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Six-year longitudinal study of *Fasciola hepatica* bulk milk antibody ELISA in the dairy dense region of the Republic Ireland

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ABSTRACT

Completion of the *F. hepatica* lifecycle is dependent on suitable climatic conditions for development of immature stages of the parasite, and its snail intermediate host. Few investigations have been conducted regarding temporal variations in *F. hepatica* status in Irish dairy herds. The current study aimed to conduct a longitudinal study examining annual and seasonal trends in bulk milk seropositivity over six years, while also investigating associations with soil temperature, rainfall and flukicide treatment. Monthly bulk milk samples (BTM) were submitted by 28 herds between March 2009 and December 2014. In all, 1337 samples were analysed using a Cathepsin L1 ELISA. Soil temperature, rainfall and management data were obtained for general estimating equation and regression analyses. A general decrease in milk seropositivity was observed over the six year study period and was associated with an increased likelihood of treating for liver fluke (OR range = 2.73–6.96). Annual and seasonal analyses of rainfall and *F. hepatica* BTM status yielded conflicting results. Higher annual rainfall (>1150 mm) yielded a lower likelihood of being BTM positive than annual rainfall of <1000 mm (OR = 0.47; $P = 0.036$). This was most likely due to farmers being more proactive in treating for *F. hepatica* in wetter years, although a 'wash effect' by high rainfall of the free living stages and snails cannot be ruled out. Higher seasonal rainfall (>120 mm), however, was associated with increased ELISA S/P% values (Coefficient = 9.63S/P%; $P = 0.001$). Soil temperature was not found to influence *F. hepatica* to the same extent as rainfall and may reflect the lack of severe temperature fluctuations in Ireland. Flukicides active against both immature and mature *F. hepatica* were approximately half as likely to record a positive *F. hepatica* herd BTM status than a flukicide active against only the mature stage of the parasite (OR ≅ 0.45; $P < 0.01$). This study highlights the importance of examining both annual and seasonal *F. hepatica* data, which can vary significantly. Additionally, it highlights the progress that can be achieved in fluke control by application of a continuous BTM monitoring program.

1. Introduction

Fasciola hepatica, commonly known as liver fluke, is an helminth parasite of mammals of the Phylum Platyhelmintes, Class Trematoda (Urquhart et al., 1996; Borgsteede, 2011). Hosts include cattle, sheep, goat, horse, deer and humans (Taylor et al., 2007). This parasite has a worldwide distribution across these species, although the impact on the human population is highly variable and very much related to socio-economic factors (Mas-Coma et al., 2009). It is considered an important disease of domestic livestock, especially in temperate climatic zones (Bennema et al., 2011), and has been estimated to generate annual losses of €90 million in Irish livestock species and €2.5 billion worldwide (AHI, 2011).

The predilection site for sexually mature *F. hepatica* in mammalian hosts is the bile ducts in the liver. From here, mature flukes shed eggs into the gastrointestinal tract which are subsequently released into the environment in faeces. Eggs hatch in the environment and develop into miracidia, a process requiring a minimum temperature of 10 °C and a reported optimum temperature of 22–26 °C (Taylor et al., 2007). Once developed, miracidia penetrate an intermediate molluscan snail host of the family Lymnaeidae, the particular species of which varies by geographical location. *Galba truncatula* acts as the main intermediate host in Europe, and this species requires moderate climatic conditions and muddy/sodden land to thrive (Thomas, 1883). Further larval stages develop in the snail host until cercariae are formed. On their release from the snail, cercaria encyst on vegetation to form the infective stage i.e.

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metacercariae. On ingestion by mammalian hosts, such as cattle and sheep, the infectious immature fluke migrate through intestinal and liver tissue until maturation in the bile ducts (Urquhart et al., 1996). The minimal period for the entire *F. hepatica* lifecycle is 17–18 weeks (Taylor et al., 2007).

Due to the environmental conditions required for fluke larval maturation, and for development of appropriate snail host habitat, temperature and rainfall have been identified as important contributory risk factors for an increased prevalence of *F. hepatica.* (Mas-Coma et al., 2009). Global warming is a widely accepted phenomenon and unprecedented changes in climate have been recorded since the 1950's. Further warming and long-lasting climate changes are expected (IPCC, 2014) with parallel increases in the risk of fasciolosis predicted (Fox et al., 2011; Van Dijk et al., 2010) due to increased cercarial production (Mas-Coma et al., 2009). The Republic of Ireland (heretofore referred to as Ireland) is located at 53.3442° N, 6.2675° W and is located in a temperate climatic zone. Between 1971 and 2010 the average monthly rainfall was 92.5 mm and average temperature was 10 °C (ECAD, 2016), appropriate conditions for completion of the fluke lifecycle as evidenced by a *F. hepatica* prevalence of 76.1% in Irish dairy herds in November 2009 (Bloemhoff et al., 2015). Met Éireann, the Irish Meteorological Service, has reported an increase in average air temperature in Ireland of 0.8 °C over the last 100 years and statistical prediction models indicate a rise in global temperature of 2 °C by the end of this century. Met Éireann (2016) also reports an approximate increase in rainfall of 5% over the last 30 years, and an increase in the number of 'very wet days' (days with rainfall >20 mm). These ongoing and predicted weather changes in Ireland may present even more favourable conditions for *F. hepatica* development.

The differences in exposure to *F. hepatica* in Irish dairy herds have previously been shown to be strongly dependent on the soil type. Soils on farms that did not record fluke exposure were deep and shallow well-drained soils, while poorly drained soils were the main soil types on farms where herds recorded exposure to the parasite (Selemetas et al., 2014). Little evidence has been presented, however, regarding annual variations in exposure to *F. hepatica* in Irish dairy herds and the impact of annual weather variability on the risk of exposure. In that regard, the current study aimed to conduct a longitudinal study to examine trends in bulk milk seropositivity over time. The study also aimed to investigate associations between *F. hepatica* bulk milk seropositivity, two weather-related variables (soil temperature and rainfall), and additional risk factors.

