Final report

Development of a dipstick method for on-farm diagnosis of Haemonchus infections in ruminants

Project code: B.AHE.0003

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Executive summary

The project developed a method to remove the effect of pasture on scores in the Haemonchus field test for faecal occult blood by boiling diluted samples for 15 minutes, or placing diluted samples in an esky containing boiled water for 30 minutes. When applied to experimentally infected sheep running at pasture in Armidale a score of 4 was associated with haematocrit < 22 and (worm egg count) WEC > 20,000 indicating imminent risk of death from haemonchosis. A score of 3 was associated with a significantly lower haematocrit than occurred in sheep with lower test scores and with WEC > 5000, indicating the need to drench sheep for Haemonchus infection. The test was applied to experimentally infected sheep running at pasture near Albany WA before the development of the boiling method. Nonetheless, there was little effect of pasture greenness on scores in uninfected sheep. More importantly, WECs and haematocrits indicative of a need to drench sheep for haemonchosis were recorded at lower test scores than seen in Armidale. A trial conducted by QDPI&F on 2 properties in Queensland gave comparable results to those seen in Armidale.

A trial of the test on boiled samples showed that infection with *Fasciola hepatica* resulted in scores less than 3. Experimental infection with Mycoplasma ovis did not cause positive test results.

The project achieved its objectives with the exception of a consistent test score for the decision to drench. These data were obtained from sheep undergoing experimental infections on two research properties. A meeting with Ancare, MLA, AWI and research provider representatives on 15th May 2007 concluded that further results are required from application of the test to samples collected from farms in diverse geographic and environmental regions to confirm the scores from which final drench decision advice can be formulated. The study is currently being designed, together with a short study on the effect of storage of whole faeces on results obtained with the boiling method. The review expressed a strong view that the test continued to show good evidence of final suitability for use in the field, provided the field trial gave satisfactory results. The final drench decision advice would be determined by performance of the test in the field trial. The ability to remove green pasture effect by boiling samples diluted for assay should greatly simplify the drench decision aid from earlier drafts that included complex corrections for pasture colour.

Examiners queries on the patent have been satisfied and we are advised by Spruson and Ferguson that a patent will be issued.

The project addressed the following issues.

1. Examine the capacity of the Haemonchus field test to identify sheep at risk of death from haemonchosis
2. Refine decision points for interpretation of the test results
3. Provide data for input to a drench decision aid
4. Develop methods for removing the non-specific effect of green pastures on test results
5. Assess cross reactivity of experimental *Mycoplasma ovis* infection with Haemonchus field test in a pen experiment. Assess interference from liver fluke infection in the Haemonchus field test

To address these issues, a field trial was conducted in parallel in Armidale and Albany. Studies on the interaction between liver fluke and Haemonchus test scores were undertaken at EMAI and a field study of Haemonchus test scores on commercial properties was undertaken by QDI&F.

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MLA is committed to investing in top quality scientific research, performed by suitably qualified, experienced and registered researchers and organisations. In experiments that involve livestock, MLA acknowledges that such research needs to be done under the auspices of a recognised Animal Care and Ethics Committee (AEC). The responsibility for obtaining AEC approval lies with the researcher. MLA has in the past not specifically asked for evidence that such AEC approval had indeed been obtained.

1. CSIRO Armidale field trial

Twenty 7-month-old Merino weaners with a light gastrointestinal nematode parasite infection acquired in the field were used. Weather conditions for the period of the experiment and the preceding months are presented in Table 1.

Table 1. Weather data for Armidale airport during the experimental period.

	Nov 06	Dec 06	Jan 07	Feb 07	To 15 March 07
Ave minimum	8.5	9.9	12.7	12.7	12.4
Ave maximum	24.1	24.5	27.7	25.9	24.3
Rainfall mm	111.2	64.2	98.6	120.6	68.4

To ensure heavy Haemonchus infections, 14 sheep were orally dosed with 7,000 Kirby strain Haemonchus contortus larvae on day 1 (18th January 2007). Six control sheep were drenched orally with Cydectin on day 1 to remove the field infection. Faecal samples were collected twice per week for WEC and dipstick test at 1/1000 and 1/2000 dilutions. Sheep were run on pasture dominated by couch grass which provided a short green pick, and they were supplemented with sheep pellets (300g three times/week). The green pasture induced false positive dipstick test result in worm free control sheep. This enabled us to examine the effect of boiling samples diluted for the dipstick test as a means of removing the non-specific activity that causes false positive results. In samples boiled for 15 minutes from sheep with WEC = 0 the non-specific activity due to pasture was decreased from 2.87 ± 0.095 to 1.34 ± 0.122 (n= 31, P<0.000, students paired t test, Figure 1). The method proved to be a reliable way of reducing the background effect of pasture on dipstick scores. A one ml aliquot of sample at the final dilution (1/1000 and 1/2000) in water for use in the test was placed in a 2ml Eppendorf tube and floated in a foam raft on the top of boiling water in an electric saucepan with a thermostat (deep fryer) and boiled for 15 or 20 minutes. The samples were removed from the saucepan and allowed to cool for 5 minutes before the test was performed (See appendix for standard operating procedure). Placing the raft of samples in freshly boiled water in an esky for 30 minutes was equally effective. Temperature in the esky after 30 minutes was 95C. Dipstick scores for samples boiled for 15 or 20 minutes or held in the esky for 30 minutes were not significantly different from each other but all differed from unheated samples (n=20, WEC ranged from 0 to 18,200; Figure 2). Objective 4 was therefore achieved.

Figure 1. Effect of boiling the final dilution of sample on dipstick scores

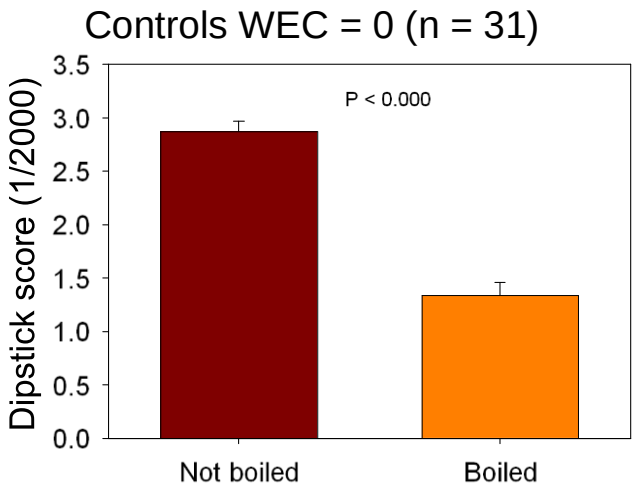
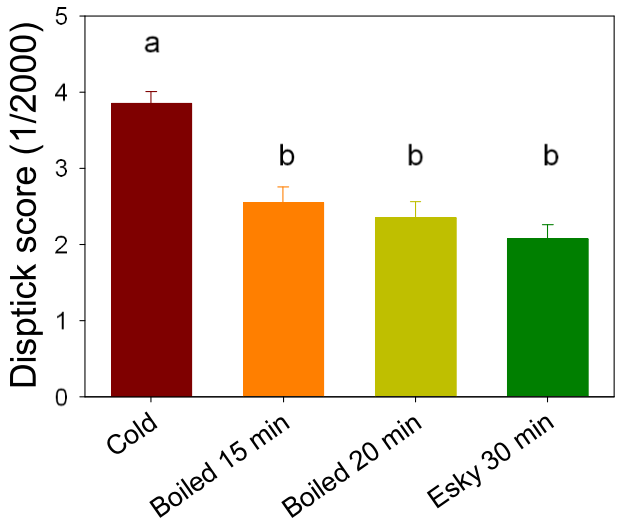
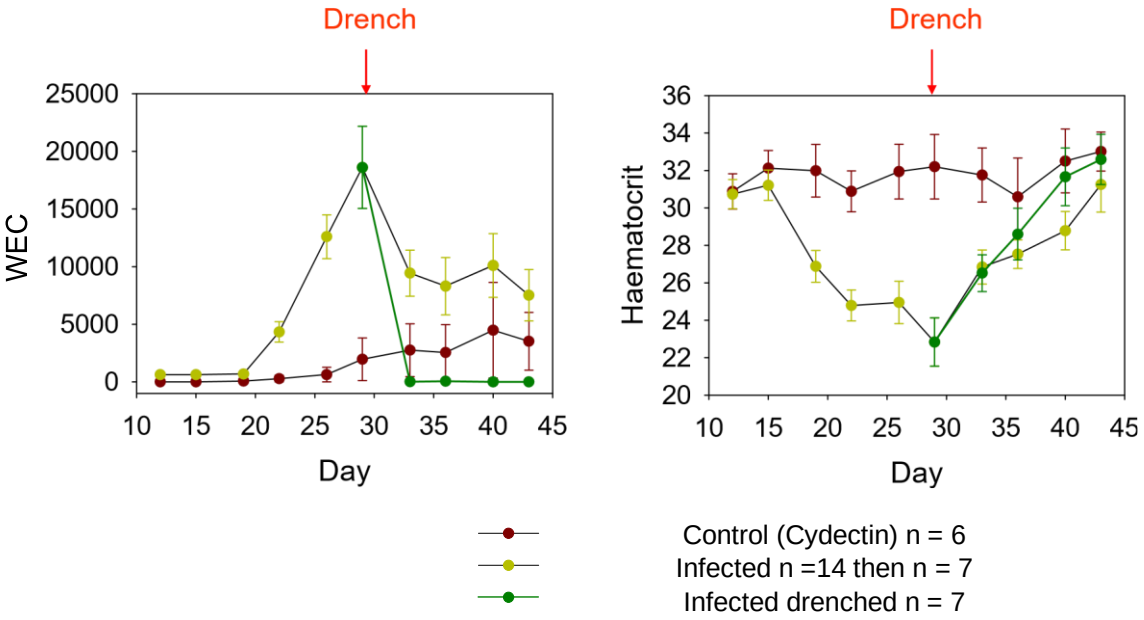


Figure 2. Effect of various methods of heating samples on dipstick scores. Columns with different superscripts are significantly different (P<0.05)



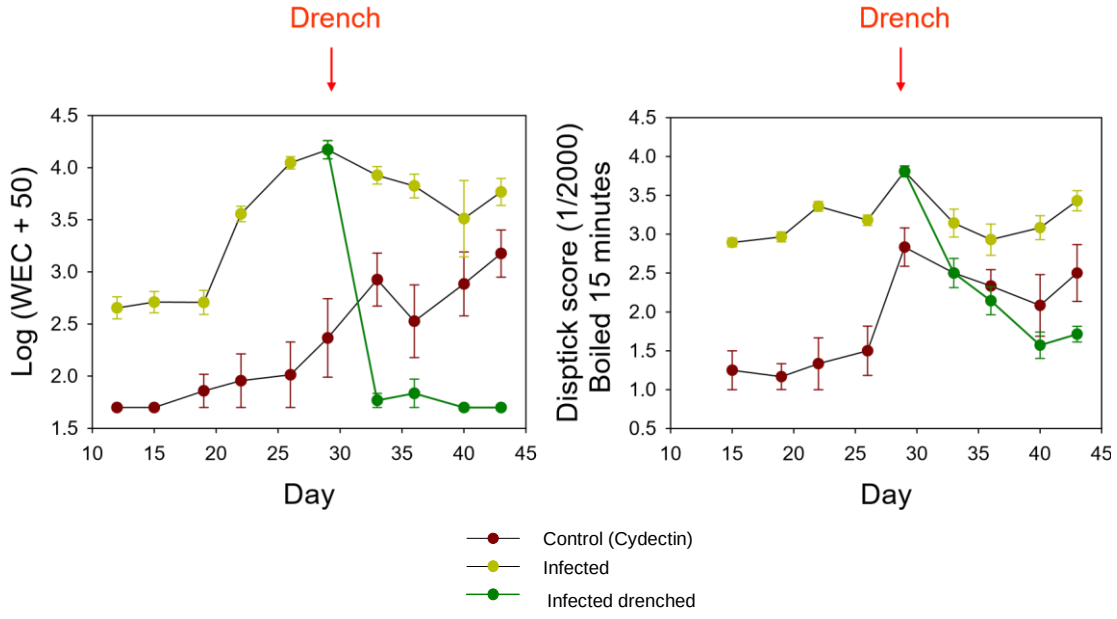
The experimental infection led to very high egg counts and an associated drop in haematocrit. On day 29, the 7 sheep with the highest egg counts were drenched orally with Cydectin. Despite no drench treatment, the remaining 7 infected sheep coped well with their heavy infections, and haematocrits in these animals and in the drenched sheep increased over the subsequent 2 weeks (Figure 3). Control sheep which had been drenched at entry to the trial picked up field infections during the course of the study and at least some of this group had positive egg counts from day 23.

