MEASURING TRICLABENDAZOLE RESISTANCE

AS PART OF A WHOLE FARM STRATEGY FOR THE CONTROL OF LIVER FLUKE IN SHEEP AND CATTLE

Final report

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ABSTRACT

Data from eight farms across Wales on liver fluke were collected by IBERS during a twelve month project sponsored by HCC (Hybu Cig Cymru). The project analysed data on the intermediate liver fluke host, the mud snail *Galba truncatula*, monitored livestock on the farms for infection, reviewed slaughter data and assessed the influence of infection on the natural behaviours of cattle and on the grazing behaviours of sheep in fields with known mud snail habitats. A major aim of the project was to measure the level of triclabendazole (TCBZ) resistance on the study farms and contribute to the development of a whole farm liver fluke control strategy.

The project found snail populations to have increased in numbers between June to August 2011 with snail numbers reducing in September 2011. However, in this study the number of snails infected with liver fluke did not rise in parallel with the increasing snail numbers, indicating that snail populations appeared to respond with more plasticity than the parasite to changes in the habitat climate during 2011.

The host –parasite behaviour studies showed that sheep grazing fields containing snail habitats covering 15% of the field area, or less, spent significantly less time grazing in snail habitats than sheep in a field containing snail habitats covering 40% or more of the field.

No livers were reported condemned from six of the farms and only one farm, farm 2, reported multiple liver condemnations. Farm 2, therefore, was the only farm to produce production based data. On farm 2 it was confirmed by new molecular tests that triclabendazole resistant liver fluke were present on the farm. It was also evident that the farm needed to dose in July and not to wait until November since the monthly faecal egg counting highlighted an early year rise in liver fluke egg counts from the sheep.

Farm 2 also illustrated that 2011/2012 showed a drop in the average number of liver fluke found in the bile ducts of condemned livers, compared to 2010/2011.

The project findings showed that it is realistic to develop a whole farm plan for the control of liver fluke by the combination of new methods for testing TCBZ resistance and more traditional monitoring techniques such as reviewing liver condemnations, undertaking regular faecal egg counting, and identifying snail habitats in relation to grazing management. This project concluded that with these datasets, the farmer, with support from a veterinarian, should be able to put in place an effective strategy for the long term control of liver fluke.

The findings of this project have been incorporated into a HCC sponsored booklet as part of the dissemination aims of the project.

1.1INTRODUCTION

1.1.1 Liver Fluke (*Fasciola hepatica*)

Liver fluke is a parasitic worm that can be found in sheep and cattle that graze wet or waterlogged pastures. The parasite has an indirect lifecycle that first involves infection within an intermediate mud snail host (*Galba truncatula* in the UK*)*. The parasite develops and multiplies within the snail host, emerges and usually attaches to herbage within the snail's habitat, in order to await ingestion by a grazing sheep or cow. Thus, as a consequence of this complicated lifecycle, the liver fluke parasite only thrives on farms with overlap in habitat between the mud snail and livestock.
Liver fluke has traditionally been a problem on many Welsh sheep and beef farms as Wales has a wet temperate climate suited to the survival and development of intermediate snail hosts. Recently, liver fluke appears to be establishing on farms without a previous history of liver fluke. Currently in Wales, liver fluke causes approximately 25% of cattle livers and 5% of sheep livers to be condemned at slaughter per year, with the majority of liver condemnations occurring in the winter and spring months.

Although currently farmers are not directly financially penalised for condemned livers, a damaged liver will have a negative impact on production, with liver fluke infected animals losing up to 1.2kg weight per week. In Wales, the succession of wet summers and mild winters that occurred between 2006 and 2009 allowed for a dramatic increase in the number of cases of acute liver fluke. This increase was due to the successful overwintering of eggs coupled to the ideal conditions for survival of the intermediate snail host. However, with the relatively cold conditions during the winters of 2009/2010 and 2010/2011, the number of liver fluke eggs successfully overwintering on pastures was apparently reduced. In addition, the relatively dry spell during the spring of 2011 may also have reduced the number of snails present for the surviving liver fluke eggs that subsequently would have infected snails as larvae. Thus, the knock-on effect of the cold temperature has led to reduced cases of acute infections during the winter of 2011/2012. However, with long term climate trends predicted for Wales of milder winters and wetter summers due to climate change, periods of intense infection similar to 2006-2009 are predicted to occur more frequently in the future, and the winters of 2009-2011 are the exceptions.

1.1.2 Triclabendazole Resistance

Triclabendazole, TCBZ (FasinexTM) is the only commercial agent that kills young pathogenic liver fluke, and is considered an achilles heel in the overall control of liver fluke (Brennan et al., 2007). Unfortunately, suspected cases of liver fluke parasites resistant to TCBZ have been reported for over ten years in Welsh

sheep and cattle (Thomas et al., 2000) and without intervention resistance is likely to establish as outbreaks of liver fluke continue to spread (VLA reports 1995-2010). There is likely to be only a narrow window of opportunity to avoid drug resistant liver fluke populations being as widespread as drug resistant gastro-intestinal nematodes. The caveat is that resistance to liver fluke is not directly measured, and the only method directing veterinarians to suggest liver fluke resistance, is the Faecal Egg Count – Reduction Test (FEC-RT). This test can be technically problematic and only confirms that adult fluke have likely survived TCBZ treatment. FEC-RT does not confirm that the liver fluke are *actually* resistant to the drug. In addition, best intentioned, but often misguided media reports, and subsequent uninformed discussion about '*TCBZ resistance'* may have lead some Welsh producers to already switch to inferior products that fail to kill young pathogenic fluke, or lead some farmers to stop treating for the disease.

The failure of TCBZ to kill liver fluke could be due to several factors ranging from problematic drug delivery, reduced host liver metabolism of TCBZ to active pro-drug, or management practices that select for TCBZ resistant parasites. The inability of the FEC-RT to indicate why the drug has failed means that veterinarians cannot fully advise on the spectrum of potential solutions. Thus, current advice if egg counts fail to fall after TCBZ treatment is to switch to an alternative but less effective drug and recommend that TCBZ dosing is suspended to eliminate threat of *'resistant'* parasites causing greater production losses.

1.2 Project history

IBERS scientists via recent BBSRC, DEFRA, EU, Levy Board and Welsh Government funding have optimised two laboratory based tests that can either differentiate if liver fluke are resistant to TCBZ treatment, or predict that the activated form of the drug has failed to reach the fluke at the required lethal dose via host tissues.

The first test was based on research that used previously well characterized liver fluke isolates, with known TCBZ response history, to create a reference pool that allows the classification of any individual liver fluke sample with unknown TCBZ susceptibility as either resistant or susceptible to the drug. The test is based upon comparing the proteome (protein fingerprint) of the test liver fluke to the reference library.