2. Materials and methods

2.1. Sample population

A total of 28 herds were included in the study. Of these, 22 were commercial dairy farms, all members of the Dairy Management Information System ('DairyMIS') discussion group coordinated by Teagasc (Irish Agriculture and Food Development Authority). The remaining six were Teagasc research herds. All farms were located in the southernmost Munster region in Ireland. These herds were initially recruited in 2009 to a *F. hepatica* prevalence study (Bloemhoff et al., 2015). Sampling and data collection were subsequently extended for the purposes of the current research.

2.2. Sampling

Each study farm was requested to submit a monthly bulk tank milk (BTM) sample over the course of each lactation between March 2009 and December 2014. For logistical reasons, monthly samples were not available for spring and summer seasons in 2010. Samples were submitted to Teagasc by post in a standardised sampling kit, which contained a sample bottle containing broad spectrum Microtab II milk preservative tablets (D&F Control Systems Inc., USA). All samples were received within 48 h of sampling. On receipt at the laboratory, BTM samples were dispensed into 2.5 mL micro tubes (Sarstedt, Germany), centrifuged at 20,000*g* for one minute, de-fatted, and the supernatant transferred to 1.5 mL microtubes (Sarstedt, Germany). De-fatted samples were frozen at −80° C until analysed.

2.3. Sample analysis

All samples were analysed using a commercially available ELISA kit as previously described by Selemetas et al. (2014). Briefly, the kit is based on the recombinant mutant Cathepsin L1 antigen (rmCL1) (Ildana Biotech, Dublin, Ireland; Collins et al., 2004) and has a documented sensitivity (Se) and specificity (Sp) of 98% (Bloemhoff et al., 2015). It has been previously shown that F. hepatica antibodies detected by this test decrease three to four months following treatment with a flukicide (Bloemhoff et al., 2015).

As per kit manufacturer's instructions, the level of antibodies present in each BTM sample was expressed as S/P%, calculated using the formula S/P% = (sample optical density (OD)/mean OD of positive control) × 100. Samples yielding S/P% values of >15 were classified as positive for exposure to *F. hepatica*.

2.4. Weather data

Daily rainfall (mm) and soil temperature at depths of 5 cm, 10 cm, and 15 cm below ground level (°C) were recorded by an automated weather measurement station (Campbell Scientific Ltd. Loughborough, UK) based at Teagasc Moorepark, Fermoy, Co Cork, Ireland. This weather measurement station was located within 30 miles of study farms. Daily rainfall measurements were summated to yield monthly values (mm), these values were subsequently averaged to yield seasonal (spring (January, February, March), summer (April, May, June), autumn (July, August, September), winter (October, November, December)) and annual (2009–2014) mean monthly rainfall. Soil temperatures were recorded every five minutes and subsequently averaged to yield seasonal and annual soil temperature values (°C). The 5 cm depth soil temperature reading was chosen for inclusion in the current data analysis as soil temperature is highly correlated with air temperature (Charlier et al., 2014) and this soil depth more closely reflects both fluke larvae and mollusc environments.

2.5. Farm and fluke management data

Farm and fluke management data were collected using a web-based survey tool (https://www.surveymonkey.com). Data collected included predominant dairy cow breed in each herd, farmer self-reported land classification, and whether or not an annual flukicide treatment was administered. Additionally, flukicide product(s) used in each year of the study, and how many times the product was administered in a particular year, was recorded. Participation in the study was non-incentivised other than reporting of results of monthly BTM testing in October each year to inform each farmer's annual *F. hepatica* control programme.

2.6. Herd classification

Each study herd was categorised by herd size (small (71–130 cows), medium (131–190 cows, large (>190 cows)), predominant breed in the herd (Holstein-Friesian (HF), Jersey cross (JEX), Norwegian Red cross (NRX)), and farmer self-reported land classification (dry (predominantly dry paddocks on the farm), occasionally waterlogged (some paddocks prone to becoming wet following

rainfall), wet (predominantly wet paddocks on the farm)). With regard to flukicide treatments, herds were classified as 'treated' or 'not treated' on the basis of whether at least a single treatment was administered in a particular year. The flukicide products administered were classified into five categories depending on the spectrum of activity of the product used and the number of times a product was administered in a particular year. Category 1 farms administered a single dose of a product active against mature *F. hepatica* alone i.e. albendazole, oxyclozanide, clorsulon. Category 2 farms employed a two dose programme using only products active against mature *F. hepatica*. Category 3 farms administered a single dose of a product active against both mature and immature *F. hepatica* i.e. triclabendazole, nitroxynil. Category 4 farms used a combination of flukicide products with differing activity spectra in a multi-dose programme. Finally, category 5 farms included those that did not administer a flukicide product in a particular year. Herd categorisations on the basis of flukicide treatment programme were assigned annually. Finally, each farm was classified as *F. hepatica* BTM antibody 'positive' or 'negative' on the basis of monthly BTM ELISA S/P% values.

2.7. Data analysis

Data collation and descriptive analyses were completed in Excel (MS Office 2010). Normality of the data was assessed using ladder of powers histogram, which showed visually different power transformations promoting symmetry of the data, normality of residuals was assessed using normal probability plots both constructed in Stata version 11 (StataCorp, USA). Graphical representations, Mann-Whitney test, and *t*-test were completed in GraphPad Prism 6 (GraphPad Software Inc., 2016). Additional statistical analyses, including Pearson's chi-squared, generalised estimating equations (GEE) and regression analyses, were completed using Stata version 11 (StataCorp, USA).

In order to complete the most comprehensive study of the variability in exposure to *F. hepatica* over time, both annual (2009–2014) and seasonal (spring, summer, autumn, and winter of each study year) datasets were compiled. Additional independent variables investigated included categorical seasonal soil temperature (low (<5 °C), borderline low (>5–8 °C), moderate (>8–12 °C), boderline high (>12–15 °C), high (>15–18 °C)), annual soil temperature (<10 °C, >10 °C), seasonal rainfall (low (41–80 mm), moderate (>80–120 mm), high (>120 mm)), and annual rainfall (low (850–1000 mm), moderate (>1000–1150 mm), high (>1150 mm)). Categorisation for each variable was defined to best represent the spread of data recorded over the duration of the study.

