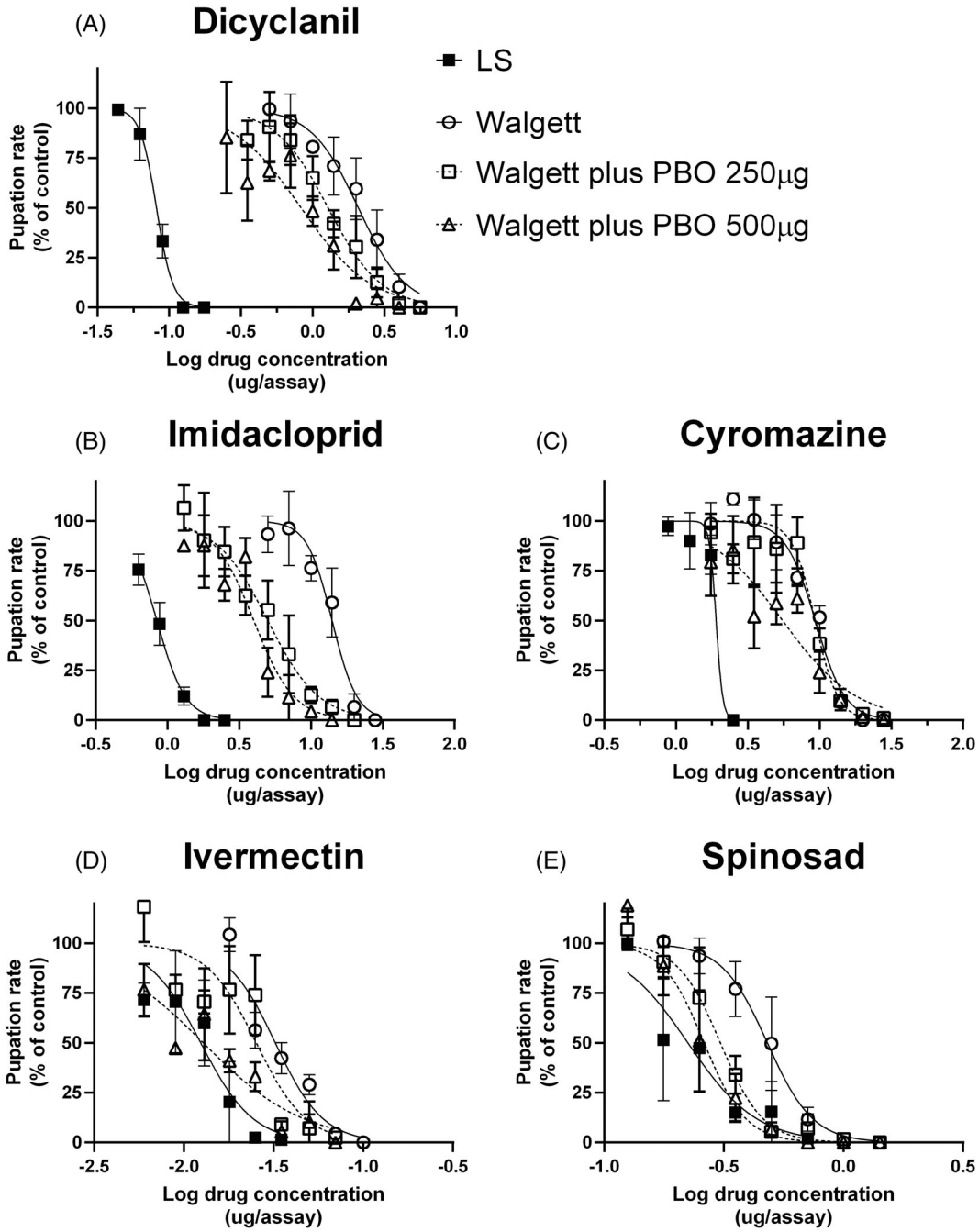

Figure 2. Effect of co-treatment with piperonyl butoxide (PBO) on dose responses of Walgett 2019 G9–10 larvae to dicyclanil (A), imidacloprid (B), cyromazine (C), ivermectin (D) and spinosad (E). Dose responses for LS larvae in the absence of PBO (from Fig. 1) also are shown for comparison. Each data point represents mean ± SE, $n = 3$ separate experiments, each with single assays at each insecticide concentration.

