Development of an insect development inhibitor for the control of *Bovicola ovis* in sheep

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Summary

Coopers Magnum® IGR, an aqueous based pour-on formulation containing 25g/L diflubenzuron, developed by Schering-Plough at their Prospect Research Laboratories, was registered as an off-shears sheep lice treatment in 1999. The extensive research work on this off-shears pour-on commenced in Australia in 1996 but has not been reported outside the National Registration Authority. The following contribution compiles data generated from formulation selection, dose determination, effect of wetting on efficacy, field trials, period of protection and post-treatment non infection, wool and tissue residues. Scourability and target host, skin and wool safety are presented as are details of some extensions to claims in relation to use on lambing ewes.

Keywords

Diflubenzuron, Magnum, sheep lice, sheep, off-shears lousicide

Introduction

Chitin synthesis inhibitor's (CSI's) are broadly classified in the group of compounds known as Insect Growth Regulator's (IGR's) and are used for the control of numerous insect pests. IGR's were developed from the study of juvenile hormone, the hormone responsible for regulating the advancement in development of the insect larvae to the adult form, in the 1960's in Cambridge. These juvenile hormone analogues were based on the chemical structure of juvenile hormone and were used to prevent moulting and arrest development in insect larvae, thus being termed insect growth regulators.

Two main concepts underlie the success of the development of a CSI. Firstly, emerging resistance to existing chemicals has lead to the need to identify products that target the insects' natural growth pattern. Secondly and more importantly, is the need to develop chemicals that have high target pest specificity, both in terms of preventing toxicity to beneficial arthropod and mammalian organisms and, to provide a satisfactory chemical for human use from an occupational health and safety perspective.

Diflubenzuron (N-[[(4-chlorophenyl) amino] carbonyl]-2,6-difluorobenzamide) was first released as Dimilin®, in 1972 by the Dutch company Duphar. It has since been shown to control mosquitoes and other Diptera, certain Lepidoptera, Coleoptera and Pthiraptera. In Australia Hoechst Roussel Vet released diflubenzuron (DFB) as a suspension concentrate for the control of sheep body lice as Fleececare® for plunge and shower dipping in 1993 and Schering-Plough Limited (SPAH) as Coopers followed with Strike® Sheep Dip being registered in 1996.

This contribution will attempt to collate the development work conducted from 1996 through to 2001 that has assessed the safety and efficacy of a DFB based pour-on formulation marketed by Schering-Plough Ltd as Coopers Magnum® IGR pour-on.

Diflubenzuron mode of action

DFB belongs to a class of compounds called benzoyl phenyl ureas (BPU's). It is considered essentially non toxic to mammals with rat oral and dermal LD_{50}'s reported as >4640 mg/kg and >10000 mg/kg respectively (Mallinckrodt Veterinary Ltd., 1997). Benzoyl phenyl ureas in general are believed to act as insecticides by inhibiting the enzyme chitin synthetase (Ivie, 1978). While normal chitin production in the parasitic nematode *Ascaris suum* is disrupted by DFB, it also disrupts the development of *Haemonchus contortus* larvae, which lack chitin (Fetterer *et al*., 1989). Nakagawa *et al*. (1996) concluded from work with *Periplaneta Americana* that DFB was able, through phosphorylation, to specifically disrupt integumental proteins critical for insect structural integrity and not just development.

This finding is supported by the many DFB trials that comprise the development discussed here, where the rapid falls in lice numbers can only be explained by significant losses not only in the nymph population, but in the adults as well. Thus, DFB may disrupt more functions within *Bovicola ovis* than chitin synthetase production.

Relative and absolute DFB efficacy - Pen trials 1996 -1997

By 1996 there had been dramatic increases in the incidence of sheep lice resistant to the synthetic pyrethroid classes of chemicals delivered as pour-ons (Morcombe and Young, 1995). The need for treatments delivering greater efficacy was apparent, as strategies for management of lice had traditionally been pyrethroid or organophosphate pour-on or dips. At that time, the recent release and favourable results of the chitin synthesis inhibitor (CSI) triflumuron sold as Zapp® by Bayer, had indicated the markets preparedness to accept treatments that did not possess rapid knockdown in lice numbers.