Study herd demographics, number of samples per farm, and monthly S/P% values per farm were tabulated, as well as annual and seasonal S/P%, rainfall and soil temperature summary statistics. The Mann-Whitney test used to determine significant differences between monthly S/P% across each year of the study using 2009 as the reference year, as in this year test results were based on farmers' own treatment program. Similarly, a *t*-test was used to examine differences in normally distributed rainfall and soil temperature, again using 2009 as the reference year. Boxplots and line graphs of annual and monthly BTM S/P%, mean monthly rainfall, and mean soil temperature, were constructed to allow clearer visualisation of these data. Finally, the frequency of annual use of anthelmintics (flukicides) was computed and plotted.

2.7.1. Generalised estimating equations (GEE)

A univariable generalised estimating equation (GEE) analysis, was used to investigate seasonal differences in *F. hepatica* S/P% values across each year of the study, using spring 2009 as the reference variable. Herd was included as a repeated measure, and a Gaussian distribution, identity link, and exchangeable correlation used to generate GEE coefficients. Coefficients were subsequently plotted to allow visual assessment of seasonal and annual trends in *F. hepatica* BTM antibody over the entire duration of the study.

For the purposes of constructing the multivariable GEE model, two-way associations between categorical independent variables were initially identified by Person's chi-squared. Variables recording P values ≤ 0.15 in this analysis were investigated as potential interacting variables in the multivariable GEE model. BTM results were not included in this analysis due to the repeated nature of the data. Herd *F. hepatica* BTM antibody status, both categorical (positive vs. negative) and continuous (ELISA readings), was the dependent variable in all multivariable GEE analyses. Independent variables included time (year or season), weather (rainfall, soil temperature) flukicide treatment and herd management data. In final models, herd was included as a repeated measure, a Gaussian distribution and identity link function was used for continuous data, and a binominal distribution and logit link function assumed for categorical data. An exchangeable correlation was used for both continuous and categorical GEE analyses. A manual backwards elimination with a forward step was used to build final models with variables recording P values of ≤ 0.05 maintained. Second level interactions between independent variables were examined for significance and retained if significant at the 5% level.

2.7.2. Logistic regression

Multivariable logistic regression was performed to examine associations between flukicide treatment (dependent variable) and independent variables identified as significant in the Pearson chi-squared analysis conducted for GEE. Again, a manual backwards elimination with a forward step was used to build the final model with variables recording P values of ≤ 0.05 maintained. Second level interactions between independent variables were investigated and included in the final model at a P value ≤ 0.05.

3. Results

3.1. Descriptive analysis

A total of 1337 samples from 28 herds were collected and analysed over the entire study period (2009–2014). The mean number of samples per farm per year was 8 (range 0–11 samples). The numbers of samples submitted by each study farm and their mean annual S/P% value each year over the study period are included in Table 1. The majority of herds ($n = 27$) reported having a spring calving system i.e. the majority of cows calved between February and May of any year. A single herd operated an autumn-calving system during the first four years of study, and subsequently reduced to 60% of the herd calving in autumn for the last two years of the study. All study herds were specialist dairy enterprises with no additional livestock species (e.g. fattening cattle or sheep) present on the farm. All cows had full access to pasture from at least March until November and all were housed in December and January of each year. Housing periods extended to varying degrees into November and February of any particular year depending on grass availability and weather conditions.

The mean herd size across the 28 study herds was 161 cows (range 71–310 cows). Of these, 8 herds contained <100 cows and 13 contained >150 cows. The predominant breed in the majority of herds was Holstein-Friesian (78.6%). The majority of farmers (60.7%) reported having dry land across their farms, the remainder stating their farms were either 'wet' or occasionally waterlogged. More detailed herd demographic data are included in Table 2.

Table 1
Number of samples collected from study herds in each year of the study and their mean S/P% values.

Herd	2009		2010		2011		2012		2013		2014		Total per farm	Mean S/P% 2009 to 2014
	n	Mean S/P%	*n*	Mean S/P%	*n*	Mean S/P%	*n*	Mean S/P%	*n*	Mean S/P%	*n*	Mean S/P%	*n*	
1	10	0.8	5	4.6	9	0	9	0.1	8	0.1	8	−0.9	49	0.54
2	10	126.8	6	134.5	10	101.2	11	58.2	9	54	10	17.9	56	78.46
3	10	36.9	4	93	10	45.3	9	19.7	8	25.9	9	7	50	32.84
4	10	1.3	5	9.4	9	0.6	9	3.6	9	1	8	−0.3	50	2.16
5	10	74.8	4	27.8	8	2.8	9	21.6	9	4.2	8	0.6	48	23.36
6	10	135.8	5	156.4	9	98.6	9	94.4	10	93	8	53.8	51	102.66
7	10	70.2	6	138.7	10	95.2	9	49.7	9	46.8	9	9.4	53	64.91
8	10	1.4	4	3.8	8	6	9	16.4	9	33	9	25.8	49	15.31
9	9	2.8	5	8.2	9	1.3	9	−0.8	9	0.8	7	−0.1	48	1.63
10	10	118.3	5	143.4	10	38.7	10	27.8	9	9.9	8	0.3	52	51.05
11	10	75.7	4	123.8	9	82.1	9	91.1	10	71.7	9	40.7	51	76.26
12	10	13.5	6	9.3	10	3.2	10	14.8	9	9.2	8	−0.8	53	8.45
13	10	91.8	5	133	6	71.7	6	49	3	13.3	0		30	78.24
14	10	103.7	5	129.2	9	54.2	10	23.8	9	6.1	8	−0.6	51	48.21
15	9	9.8	5	4.2	9	3.7	9	17.3	8	17.1	8	20	48	12.45
16	10	2.8	5	0.8	9	0.2	10	0.2	10	1.3	9	0.8	53	1.01
17	10	28.5	4	35.5	9	31.4	9	18	7	47.4	7	15.6	46	28.59
18	9	123.8	5	135	11	95.2	9	78	10	59.9	8	27.6	52	83.78
19	10	134.3	6	94	10	31.1	11	20.5	10	7.5	9	7.9	56	46.19
20	10	9.5	6	25.8	11	0.7	9	15	8	11.1	8	4.6	52	9.97
21	9	14.4	5	38.2	9	34.1	9	22.4	7	36	4	13.8	43	26.45
22	10	101.1	4	128.3	9	76.7	8	36.1	7	55.6	6	12.2	44	67.33
25	10	93.5	6	89.8	11	86.8	11	53.1	9	75.3	10	38.4	57	71.5
26	10	5.1	4	8.8	10	31.4	8	0.3	4	31.3	1	28	37	14.91
27	10	26.4	3	57	2	74	6	0.3	10	48.7	8	28.6	39	33.36
28	10	24.8	6	73.7	10	92.7	11	75.9	9	70.4	10	10.1	56	56.96
29	10	29.5	5	69.4	7	79	8	64.8	8	84.3	6	65.3	44	63.26
32	0		0		0		0		10	36.7	9	0.8	19	19.73
Mean S/P% (all farms)		54.2		70.7		45.5		32.8		34.7		15.2		
Total samples (*n*)	266		133		243		246		237		212		1337	39.98