Figure 3. Egg counts and haematocrits for control and infected sheep at pasture



On day 15, WEC in infected group (from prior field infection) was 620 and dipstick score was 2.89 (Figure 4). The dipstick score at this time point would have included blood loss due to the pre-existing field infection and from prepatent worms resulting from the experimental infection. Previous studies have shown blood in faeces can be detected by the dipstick test from around day 11 of infection.

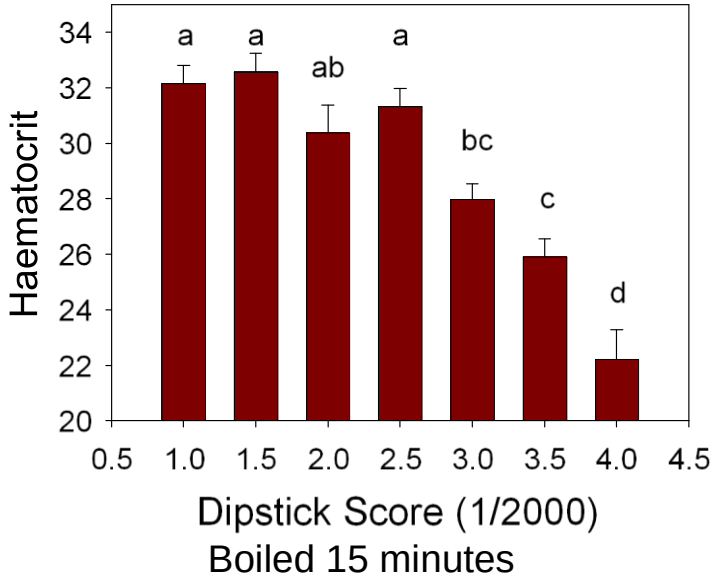
Figure 4. WEC and dipstick score for infected and control sheep at pasture



Dipstick scores in control sheep increased rapidly between day 26 and day 29 which corresponded to the time when egg counts from infections acquired from pasture also increased rapidly in these animals. Animals in the experimental infection group with haematocrits ranging from 15 to 22 were selected for drenching on day 29. These animals had dipstick scores of 4 (the maximum recorded in the study) except the animal with a haematocrit of 22 which had a dipstick score of 3.5. Following drenching of infected sheep, dipstick scores took around 10 days to fall to the level seen in control

sheep without acquired field infections. Animal Ethics Committee approval for the conduct of parasitology studies in sheep require animals with a haematocrit of 15 or lower be drenched to avoid death. There was a significant effect of dipstick score on haematocrit (P<0.000). The association between haematocrit and dipstick score is presented in Figure 5. The results indicate that a dipstick score of 4 indicates a high risk of death from haemonchosis and the need to drench. The first objective was therefore achieved.

Figure 5. Association between haematocrit and dipstick score. Bars with different superscript are significantly different (P<0.05)



1.1 Objectives 2 and 3

The association between dipstick score and WEC was examined to see if scores could be used to identify the need to drench sheep for Haemonchus infection. As the dipstick test was conducted on samples diluted at 1/1000 and 1/2000 throughout the study, the results enable analysis of the best dilution for conduct of the test and also enable the impact of dilution per se on dipstick scores to be examined.

It is noteworthy that the colour scale used for interpretation of the reaction of the reagent patch on the Hemastix test strip is based on a logarithmic scale. This is illustrated in Figure 6. Not surprisingly then, the association between WEC and dipstick score also increases in a logarithmic fashion (Figure 7).

For both test dilutions, a dipstick score of 1 was only ever returned for sheep with WEC = 0, although some sheep with WEC = 0 returned higher scores. This could arise from blood loss prior to emergence of eggs in faeces. The greatest increase in WEC appeared to occur between dipstick scores 2.5 and 3 for samples diluted 1/2000 and between 3 and 3.5 for 1/1000 (Figure 7). For samples diluted 1/2000, dipstick score 3 was also associated with a significantly lower haematocrit than lower dipstick scores (Figure 5). The average WEC for samples scoring 3 at 1/2000 dilution (n = 53) was

5003 epg, or 2137 epg when the mean log WEC is back-transformed. Values for score 2.5 (n = 29) are 1114 and 269 respectively. The distribution of WECs for each dipstick score is presented in Figure 8. The y axis scale is enlarged to show the sensitivity of the test to detection of Haemonchus egg counts at a hypothetical cut-off of 2000epg. For samples diluted 1/2000, a dipstick score of 3 yielded 2.2% false negative results, and for samples diluted at 1/1000 a dipstick score of 3.5 yielded a comparable proportion of false negatives (3.3%).

Figure 6. The sensitivity of the regent patch to haemoglobin is distributed on a logarithmic scale

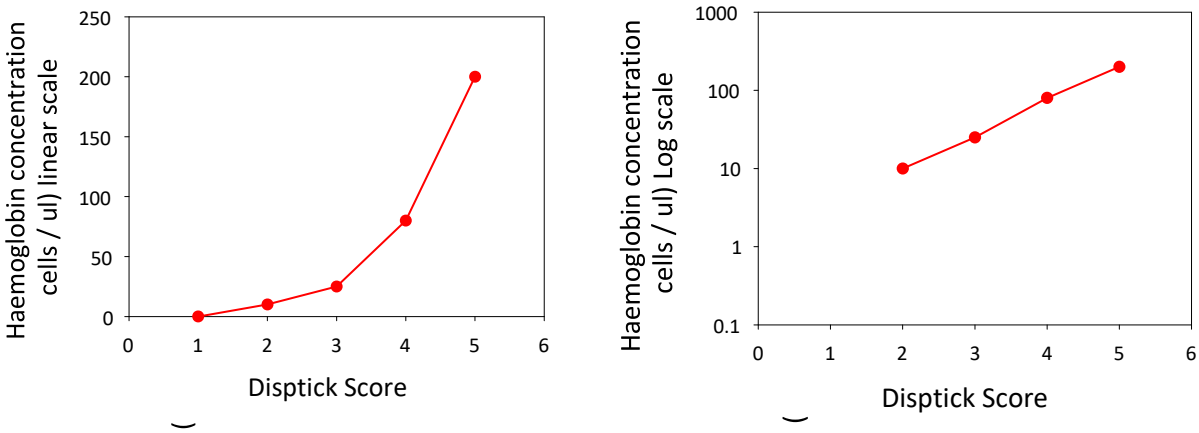


Figure 7. Association between dipstick score and egg count (n=199)

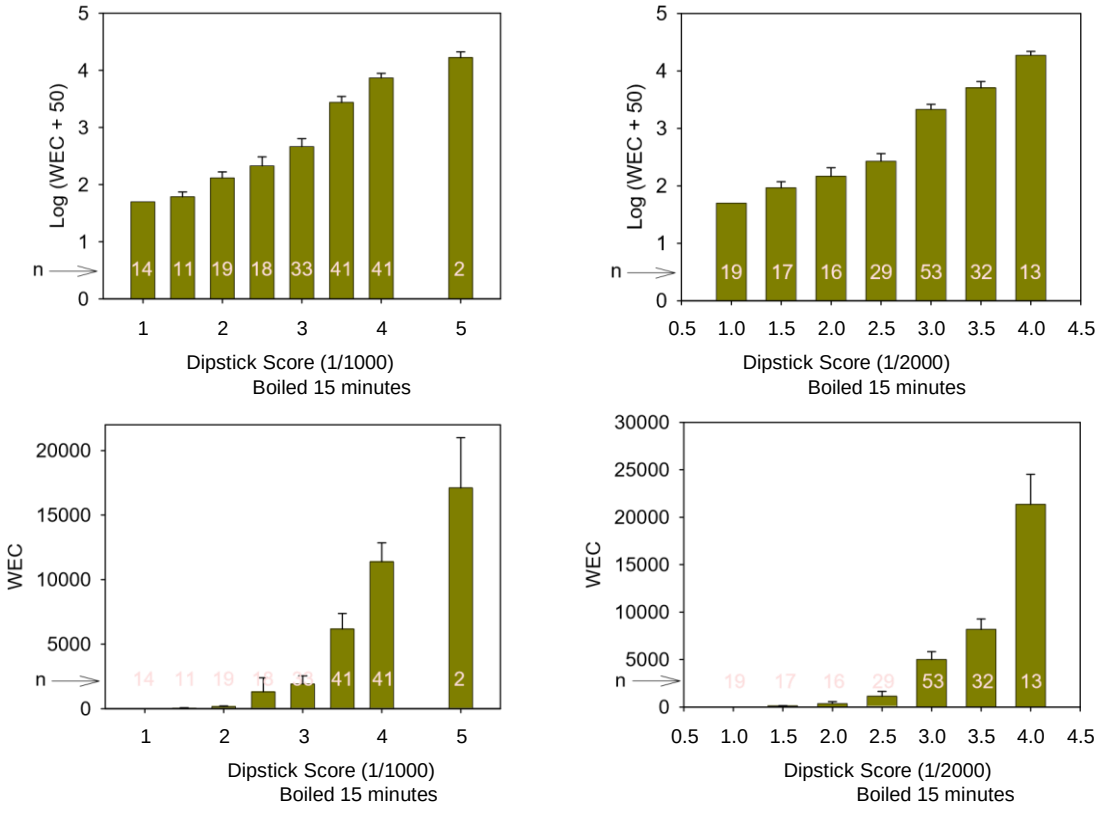
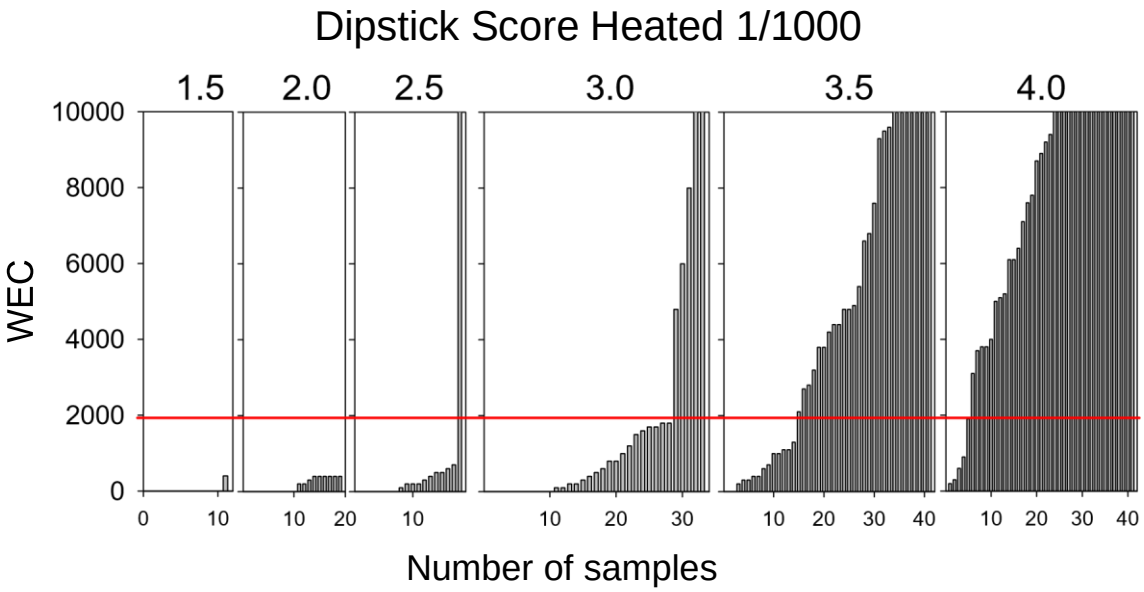
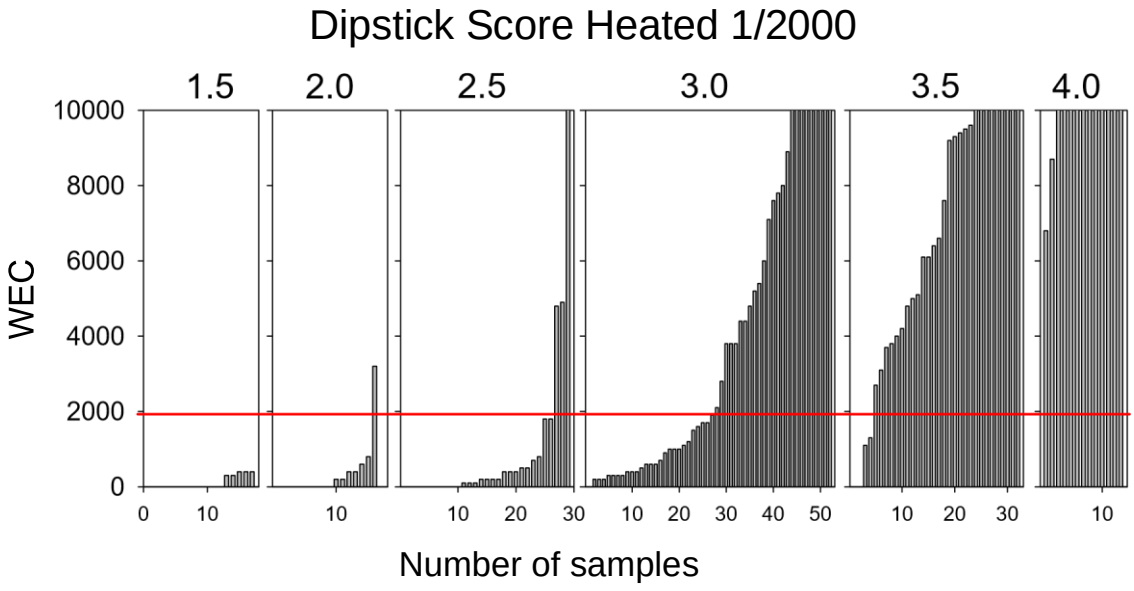