The second test developed from *in vitro* culturing research on drug response (Chemale *et al.*, 2010) and involves culturing of liver fluke in the active form of TCBZ drug for 24 hours under defined laboratory conditions that mimic the host bile environment. During the culturing the motility of the liver fluke is recorded to

quantify the susceptibility of the parasite to the active form of the drug using mobility data from reference isolates with known response to TCBZ.
Both tests have the advantage of removing the possibility of drug failure being the cause of liver fluke survival *in vivo*. However, both tests do require collection of liver fluke from the bile ducts of the infected host.

1.3 Project aim

The overall aim of this project was to measure liver fluke on 12 farms across Wales to support the development of a whole farm strategy for the control of liver fluke on Welsh farms. The individual objectives for the project were:

Aim 1: Confirmation of level of TCBZ resistance on Welsh Farms

- Confirm collaboration farms.
- Faecal egg count reduction test to screen potential problems with TCBZ on collaboration research farms.
- Adult liver fluke to be collected from animals at slaughter and a newly devised in vitro assay to confirm if parasites are resistant or susceptible to TCBZ (Chemale et al., 2010). TCBZ is the first test priority, other drug groups (closantel and nitroxyl) to be tested to confirm that multi-drug resistance is not present, and that TCBZ resistant liver fluke adults are sensitive to other drugs.
- Liver scores taken at time of slaughter to correlate with intensity of infections.

Aim 2: Behavioural Test to determine the time spent grazing in potentially infective areas

- Ethogram development using grazing animals on university farm.
- Validation of ethogram using 3 flocks of sheep on at least 3 farms in the project on 3 consecutive days.
- Determination of behaviour differences due to infection levels in cattle.

An ethogram is a list of an animal's repertoire of behaviours that can be used to measure the response to a challenge in its natural habitat such as parasites on pasture.

Aim 3: Management & Dissemination

- Weight to be measured weekly of each animal in an observation group and just before slaughter (to calculate live weight gains).

- Condition scores.
- Carcass classification (to confirm if carcass classification is affected by infection).
- Liver condemnations (tied in with Aim1).

The following measurements are to apply whole farm data and help in the formation of a whole farm plan

- Soil maps.
- Soil temperature.
- Rainfall.
- Drainage.
- Grazing and sward type.
- Snails will be collected on each farm from fields identified as high risk of infection and checked for infection.
- Dosing history and dosing recorded over 12 months of project.

Dissemination

- At the end of the project summaries will be sent to each participating farm on the data collect from their farm.
  The researcher will attend two agricultural events and present talks when requested by HCC.

2 METHODS

2.1 Farm Selection

Twelve farms were identified and confirmed as suitable for the project according to the history they could provide on liver fluke infections in their livestock. Only eight of the twelve farms were subsequently able to provide data for the project, due to the other four farms requesting, at various time points not to continue to contribute to the project.

In brief, the farms were identified by the following procedure. Leaflets were distributed to the agricultural community and farms were also directly provided with study details, with the onus placed on the farmers to subsequently contact the project researcher directly. Thus, upon farmer-project contact, the researcher would explain what the project would involve and, if the farmer was interested, the researcher would organise an initial visit to the farm. The initial visit would involve a more detailed discussion between the researcher and the farmer about the project. To ascertain what information the project could obtain from each farm. The farms were located around Wales but with a bias towards the south (Figure 1).

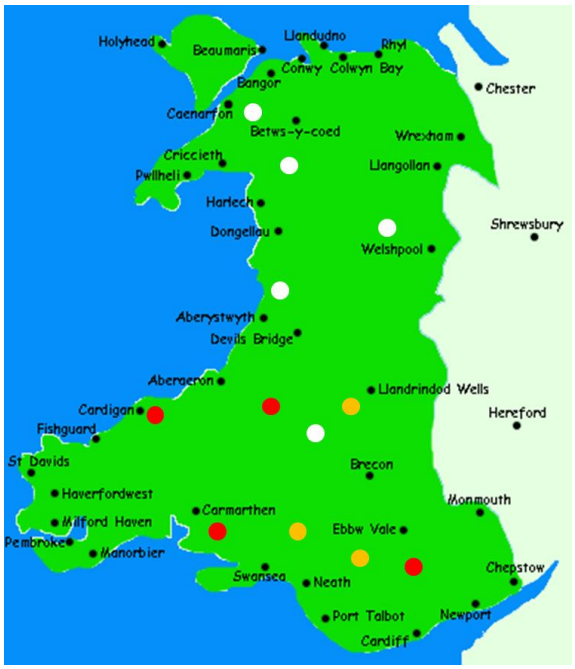

Figure 1: A map of Wales showing the participating farms locations. White dots indicate the farms have never reported any problems with the efficacy of TCBZ. Yellow dots indicate the farms which think they may have a problem with the efficacy of TCBZ, and red dots indicate the farms which know they have a problem with the efficacy of TCBZ

2.2 Snails

Four suitable snail habitats were selected and visited once a month from June to September 2011. Within each habitat a suitable area was found to carry out a 10 minute collection of snails using established manual collection methods. Collected snails were transported to the laboratory in water from their natural habitat.

Each snail was subsequently dissected to determine level of infection with liver fluke. A sensitive molecular test was used to identify *Fasciola hepatica* and avoid measuring a related flatworm *Cercaria cambrensis*. Thus, PCR based screening with species specific *F. hepatica* gene primers was routinely completed using a sub-set of randomly selected snails determined as infected by dissection.

The temperature and pH were also recorded at each site using a portable temperature probe and litmus papers (Fisherbrand, U.K.).

2.3 Behaviour Data

Three of the study farms were visited on three consecutive days (nine days in total). Each day on each farm a group of sheep in a field were observed for three hours at five minute intervals. Every five minutes the number of animals grazing

in areas suspected as being snail habitats (labelled IZ) and the number of animals in areas believed to be unsuitable for the snail (labelled NIZ), were counted. The data was then analysed by Man-Whitney U statistical test.

Also on each day, on each farm, three individual heifers of between 18-22months of age were marked and observed for two hours with their behaviour noted every 5 minutes. A faecal sample from each animal was then taken back to the lab to determine if they were infected with liver fluke. The behaviour data was then analysed by Man-Whitney U statistical test.

2.4 Production Data

Live weights were taken monthly on a specific group of sheep. After weighing, the sheep were kept in a pen with no fresh faecal deposits for one hour. At the end of the hour the sheep were released and a pooled faecal sample was collected.

Pooled cattle faecal samples were collected monthly from the same group of cattle by taking the first six faecal deposits produced by six different animals within the group.

All pooled faecal samples were tested by the Veterinary Laboratory Agency's method of sedimentation. In brief, 20-40 grams of faeces were washed initially through a 300µm sieve followed by a 53µm sieve until the waste water is judged clear by eye. The material retained on the 53µm sieve is rinsed into a 1 litre measuring cylinder and allowed to settle for 8 minutes. After this settling period the water is removed by a water suction hose down to the 300ml mark on the cylinder and the measuring cylinder is refilled to the 1 litre mark with fresh water. This is repeated until the water layer is completely clear. The sample is t transferred to a 5cm diameter counting chamber and the number of eggs per 1 cm^2 area are counted. The average from each square is then multiplied by the total area of the dish (19.62cm^2) and then divided by the original weight of the faecal sample to establish the eggs per gram (epg) of the pooled sample. Each pooled sample was analysed at least 3 times to ascertain an average epg for each sample.