less in each case at the lower PBO concentration, with no synergism observed for cyromazine and ivermectin. PBO did not affect the toxicity of dicyclanil to LS larvae. On the other hand, the synergist had a similar effect on toxicity of imidacloprid to LS larvae as observed for the Walgett strain at this PBO level (SRs of 2.1 and 2.8, respectively).

While performing initial experiments using a wider range of synergists we noted a change in the response of the Walgett strain to several of the insecticides compared to the earlier assays. We investigated this further by measuring sensitivity of Walgett 2019 larvae to dicyclanil and imidacloprid in larvae after three periods of time in laboratory culture (Table 3). The resistance to dicyclanil decreased over time in culture from 26-fold to 12-fold. The resistance to imidacloprid decreased slightly between the first two time periods, however did not differ between the initial and final populations tested (17-fold compared to 13-fold).

Assays with a greater range of potential synergists were performed using Walgett 2019 G18–21 larvae. As described previously for PBO, preliminary experiments determined the concentration of each synergist that resulted in the pupation rate

Table 3. Sensitivity of different generations of Walgett 2019 larvae to dicyclanil and imidacloprid

Insecticide	Blowfly strain[†]	IC_{50} (μg/assay)	95% CI	Resistance factor[‡]
Dicyclanil	LS	0.081	0.076–0.088	—
	Walgett G9-10	2.08	1.73–2.49	26 a
	Walgett G18-21	1.42	1.10–1.81	18 ab
	Walgett G27-29	1.00	0.91–1.10	12 b
Imidacloprid	LS	0.84	0.77–0.92	—
	Walgett G9-10	14.0	12.1–16.3	17 a
	Walgett G18-21	9.36	8.48–10.32	11 b
	Walgett G27-29	11.3	10.2–12.5	13 ab

[†] G = number of generations in laboratory culture.
[‡] Resistance factor = IC_{50} resistant strain/IC_{50} susceptible strain; within an insecticide, resistance factors followed by the same letter are not significantly different, as judged by nonoverlap of 95% confidence intervals for the Walgett IC_{50} values from which the resistance factor values were derived.

being maintained at >75% (Fig. S2). Each synergist then was tested in combination with dicyclanil and imidacloprid. As initial results showed a high level of synergism with aminobenzotriazole, this synergist also was examined in combination with dicyclanil and imidacloprid against LS larvae. Aminobenzotriazole was the most effective of the synergists tested, with synergism ratios of 7.2 and 6.1 for dicyclanil and imidacloprid, respectively (Table 4; Fig. 3). Resistance factors decreased from 18-fold to 2.6-fold for dicyclanil, and from 11-fold to 1.8-fold for imidacloprid after co-treatment with aminobenzotriazole. Aminobenzotriazole did not affect the sensitivity of LS larvae to dicyclanil, whereas the sensitivity to imidacloprid was increased 2-fold. MGK264, ketoconazole and tranycypromine resulted in significant levels of synergism for dicyclanil; however, the magnitude of these effects (synergism ratio values) was much less than for aminobenzotriazole. MGK264 showed a low level of synergism with imidacloprid. The glutathione transferase inhibitor, diethyl maleate, did not show any synergism with dicyclanil or imidacloprid.