Figure 8. Distribution of egg counts for each dipstick score in samples diluted 1/1000 and 1/2000. Y axis is enlarged to illustrate the number of samples falling each side of a hypothetical target egg count of 2000 epg, shown by the horizontal red line, for diagnosis of infection



False negatives: Dipstick score < 3.5 and WEC > 2000 = 3.3%

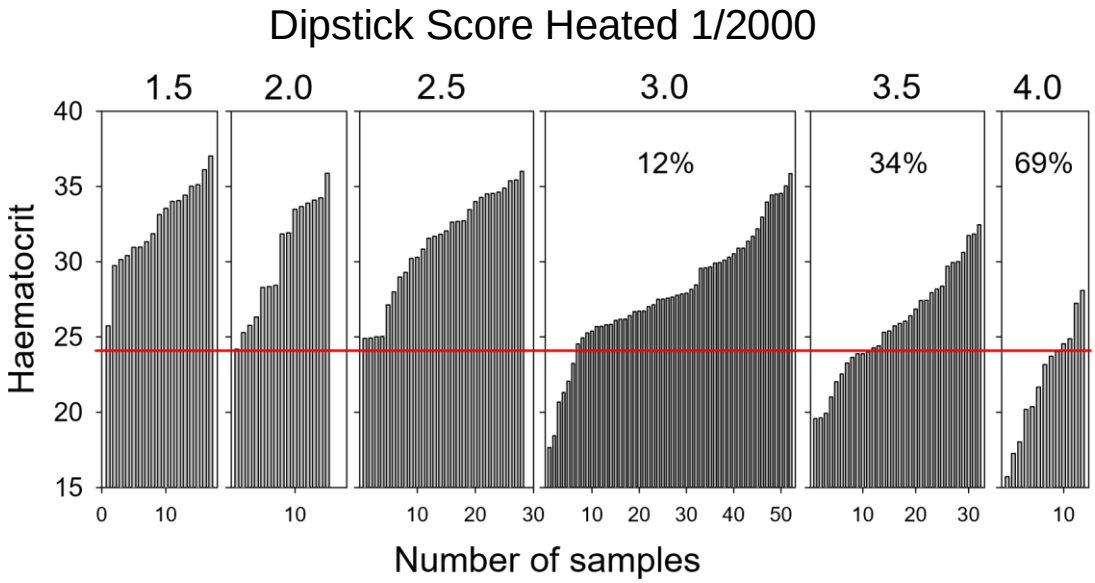


False negatives: Dipstick score < 3 and WEC > 2000 = 2.2%

The distribution of haematocrits for each dipstick score in samples diluted 1/2000 is illustrated in Figure 9. For the 79 samples with dipstick score less than 3, the lowest haematocrit was 24.2%, which is not a value that places a sheep in imminent danger of death.

The results suggest that a dipstick score of 3 for samples diluted 1/2000 provided a good indication that sheep required drenching in this study. The score of 3 was also associated with reduced mean haematocrit. The logarithmic distribution of colour changes on the test strip appears to be advantageous for giving sensitivity to the test in the critical egg count range between several hundred and a couple of thousand eggs per gram. Similar sensitivity occurs between scores 3 and 3.5 for samples diluted 1/1000. Using a whole number rather than a half score as the cut-off point seems desirable for application of the test in the field where some users may lack confidence in identifying half-unit scores, thus a test dilution of 1/2000 is recommended. The data from this study suggest that a dipstick score of 3 may be able to be used as the decision point for deciding whether or not to drench sheep.

Figure 9. Distribution of haematocrits for each dipstick score in samples diluted 1/2000. The red line indicates the lowest haematocrit (24.2%) detected in samples with a dipstick score less than 3. Percentages are the proportion of samples in each class with haematocrits less than 24.2%.



1.2 Effect of faecal moisture on dipstick scores

We recently demonstrated that an increasing moisture content in faeces dilutes eggs within the bowel contents. The in vivo dilution by faecal moisture decreases WECs in a linear fashion (Le Jambre et al Vet Par 2007). Classification of faeces into 5 faecal consistency classes on the basis of their appearance, then multiplying WEC by an appropriate factor for each class provides an accurate method for adjusting WEC to a standard count representative of normal faeces. Adjustment factors for WEC are shown in Figure 11 (top panel). Unfortunately, faecal consistency scores were not recorded in the current study; however, the study results for samples diluted 1/1000 and 1/2000 illustrate the impact of dilution on test results and let us predict the impact of dilution in vivo by faecal moisture on test results. Figure 10 illustrates the sigmoidal relationship between log WEC and dipstick score for samples diluted 1/1000 and 1/2000. From the difference between these curves which represent a two fold dilution and the known in vivo dilution factors for faecal consistency scores,

which have a maximal dilution of three fold for loose stool associated with diarrhoea, a set of adjustment factors were calculated (Table 2). The Table shows that faecal consistency scores of 2 and above could lead to underestimation of dipstick scores. In reality, this effect would only be critical near the decision point of 3 and a simple recommendation could be made for samples from sheep with loose faeces that have a dipstick score of 2.5. Figure 11 provides a potential example of how the advice could be provided to users of the test.

Figure 10. Curves of best fit for the association between dipstick score and Log WEC in samples diluted 1/1000 and 1/2000

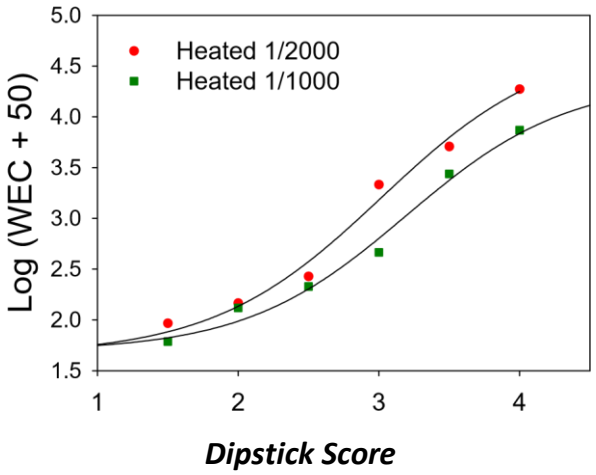
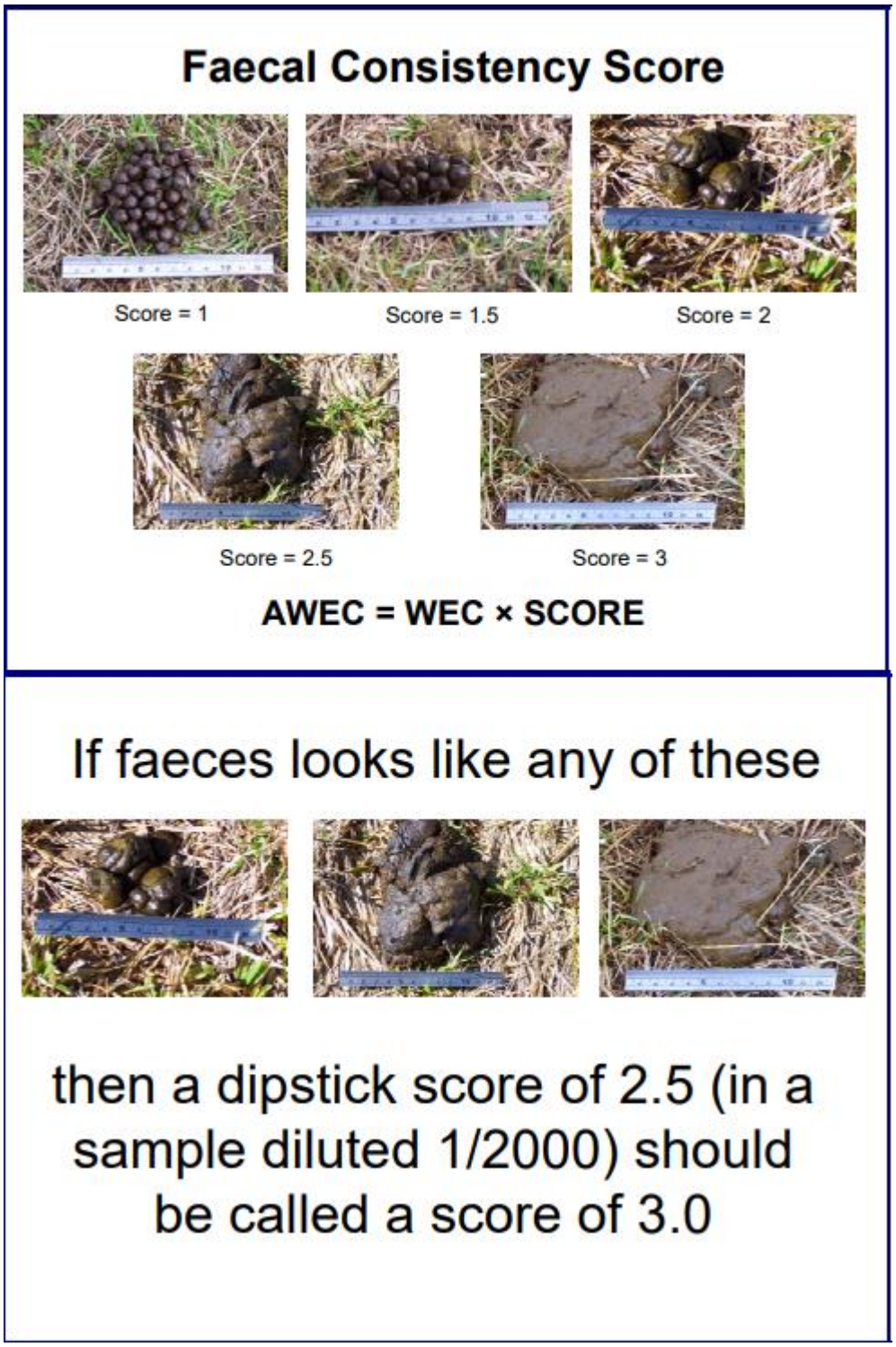


Table 2. The potential effect of dilution of blood by moisture in loose faeces on dipstick scores. Samples with raw dipstick score of 2.5 and faecal consistency score of 2 or greater (pink cells in table) fall in a danger zone where dilution of blood in faeces by faecal moisture could provide a test result that underestimates the importance of the worm burden. The impact of faecal dilution on scores greater than 3 is inconsequential if 3 is used as the decision point for drenching sheep. The recommendation for scores greater than 3 would be "drench regardless of FCS".