For faecal egg count reduction tests, 40 animals were selected and split into 4 groups. A pooled sample was taken from each group before treating each group with either: no drug, FlukiverTM (Closantel), TrodaxTM (Nitroxynil) or FasinexTM (Triclabendazole). Each group was then marked and released. 21 days later pooled faecal samples were taken from each group.

Each sample was also tested by the 'Modified McMaster' flotation method for nematode eggs to ensure the majority of other parasites were also being controlled and unlikely to cause major production effects.

The results from each sample were given within 48 hours to each farmer except when the sample was taken on a Saturday.

2.5 Resistance Data

Participating farmers were requested to inform the researcher if any livers were condemned for fluke at slaughter. If condemned livers were reported the researcher would organise to be present at the slaughtering of the next batch of animals to be slaughtered from that farm.

The number of liver fluke found in each liver was counted and collected liver fluke were cultured in culture media for 2 hours at 39˚C during transport back to the laboratory. The liver fluke were then individually cultured for 24 hours at 39˚C in media either containing: solvent only, 10µg/ml research grade triclabendazole-sulphoxide (TCBZ-SO) dissolved in solvent or 40µg/ml TCBZ-SO dissolved in solvent.

Every four hours the motility of the liver fluke was scored using a motility scale previously developed in IBERS in order to determine individual liver fluke susceptibility to the pro-drug TCBZ-SO.

Adult liver fluke were collected at slaughter and analysed through two-dimensional protein separation followed by multivariate analysis, before classification using a pre-existing TCBZ resistant/susceptible database in order to determine if a farm had TCBZ resistant liver fluke,

3 RESULTS

Due to the reduced incidence of liver fluke encountered on the project farms only the snail data and behaviour data for the sheep trials is presented here. However, farm 2 did produce production data on the sheep enterprise and this will also be presented in the report.

3.1 Snails

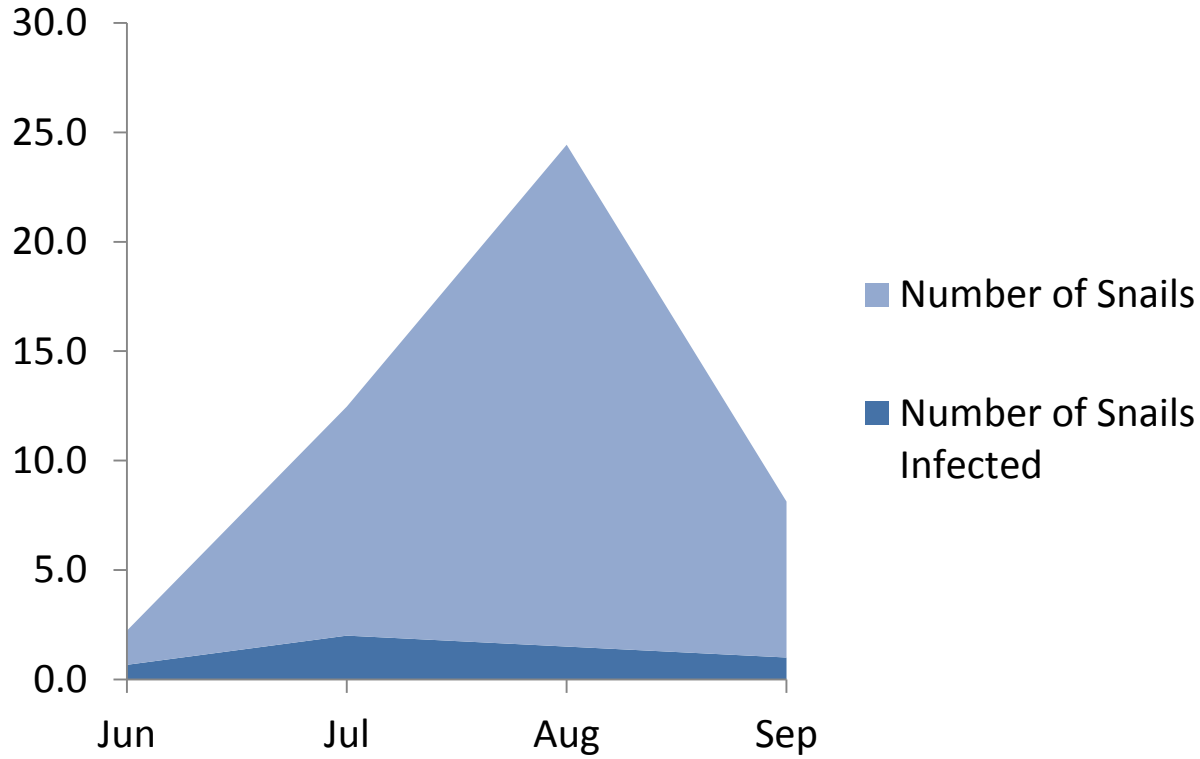

Figure 2: The average number of snails (activity) collected from each site in the project and the number of those snails found to be infected by dissection between the months of June and September

The snail numbers (activity) found in June 2011 was considered low for the time of year, probably due to the dry spring encountered during the project. Interestingly, despite a later rise in snail activity in August 2011, the number of infected snails did not rise which appears consistent with the parallel lack of infection seen in sheep and cattle on most farms (Figure 2). The decrease in snail activity in September 2011 was possibly due to the cooling temperatures seen at generally all sites visited that month (Figure 3).