Sensitivity to dicyclanil and imidacloprid was measured in larvae collected from the Walgett property over different seasons (Table 5). The comparison involved strains that had been maintained in culture for different lengths of time (number of

Table 4. Effect of various synergists on dicyclanil and imidacloprid IC_{50} values and resistance factors with LS and Walgett 2019 G18-21 larvae

Insecticide	Blowfly strain	Synergist	IC_{50} (μg/assay)	95% CI	Resistance factor †	Synergism ratio ‡
Dicyclanil	LS	None	0.081	0.076–0.088		
		ABT (aminobenzotriazole)	0.086	0.081–0.091	—	—
	Walgett	None	1.42	1.10–1.81	18	
		ABT (aminobenzotriazole)	0.20	0.18–0.23	2.6	7.2
		MGK264	0.82	0.69–0.98	10	1.7
		Ketoconazole	0.82	0.74–0.91	10	1.7
		SKF525A (proadifen)	1.60	1.26–2.02	20	—
		Tranylcypromine	0.85	0.72–1.02	10	1.7
		TCPPE (trichlorophenyl propynyl ether)	1.00	0.78–1.26	12	—
		DEM (diethylmaleate)	1.19	1.02–1.38	15	—
Imidacloprid	LS	None	0.84	0.77–0.92		
		ABT (aminobenzotriazole)	0.42	0.39–0.46	0.5	2
	Walgett	None	9.36	8.48–10.32	11	
		ABT (aminobenzotriazole)	1.53	1.35–1.73	1.8	6.1
		MGK264	4.35	3.26–5.81	5.2	2.2
		Ketoconazole	7.80	6.71–9.07	9.3	—
		SKF525A (proadifen)	7.82	6.86–8.90	9.3	—
		Tranylcypromine	8.80	7.03–11.00	10	—
		TCPPE (trichlorophenyl propynyl ether)	9.19	8.22–10.38	11	—
		DEM (diethylmaleate)	10.35	8.81–12.17	12	—

[†] Resistance factor = IC_{50} Walgett strain in the presence or absence of synergists / IC_{50} LS strain in the absence of synergists; Resistance factor values only shown if the difference between the two IC_{50} values was significant, as judged by nonoverlap of 95% confidence intervals.
[‡] Synergism ratio = IC_{50} in absence of a synergist / IC_{50} in presence of a synergist in the same isolate; Synergism ratio values only shown if the difference between the two IC_{50} values was significant, as judged by nonoverlap of 95% confidence intervals.

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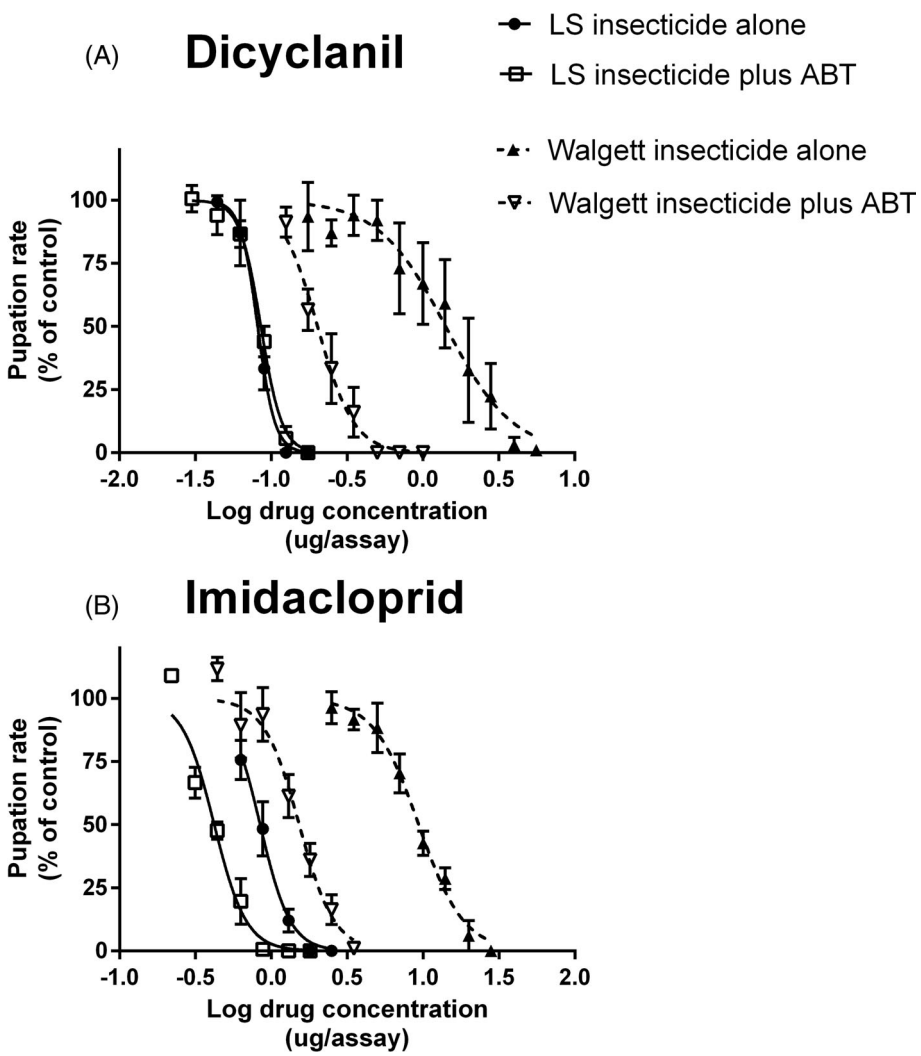