Raw dipstick score	Dipstick score after correction for FCS				
	FCS 1	FCS 1.5	FCS 2.0	FCS 2.5	FCS 3.0
2.0	2.0	2.2	2.3	2.3	2.4
2.5	2.5	2.7	2.8	2.9	2.9
3.0	3.0	3.3	3.3	3.4	3.5
3.5	3.5	3.8	3.9	4.1	4.2

Figure 11. Visual classes for scoring faecal consistency in sheep with adjustment factors for WEC derived from the moisture content of faeces (top panel) and a draft guide to users of the dipstick test to adjust dipstick test scores of 2.5 when faecal consistency is in the 3 classes most affected by faecal moisture.

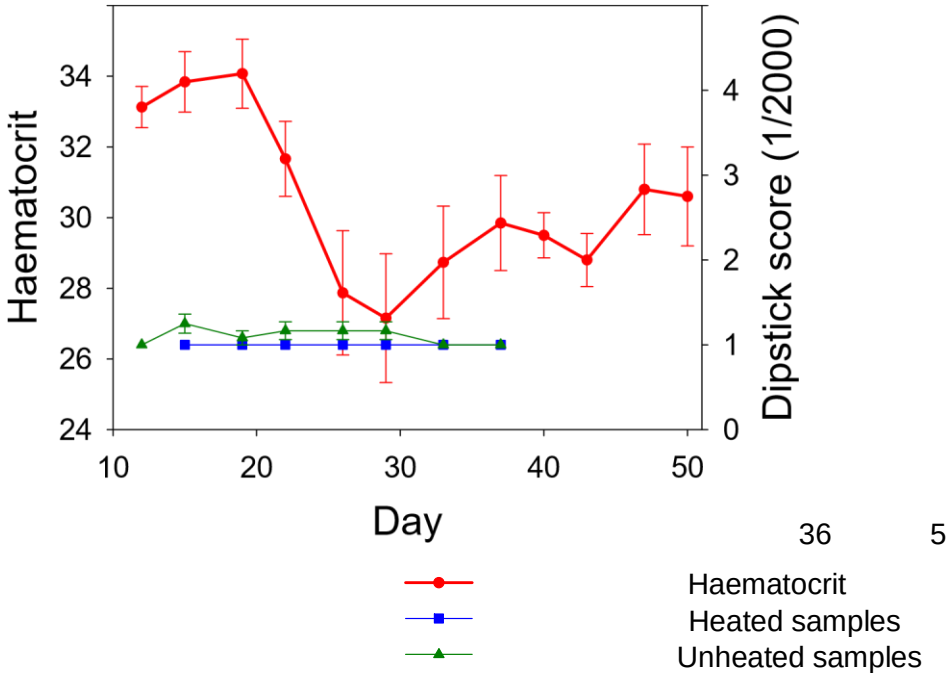


1.3 Objective 5. Effect of *Mycoplasma ovis* infection on dipstick scores

Six 7-month-older weaner sheep were drenched orally with Cydectin and placed in the animal house and fed a dry pelleted ration as per standard animal house procedures. On day 1 (18th January), the

sheep received an subcutaneous injection of 5 ml of blood from an animal previously diagnosed as infected by *Mycoplasma ovis.* The infected blood used in this trial had been stored with 10% glycerol at -80°C. Blood and faeces were collected twice weekly for laboratory analysis. Haematocrit declined from day 19 to day 29 at which time the presence of *M. ovis* organisms in red blood cells was confirmed in all animals by microscopy. The haematocrit increased over the subsequent 3 weeks without therapeutic intervention, mimicking the clinical course seen in the field (Figure 12). At no time was a positive dipstick score recorded in faeces from these animals. It is noteworthy that dipstick scores in unheated faecal dilutions from these animal house sheep were only occasionally more than 1, whereas samples assayed contemporaneously from control sheep at pasture that had WEC = 0 during the first 3 weeks following drenching with Cydectin when the drug had efficacy in preventing the establishment of new infections frequently had dipstick scores of 3 or 3.5 in unheated faecal dilutions. This observation adds further weight to the conclusion that the elevated dipstick scores in unheated samples from control sheep at pasture were due to components of the diet. We can conclude that *M. ovis* infection does not cause false positive results with the dipstick test.

Figure 12. Haematocrit and dipstick scores in sheep (n = 6) experimentally infected with *M. ovis*



1.4 Conclusions from the Armidale study

A method has been developed for removing interference in the dipstick test for blood in faeces caused by dietary components associated with green pasture. In this study, a test score of 3 in samples diluted 1/2000 indicated a Haemonchus burden that is impacting on haematocrit. A score of 4 indicated sheep at imminent risk of death from haemonchosis. A score of 3 is proposed as the decision point for drenching.

It is necessary to confirm that heat treatment of samples removes background interference from dietary components provided by other pasture types. This seems likely to be the case as the heating method has been used previously on human faeces to remove the effect of non-specific

dietary effects on faecal occult blood tests that employ the same assay principle as Hemastix (Scriven and Tapley, 1989). Further validation of the cut-off value of 3 for samples submitted through diagnostic laboratories would be prudent as current results are based on results from one trial with a bolus infection of weaner sheep in one environment. Previous studies undertaken before adoption of the boiling step were unable to clearly distinguish between the effect of pasture type and the effect of parasitism on dipstick scores. Studies on performance of the test in Albany are described below.

1.5 Reference

Scriven, A.J., Tapley, E.M., 1989. Coloscreen VPI test kit evaluated for detection of fecal occult blood. Clinical Chemistry 35, 156-158.

2. DAFWA trial Albany

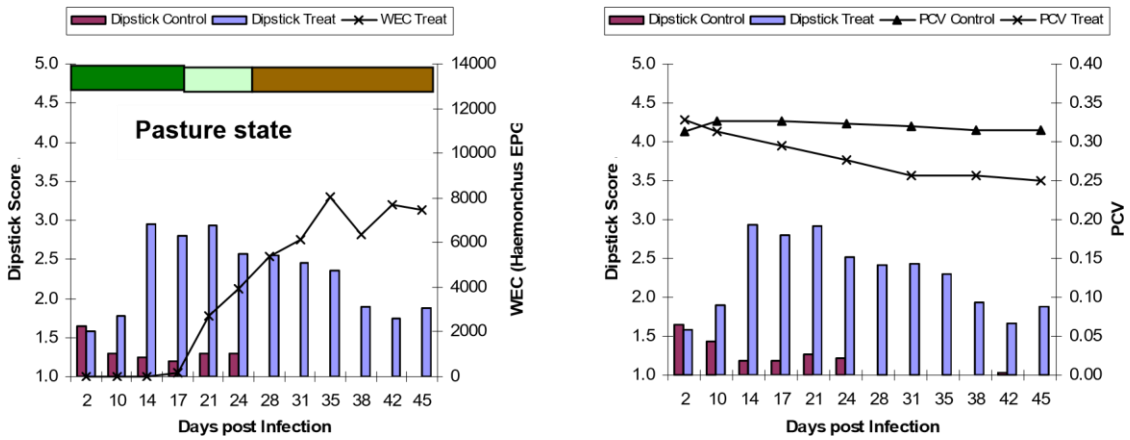
The experiment involved two treatments on 3 to 4 month old weaner sheep: a control (n=15) and a group infected with 4000 *Haemonchus* larvae (n=30). The experiment was in two stages, the first with all the sheep grazing a pasture paddock for 45 days post infection. Between day 45 and 52 a selection of 5 control sheep and 12 treated sheep were moved to Albany where they were confined on a small area of pasture until the treated sheep were slaughtered between 85 and 87 days post infection. The results from the two stages are presented separately.

Unless otherwise stated, the dipstick values in the report are from a dilution of 1/2000.

2.1 Results 1. Research station pasture (Day 0 to 45)

The *Haemonchus* Worm Egg Count (WEC) was measured for 10 of the 15 control sheep and 20 of the 30 treated sheep with the same sheep recorded each time. Figure 13(a) shows the rise in *Haemonchus* WEC over the first 45 days of the experiment, together with the change in average dipstick score for the same subsets of animals. The *Haemonchus* WEC for the control group is not shown because it was zero for all observations except two (which were less than 100). The dipstick and PCV were measured on all animals and Figure 13(b) shows how these varied over the first 45 days of the experiment.

Figure 13. Average dipstick scores together with (a) average *Haemonchus* WEC and (b) average PCV during the first stage of the experiment (significantly different from control group from Day 17).

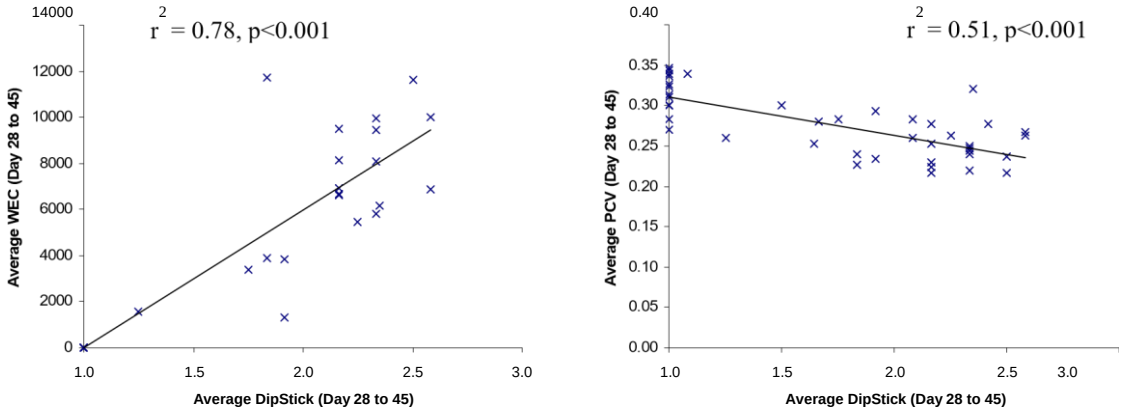


By day 10 there was a significant difference in dipstick scores between the control and treated groups and this was maintained until the end of the trial. As expected the rise in dipstick scores preceded the rise in *Haemonchus* WEC which started from day 17. Before day 24 the pasture was still green and as a result over half of the dipstick scores from the control group were greater than 1, although only one (out of 65) exceeded a score of 2. From day 28 to 45 the pasture was dry and all but one of the 84 dipstick scores from the control group were 1. The dipstick scores of the treatment group also fell (from an average of about 3 to about 2.5) as the pasture went from green to dry (day 17 to 24). While this was expected (due to the effect of green pasture) of more concern was the continued drop in dipstick scores while the pasture was dry and the *Haemonchus* WEC increased. The average of the treatment group dropped below 2 on the last three observations (days 38 to 45) and during this period 15% (13 out of 86) of the dipstick scores were 1. Of these 13 scores, 9 had corresponding *Haemonchus* WEC measurements (742, 1320, 1452, 1968, 2720, 3471, 3564, 8000, 10857).