Table 2
Study herd demographics including herd size and predominant breed.

Variable	Proportion (%)
Category	
Herd size (cows)	
71–130	35.7%
131–190	32.1%
>190	32.1%
Predominant Breed	
HF[a]	78.0%
JEX[b]	14.3%
NRX[c]	7.1%
Calving period	
Spring-calving	96.4%
Non-spring-calving	3.6%
Enterprise	
Dairy only	100.0%
Farmer land classification[d]	
Dry	60.7%
Occasionally waterlogged	14.0%
Wet	25.0%

[a] Holstein-Friesian.
[b] Jersey crossbreed.
[c] Norwegian Red crossbreed.
[d] Farmer self-reported classification of predominant land type on each study farm.

3.2. F. *hepatica* S/P%, rainfall and soil temperature values

Ladder of powers histograms revealed a normal distribution for weather variables, S/P% values presented a positive skewness and normal probability plot ensured the normality of residuals.

Annual and seasonal BTM seropositivity interquartile range (IQR) and median S/P% values are included in Table 3 and highlighted a general decrease in exposure to *F. hepatica* in study herds from 2010 (Table 3). The highest levels of exposure to *F. hepatica* were recorded in winter. Examination of annual trends highlighted that 2010 recorded the highest median *F. hepatica* S/P% values and 2014, the lowest. S/P% values in 2010, 2012, and 2014 were significantly different from 2009 (Table 3), being higher in 2010 and lower in 2012 and 2014. There was no visible trend in annual rainfall and soil temperature, although 2013 tended to be colder than other years. A boxplot of annual and monthly S/P% ELISA values in addition to mean soil temperature and rainfall are shown in Fig. 1.

3.3. Flukicide treatment of milking herd

Flukicide treatment protocols for all six years of the study were available for 25 herds. A single farm did not provide any treatment data and two farms did not have records of 2009 treatment protocols. In all, 134 herd treatments were administered across 27 farms over the six years of study. The majority of farmers administered at least a single treatment in every year of the study ($n = 18$; 66.7%). The number of farmers using each flukicide product is outlined in Fig. 2a with the frequency of herds in each product treatment category (categories 1–5) by year included in Fig. 2b. The median S/P% of farms that used or did not use treatment across the entire duration of the study was 45 and 28 S/P%, respectively, and the difference was significantly different ($P = 0.002$).

3.4. GEE and logistic regression analyses

Univariable GEE analysis of BTM ELISA status results over time are outlined in Supplementary Table S1 and highlighted significant differences between seasons. A graphical representation of the difference in S/P% values in each season compared to spring 2009 is outlined in Fig. 3 and exhibits an overall downward trend in BTM result over the course of the study. Univariable associations

Table 3
Significant differences between S/P%, mean monthly rainfall, and soil temperature across study year (2009 used as reference year) and season (winter used as reference season).

Variable	S/P ratio			Mean monthly rainfall (mm)				Soil Temperature (°C)		
	Median	IQR	Range	Mean	SD	CI (95%)	Total annual	Mean	SD	CI (95%)
Year										
2009	25.50	92.75	0, 194	107.80	66.32	101.00, 114.50	1293.6	11.03	5.26	10.50, 11.57
2010	66.00[a]	112.00	0, 179	72.40	34.89	68.84, 75.96	868.8	10.23	6.14	9.50, 10.85
2011	30.50	84.00	0, 135	71.30	34.99	67.73, 74.87	855.6	11.2	4.53	10.74, 11.66
2012	13.00[a]	64.00	−8, 125	91.45	57.36	85.60, 97.30	1097.4	10.83	4.49	10.37, 11.28
2013	29.00	52.50	0, 123	78.83	42.85	74.46, 83.19	945.9	6.65[b]	5.16	6.12, 7.18
2014	6.00[a]	21.00	−2, 93	103.20	57.35	97.39, 109.10	1238.8	11.38	5.48	10.82, 11.93
Season										
Spring	15.50	70.25	−3, 159	85.73	58.97	80.82, 90.63	n/a	4.97	2.44	4.76, 5.17
Summer	10.00	50.00	−2, 147	73.55	39.56	70.26, 76.84	n/a	13.25	4.18	12.90, 13.6
Autumn	14.00	59.00	−8, 176	75.53	46.48	71.66, 79.39	n/a	15.37	3.51	15.07, 15.66
Winter	48.00	92.25	−1, 194	115.20	51.54	110.90, 119.50	n/a	7.29	3.39	7.01, 7.57

[a] Significantly different at $P < 0.01$ using Mann-Whitney test.
[b] Tendancy toward significance at $P > 0.05 < 0.10$ using t-test.