Figure 3. Effect of co-treatment with aminobenzotriazole (ABT) on dose responses of LS (solid lines) and Walgett 2019 G18-21 (dashed lines) larvae to dicyclanil (A) and imidacloprid (B). Each data point represents mean ± SE, $n = 3$ separate experiments, each with single assays at each insecticide concentration.

generations) before the assays being performed. Walgett 2020 (G3) and 2021 larvae (G3) showed a slightly lower level of resistance towards dicyclanil compared to Walgett 2019 (G9-10) (14–15-fold *versus* 26-fold). Resistance to imidacloprid decreased slightly in Walgett 2020, however, did not differ between the Walgett 2019 and Walgett 2021 collections.

An examination of transcription patterns for the four *cyp* genes across life stages for Walgett 2019 G22 and LS larvae showed that *cyp12d1* was the only gene that differed between the two strains at any of the time points examined (Fig. 4). Transcription of *cyp12d1* did not differ between eggs of the two strains, however it was elevated between 20- and 45-fold in larvae at the 24–96 h

Table 5. Sensitivity to dicyclanil and imidacloprid in Walgett larvae collected from the field over three seasons

Insecticide	Year collected[†]	IC_{50} (µg/assay)	95% CI	RF[‡]
Dicyclanil	2019	2.08	1.73–2.49	26 a
	2020	1.21	1.13–1.29	15 b
	2021	1.10	1.01–1.20	14 b
Imidacloprid	2019	14.0	12.1–16.3	17 a
	2020	7.63	7.00–8.33	9 b
	2021	11.9	10.7–13.2	14 a

[a] 2019 collection was assayed at G9-10; 2020 and 2021 collections were assayed at G3.
[b] Resistance factor = IC_{50} Walgett strain/IC_{50} LS strain; within an insecticide, resistance factors followed by the same letter are not significantly different, as judged by nonoverlap of 95% confidence intervals for the Walgett IC_{50} values from which the resistance factor values were derived.