The continued drop in dipstick score even when the pasture was visually dry may reflect a continuing loss of all green material from niche areas, although sheep are likely to have preferentially sought green pasture where present. However, the very low dipstick scores despite totally dry pasture are at odds with pen observations (CSIRO) of positive (although lower) score in *Haemonchus* infected sheep in pens on dry rations. Of major concern, the obvious presence of blood in faeces indicated by high WECs and paralleled by the continuing decrease in PCVs was associated with very low dipstick scores.

Despite this Figure 14 shows good relationships between the average dipstick score and average *Haemonchus* WEC and average PCV for the time the sheep were on the dry pasture. Both relationships were significant ($p<0.001$).

Figure 14. Relationship between average dipstick scores and (a) average *Haemonchus* WEC and (b) average PCV during the initial period of grazing dry pasture. Each point represents one sheep.



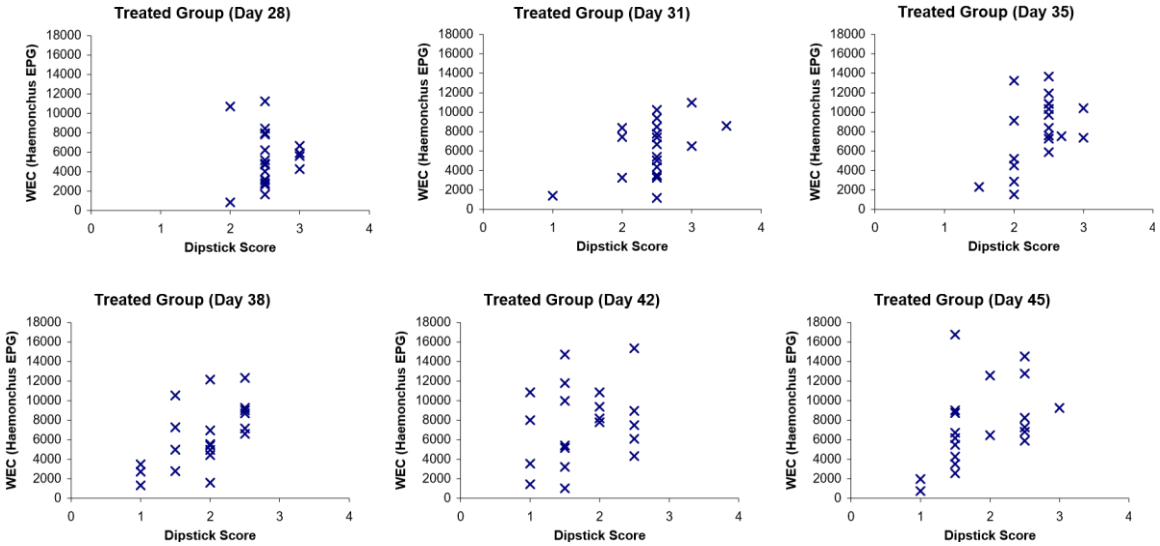
2.2 Dipstick decision point

Due to the known effect of green pastures on the dipstick scores, the data up to day 24 provides little guidance as to an appropriate decision point (minimum dipstick score that indicates high *Haemonchus* and the need for a drench). (This emphasizes the importance of a procedure to remove the “green pasture effect”.)

The scores recorded between days 28 and 45 were on dry pasture, however the drop in dipstick scores over this period also makes discussion of an appropriate cutoff difficult. Figure 15 shows how the relationship between dipstick scores and *Haemonchus* WEC changed on the six sampling occasions between day 28 (mostly scores of 2.5) and day 45 (mostly scores of 1.5) for the treatment group. The control group is not shown as these consistently had a zero *Haemonchus* WEC and a dipstick score of 1.

A decision point of 3 (as suggested by the report of a similar exercise by CSIRO) is clearly inappropriate for this dataset because it gives more than 70% false negatives (animals with *Haemonchus* WEC>2000 but a dipstick score below the cutoff) at each of the six sampling dates. It is not known if the use of boiling (as done in the CSIRO experiments) to remove the effect of green pasture would also has any impact on samples collected from sheep grazing dry pasture. A decision point of 2.5 would have resulted in false negatives of 5% for day 28 (1 out of 20 animals with *Haemonchus* WEC>2000 not detected) rising to 65% for day 42 (13 out of 20). Even the lowest possible decision point of 1.5 still gave 15% (3 out of 20) false negatives for day 42.

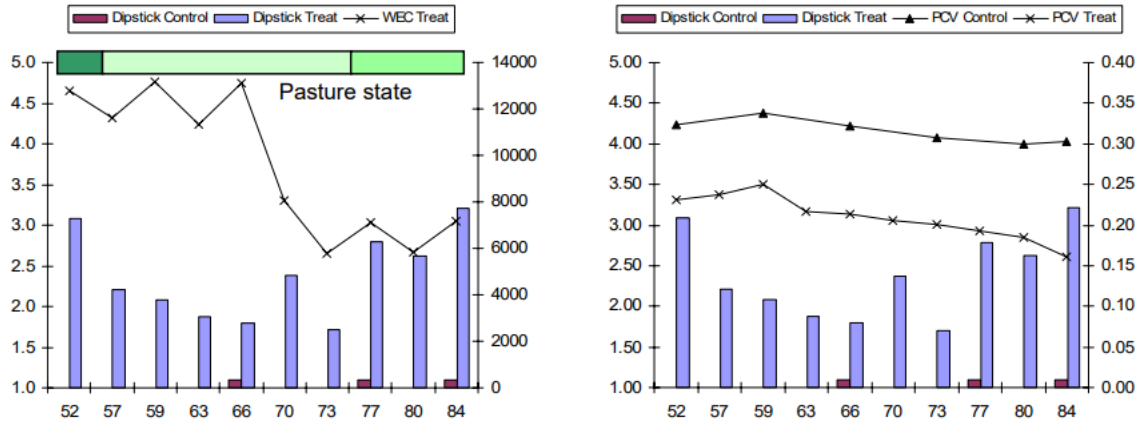
Figure 15. Relationship between dipstick scores and *Haemonchus* WEC for the treated group during the initial period of grazing dry pasture.



2.3 Results 2. Laboratory paddocks (Day 52 to 84)

Between days 45 and 52 a selection of 12 treated sheep and 5 control sheep were moved from the dry pasture to a small area of green pasture in Albany. The sheep from the two groups were moved on separate days and the relocation is likely to have caused stress to the animals. Figure 16 shows how the average dipstick score, PCV and *Haemonchus* WEC varied during this second stage. The *Haemonchus* WEC for the control group is not shown as it remained zero throughout.

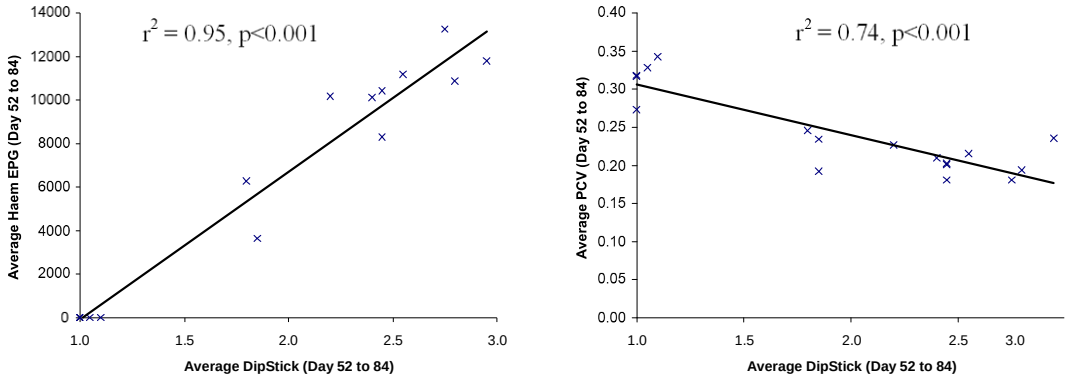
Figure 16. Average dipstick scores together with (a) average *Haemonchus* WEC and (b) average PCV during the second stage.



The increase in average dipstick score and *Haemonchus* WEC on Day 52 makes sense given the combined effects of green pasture, relocation and sheep selection method. The variation in dipstick scores during the second stage could possibly be explained by several changes in the greenness of the small grazing area in Albany: rapidly eaten out and hence dry within a few days, but re-growth later resulting from several summer rainfall events. The drop in *Haemonchus* WEC from day 70 may reflect the development of resistance of sheep to the worm infection.

Figure 17 shows the relationship between the average dipstick score and average *Haemonchus* WEC and average PCV. The relationships were again significant ($p<0.001$) and are even more clear in this second stage.

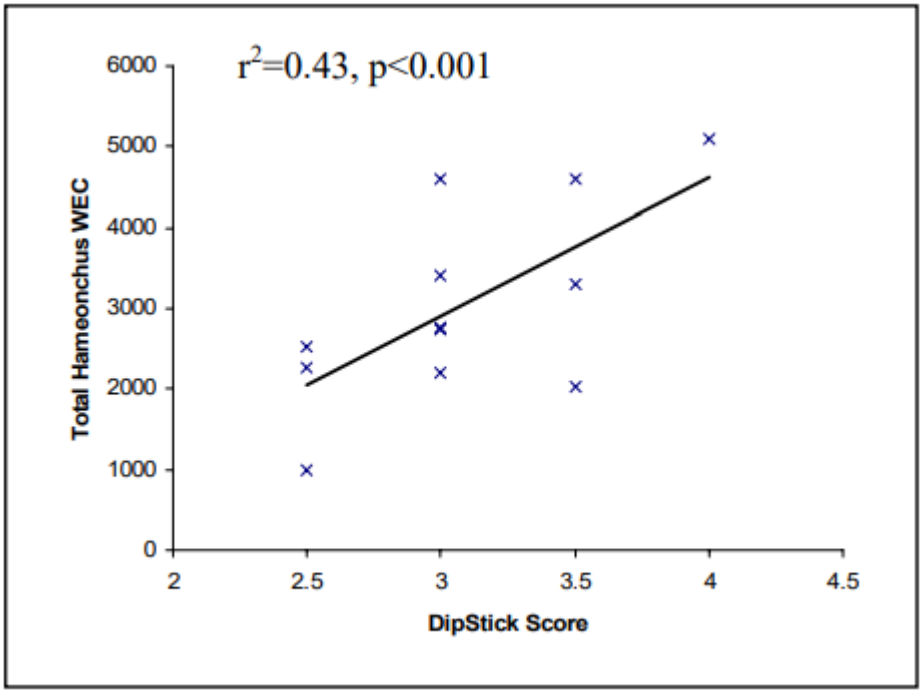
Figure 17. Relationship between average dipstick scores and (a) average *Haemonchus* WEC and (b) average PCV during the second stage. Each point represents one sheep.



2.4 Total worm count (Day 85-87)

Twelve sheep were slaughtered for total worm egg counts on days 85 and 87. At slaughter a sample was taken for a dipstick score. Figure 18 shows a significant relationship between the dipstick score and total *Haemonchus* WEC ($p=0.02$).