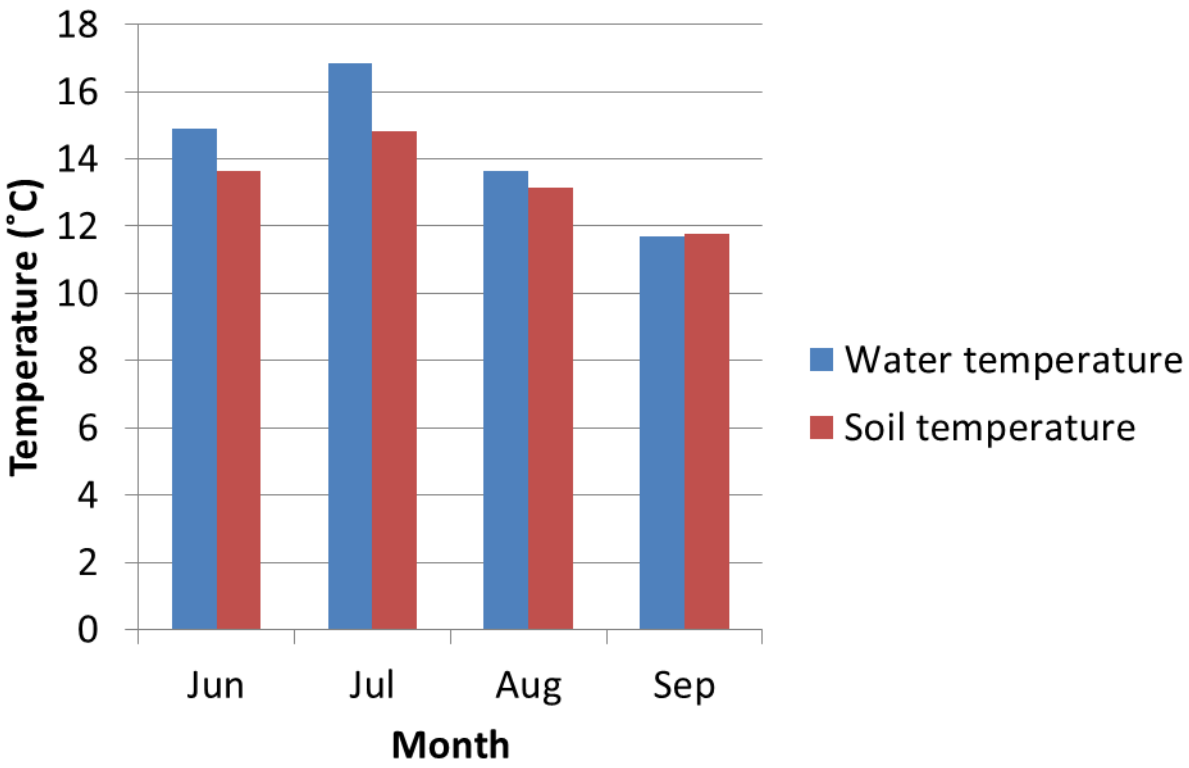

Figure 3: The average water temperature in the mud snail habitat and soil temperatures per month across all sites monitored in the project

All the temperatures recorded were above threshold of 10^{o}C for snail and liver fluke development.

3.2 Behaviour Data

The sheep behaviour tests showed that sheep in a field with 40% of the field covered with IZ (areas considered to be snail habitats) areas spent significantly more time grazing in those areas than sheep in fields containing 15% or 10% cover with IZ areas. However, there was no significant difference between 10 and 15% cover. This indicates that animals grazing fields where greater than 15% of the field contain potentially infective areas, should be monitored for infection and may require more treatments for liver fluke than animals only grazing fields with less than 15% potentially infective areas.

The cattle monitored for behaviour showed no signs of infection for liver fluke as judged by faecal egg analysis. However, the cattle on one farm all had confirmed infections with rumen fluke. There was no significant difference observed in the behaviour of the rumen fluke infected cattle compared to the other two groups of cattle.

3.3 Production Data

As discussed above, due to the lack of liver fluke infection recorded on the farms taking part in the project, only farm 2 produced production data in their sheep enterprise.

Farm 2 normally treated sheep in October 2011 (ewes) and never treated the lambs because of the withdrawal time needed before slaughter. A group of the ewe lambs were monitored from April 2011 through to September 2011 by taking faecal egg counts once a month. Interestingly the faecal egg count increased in July 2011 and August 2011 indicating adult stage infection was occurring much sooner than previously realised by the farmer (Figure 4). This increase in egg producing adult stage infection was not seen in the cattle. This was probably due to the cattle being kept on drier fields than the ewe lambs. Interestingly, in August 2011 a small decrease in live weight was observed in sheep followed by an increase in September 2011 after treatment for liver fluke. This change in live weight may have been due to liver fluke. However, at this stage of reporting other factors such as grazing quality may also have had an influence (Figure 5).

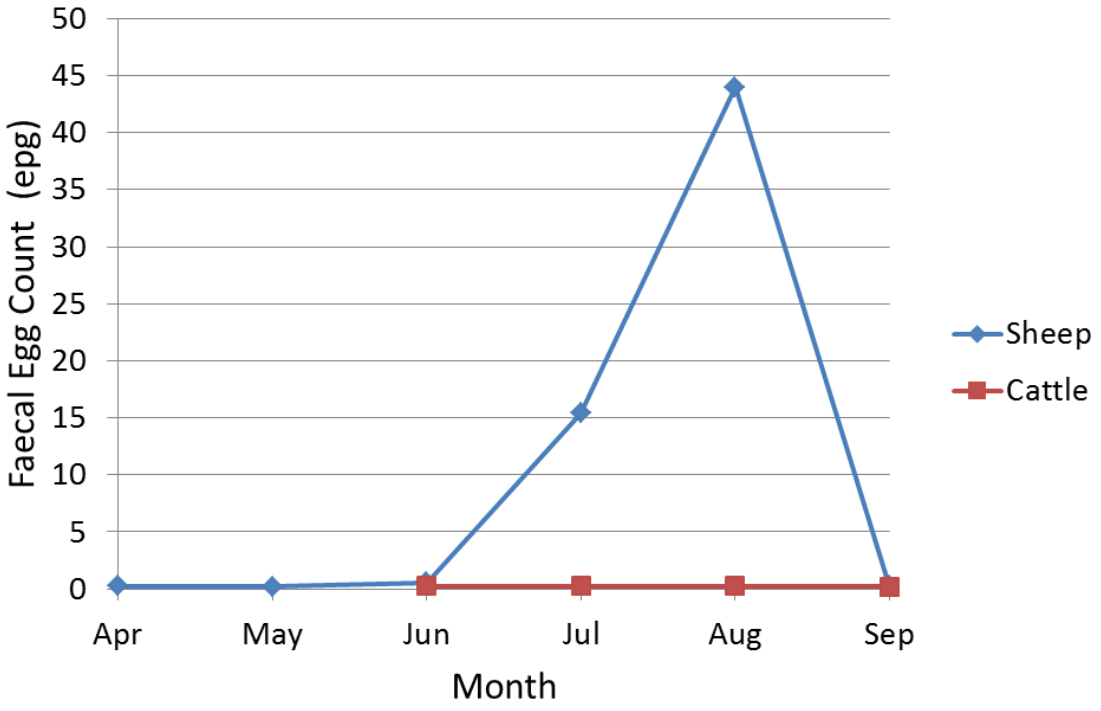

Figure 4: The pooled faecal egg counts for the ewe lambs and the cattle on farm 2 between April and September