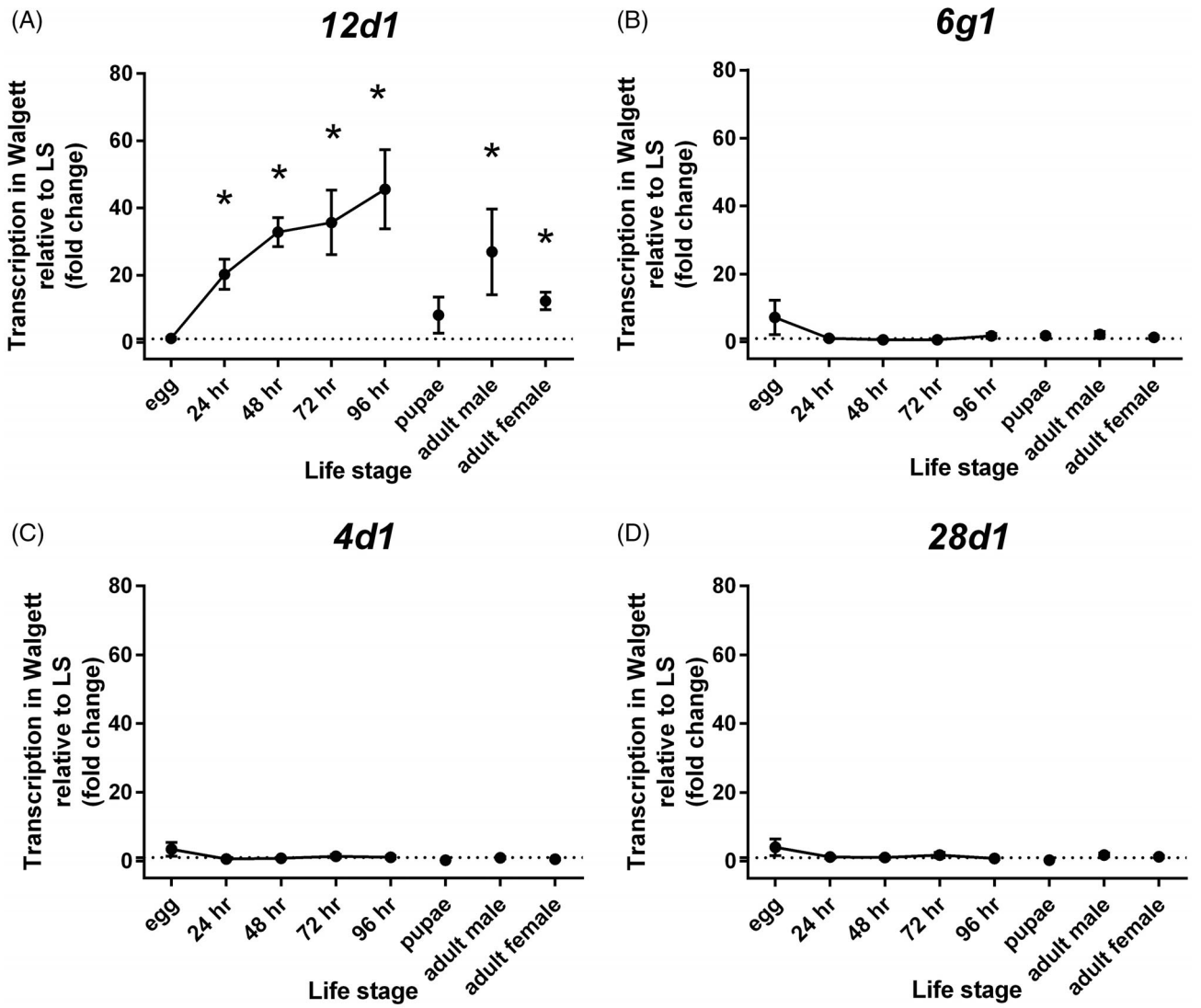

Figure 4. Transcription levels of four *cyp* genes in Walgett 2019 G22 compared to LS in eggs, larvae, pupae and adult flies: (A) *cyp12d1*, (B) *cyp6g1*, (C) *cyp4d1* and (D) *cyp28d1*. Each data point represents mean ± SE, $n = 4$ separate RNA preparations at each life stage, each assayed in quadruplicate qPCRs. * denotes that transcription level in Walgett was significantly different to equivalent life stage in LS ($P < 0.05$); dotted line shown at relative transcription level of 1 (i.e. equivalent transcription in Walgett and LS).

time points. There was no difference in transcription levels in pupae, while 27- and 12-fold increased levels of transcription were observed in Walgett male and female flies, respectively, compared to LS adult flies.