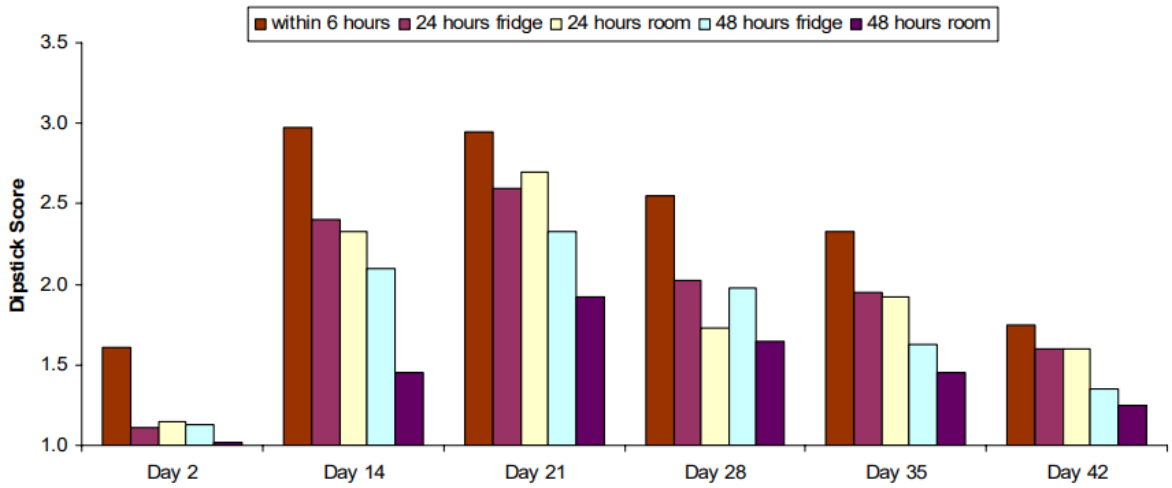
Figure 18. Relationship between dipstick scores and total Haemonchus WEC at slaughter.



Effect of Storage

An experiment was conducted to determine the effect of storage time and temperature on dipstick scores for the secondary dilution (ie, not stored faeces). On six sampling occasions four sub-samples of each sample were stored at fridge (4o) and room (20o) temperatures over one and two nights. Figure 19 shows that the Dipstick scores on each occasion were significantly lower than the scores obtained from an immediate analysis in the lab.

Figure 19. Effect of storage time and temperature on dipstick scores on six separate occasions. Each bar represents the average of 20 treated sheep.



While there were some differences between the six sampling occasions the conclusions were mostly the same: a significant drop in dipstick scores after 24 hours (but interestingly no significant difference between the room and fridge temperatures) and another significant drop between 24 and 48 hours, with the samples at room temperature significantly lower this time. As one may

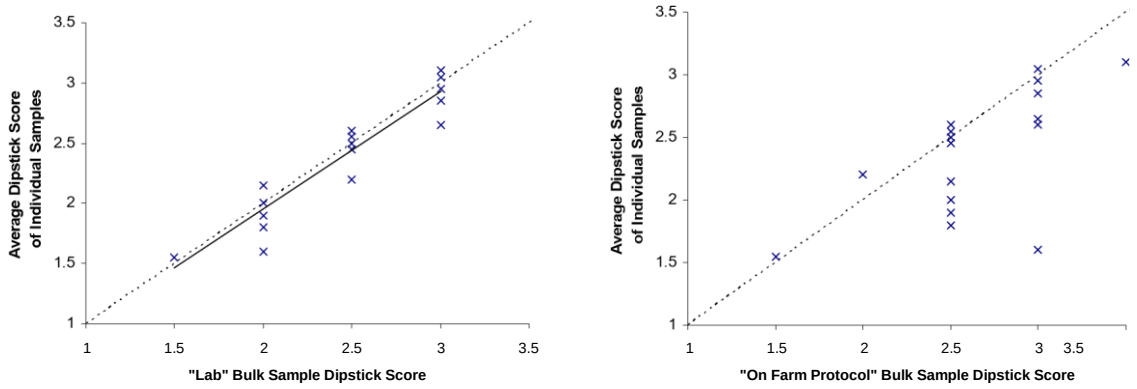
have expected there were larger decreases from the higher initial dipstick scores. The CSIRO experiments concluded that dipstick scores for the secondary dilution decreased after 80 minutes at room temperature. We recommend that samples diluted for assay be stored for no longer than 80 minutes. It has been found that bright light and sunshine can lead to decay of activity on samples over time.

2.5 Bulk samples

For each sampling occasion from day 14 to day 45, three “on farm protocol” bulk samples were obtained each from 10 sheep (two bulk samples from the treatment group and one from the control group). For each “on-farm protocol” bulk a volume of 2mLs faeces was taken from each of 10 sheep. The faeces were mashed and water added to 600mL to make a 30 fold dilution. One mL was transferred to another 65mL water, to yield a final dilution of 1:1980. Individual samples were also taken from the same sheep and after being tested, means of the results were used to give a “lab” bulk sample.

Figure 20 shows how for the treatment group the average of the 10 individual dipstick scores compares to the results of the bulk samples. The dipstick scores for all bulk samples of the control group during the period the sheep were on dry pasture were zero.

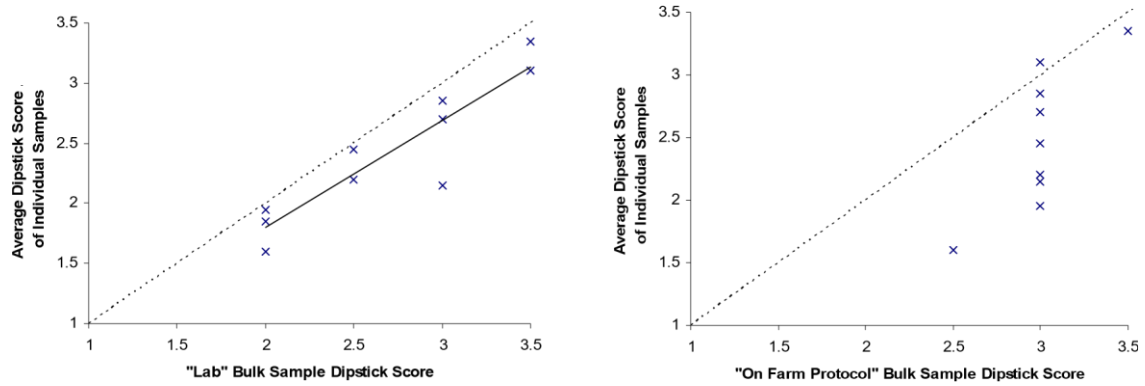
Figure 20. (a) “lab” and (b) “on farm protocol” bulk sample dipstick scores compared to the average of the corresponding individual sample dipstick scores for days 14 to 52. The dotted line represents perfect agreement.



There was no significant difference between the “lab” bulk dipstick scores and the average of the individual sample dipstick scores during the first stage of the trial. The “on farm protocol” bulk samples however were significantly higher than the individual sample scores by about one quarter of a score on average.

Likewise after day 45 one “on farm protocol” and one “lab” bulk sample were obtained from 10 sheep in the treatment group only. Figure 21 shows how the bulk sample dipstick scores compare to the average of the individual sample dipstick scores.

Figure 21. (a) "lab" and (b) "on farm protocol" bulk sample dipstick scores compared to the average of the corresponding individual sample dipstick scores for the second stage of the experiment. The dotted line represents perfect agreement.



In this period there was a significant positive bias for both the "lab" and "on farm protocol" bulk samples of about a quarter and a half of a dipstick score respectively. The positive bias could be a result of the expected curvilinear (log) relationship between dipstick score and *Haemonchus* WEC, however Figure 17 shows little evidence of this. Also in both periods there was less variation for the "lab" bulk samples compared to the "on farm protocol" bulk samples, which could be in part a result of the "lab" bulk samples being constructed directly from the individual samples.

2.6 Conclusions from Albany trial

2.6.1 Basis of test performance confirmed

- There is a good overall relationship between average dipstick scores of individual sheep (day 28 to 45 (6 data points), and day 52 to 84 (10 data points)) and their *Haemonchus* worm egg counts, and (slightly less) with total *Haemonchus* counts when sheep were killed.
- The test detected *Haemonchus* infection before it became patent.

2.6.2 Green pasture effect confirmed

- The higher scores on green pasture was very marked but not easily quantifiable (ie, could not associate a level of "greenness" with dipstick scores). As expected, dipstick scores reduced when the pasture became dry; this was reinforced when score rose again the sheep re-encountered green pastures - The effect of boiling on the samples was not tested.

2.6.3 Cut-off values (diagnostic indicators) not consistent

-For similar worm egg counts, dipstick scores were considerably lower than CSIRO found (on green pastures)
- Dipstick scores were very low on dry pasture. Of concern, some sheep with very high *Haemonchus* egg counts (and relatively low PCVs) had the lowest possible dipstick score. Is this characteristic, ie, that the test does not work on dry pasture, or on the pasture species involved?

2.6.4 Test protocols

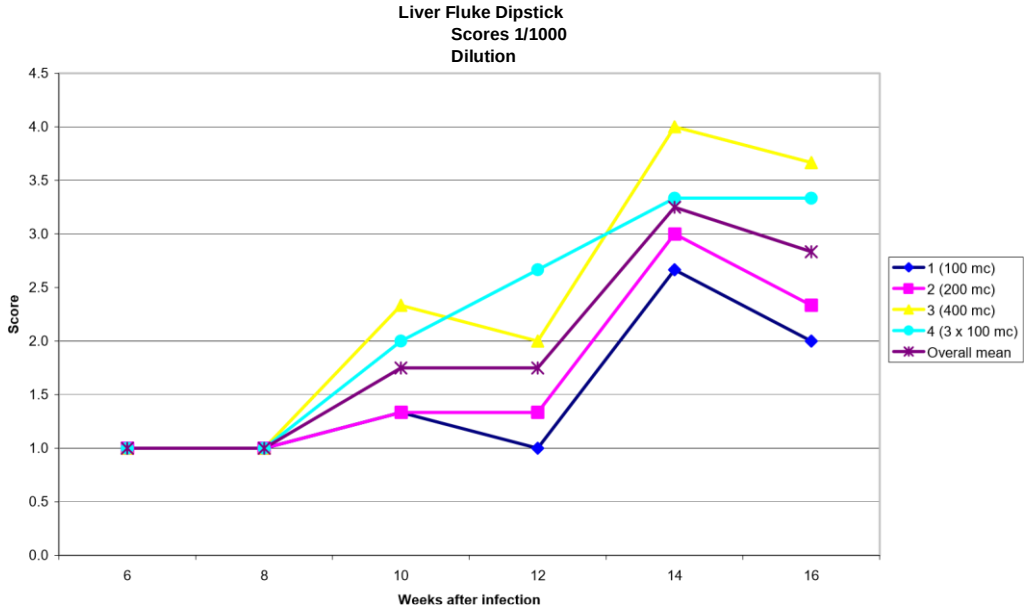
-Time of conducting tests after sampling is critical: a major difference (lower scores) when tested at 24 hours (although with the secondary dilutions, not stored faeces). The temperature of storage appeared to have less effect.
-Bulking faeces so one test is performed per flock appears to be acceptable (similar dipstick scores to means of individual sheep scores).
-An "on-farm bulk" procedure (volume based) gave a more variable result from the "lab bulk": difficult to explain at this point
-Faecal consistency was not considered in calculating dipstick scores (as suggested by CSIRO), but would not have raised scores sufficient to be consistent on dry pasture to CSIRO ones for equivalent WECs.

3. NSW DPI EMAI liver fluke studies

3.1 Background

Previous studies at NSW DPI have shown that acute artificial infections with liver fluke, *Fasciola hepatica*, in worm-free sheep housed and fed a dry pellet ration with ad lib hay and chaff, elevated dipstick scores using a 1/1000 dilution from a low background (1units) up to 4 depending on numbers of metacercariae (mc) given. This rise corresponded with arrival of flukes in the bile ducts and their maturity and egg production, from 10 weeks postinfection. How long these elevated scores would continue was not able to be determined in this trial as the animals were slaughtered at 16 weeks post-challenge for total fluke counts and fluke egg counts (Figure 22).