The first study of the DFB pour-on development (SPAH Report 98/0467) examined a range of formulations' efficacy against a significantly pyrethroid resistant strain (Resistance Ratio compared to Peak Hill strain of 18) of *Bovicola ovis*. To differentiate between formulations, an expectedly sub lethal dose of 125 mg DFB per head was used in one of three formulation types; either an aqueous based suspension, a Zapp® type solvent system or a Zapp® type solvent system with water miscible added surfactants. Lice burdens on the sheep were assessed pre treatment and 2, 6 and 10 weeks post-shearing and treatment. At 10 weeks after treatment the lice reductions recorded for the aqueous, solvent and solvent plus surfactant formulations were 93.3%, 78.5% and 73.8% respectively.

The subsequent study (SPAH Report 98/0468) examined the aqueous and solvent plus surfactant formulations at a dose range titration of DFB from placebo through to 500 mg/hd. Twelve groups of 5 heavily lice infected sheep were treated with a range of concentrations of DFB off-shears and lice burdens assessed 2, 5, 10, 15 and 20 weeks after treatment. The mean bodyweight of the experimental sheep was

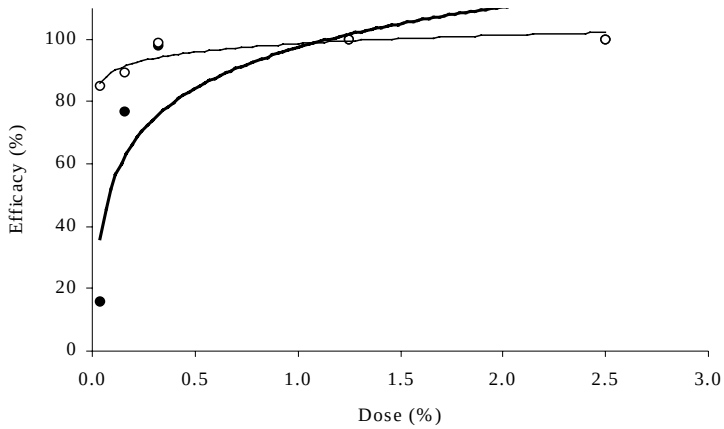

Figure 1. Efficacy of DFB (corrected % reductions) with either a Zapp type solvent (Group C) (○) or an aqueous based solvent (Group D) (●).

40.1 kg at study commencement and at completion 47.7 kg. As the lice reductions with both formulations were found to be bioequivalent (see Figure 1) and, on the grounds of potentially higher handler safety, lower wool and meat residues, cost and marketability, the decision was made to continue the development with the aqueous formulation. The data from this lead formulation is presented in Table 1.

With a predicted LD_{95} of less than 125 mg per head in these adult sheep, the development proceeded with some confidence that a target dose of 500 mg per 50 kg sheep would allow a satisfactory efficacy use margin to be confirmed by the field use phase of the project. As this was the first aqueous based IGR pour-on formulation to be developed it is the subject of an international patent application.

Table 1. Percentage reductions in lice numbers over time after treatment with increasing concentrations of DFB in an aqueous formulation (corrected percentage lice count reductions) [1].

Formulation concentration	DFB mg/hd	14 DAT[2]	36 DAT	70 DAT	108 DAT	140 DAT
2.5%	500	66.7	83.4	94.8	96.4	100.0
1.25%	250	91.0	94.9	98.9	100.0	100.0
0.625%	125	86.9	93.7	98.8	99.4	98.3
0.156%	31.2	81.7	93.0	96.6	86.3	70.8
0.039%	7.80	70.0	61.1	73.0	81.1	29.6

[1]corrected % lice count reductions = [1-(T2/T1 x C1/C2)] x 100 where T = Treatment mean, C = Control mean
and 1 and 2 refer to pre and post treatment mean lice counts respectively
[2]DAT = days after treatment

Rainfast efficacy – Pen trial 1997

Rainfast efficacy was examined during a pen trial (SPAH Report 98/0291) using five groups of six heavily lice infected adult Merino sheep. Sheep were either untreated and left dry, untreated and wetted, wetted before treatment with 500 mg DFB/head, wetted after treatment with 500 mg DFB/head or treated and left dry. 'Wetting' consisted of the sheep being exposed to 25 mm of artificial rain over a period of an hour simulating storm type rain. At the final sheep examination 20 weeks after treatment, both wetted groups demonstrated post treatment lice reductions of 100%, confirming a rain fast claim for the product.