Exposure of Walgett 2019 G26 larvae to dicyclanil for 24 or 48 h did not result in any significant changes in transcription levels for the four *cyp* genes (Fig. S3).

4 DISCUSSION

The present study has described insecticide resistance in a field-derived strain of the sheep blowfly and used synergists and gene expression measurements to investigate the resistance mechanism(s). The Walgett 2019 strain showed reduced sensitivity to each of the five insecticides tested in the *in vitro* assays, with the levels of resistance towards dicyclanil and imidacloprid being of the greatest magnitudes (26- and 17-fold, respectively, at the IC_{50}) whereas those towards cyromazine, ivermectin and spinosad were of only low magnitude (<5-fold). Co-treatment with aminobenzotriazole resulted in significant synergism with both dicyclanil and imidacloprid, implicating cytochrome P450 in resistance to both compounds. The *cyp12d1* gene was expressed at significantly higher levels in Walgett larvae compared to the susceptible LS larvae throughout the larval life stages, suggesting a role for the CYP12d1 enzyme in the observed resistances. Cytochrome P450 enzymes previously have been implicated in resistances to diflubenzuron, deltamethrin and butacarb in laboratory-pressured and field-collected strains of the sheep blowfly.[22–24]

Blowfly control chemicals are most commonly used as prophylactic treatments applied to sheep in advance of expected fly waves. They are described on product labels as being able to protect sheep from flystrike for periods up to specified maximum lengths of time. These protection periods range from 4–6 weeks for spinosad, up to 10–14 weeks for cyromazine, imidacloprid, ivermectin and cypermethrin, and low-concentration dicyclanil products, and up to 29 weeks for a high concentration dicyclanil

product. The impact of resistance in the sheep blowfly on the performance of insecticides in the field is measured in terms of its impact on these protection periods. The question therefore arises as to what impact the observed *in vitro* resistances shown by the Walgett strain might have on product protection periods of dicyclanil and imidacloprid products in the field. Sales *et al.*[5] recently measured protection periods for dicyclanil-, cyromazine- and ivermectin-based products against artificial infections on sheep (larval implants) with resistant blowfly strains. The larvae used for these implant trials showed *in vitro* resistance factors of 32.5-fold and 46.5 fold, which are only marginally higher (1.25–1.79-fold) than the 26-fold resistance shown by Walgett G9–10 in the present study. Given the very significant reductions in protection periods reported by Sales *et al.*[5] (for example, a high-concentration dicyclanil product gave a protection period of <9 weeks compared to the maximum label guideline of up to 29 weeks), it is likely that the Walgett strain also would show reduced protection periods *in vivo*, although this remains to be tested. A reduced protection period is in accord with the field observations made on the property in November/December 2019 (as described in section 2.1), however it has not been confirmed in a controlled *in vivo* trial. It is not possible to determine the impact of the *in vitro* imidacloprid resistance level observed in the present study on the performance of this chemical in the field as there have been no *in vitro* / *in vivo* performance comparisons reported for imidacloprid with blowfly stains showing resistance in *in vitro* assays. It will be important to determine the impact of *in vitro* imidacloprid resistance reported in the present study on protection periods for products based on this chemical.

The resistance to dicyclanil and imidacloprid in Walgett 2019 flies appeared to be quite stable in the absence of insecticide exposure. Maintenance in laboratory culture over a period of approximately 20 generations (from G9–10 until G27–29) in the absence of any exposure to insecticide did not result in any decrease in resistance to imidacloprid, while resistance to dicyclanil decreased only two-fold. Insecticide resistance often is unstable in insect cultures maintained in the absence of insecticide exposure owing to fitness costs associated with the resistance mechanism,[25–27] and influenced by the homozygosity of the population.[28] The observed stability may be a consequence of the Walgett 2019 strain representing an inbred population originating from homozygous resistant survivors of the insecticide treatment in the field (absence of susceptible alleles), rather than representing any lack of fitness costs associated with resistance. Importantly, the resistance stability in the present study is an observation based on maintenance of blowflies under laboratory conditions only. Further experimentation will be required to determine the implications of this observed resistance stability on the resistance patterns shown by field populations of the blowfly.