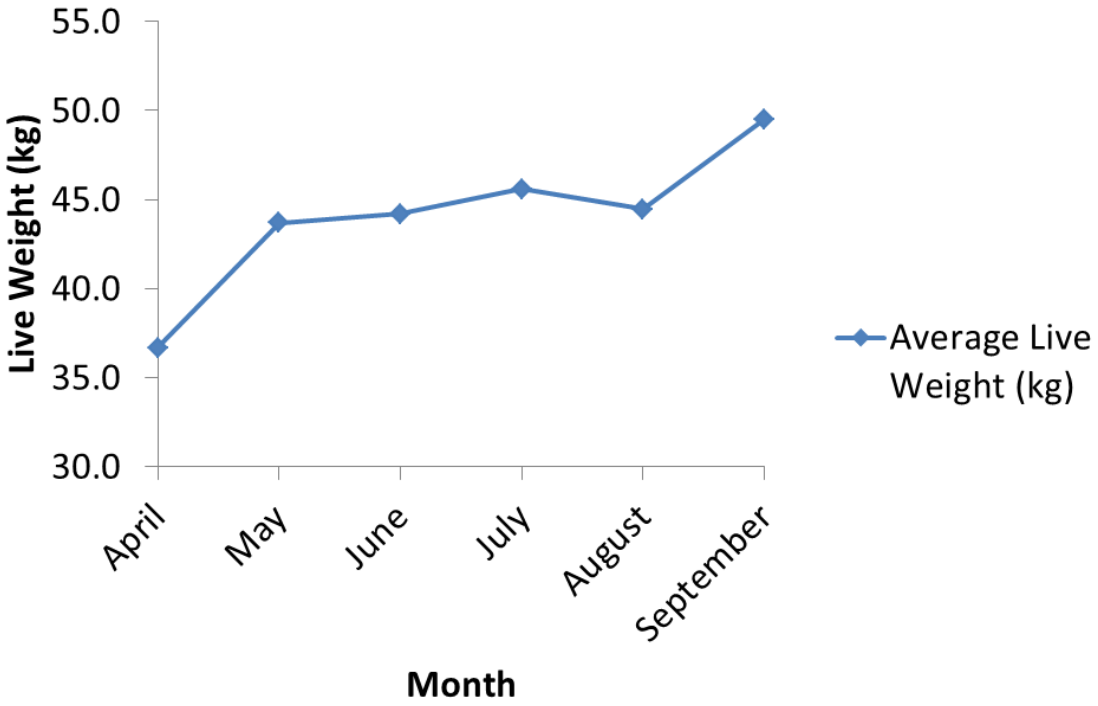

Figure 5: The live weight's of the ewe lambs on farm 2 between April and September

3.4 Resistance Data

A faecal egg count reduction test was undertaken due to the increase in adult (egg producing) infection in July 2011 and again in August that year. The test indicated that TCBZ was not as effective against adult egg producing infections as closantel and nitroxynil (Table 1). Liver fluke were harvested at slaughter and tested using the two resistance tests developed at IBERS in order to determine if this was due to liver fluke being resistant to TCBZ and not failure of the drug to reach the liver fluke in the correct dose and form to kill the liver fluke.

Table 1: Faecal egg count reduction test on four groups of ten ewe lambs. Control were not treated for liver fluke

Treatment	Day 0 (epg)	Day 21 (epg)	Percentage change
Control	20.5	44.0	0
Fasinex (TCBZ)	16.4	1.4	91.5
Flukiver (Closantel)	4.8	0	100.0
Trodax (Nitroxynil)	10.6	0	100.0

The failure of TCBZ treatment appears to be due to the presence of liver fluke resistant to TCBZ. This is shown by the average motility score of greater than 1 shown by the liver fluke after 24 hours of culturing in a normally lethal

concentration of TCBZ-SO (Figure 6). Also liver fluke from the farm were also statistically classified as having a global proteome significantly ($p>0.05$) more similar to a TCBZ resistant liver fluke than a TCBZ susceptible liver fluke.

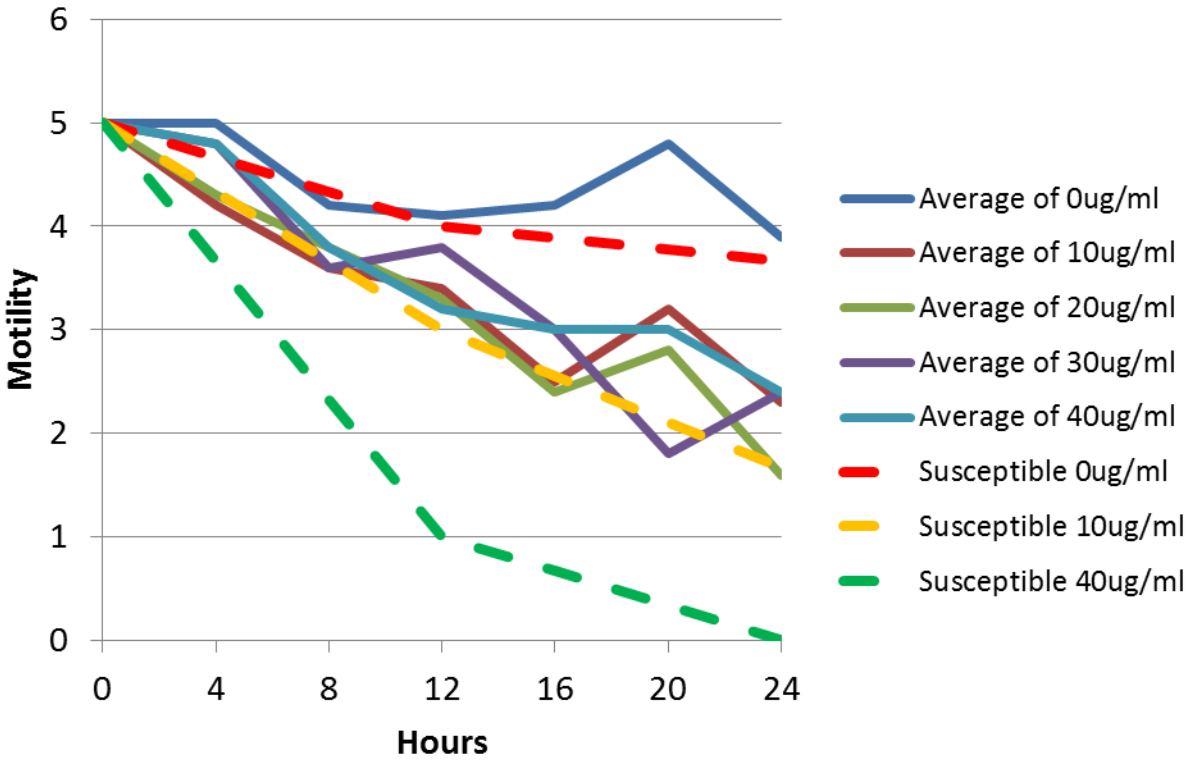

Figure 6: Relative motility of adult liver fluke collected from farm 2 and cultured in the presence of the activated pro-drug TCBZ-SO for up to 24 hour period in the laboratory

The activated pro-drug form of TCBZ-SO decreased as expected the mobility of control TCBZ sensitive liver fluke to zero by 24 hours at 40ug/ml in laboratory culture (Chemale et al 2010). None of the liver fluke from Farm 2 showed zero motility (death) over the identical 24 hour assay period in the presence of the anti-liver fluke drug as shown by the average mobility scores. Thus, liver fluke sampled from Farm 2 score as resistant to TCBZ in this test compared to a drug sensitive liver fluke control.