Field efficacy – 1997 - 1998

To confirm earlier formulation findings and, to examine product use in the field in a range of sheep types, climatic regions and husbandry situations, field trials were conducted on farms from the Northern Tablelands of NSW to the Mallee of South Australia. The 6 trials treated a total of 6996 lice infected Merino sheep ranging from strong wool strains to superfine with the selected aqueous 25 g DFB/L pour-on formulation, now called Magnum®. At each site 25 tracer sheep were identified and assessed for lice at forty fleece partings before treatment and 6, 12 and 20 weeks post treatment. Table 2 presents features of each trial site.

Table 2. Details of each field lice efficacy site.

Report number	Location	Sheep type	Wool type	Number
98/0287	Uralla NSW	Wethers	Fine 19 micron	1309
98/0289	Crookwell, NSW	Wethers	Fine 20 micron	680
98/0293	Guyra	Ewes, wethers	Superfine 17 micron	2000
98/0294	Coonalpyn, VIC	Ewes	Medium 23 micron	1101
98/0295	Lucindale, SA	Ewes	Strong 26 micron	1094
98/0466	Lismore, VIC	Wethers	Fine 20 micron	812

At each field site (see Table 3) greater than 99.6% reductions over pre-treatment counts were seen within 6 to 7 weeks of treatment as presented in Table 3.

Table 3. Details of % lice efficacy of a Magnum® pour-on formulation at each of 6 field sites.

Report number	Location	Assessment weeks post treatment		
		6 WAT	12 WAT	20 WAT[1,2]
98/0287	Uralla NSW	99.9[3]	100	100
98/0289	Crookwell, NSW	99.6	100	100
98/0293	Guyra	100	100	100
98/0294	Coonalpyn, VIC	99.6	100	100
98/0295	Lucindale, SA	99.7	100	100
98/0466	Lismore, VIC	99.6[3]	100	100[4]

[1]WAT = weeks after treatment
[2]Data from 50 sheep, as during the final inspection at each site when and if no lice were found on the 25 tracers a further 25 sheep selected on sign of wool derangement were examined.
[3] Inspection conducted 7 WAT [4] Inspection conducted 23 WAT

Scourability of treated wool – 1997

Colouring agents are included in the Magnum® formulation to allow verification that all sheep have been treated. In order to maintain the processing quality of the wool from treated sheep, it is important that the colouring agent fades and that any residual dye is able to be scoured out of the wool during processing procedures. A study examining the rate of colour fading and scourability of the food grade soluble dyes included in the Magnum® formulation involved 40 sheep divided into 4 groups of 10 sheep per group (SPAH Study 98/0290). Three groups of sheep were treated and one group was left untreated off-shears and used to provide wool for comparison. Wool samples were taken in strips at the treatment site along the backline between the poll and the rump. Testing was carried out according to AS 4054-1992 by The Australian Wool Testing Authority in Melbourne. Results are expressed on a Staining Scale Rating where 1 is severe staining and 5 is no staining. While no dye was visible during wool collection from sheep 30 days after treatment, of the 10 samples collected, after scouring 8 scored 5 (no staining) and 2 scored 4-5 (very little staining). In the 60 days post treatment sampling group all 10 scoured samples rated 5. The 90 days after treatment group were not sampled, as the results had already demonstrated that the dye used had suitable degradation and scouring characteristics.

Tissue residues - 1997

Forty merino sheep were divided into 7 groups of 5 sheep, shorn and treated with the Magnum® pour-on at the maximum dose rate possible from the dose break table, equal to 20 mg DFB/kg (SPAH Report 97/0166). One group of sheep was slaughtered on each of days 1, 3, 7, 14, 21, 42 and 84 days post treatment and tissue samples of muscle, subcutaneous pre femoral fat, peri-renal fat, lumbar fat, liver and kidney were collected for residual DFB analysis.

The maximum residue limit for diflubenzuron in sheep meat or edible offal is 0.05 mg/kg.

Table 4 presents the results of the tissue analysis for DFB by high performance liquid chromatography using UV spectrophotometric detection.

All samples of Lumbar fat were found to be below the Limit of Detection 84 days after treatment.

The tissue levels were compared to the Maximum Residue Limit for DFB and the Withholding Period and Export Slaughter Interval was set at 42 days.

Table 4. Residues of DFB (mg/kg tissue) in 5 samples of tissue in sheep treated with Magnum®.