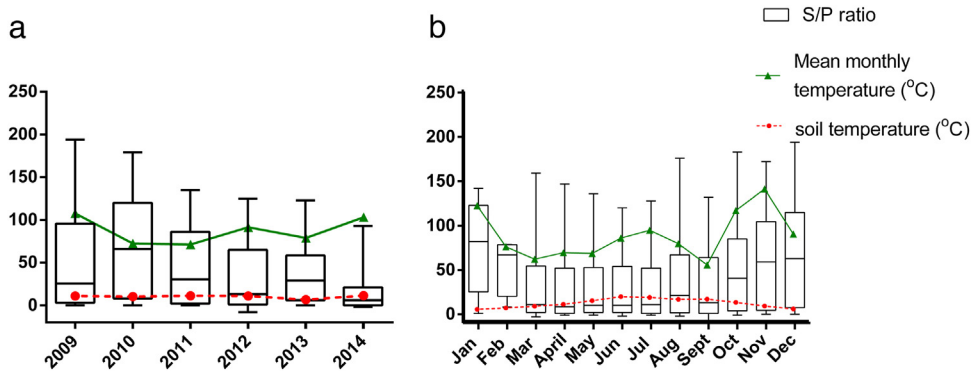

Fig. 1. Boxplot of annual (a) and monthly (b) mean S/P% across all study farms. Superimposed line graphs represent mean soil temperature and mean monthly rainfall in each year and month of the study.

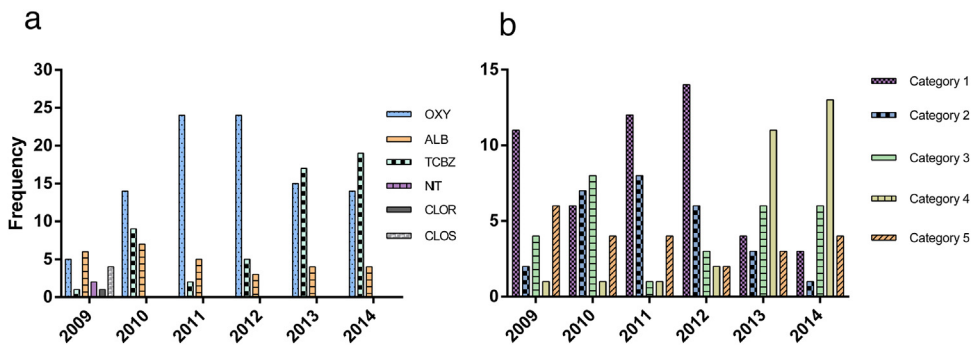

Fig. 2. Frequency of use of different flukicide active ingredients during each year of the study (a) and frequency of use based on activity of each active ingredient (categories 1–5) (b).
OXY = oxyclozanide, TCBZ = triclabendazole, ALB = albendazole, NIT = nitroxynil, CLOR = clorsulon, CLOS = closantel
Category 1: a single dose of a product active against mature liver fluke only
Category 2: a two-dose programme using only products active against mature liver fluke
Category 3: a single dose of a product active against both mature and immature liver fluke
Category 4: a combination of flukicide products with differing activity spectra in a multi-dose programme
Category 5: no flukicide product administered.

between additional independent variables are outlined in Supplementary Table S2. Multivariable GEE analyses of both annual and seasonal data are outlined in Tables 4 and 5 (categorical data) and Supplementary Tables S3 and S4 (continuous data). Annual and seasonal analysis of the association between rainfall and *F. hepatica* BTM yielded contradictory results. Annual rainfall >1000 mm was associated with a lower risk of *F. hepatica* (OR < 0.5; $P < 0.05$), while seasonal rainfall over 120 mm (winter 2009, winter 2013 and spring 2014) increased the risk (OR = 1.37; $P = 0.059$). Mean annual soil temperature was not significantly associated with *F. hepatica* status while, in general, seasonal temperatures less than 8 °C and greater than 12 °C reduced the risk of being BTM antibody positive (Table 5). More specifically, a soil temperature between 8 °C and 12 °C increased the odds of being *F. hepatica* BTM positive by a factor of two compared to lower soil temperatures ($P = 0.001$). An exception to this was the increased tendency towards being pos-

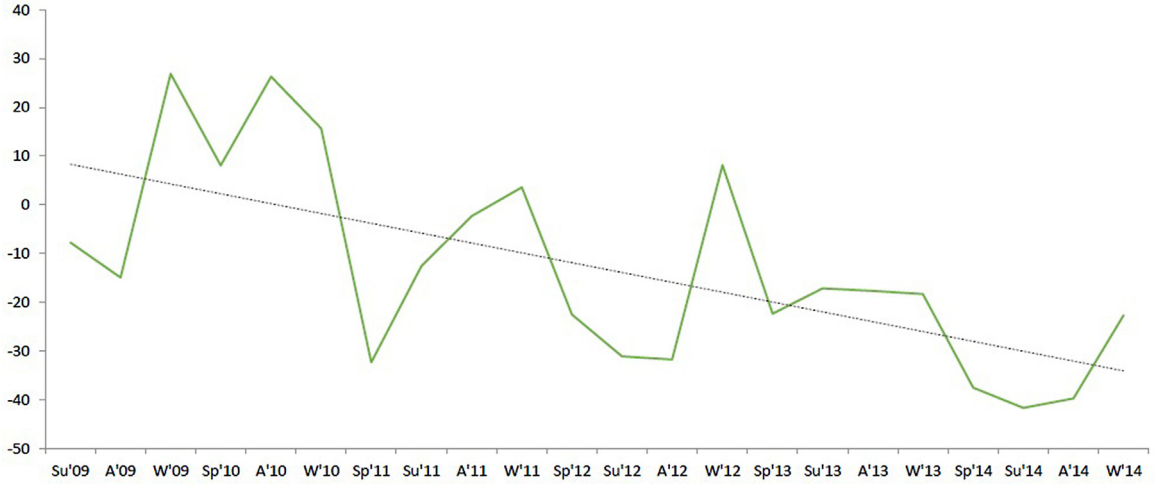

Fig. 3. Line graph representing the difference in S/P% across seasons in each study year using a univariable GEE. Spring 2009 was used as the reference season for GEE analysis. A trend line (- - -) has been included for ease of interpretation. Sp = spring; Su = summer; A = autumn; W = winter.

Table 4
Multivariable GEE of annual *F. hepatica* BTM antibody status (categorical data).