The years 2011 and 2012 from Farm 2 were compared with respect to liver fluke levels in hoggets using data from two separate visits to the slaughterhouse. Table 2 shows that the percentage of livers condemned from each group has fallen and, more importantly, the number of liver fluke found in each liver has also fallen between 2011 and 2012, from an average of 23 liver fluke per liver in 2011 to an average of 4 liver fluke per liver in 2012.

Table 2: Comparison of the general slaughter data collected for farm 2 at the beginning and the end of the 12 month project

	2011	2012	Difference
No. of Animals Slaughtered	25	18	7
No. of Livers Condemned	25	14	11
Percentage of Livers Condemned	100	78	22
No. of Condemned Livers without Fluke in Bile Ducts	4	7	-3
No. of Condemned Livers with Fluke in Bile Ducts	21	7	14
Average No. Fluke per Liver	23	4	19
Maximum No. of Fluke per Liver	92	15	77
Minimum No. of Fluke per Liver	0	0	0
No. of Livers with 1 to 10 Fluke	11	3	8
No. of Livers with 11 to 20 Fluke	4	4	0
No. of Livers with21 to 30 Fluke	0	0	0
No. of Livers with 31 to 40 Fluke	0	0	0
No. of Livers with 41 to 50 Fluke	1	0	1
No. of Livers with >50 Fluke	5	0	5

4 DISCUSSION

The relatively low levels of liver fluke infection observed in the project are likely due to the atypical cold weather that affected Wales during the winters of 2009/10 and 2010/11. These long cold spells will have reduced the number of liver fluke eggs surviving to the following spring and subsequently the numbers infecting mud snails. During the project a dry period of lower than average rainfall was experienced on many of the farms between late March 2011 and early May 2011.

The low levels of liver fluke infection observed in snails despite the snail population increasing was interesting, tentatively indicating that the snail population may recover faster that the parasite population to detrimental climatic changes. However, the data collected as part of this project cannot be considered as conclusive evidence, as it only represents one year of data. The snail data does indicate that dramatic changes in the weather, at key points in the year, will have dramatic effects on the liver fluke population's ability to complete its life cycle.

However, production data from farm 2 clearly shows that despite a drop in infection intensity, a critical percentage of the parasite population have completed

the life cycle supported by successful survival of the snail population. Thus, natural selection in parasites appears to favour a small number of parasite survivals in severe winter temperatures in order to maintain parasite populations and, thus, the potential remains on the farm for the parasite population to increase in number in the future.

The data collected here shows the combination of the following data is needed to fully plan the control of liver fluke on Welsh farms:

1. A full and detailed report of liver condemnations from every batch of animals sent to slaughter. The ideal would be the linking of tag numbers to each liver allowing an individual report on each animal's liver to be provided by the MHS through the slaughterhouse. Allowing each farmer to identify where the majority of infection is being picked up through their own grazing and treatment records.
2. The collection of faecal samples to check treatment is occurring at the correct time of the year.
3. The identification of potential snail habitats to look at possibilities of removal or separation from the grazing animals, by drainage or fencing.
4. The adjustment of grazing to avoid grazing fields with large areas of potential snail habitats during the summer months.
5. And finally, in combination with all data from points 1-4, the designing of a treatment schedule to reduce recontamination of grazing with eggs. This must be done with a veterinarian.

5 CONCLUSIONS

The findings of the project indicate the level of liver fluke infection in Wales has been lower overall in 2011/2012 than in previous periods. Although some slaughterhouses continue to report high percentage of liver condemnations it is clear from this projects data and from the drop in the number of submissions to the Veterinary Laboratory Agency's being diagnosed as acute cases of fasciolosis, that the intensity of infection per sheep has been reduced. This may have led to lower production losses as evident from live weight gains and less condition loss on the farm. This project did not resolve if this finding was liver fluke related. However, despite the possible benefits to the farmer of lowered intensity of infection the high percentage of livers currently being condemned possibly indicate that such benefits will not be seen by slaughterhouses and meat processors from reduced losses on the fifth quarter.

This project has supported the view that that it is feasible to develop a whole farm plan for the control of liver fluke by incorporating the new IBERS methods for testing TCBZ resistance work in the field. The findings of this project have been incorporated into a HCC sponsored booklet as part of the dissemination aims of the project.

Appendix 1: Project Data summary

1. SUMMARY DATA FOR SMALL SCALE INTEGRATED STUDY ON IMPACT OF *FASCIOLA HEPATICA* IN SHEEP IN WALES

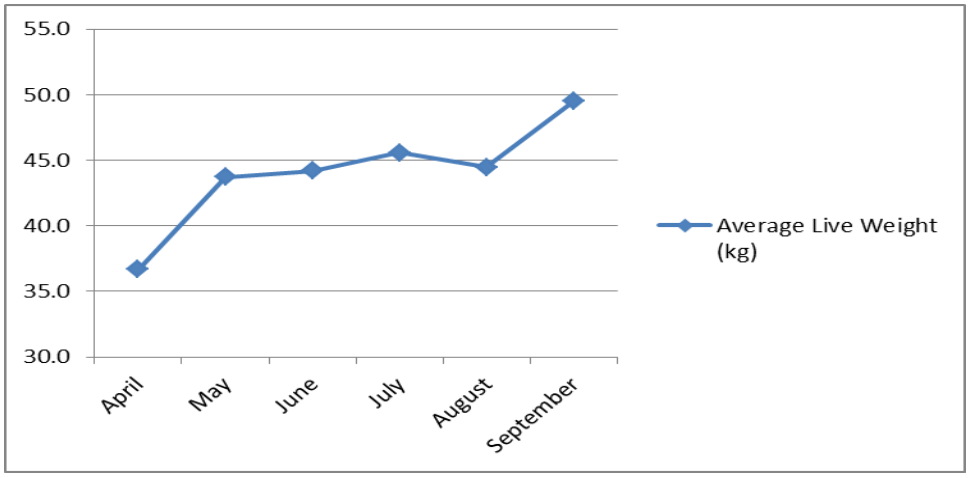

Figure 1 Live weights of 25 ewe lambs were taken over 5 months, the average of the animal's weights are shown below and tabulated in Table 1.

Table 1 *Live weights* of 25 ewe lambs were taken over 5 months, the average of the animal's weights are shown below and in shown graphically in Figure 1.