Tissue	1 DAT[1]	3 DAT	7 DAT	14 DAT	21 DAT	42 DAT
Liver	1 sample 0.02 the rest < LOD	All samples < LOD	All samples < LOD	1 sample 0.03 all the rest < LOD	All samples < LOD	All samples < LOD
Kidney	All samples < LOD	All samples < LOD	All samples < LOD	All samples < LOD	All samples < LOD	All samples < LOD
Muscle	All samples < LOD	All samples < LOD	All samples < LOD	1 sample 0.02 the rest < LOD	All samples < LOD	All samples < LOD
Peri renal fat	2 samples 0.03, 1 sample 0.02 and 2 < LOQ	1 sample 0.02 and the rest < LOQ	2 samples at 0.02 and the rest < LOQ	1 sample at 0.05, 2 < LOQ and 2 < LOD	1 sample < LOQ and the rest < LOD	All samples < LOD
Pre femoral fat	2 samples at 0.02 and 3 samples < LOQ	1 sample at 0.04, 2 at 0.02 and 2 < LOQ	1 sample at 0.03, 2 < LOQ and 2 <LOD	1 sample at 0.36, 1 at 0.02, 1 < LOQ and 2 < LOD	1 sample at 0.05 and the rest < LOQ	All samples < LOD
Lumbar fat	All samples < LOQ	1 sample at 0.04, 1 at 0.02, 2 < LOQ and 1 < LOD	1 sample at 0.03, 1 at 0.02 and 3 < LOQ	1 sample at 0.03, 3 at 0.02 and 1 < LOQ	1 sample at 0.03, 2 < LOQ and 2 < LOD	2 samples < LOQ and 3 < LOD

[1]DAT = days after treatment
LOQ = limit of quantitation = 0.02 mg/kg
LOD = limit of detection = 0.005mg/kg

Target host safety studies – 1998

Indirect target host product safety data is obtained by examining all animals on each occasion of treatment application during the course of a development. By the end of 1998, 7220, primarily Merino ewes, wethers and lambs, had been treated with the Magnum® formulation. These studies were conducted under a range of environmental conditions, seasons and farm managements. Investigators observed all sheep for 10 minutes after treatment and inspected, in conjunction with co-operators, both the skin and wool of the treated sheep at each handling event. Over the course of 14 pen and field trials no adverse reactions to the treatment were reported.

A specific safety study (SPAH Report 98/0292) was conducted to examine the effect of overdosing with Magnum® using 4 groups of 10 uniform Merino wethers on the point of shearing well acclimatized to a feeding and housing regime. Baseline observations of behaviour associated with skin irritancy were made on days –14, -7 and –1. On day 0, each group of sheep was shorn and the same observations were made 1, 3, 6 hours, 1, 2, 7 and 14 days post treatment with either a placebo, 1x, 3x or 5x the recommended dose of Magnum®. Feeding behaviour and body weights were also recorded. In the highest treatment level group (5x the recommended dose) there was no evidence of reaction to the treatment, nor were there any lesions or skin damage at the site of treatment application. Animal feed intakes were normal and there was no difference between the weight gain of the treated and untreated animals during the study period.

Wool residue studies - 1998

Wool residue studies have been conducted both in house by Schering-Plough (SPAH Report 97/0168) and externally as cited by Savage (1998) and are expected to be reported elsewhere in these proceedings.

Quarantine period – 2000

There are two stages to the establishment of a *Bovicola ovis* infection on a recipient sheep. Initially, sufficient numbers of lice must transfer. All stages are capable of transferral, but adult and stage III nymphs predominate (Murray, 1968). Once on the new host, these lice must then succeed in reproducing. Whilst DFB has more knock down capability than most IGRs, lice do persist for a period on treated sheep. Are persisting lice capable of transferral from treated sheep and can they establish an infection on lice free untreated sheep?

To examine the infectivity and then define a suitable quarantine period required for Magnum®, lousy sheep were shorn and treated off-shears and confined for 10 days with separate untreated, lice free recipient sheep, at either 3, 6 or 9 weeks after treatment. The mean arithmetic lice counts of the 10 sheep treated with Magnum® were 155.4 pre-treatment and decreased to 4.3, 2.5, 0.7, 1.1 and 0.0 at 3, 6, 9, 12 and 20 weeks after treatment respectively (SPAH Study 00/026). Whilst low numbers of lice were found on sheep in the 6 weeks after treatment group at one post-challenge inspection (Table 5), no lice were able, ultimately, to establish in any of the potential recipient sheep.