Dependent variable Independent variable	Odds ratio	Confidence interval (95%)	*P*- value	Model (*P*-value)
F. hepatica status (Positive vs. Negative)				
Annual rainfall				
High vs. moderate	0.33	0.12, 0.91	0.003	Annual rainfall, herd size, farmer land classification, year ($P = 0.032$)
High vs. low	0.47	0.24, 0.95	0.036	
Herd size				
Medium vs. large	0.19	0.04, 0.88	0.033	
Farmer land classification				
Wet vs. dry	3.56	0.78, 16.19	0.100	
Year	0.79	0.62, 1.01	0.059	

Annual rainfall: low: 850–1000 mm; moderate: >1000–1150 mm; high: >1150 mm.
Herd size: small = 71–130 cows; medium = 131–190 cows; large = > 190cows.
Only significant associations ($P \leq 0.05$) are reported. Tendencies are reported at $P > 0.05 < 0.10$.

itive (OR = 1.47; P = 0.067) at soil temperatures between 15 °C and 18 °C compared to a soil temperature of between 12 °C and 15 °C.

Additional findings in multivariable GEE included an increased S/P% value (20.20 S/P%, $P = 0.027$ (Supplementary Table S3)) in herds that treated with a flukicide, and a decreased likelihood (OR ≅ 0.50) of being BTM positive in herds that included a flukicide active against mature and immature fluke (categories 3 and 4) in their dosing regimen (Table 4). Finally, depending on the model and time variable used, wetter farms were either more likely (OR = 3.88; $P = 0.045$ (Table 5)) or tended to be more likely (3.56; $P = 0.10$ (Table 4)) to record a BTM antibody positive result.

Compared to 2009, multivariable logistic regression of whether a flukicide treatment was administered or not, indicated that increased levels of dosing were practiced over subsequent years of the study. Larger herds (>190 cows) were most likely to administer a treatment compared to other herd sizes (Table 6) and a predominant breed in a given herd was also an influencing factor. Predominantly JEX herds were less likely (OR = 0.37; $P = 0.004$) to administer a flukicide than HF herds. Finally, herds administering a treatment were almost twice as likely to be *F. hepatica* BTM antibody positive (OR = 1.74; $P = 0.040$).

4. Discussion

The aim of this study was to examine trends in exposure to *F. hepatica* over a six year period in a sub-set of Irish dairy herds, while also investigating the relationship between soil temperature, rainfall, flukicide treatment and *F. hepatica* status. Annual weather variations were found to have relatively minor impact on exposure to *F. hepatica*, with seasonal weather patterns proving to be more influential. Additionally, the use of a flukicide treatment active against both mature and immature stages of *F. hepatica* was very much associated with reduction in bulk milk antibody status of herds compared with treatments active against mature *F. hepatica* only.

Recently, the application of ELISA methodologies to detection of *F. hepatica* infections has been shown to improve the sensitivity of diagnosis over more conventional corprological techniques (Charlier et al., 2007, 2008). In turn, serum ELISA has been shown to yield greater sensitivity than bulk milk antibody ELISA (Duscher et al., 2011). The majority of *F. hepatica* infected herds will nonetheless be detected using BTM samples (Bloemhoff et al., 2015), and the practicality of collecting BTM and determination of whole herd status on the basis of a single pooled sample, makes BTM antibody ELISA an attractive alternative to serum ELISA (Salimi-Bejestani et al., 2007; Charlier et al., 2008). In the case of bovine leukaemia virus, it has been shown that repeated measurements improve the diagnostic sensitivity of a bovine leukaemia virus BTM antibody ELISA (Nekouei et al., 2015). Use of repeated monthly samples in the current study, similarly, should provide an effective method of determining whole herd *F. hepatica* status, and the noteworthy compliance rates achieved in sample submission over a six year period highlights the marked benefits of using BTM samples as a research tool. It should be noted that although compliance rates for sample submission appear lower in 2010, this was due to a suspension of the project for logistical reasons. The majority of

Table 5
Multivariable GEE of seasonal *F. hepatica* BTM antibody status (categorical data).

Dependent variable Independent variable	Odds ratio	Confidence interval (95%)	P-value	Model (*P*-value)
F. hepatica status (Positive vs. Negative)				
Seasonal soil temperature				
Moderate vs. borderline low	1.95	1.32, 2.88	0.001	
Moderate vs. borderline high	2.86	1.79, 4.55	0.000	
Moderate vs. high	1.92	1.24, 2.94	0.003	
Borderline high vs. low	0.45	0.26, 0.80	0.006	
Borderline high vs. borderline low	0.69	0.47, 1.02	0.062	
High vs. borderline high	1.47	0.97, 2.23	0.067	
Seasonal rainfall				Soil temperature, rainfall, farmer land classification, flukicide treatment, product type, season ($P < 0.001$)
High vs. low	1.37	0.99, 1.91	0.059	
Flukicide Type				
Category 1 vs. category 5	2.11	1.31, 3.41	0.002	
Category 2 vs. category 5	2.19	1.26, 3.79	0.005	
Category 3 vs. category 1	0.43	0.26, 0.74	0.002	
Category 4 vs. category 1	0.47	0.29, 0.76	0.002	
Category 3 vs. category 2	0.42	0.22, 0.79	0.007	
Category 4 vs. category 2	0.46	0.26, 0.79	0.005	
Farmer land classification				
Wet vs. dry	3.88	1.03, 14.58	0.045	
Season	0.94	0.92, 0.97	<0.001	

Seasonal soil temperature: Low = <5 °C; borderline low = >5–8 °C; moderate = > 8–12 °C; boderline high = >12–15 °C; high = >15–18 °C.
Seasonal rainfall: low = 41–80 mm; moderate = > 80–120 mm; high = > 120 mm.
Flukicide categories: Category 1: a single dose of a product active against mature liver fluke only; Category 2: a two-dose programme using only products active against mature liver fluke; Category 3 a single dose of a product active against both mature and immature liver fluke; Category 4: a combination of flukicide products with differing activity spectra in a multi-dose programme; Category 5: no flukicide product administered.
Only significant associations ($P \leq 0.05$) are reported. Tendencies are reported at $P > 0.05 < 0.10$.

Table 6
Multivariable logistic regression of flukicide treatment (dependent variable) across year, herd size, breed and *F. hepatica* status (independent variables).