Figure 22. Change in Haemonchus field test scores (unboiled samples) with time during experimental infection of sheep with liver fluke



Studies conducted in the current period have been aimed at determining:

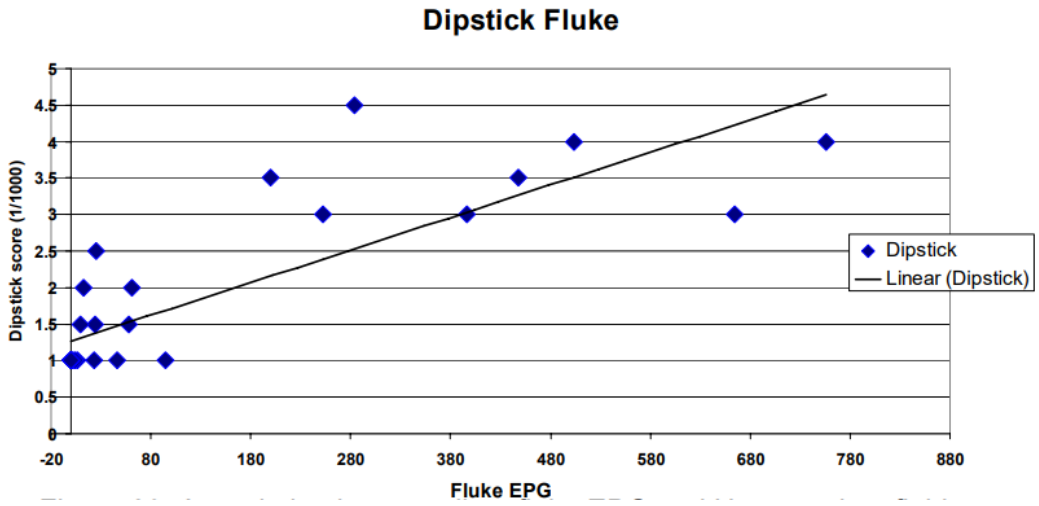
a) If there is a relationship between fluke egg counts and dipstick scores in chronic infections, b) whether acute *Haemonchus* infections superimposed on chronic liver fluke infections would cause a discernable elevation in dipstick scores, and c) dipstick scores in field cases of natural fluke infections submitted to a regional veterinary diagnostic laboratory for liver fluke egg counts.

3.2 Relationship of fluke egg counts with dipstick scores in chronic fluke infections.

A total of 31 grazing wethers aged 3-5 years old, including 20 artificially infected with approximately 200-300 metacercariae were available. The length of infection ranged from 1-3 years and individual fluke egg counts were 4-756 epg. It should be noted that there is no direct relationship between fluke egg counts and adult fluke numbers. Low numbers of nematode eggs (<200epg) were recorded in the sheep indicating that they had naturally acquired low level mixed infections from pasture.

Of 11 fluke-uninfected sheep the average score at 1/1000 dilution using ethanol was 1.0, while in the 20 fluke-infected sheep the average score was 2.28 (range 1-4.5). Of these 6 had a score of 1, three with 1.5, two with 2, and 9 with 3 or more (Figure 23).

Figure 23. Association between liver fluke EPG and Haemonchus field test scores



3.3 Superimposed *Haemonchus* on pre-existing fluke infections with "high" and "low" dipstick scores

Three groups each of 4 sheep were selected from the sheep studied in a). These were divided into:

Uninfected with fluke (average score 1.0 at 1/1000 dilution)

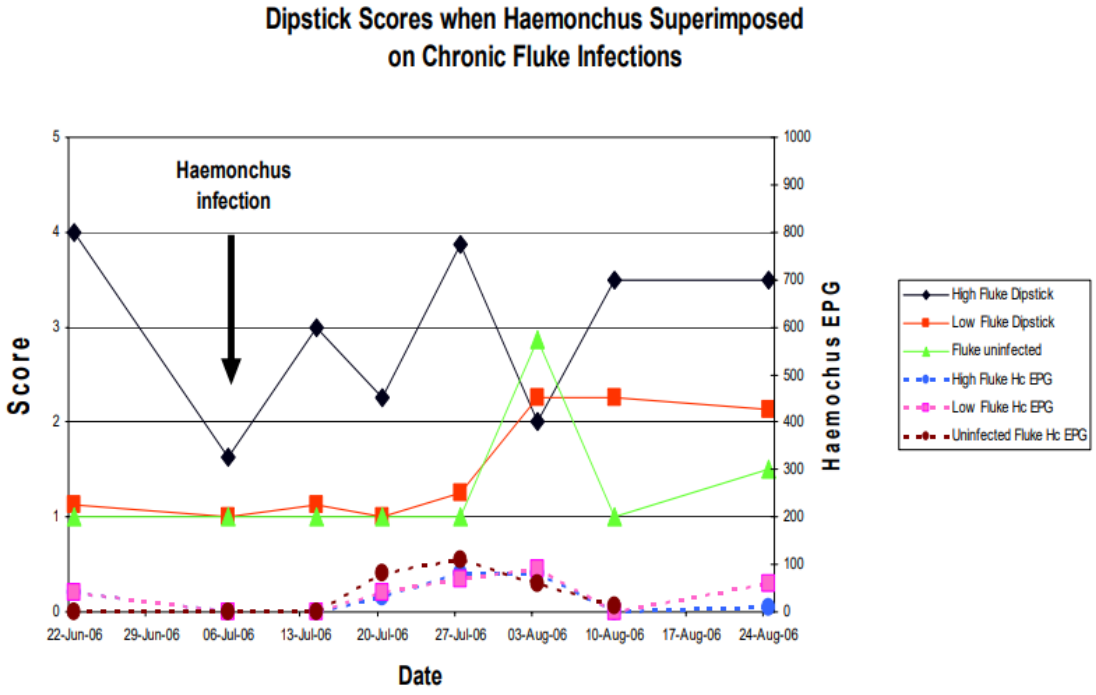
Fluke infected with Low Dipstick (average score 1.1 at 1/1000 dilution**)**

Fluke infected with High Dipstick (average score 4.0 at 1/1000 dilution)

All sheep were given a clean out drench with ivermectin (to remove any pre-existing nematodes) and zero faecal egg counts confirmed this. They were then infected 14 days later with 10,000 *H.contortus* (low pathogenicity fully susceptible McMaster strain). Faeces were collected at weekly intervals for Dipstick and FEC for the following 7 weeks.

Results are shown in Figure 24. Both low dipstick and uninfected fluke groups remained at negative values (score 1) until 4 weeks after infection with *Haemonchus*.

Figure 24. Haemonchus test scores in sheep with fluke and Haemonchus infections



A temporary peak up to score 3 was recorded in the fluke-uninfected group at week 4 postinfection (PI) and low dipstick group increased to a plateau of a little over score 2 until the experiment was concluded at week 7 PI. The high dipstick fluke group recorded variable scores throughout the post-*Haemonchus* infection period, with no clear relationship to the nematode infection. Faecal nematode worm egg counts (WECs) became positive as soon as 14 days after *H.contortus* infection in all three groups but never reached any significant level above about 100 epg. It was concluded that the age of the sheep, their previous exposure to natural nematode infections and the relatively low dose of infective larvae and the limited pathogenicity of the McMaster strain all contributed to limited response of dipstick scores.

3.4 Dipstick scores in field cases of liver fluke positive faecal samples

Various samples from a total of 52 sheep submitted to the EMAI diagnostic lab for fluke egg counts were investigated (Figure 25). They included 17 chronic adult infections. The method used the now standard1/2000 dilutions with unheated samples plus some heated at 100°C for 20 min in a block heater (see Figure 26). This was to remove any potential high background caused by green feed. In all previous liver fluke infected animals had been fed dry pellets, and high backgrounds had not been a problem.

Faecal consistency for each sample was noted but no compensation for different faecal moisture content was varied from hard pellets, tary (Figure 27) through to soft, and fluke egg counts from zero to 424 epg.

In a group of 6 yo. ewes from Young RLPB district submitted for paired fluke counts had counts of 0-28 epg. Pellets were from dry to soft and tary. Dipstick scores were untreated = 1.25, reducing to 1.20 when boiled. While in 3 yo ewes from Yass, pooled fluke counts were all zero, soft to normal pellets had mean unheated values of 1.5, which reduced slightly when boiled to 1.0. A second group of 18 mos. ewes from Yass had low pooled fluke egg counts of only 3-4 epg. Consistency of pellets ranged from soft to soft pellets, and unheated dipstick scores averaged 1.40, which was reduced to 1.0 after heating.

A group of rams, from the Hay plain, individual fluke counts were all 0 epg. The contaminating red dirt (see Figure 28) caused no interference with the dipstick result as it remained at 1.0 both in the unheated and after heating.

Finally, a group 17 two to five yo. wethers, at EMAI were tested. They were being grazed on haying off green feed, and had normal faecal pellets. Individual fluke counts were 1-424 epg (mean 97 epg). Mean unheated dipsticks of 3.9, were reduced to 1.65 on heating.

Figure 25. Dipstick and fluke EPG for liver fluke field samples

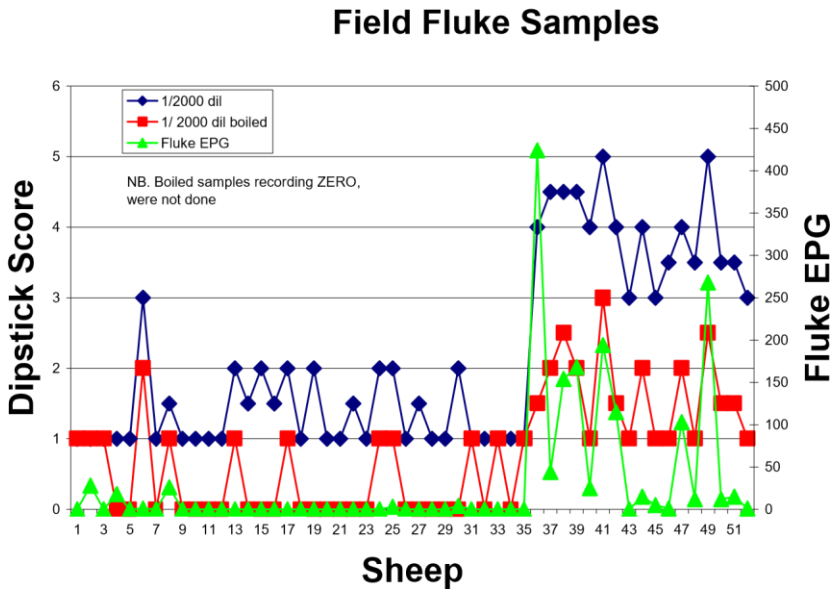


Figure 26. Dry block heater with diluted samples in 1.5 ml Eppendorf tubes.

Figure 27. Tary faeces and red dirt from Hay district.

Figure 28. Red dirt faecal pellets from Hay plain

3.5 Conclusions for liver fluke studies

From these studies on liver fluke infection it was concluded that:

- At 1/2000 dilution low level field fluke infections did not greatly elevate dipstick scores
- Scores above 3 were mostly related to feed
- Scores after boiling to remove “Green feed” background were no more than 3.0

- Red dirt pellets did not cause increased scores (no false colour reaction)
- Except in severe acute infections fluke not likely to cause false positives if diluted 1/2000 and boiled.

4. On-farm diagnosis of haemonchosis

4.1 Queensland monitor properties

Two properties agreed to a monitor situation whereby dung samples were collected every 2 weeks between January and March 2007 for submission to the laboratory for worm egg count, larval culture and dipstick testing. Dung from 10 different animals was collected with each sample placed into one of 10 bottles. A dipstick was also conducted on farm before samples were sent to the laboratory.