Table 5. Arithmetic mean lice counts in untreated recipient sheep challenged with sheep treated off-shears with Magnum® at either 3, 6 or 9 weeks after treatment.

Challenge time after treatment	Pre-challenge lice counts	Assessment of recipient sheep (weeks post challenge)			
		1 WAC	6 WAC	12 WAC	20 WAC[1]
3 Weeks	0.0	0.0	0.0	0.0	0.0
6 Weeks	0.0	0.2	0.2	0.2	0.0
9 Weeks	0.0	0.0	0.0	0.0	0.0

[1]WAC = weeks after challenge

During a field study at Uralla (SPAH Report 00/0251) 750 Merino pregnant and lice infected ewes were treated with Magnum® off-shears. Pre-treatment mean lice counts for these ewes were 29.6 pre-treatment and 0.08, 0.16 and 0.08 when inspected 9, 14 and 20 weeks post treatment respectively. Lambing commenced 10 days after shearing and treatment of the ewes and continued for 6 weeks. The first lice counts were conducted on the mixed age lambs at the 14th week after treatment assessment of the ewes. No lice were found on this occasion, at the 20th week inspection, or at a final inspection, when the lambs were approximately 7 months of age.

Protective period studies – 2000 - 2001

In addition to defining lice transferral and a likely 3 week quarantine period after treatment, the potential for transferral of lice from lousy untreated sheep to treated sheep at various times after treatment (protection) was also examined.

The protection period offered to sheep treated with Magnum® was assessed by preparing 3 groups of 10 sheep treated with Magnum® and 3 groups of 10 shorn but untreated sheep. Each group of 10 treated or untreated sheep were confined (challenged) for 10 days with 5 moderately lice infested sheep at either 8, 12 or 16 weeks after treatment and monitored for 20 weeks after challenge (SPAH Report No. 00/0300). The results (Table 6) suggest that sheep can be challenged with lice infested sheep for at least 12 weeks after treatment without the lice population being able to establish on the treated sheep.

Table 6. Arithmetic mean lice counts and % reductions from untreated controls for sheep treated off-shears with Magnum® and challenged with lousy sheep at either 8, 12 or 16 weeks after treatment.

Challenge time after treatment	Treatment		Pre-challenge lice counts	Assessment weeks post challenge			
				3 WAC[2]	6 WAT	12 WAT	20 WAT
8 WAT[1]	UTC[3]	Mean	0.0	2.7	6.4	39.6	328.4
8 WAT	DFB	Mean	0.0	1.5	0.9	1.1	1.8
		% Reduction		**44.4**	**85.9**	**97.2**	**99.5**
12 WAT	UTC	Mean	0.0	3.0	10.5	51.3	208
12 WAT	DFB	Mean	0.0	0.4	0.7	0.6	3.0
		% Reduction		**86.7**	**93.3**	**98.8**	**98.6**
16 WAT	UTC	Mean	0.0	1.9	19.2	71.5	342.4
16 WAT	DFB	Mean	0.0	1.0	1.1	10.0	92.1
		% Reduction		**47.4**	**94.3**	**86.0**	**73.1**

[1]WAT = weeks after treatment [2]WAC = weeks after challenge [3]UTC = Untreated control

Both groups of this study challenged at 12 WAT are being maintained to determine the relative lice counts and the economic value of the fleeces at the following shearing.

Another source of challenge to treated sheep is in ewes that are challenged from their lambs that are lousy at the time that their mothers are treated, but themselves are not treated. A pen efficacy study was designed to examine the possible transferal of lice from untreated lousy lambs to ewes after treatment with a Magnum® formulation (SPAH Report No. 00/0266). This represents a constant challenge from lousy sheep for 20 weeks after treatment. Lice were not able to transfer from lousy lambs to ewes and establish an infestation up to 20 weeks after treatment of the ewes (Table 7).

Table 7. Arithmetic mean lice counts in ewes treated off-shears with Magnum® and challenged for up to 20 weeks after treatment by their untreated lambs.