Dependent variable Independent variable	Odds ratio	Confidence interval (95%)	*P*- value	Model (*P*-value)
Flukicide treatment vs. no treatment				
Year				
2010 vs. 2009	2.73	1.27, 5.90	0.011	
2011 vs. 2009	2.86	1.41, 5.80	0.004	
2012 vs. 2009	6.96	2.94, 16.43	<0.001	
2013 vs. 2009	4.29	2.01, 9.17	<0.001	
2014 vs. 2009	3.47	1.67, 7.20	0.001	
2012 vs. 2010	2.43	0.91, 6.54	0.078	Year, herd size, breed, *F. hepatica* bulk milk antibody status ($P < 0.001$)
2012 vs. 2011	2.38	0.16, 1.05	0.064	
Herd size				
Large vs. medium	6.68	3.18, 14.03	<0.001	
Large vs. small	2.90	1.36, 6.20	0.006	
Medium vs. small	0.43	0.25, 0.75	0.003	
Breed				
Jersey cross vs. Holstein Friesian	0.37	0.19, 0.72	0.004	
F. hepatica status				
Positive vs. negative	1.74	1.02, 2.94	0.040	

Herd size: small (71–130 cows); medium (131–190 cows); large (>190 cows).
Only significant associations ($P \leq 0.05$) are reported. Tendencies are reported at $P > 0.05 < 0.10$.

samples submitted in 2010 were submitted in the latter half of the year, which accounts for the significantly higher S/P% median value recorded in that year (Bloemhoff et al., 2015).

A disadvantage of using a serological technique to monitor fasciolosis is that it indicates only the presence (or absence) of antibodies. This does not allow differentiation between current/active and historical (e.g. post-treatment) exposure (Sekiya et al., 2013). The vast majority (>80%) of Irish dairy farmers have previously been shown to treat cows for *F. hepatica* in winter only, however, when cows in spring-calving herds are housed and not lactating (Bloemhoff et al., 2014). The BTM ELISA used in the current study can detect seasonal changes in exposure to *F. hepatica* (Bloemhoff et al., 2015) when applied to dairy herds that are predominantly spring-calving and this minimises the disadvantages of using a serological technique in Irish dairy herds.

The seasonal variation in *F. hepatica* infection highlighted in the current study, indicating higher *F. hepatica* exposure in autumn and winter, is similar to that previously reported (Ollerenshaw and Smith, 1969; Relf et al., 2011; Bloemhoff et al., 2015). Conflicting evidence is presented in the literature, however, with regard to the impact of weather conditions on the prevalence of *F. hepatica,* and its intermediate snail host. Ollerenshaw and Smith (1969), Yilma and Malone (1998), Morley and Lewis (2008), Cruz-Mendoza et al. (2011), Relf et al. (2011), Charlier et al. (2014), Selemetas et al. (2014) and Ducheyne et al. (2015) have all reported significant impacts of weather on the prevalence of *F. hepatica*. Others, however, report only very minor or weak associations (Bossaert et al., 1999; McCann et al., 2010; Bennema et al., 2011; Kuerpick et al., 2013; Novobilský et al., 2015). This lack of consistency across studies may be as a result of differences in the time variables used (e.g. seasonal, annual), the statistical models applied, and the jurisdictions in which studies were undertaken. Some studies included weather patterns alone (Ollerenshaw and Smith, 1969; Yilma and Malone, 1998; Bossaert et al., 1999; Morley and Lewis,

2008; McCann et al., 2010; Bennema et al., 2011; Relf et al., 2011; Kuerpick et al., 2013; Selemetas et al., 2014; Ducheyne et al., 2015; Novobilský et al., 2015), while others also include on-farm management practices such as treatment regimens (Cruz-Mendoza et al., 2011). In the current study, we aimed to provide a comprehensive analysis of six years of monthly data, which, as far as the authors are aware, has not been previously been reported. This afforded a unique opportunity to study both annual and seasonal variations in *F. hepatica* exposure, their relationship with variations in weather, and finally, the relationship with flukicide treatment practices.

Annual and seasonal analyses of rainfall and exposure to *F. hepatica* yielded conflicting results. Unexpectedly, years recording annual rainfall of over 1150 mm were associated with an apparent lower risk of *F. hepatica* (OR = 0.47) compared to years recording <1000 mm of rain. Analysis of seasonal data yielded a more typical outcome, i.e. higher seasonal rainfall associated with higher levels of *F. hepatica* (OR = 1.37). As mentioned previously, Irish dairy farmers typically treat adult cows for *F. hepatica* at housing. This allows detection of seasonal variations in *F. hepatica* largely independent of interference from flukicide treatments. In the case of annual data, however, farmer treatment interventions become important. Anecdotally, farmers often assume a higher risk of *F hepatica* infection in a year that they perceive as 'wet'. Levels of perceived risk are known to influence behaviour (Sayers et al., 2013) and it is likely that higher annual rainfall may lead to increased levels of treatment in certain years, manifesting as an apparent reduced risk of fluke in wetter years. This was not highlighted in the logistic regression analysis, however, which examined influences of rainfall on the administration of flukicides. Further investigations are required to examine the relationship between perceived farmer risk and actual weather patterns to further elucidate our findings.

The possibility that an additional factor reduces the risk of exposure to *F. hepatica* in wetter years cannot be ruled out. In that regard, Rapsch et al. (2008) have reported an important decrease in relative risk of infection at a monthly rainfall threshold of 210 mm whereby *G. truncatula* and the free-living stages of *F. hepatica* ('wash effect'). The mean monthly rainfall in the current study was 87.5 mm which may support a lower threshold for the spread of *F. hepatica* free living stages and the intermediate host, also suggested previously by Bennema et al. (2011), although our seasonal analysis would not support this hypothesis. These data highlight the difficulties in interpreting the parasite risk related to variations in weather, especially when external interventions (treatments) are also potentially influenced by annual weather conditions. Although significant interactions between weather variables and treatment variables were not detected in GEE models, it is both possible and plausible that farmer interventions present a greater influence on annual *F. hepatica* herd status than annual weather variations. This is supported by the fact that, although the mean annual temperature, and mean monthly rainfall in 2014, were marginally but not significantly higher (11.38 °C vs. 11.03 °C; $P > 0.10$) and marginally but not significantly lower (103.2 mm vs. 107.8 mm; $P > 0.1$) than 2009, respectively, *F. hepatica* BTM antibody S/P ratio was significantly lower in 2014 ($P < 0.01$) and the likelihood of administering a flukicide greater (OR = 3.47). It has been reported that the inclusion of management factors can substantially improve spatial distribution models for *F. hepatica* in temperate climatic zones (Bennema et al., 2011). Certainly the current study would support the addition of flukicide treatments, at a minimum, in *F. hepatica* modelling studies.