The ability of P450 inhibitors to synergize different insecticides can vary considerably due, at least partly, to differences in the interactions of the inhibitors with different P450 enzymes.[15,29] Hence, the effects of a number of different inhibitors on toxicity of dicyclanil and imidacloprid to blowfly larvae were examined in the present study. Aminobenzotriazole was the most effective synergist tested, and was able to reduce the resistances towards both dicyclanil and imidacloprid to very low levels (2.6- and 1.8-fold, respectively), with synergism ratios of 7.2- and 6.1-fold for the two drugs, respectively. Aminobenzotriazole is a pan-specific, mechanism-based inhibitor of cytochrome P450 from animals, insects, plants and micro-organisms, inhibiting a broad range of cytochrome P450 isoforms.[30] Interestingly, while the compound significantly reduced the dicyclanil resistance shown by Walgett larvae, it had no effect on the sensitivity of LS larvae to this compound, suggesting that this susceptible reference strain has a negligible ability to metabolize the insecticide. On the other, although aminobenzotriazole showed a similar synergistic effect with imidacloprid and Walgett larvae as that observed with dicyclanil (synergism ratios of 6.1 and 7.2, respectively), it also showed some synergism with the former insecticide and LS larvae (synergism ratio of 2), suggesting that some level of P450-mediated metabolism of imidacloprid occurs in the susceptible reference LS strain. Importantly though, the level of synergism of imidacloprid in Walgett was significantly higher than observed with LS (synergism ratios of 6.1 *versus* 2, respectively), indicating an increased level of metabolism of the drug in the former strain. The ability of LS larvae to metabolize imidacloprid compared to the negligible metabolism of dicyclanil has potential implications for the development of resistance. A pre-drug-exposure ability to metabolize a chemical may more readily facilitate subsequent increases in activity under insecticide selection pressure compared to the response to selection pressure by a chemical towards which an organism shows a negligible level of metabolism before the initial drug-exposure.

Piperonyl butoxide had some effect on the sensitivity of Walgett larvae to both dicyclanil and imidacloprid, however the synergism ratios were less than for aminobenzotriazole. The larvae treated with piperonyl butoxide and either dicyclanil or imidacloprid retained significant levels of resistance to both insecticides (10-fold and 4.7-fold, respectively). Interestingly, as observed with aminobenzotriazole, piperonyl butoxide synergized imidacloprid, but not dicyclanil, with LS larvae, again suggesting an ability of the susceptible larvae to metabolize the former compound only. Although piperonyl butoxide is commonly used as a synergist in commercial insecticide formulations,[31] the levels required to synergize dicyclanil and imidacloprid in the present study (6000- and 600-fold higher than the IC_{50} values against the LS strain), and the significant levels of resistance retained in co-treated Walgett larvae, argue against the commercial use of the compound to restore sensitivity to dicyclanil and imidacloprid in blowflies.