The laboratory used a composite egg counting technique (Baldock and Lyndal-Murphy 1990) and from each group of 5 bottles a worm egg count and dipstick reading was produced resulting in two worm egg counts and 2 dipstick readings for each submission. From February boiled dipstick readings were also set- up. One bulk larval culture was set up per 10 bottles.

Producers quickly decided that their dipstick test results coincided with those of the laboratory and ceased the activity.

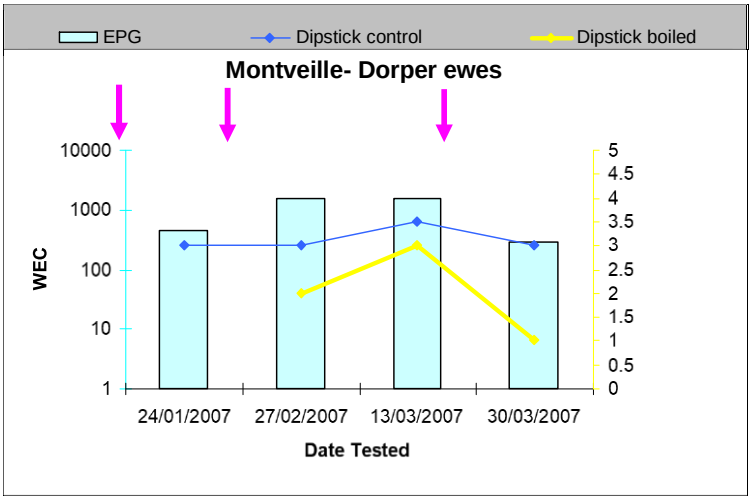
Property 1 Montville Property

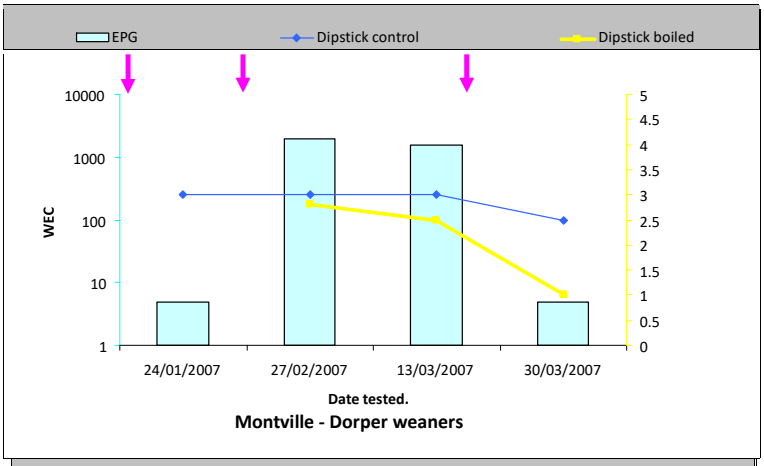
This property is a small, but a very profitable dorper sheep stud.

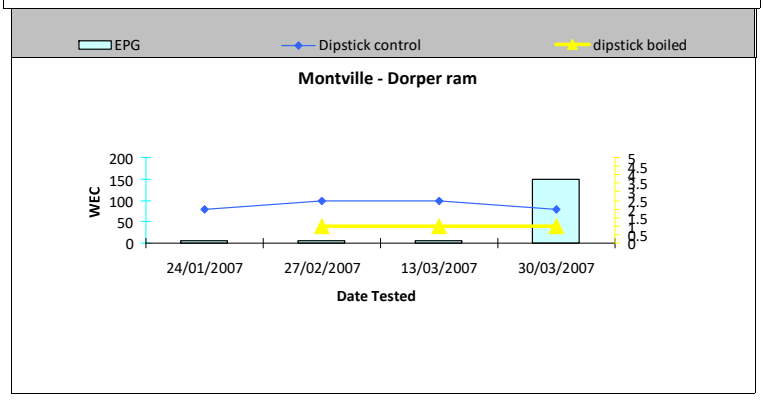
Rainfall is uniform receiving at least 100mm rain each month of the test period with 289mm in February. Temperature ranges were from about 14^{o}C to 29^{o}C with the highest temperature of 34^{o}C recorded in March. Grass was lush and in good supply.

Samples arrived at the laboratory within 48 hours. Cultures identified the worm populations as 99% H. contortus.

Three groups were tested; ewe, weaner and the ram. Data from bottles 1-5 are shown. Only one occasion did the boiled dipstick reading move close to category 3 which coincided with an elevation in the dipstick control reading and elevated worm egg counts. The wide difference between the dipstick types on 30 March correlated with the range (440 – 1560 epg) of worm egg counts within the group. Pink arrows are drench dates.







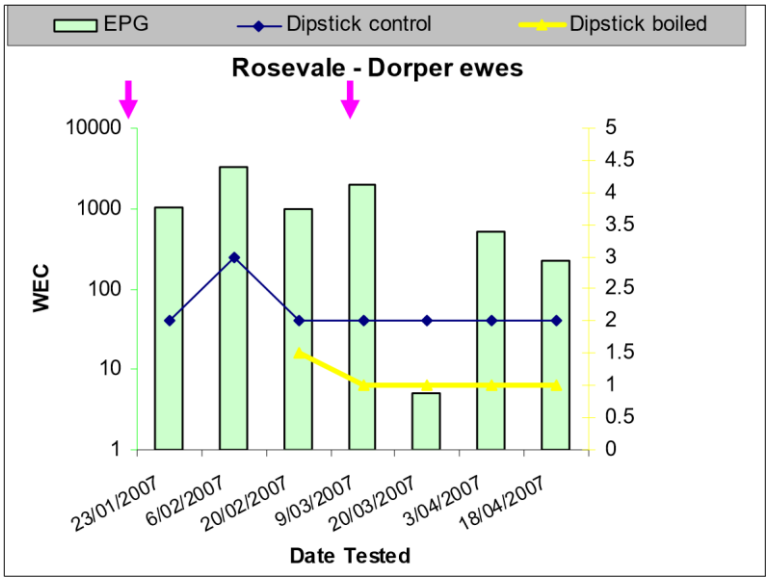
Property 2 Rosevale Property

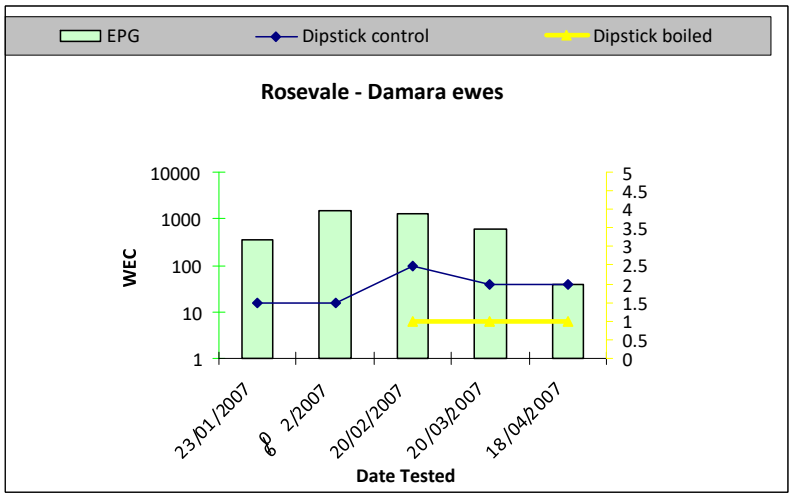
This property is a medium size and runs a dorper and damara stud. Rainfall is poor and temperatures range from 13° to 38°C in March.

Samples arrived at the laboratory within 24 hours. Cultures identified the worm populations as 97% H. contortus.

Two groups were tested; dorper ewes and damara ewes. Data from bottles 1-5 are shown. Both groups were supplemented with mineral supplement containing copper.

Pink arrows are drench dates.





5. Review meeting 15th May 2007

A meeting with Ancare, MLA, AWI and research provider representatives on 15th May 2007 concluded that further results are required from application of the test to samples collected from farms in diverse geographic and environmental regions to confirm the scores from which final drench decision advice can be formulated. The study is currently being designed, together with a short study on the effect of storage of whole faeces on results obtained with the boiling method. The review expressed a strong view that the test continued to show good evidence of final suitability for use in the field, provided the field trial gave satisfactory results. The final drench decision advice would be determined by performance of the test in the field trial. The ability to remove green pasture effect by boiling samples diluted for assay should greatly simplify the drench decision aid from earlier drafts that included complex corrections for pasture colour.

6. Conclusions

The project has identified a method for removing the effect of green pasture on scores with the Haemonchus field test. When performed in Armidale on sheep with an experimental Haemonchus infection superimposed on a light field infection, the test provided an indication of the need to drench at score 3 and a risk of death at score 4. When conducted in Albany on sheep with an experimental infection of Haemonchus parasitological and haematological indicators of the need to drench or risk of death occurred at lower test scores. The review meeting in May concluded that further studies of experimental infections were of little value to resolving this discrepancy. Rather the way forward was to examine samples from many properties in a range of geographical and climatic zones to establish performance of the test in field infections. The review expressed a strong view that the test continued to show good evidence of final suitability for use in the field, provided the field trial gave satisfactory results. The final drench decision advice would be determined by performance of the test in the field trial. The ability to remove green pasture effect by boiling samples diluted for assay should greatly simplify the drench decision aid from earlier drafts that included complex corrections for pasture colour.

On 25th May, verbal advice was received from Spruson and Ferguson that the patent would be issued

7. Appendix

7.1 Standard operating procedure for Hemastix assay of faecal occult blood for diagnosis of Haemonchus infection in sheep

Diluting faeces – At Armidale:

Mix faeces as per standard egg count – That is: 2 g faeces mixed in 10ml water then add 40 mls saturated salt solution. This brings the mixture to the neck of a green bottle. Then 100µl is pipetted into a 5ml screw cap tube containing 3.9ml of water. This gives a 1000:1 dilution. To get a 2000:1 dilution, take 1ml of the 1000:1 and add it to a second 5ml screw cap vial that contains 1ml of water.

Tips: Use a P 1000 pipette to take the 100µl from the egg count bottle. Cut the tip off the blue pipette tip otherwise the pipette tip opening is too small and it will become clogged. Don't change tips between samples, rinse the pipette tip between each sample in de ionized water. When the 100µl is delivered into the 5ml vial, pipette the liquid up and down several times to mix the sample.

Heating technique

Dilute faeces made up for WEC to 1:1000 and 1:2000 as per usual. From the 1:1000 and from the 1: 2000 dilutions pipette 1ml into 2ml snap-top microfuge tubes. Then close lids on microfuge tube and poke a hole into each lid with a syringe needle. The microfuge tubes are then placed in a styrofoam floatee then placed in a beaker of boiling water for 15 or 20 minutes. When the boiling step is finished the floatee with the microfuge tubes is placed in container of room temperature water till cool (5min). Then the heated samples are ready for the dipsticks.

For best results do not let the indicator patch on the dipstick touch any of the condensation water on the side of the microfuge tube. If you do, that portion of the indicator patch will not change color or if it does it will not be as dark as the portion of the patch that missed the condensation and was submerged in the faecal solution.

An alternative technique to boiling is to add boiling water to an eskie or thermos and place the floatee containing the vials in it for 30 minutes. The water in the eskie should be above 94°C for the entire 30 minutes.

We find that edge blotting produces more consistent response. Once the dipstick is withdrawn from the test solution, it is held vertically and the bottom edge is touched to a blotter on the bench top. This blotter must be inert. Paper towels, tissues and some lab bench covers contain something that reacts with the indicator patch and you get a blue spot moving up from the bottom edge when these are used as blotters.

Labware used in heating faecal dilutions.

Picture 1. Two ml microfuge tubes in floatee and a 5 ml ependorf pipette.

Picture 2. Floatee with microfuge tubes in chip heater.

Picture 3. Thick walled eskie used to maintain samples at 95oC for 30 minutes

Picture 4. Eskie closed with thermometer to monitor temperature