Month	April	May	June	July	August	September
Live weight (kg)	36.7	43.7	44.2	45.6	44.5	49.5
Live weight Gain (Kg/d)		0.26	0.01	0.05	-0.04	0.13

Table 2: *Faecal Egg Counts* Pooled faecal samples were taken monthly from the ewe lambs that were weighed on the same days. *epg = eggs per gram of faeces

Month	April	May	June	July	August	September
Ewe Lambs (epg)	0.25	0.20	0.54	15.43	44.00	0.40

Table 3: *Faecal Egg Count Reduction Test.* 40 ewe lambs were split into 4 groups of 10 and each group was either not treated for live 40 ewe lambs (including the 25 weighed for the production data) were treated as part of a faecal egg count reduction test study. The testing was completed in August after the faecal egg counts for that particular month. The live weight gains of the ewe lambs, in August, were also reduced. As the faecal egg counts dropped again in September the live weight gains increased. Ewes were treated with either: Trodax (Nitroxynil), Flukiver (Closantel) or Fasimex (Triclabendazole). Faecal samples were taken just before treatment and

taken again 21 days later. Efficacy of between 90-95% is considered effective by the World Association for the Advancement of Veterinary Parasitology (WAAVP), it is noted that closantel and nitroxynil achieved 100% reduction in egg counts in this study.

Treatment	Day 0 (epg)	Day 21 (epg)	Percentage change
Control	20.5	44.0	0
Fasinex (TCBZ)	16.4	1.4	91.5
Flukiver (Closantel)	4.8	0	100.0
Trodax (Nitroxynil)	10.6	0	100.0

Table 4: Independent *Faecal egg count reduction test* for closantel on two groups of 10 ewe lambs:

Group	Day 0 (epg*)	Day 21 (epg*)	Percentage change
Undosed	24	28	0
Dosed	30	0	100.0

*epg = eggs per gram of faeces

The results of the sedimentation faecal egg counts appear to also show closantel to be 100% effective against adult liver fluke. Recommendation with this finding to consult farm vet about regular repeats of testing to ensure closantel's efficacy continues to hold.

Table 5: *Slaughterhouse data on liver fluke levels:* Two groups of hoggets from same farm study were followed through slaughter. The first group were slaughtered on the March 2011 and the second on the January 2012. Table 5 shows a summary of the general data obtained about the animals.

	2011	2012	Difference
No. of Animals Slaughtered	25	18	7
No. of Livers Condemned	25	14	11
Percentage of Livers Condemned	100	78	22
No. of Condemned Livers without Fluke in Bile Ducts	4	7	-3
No. of Condemned Livers with Fluke in Bile Ducts	21	7	14
Average No. Fluke per Liver	23	4	19
Maximum No. of Fluke per Liver	92	15	77
Minimum No. of Fluke per Liver	0	0	0
No. of Livers with 1 to 10 Fluke	11	3	8
No. of Livers with 11 to 20 Fluke	4	4	0
No. of Livers with21 to 30 Fluke	0	0	0
No. of Livers with 31 to 40 Fluke	0	0	0
No. of Livers with 41 to 50 Fluke	1	0	1
No. of Livers with >50 Fluke	5	0	5

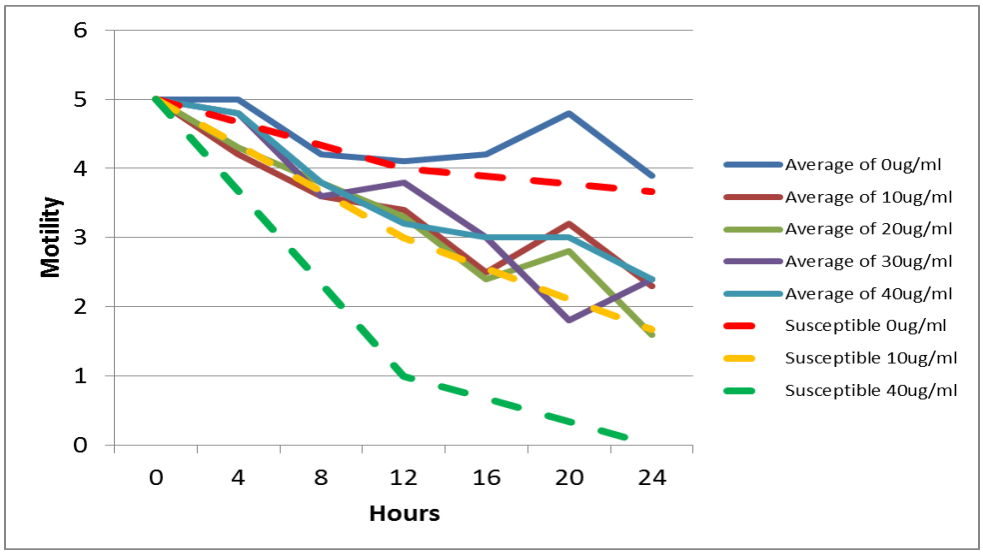

Figure 2: *IBERS resistance testing* on liver fluke from January 2012 from Table 5. In brief, liver fluke were collected and cultured in presence of TCBZ-SO for 24 hours, at 4 different concentrations. 40, 30 and 20ug/ml are considered lethal doses and 10µg/ml is considered a sub-lethal dose. The motility of the liver fluke collected were compared to the motility of liver fluke known to be fully susceptible to TCBZ-SO. The findings indicate that there were liver fluke collected on January 2012 were able to survive culturing in TCBZ-SO for 24 hours at each concentration. This appears to indicate that the failure of TCBZ to remove all eggs from the faeces of the ewe lambs in Table 3 is most likely due to liver fluke that are resistant to TCBZ. Experimental research sheep infection using TCBZ surviving fluke eggs from gall bladders is on-going in order to confirm resistance.

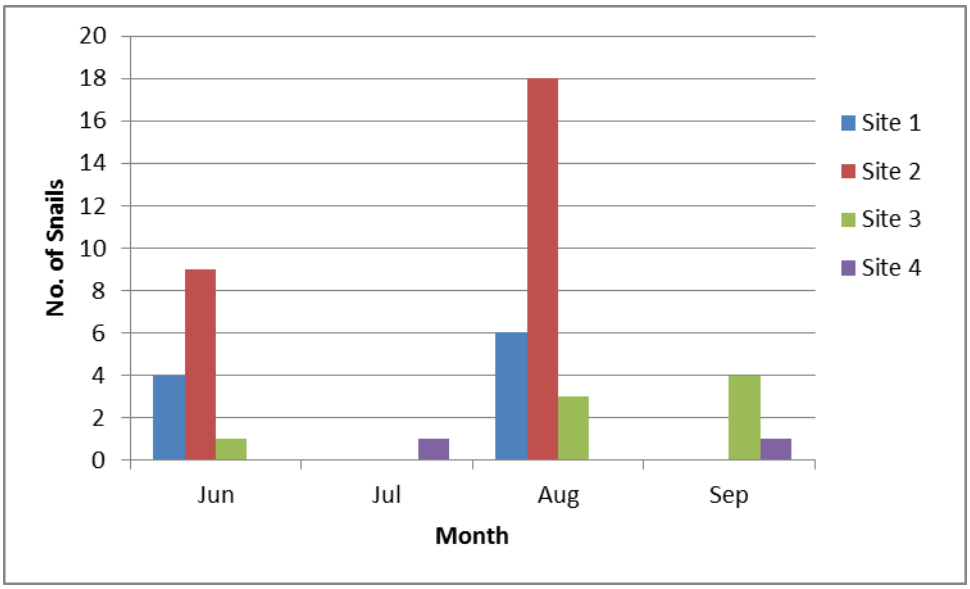

Figure 5: *Confirmation of intermediate mud snail host present in fields with infected ewes***.** Snails were collected from 4 sites identified as potential habitats for liver fluke's intermediate snail host, *Galba truncatula*. The snails were collected on 1 day per month for 10 minutes per site, between