Two cytochrome P450 genes, *cyp6g1* and *cyp12d1*, have been implicated in resistances to imidacloprid and dicyclanil in *Drosophila melanogaster*.[11–13,32–34] The present study found that *cyp12d1* was expressed at significantly higher levels in the resistant Walgett strain compared to the susceptible reference strain throughout the larval life stages. In contrast, expression of *cyp6g1* did not differ between the two strains. This result, alongside the significant impact of aminobenzotriazole on the levels of resistance to both dicyclanil and imidacloprid, suggests that the CYP12d1 enzyme plays a significant role in the observed resistances. However, it remains uncertain whether increased *cyp12d1* expression confers resistance to both drugs, or another P450 is involved in resistance to the second drug. That is, it remains uncertain if the two resistances represent cross-resistance due to a single common cytochrome P450, or independent resistances due to separate P450 enzymes. *Drosophila melanogaster* is known to possess >80 functional P450 genes,[35] and it is likely that this gene family also is extensive in the sheep blowfly. Many different P450 genes have been implicated in insecticide resistances.[15] Hence, a comprehensive study of P450 genes in sheep blowfly strains showing resistances to dicyclanil and imidacloprid is required in order to fully describe the role of this enzyme system in resistance to these two compounds.

Having demonstrated increased expression of *cyp12d1* in Walgett compared to LS larvae, we also were interested in whether

exposure to the drug itself may lead to induction of expression levels. Many cytochrome P450 genes are induced by foreign chemicals, including insecticides.[15] However, exposure of Walgett larvae to dicyclanil at a level just below that which impacted on pupation, as well as a level that reduced pupation to ≈60% of controls, did not result in the induction of any of the genes examined in the present study. The role of cytochrome P450 in resistance to dicyclanil in Walgett strain therefore appears to involve increased constitutive expression rather than induction in response to insecticide exposure, although it remains possible that P450 genes other than those examined here show a transcription response to dicyclanil exposure. Willoughby *et al*.[36] found that exposure of *D. melanogaster* larvae to a highly lethal concentration of dicyclanil for a short period did not result in any induction of the P450, glutathione transferase and esterase genes examined, including *cyp6g1* and *cyp12d1*.

Dicyclanil was released for flystrike control on sheep in 1998. Imidacloprid was released for flystrike much more recently, in 2017, however it was released for lice control on sheep in 2009. Hence, blowfly populations in the field could have been exposed to this chemical on sheep treated for lice over the last 13 years. Therefore, two possible explanations emerge for the *in vitro* resistance shown by Walgett larvae to imidacloprid despite it being used for flystrike control for a relatively short time: first, as a result of selection pressure from exposure to the chemical through lice treatments since 2009, and/or secondly, as a result of selection pressure on a common cytochrome P450-mediated resistance mechanism through many years of exposure to dicyclanil. Further work will be required to clarify these resistance-origin issues.

In conclusion, the present study adds to the earlier reports of Levot[7] and Sales *et al*.[5] in highlighting the emerging issue of resistance to the currently-used insecticides in the sheep blowfly. This issue is particularly significant for dicyclanil given its dominance in the flystrike chemical control market.[3] While the likely impact of the *in vitro* imidacloprid resistance observed in the present study on protection periods for flystrike products is unknown, a comparison of our data with those of Sales *et al*.[5] indicates that the protection period for dicyclanil would likely be significantly reduced for the Walgett strain. Given the current reliance of the sheep industry on insecticidal control of flystrike, there is clearly a need to manage the use of insecticides such that the rate at which resistance develops to the currently-available chemicals is minimized. Determining whether the P450-mediated resistance to both dicyclanil and imidacloprid demonstrated in the present study represents cross-resistance due to common P450 enzymes(s) will be important for designing drug rotation strategies to minimize the rate at which resistance develops. As described by Kotze and James,[2] a component of this capacity to manage resistance will be increased surveillance, thereby allowing chemical-use decisions to be based on knowledge of what resistances exist in localized blowfly populations in order to prevent further selection pressure on specific chemicals once resistance is detected. The present study highlights the possibility of utilizing molecular diagnostics for dicyclanil and imidacloprid resistance based on the observed increase in transcription of the *cyp12d1* gene in the Walgett strain.

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CONFLICT OF INTEREST

Several authors (SG and NR) are paid employees of Elanco Australasia Pty Limited, who provided funding for this study. All authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

SUPPORTING INFORMATION

Supporting information may be found in the online version of this article.

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