Group	Pre-treatment lice counts	Assessment weeks post treatment of ewes				
		4 WAT	8 WAT	12 WAT	16 WAT[1]	20 WAT[1]
Treated Ewes	145.1	1.6	0.7	0.1	0.1	0.1
Untreated Lambs	43.0	11.0	10.7	3.5	5.0	17.0

[1]WAT = weeks after treatment

This evidence appears to support the previous findings that sheep can be challenged with lousy sheep at least 12 weeks after treatment with Magnum® without the lice being able to establish.

Ewe and lamb studies – 2000 - 2001

As shearing and lambing intentionally and, often unintentionally, coincide, the efficacy of Magnum® in that management environment needed to be evaluated. A pen study and five field studies were conducted with the objective of examining efficacy in ewes and lambs.

The pen study (SPAH Report 00/0266) (*loc cit*) utilized 2 groups of lice infected ewes with 6 to 12 week old lambs at foot. Ewes in group 1 were shorn and treated with Magnum® along with their unshorn lambs and in the second group, the ewes were shorn and both ewes and lambs were left untreated (Table 8).

Table 8. Arithmetic mean lice counts for ewes treated off shears with Magnum® and concurrent treatment of their lambs with Magnum® or for ewes and lambs both left untreated.

Group	Pre- treatment lice counts	Assessment weeks post treatment of ewes				
		4 WAT	8 WAT	12 WAT	16 WAT	20 WAT[1]
1.Treated ewes	145.7	0.9	0.1	0.1	0	0
1.Treated lambs	48.3	1.5	0.5	0	0	0
2.Untreated ewes	150.5	37.3	37.3	19.3	68.0	126.7
2.Untreated lambs	46.8	45.8	23.8	9.8	12.1	53.0

[1]WAT = weeks after treatment

Efficacy was then examined at five field trials, with a total of 2949 lice infected Merino ewes and their 2492 lambs being treated with Magnum®. At each site 25 tracer ewes and lambs were identified and assessed for lice at forty fleece partings before treatment and at 4, 8, 12, 20 and, in one case, 25 weeks post-treatment. Features of each trial site are presented in Table 9 and the results of Magnum® use at each field site are summarized in Table 10.

Table 9. Details of each field lice efficacy site.

Report number	Location	Number of Ewes treated and type	Number of lambs treated, type and age at treatment	Time of lamb treatment
00/0251	Uralla NSW	750, Merino 19 micron	750, Merino 1.5-7.5 weeks of age	Lambs treated 64 days after ewe
00/0260	Ararat, VIC	570, Merino 20.3 micron	452, Crossbred 2-6 weeks of age	Concurrent with ewe
00/0225	Canberra, ACT	900, Merino 19-20 micron	650 Merino 4-9 weeks of age	Lambs treated 83 days after ewe
00/0228	Sandalwood, SA	560 Merino 22-23 micron	500 Merino 7-10 weeks	Concurrent with ewe
00/0203	Barry, NSW	169 Merino	140 Cross bred 2-12 weeks	Concurrent with ewe

Table 10. Arithmetic mean lice counts of ewes treated off shears with Magnum® and their lambs also treated with Magnum®.

Location	Sheep type	Assessment weeks post treatment				
		Pre treatment	4 WAT	8 WAT	12 WAT	20 WAT[1]
Uralla NSW	Ewes	41.0	NR	0.2	0.1	0.1
	Lambs	NR	0.0	0.1	NR[2]	0.0[3]
Ararat, VIC	Ewes	75.9	0.4	0.0	0.0	0.0
	Lambs	0.2	0.12	0.08	0.0	0.0
Canberra, ACT	Ewes	110.8	0.2	0.1	NR	0.0
	Lambs	0.0	NR	0.0	0.0	0.0
Sandalwood, SA	Ewes	187.2	0.2	0.1	0.0	0.0[4]
	Lambs	0.0	0.4	0.2	0.0	0.0[4]
Barry, NSW	Ewes	226.0	13.46	NR	2.7	1.1
	Lambs	27.4	5.5	NR	0.4	0.2

[1]WAT = weeks after treatment [2]NR = not recorded at this time
[3]Inspection 24 WAT [4] Inspection conducted 25 WAT

In all cases, other than at the Barry site where the ewes were not cleanly shorn, efficacy against lice was demonstrated in ewes treated with Magnum® off-shears and in lambs treated with Magnum® with up to 12 weeks wool at the time of treatment.

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