June and September to indicate the activity of the snails above ground (Figure 4). The snails were then dissected to determine if they were infected with liver fluke

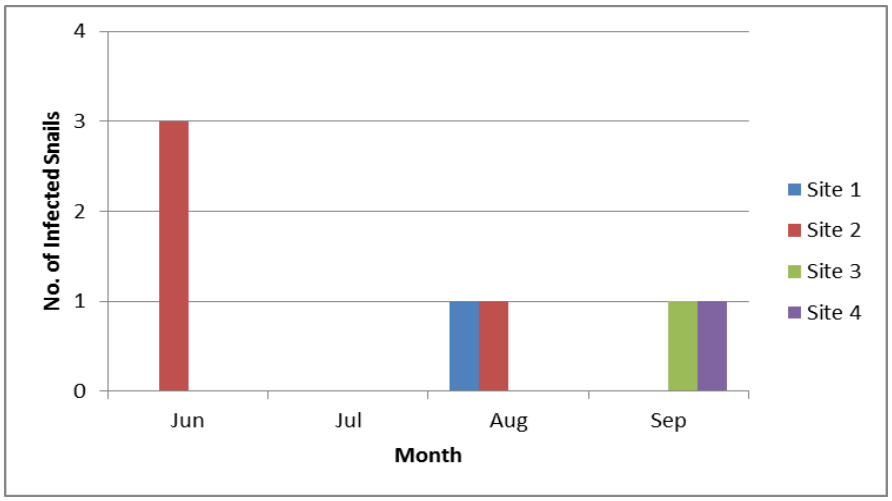

Figure 6: *Confirmation that 13% of intermediate mud snail host present in fields with liver fluke infected ewes were infected with the larval stage of the parasite.*

Conclusions : The findings indicate that depite the previous realativley cold winter the liver fluke parasite has managed to over-winter and infect intermediate snails. Also the number of snails and the number of snails infected varies between sites and month. However, snails were collected from every site in at least one of the months and in at least one of the months, an infected snail was found indicating that the sites may vary in potential to infect livestock but all sites can be infectious during the summer. It appears that infection is occurring in late spring and throughout the summer on this farm leading to patent infections being detected from July onwards in the sheep, at least.The slaughter data shows that in the hoggets at least, the level of liver fluke infection has been lower this year which could be due to the cold winter between 2010 and 2011, and the dry spell in the spring of 2011. Affecting snail reproduction which normally occurs around then, reducing the chances of any eggs that did survived the frost or were deposited after, of hatching and finding a snail host. The faecal egg count reduction test, the culturing of liver fluke in TCBZ-SO and the classification of adult liver fluke, indicates that liver fluke resistant to TCBZ containing products are present.

Appendix 2

MUD SNAILS AND LIVER FLUKE INFECTION RATES IN FARMS IN SMALL SCALE STUDY IN WALES 2011

Table 1: Summary tabulated data of Mud Snail activity (numbers) and levels of snail infection with liver fluke from June to September 2011 in Wales Farm Survey

Month	Snail number Recovered	Snails infected	% snail infected
June	22	4	18
July	56	12	21
August	166	8	5
September	41	6	15
TOTALS	**285**	**30**	**11**

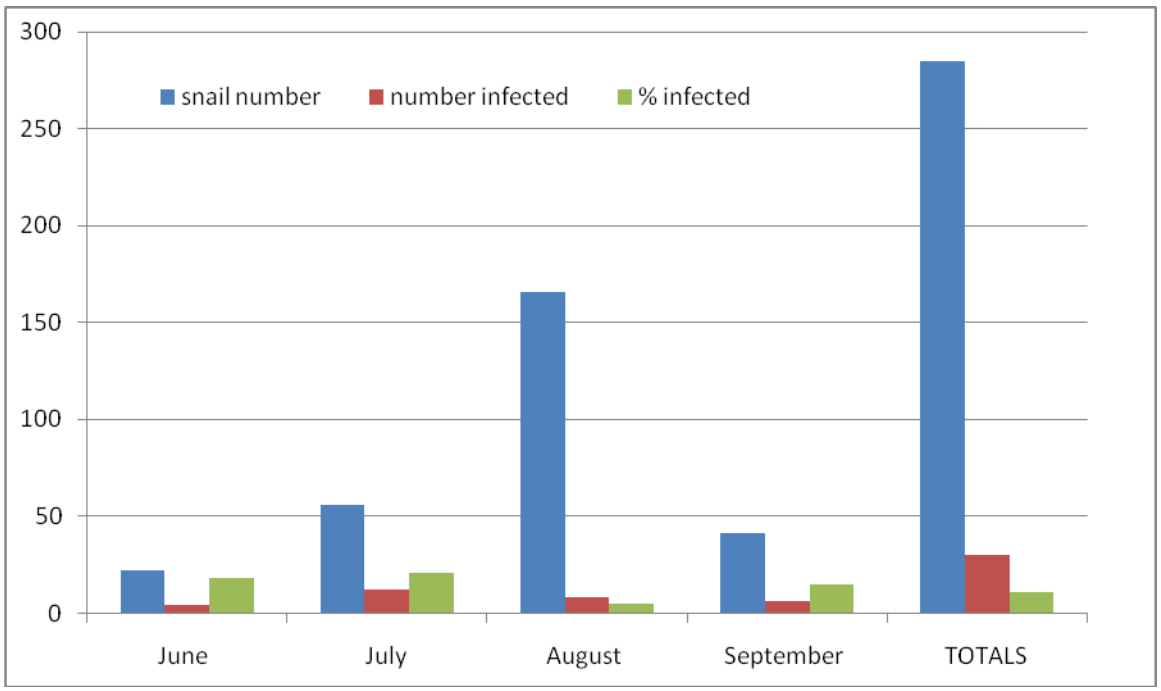

Figure 1: Graphical representation of mud snail activity (numbers) and level of snail infection with liver fluke between June and September 2011 in Wales Farm Survey

Summary: The Mud snail (*Galba truncatula*) can be collected from Welsh farms and infection rates with liver fluke determined by dissection & microscopy analysis with confirmation PCR tests in IBERS laboratories for correct species of snail and parasite. An approximate overall 10% snail infection rate between June and September 2011 is able to maintain liver fluke life cycle for livestock infection.