Soil temperature did not appear to influence *F. hepatica* BTM to the same degree as rainfall, certainly based on annual variations, where no significant association was detected. This is most likely contributed to by the minimal variability of mean annual temperatures across the study compared to seasonal temperature variations. The seasonal temperature influences on *F. hepatica* BTM antibody levels would initially appear broadly in line with the development requirements of the intermediate stages of the parasite i.e. temperatures >10 °C are optimal (Taylor et al., 2007; Borgsteede, 2011). Unusually, however, a temperature category of less than 12 °C to 15 °C was associated with a significant reduction in BTM results. This may relate to the time taken for infection to build on pastures once cows are turned out. The lifecycle of *F. hepatica* is relatively long compared to nematode lifecycles, which can be as short as 18 days (Urquhart et al., 1996). This leads to encysted metacercariae increasing on pastures later in the summer season and into autumn which may explain a mean soil temperature of between 8 °C and 12 °C appearing most favourable for *F. hepatica* infection. Rondelaud et al. (2013) and Claxton et al. (1999) found that cercarial production is increases with increasing variability in daily temperature, although many of these cercariae are not viable. Based on the results of the current study, it is unlikely that the variability in temperature in Ireland will dramatically impact on the levels of *F. hepatica* in Irish dairy cows, with seasonal rainfall being a better predictor of the risk of infection.

Based on logistic regression results for flukicide treatment, farmers were more likely to administer a treatment in the latter years of the study. In that time period, comprehensive parasite awareness programs, including *F. hepatica*, were initiated by both Teagasc and Animal Health Ireland (AHI, 2011). Additionally, in March 2010, restrictions on the use of certain flukicides in dairy herds were introduced (Bloemhoff et al., 2014), which are also likely to have made farmers more aware of flukicide treatments and their correct usage. This may also explain why larger herds in this study were more likely to administer a flukicide treatment compared to medium and small herds. Larger dairy herds in Ireland (Sayers et al., 2013), the United States (Hoe and Ruegg, 2006), and Canada (Young et al., 2010) have previously been shown to be more likely to engage in health schemes and good farm management. They could be considered the 'first responders' to awareness campaigns and may indicate a trend towards more effective fluke control in Ireland. Our study also reported that small herd categories were more likely than medium-sized herds to administer a flukicide. The majority (80%) of herds classified as 'small' in the current study contained fewer than 100 cows. Small herds lend themselves to closer day-to-day monitoring of animals and may explain the greater likelihood of smaller herds dosing compared to medium-sized herds. This highlights the importance of targeting educational messages to farmers based on farm demographics, with the owners/managers of medium-sized herds in this study lagging behind their counterparts in best practice.

As regards type of flukicide treatment administered, protocols incorporating an ingredient active against all stages of *F. hepatica* (category 3 or 4) increased throughout the years of study. Since 2012, triclabendazole is the only flukicide licensed in dairy cows in Ireland (HPRA, 2013) active against all stages of *F. hepatica* (category 3) within the final host (Fairweather and Boray, 1999). Use of a 'two-dose' regimen with a product active against mature *F. hepatica* only (category 2) likewise did not prove equivalent to treatment with triclabendazole, which was more effective. This is most likely due to the fact that Irish dairy herds operate lengthy grazing seasons incorporating a 60 to 90 day dry period (Dillon et al., 1995). The majority of flukicide treatments are administered during this housed dry period (Bloemhoff et al., 2014). If using an ingredient active against mature *F. hepatica* only, treatment is administered at the beginning and end of the housing period (AHI, 2013). As the dry period may be as short of 60 days, particularly in the south of Ireland where the current study was conducted, it is unlikely that all encysted metacercariae ingested just prior to housing will have matured to such a degree that a 'two-dose' treatment with an adulticide will be optimally effective (Fairweather and Boray, 1999).

It is interesting to note that herds which did not administer a flukicide treatment also recorded a superior *F. hepatica* status to those administering category 1 or 2 flukicide treatments. Herds that did not administer flukicides recorded the lowest S/P% values amongst study herds and can essentially be regarded as uninfected herds. Unlike category 1 and 2 herds, category 3 and 4 (i.e. triclabendazole-treated) herds did not record significantly different results to untreated herds. Category 3 treatment regimens could therefore be considered effective in reducing the burden of *F. hepatica* to the level of uninfected herds. There is a case, therefore, for using an ingredient active against all stages of *F. hepatica* alone in a single dose regimen to control *F. hepatica* in dairy herds. Generation of flukicide resistance, however, must always be a concern when promoting the use of a single active ingredient. The incidence of triclabendazole resistance in livestock is a major threat to global livestock production (Kelley et al., 2016) although reported resistance to triclabendazole in Ireland appears to be restricted to sheep flocks (Fairweather and Boray, 1999; Mooney et al., 2009; Hanna et al., 2015). Internationally, however, cases of triclabendazole resistance have been reported in cattle (Moll et al., 2000; Olaechea et al., 2011; Ortiz et al., 2013; Brockwell et al., 2014; Elliot et al., 2015) and care must be taken in promoting use of a single active ingredient.

This study highlighted that a proportion of study herds would not appear to require treatment, once continuous monitoring using an appropriate diagnostic tool is implemented for surveillance purposes. Additionally, regardless of the flukicide used, almost all study herds recorded year-on-year improvements in *F. hepatica* S/P% values. We would suggest, therefore, that continuous monitoring of *F. hepatica* status using an ELISA method capable of differentiating seasonal changes in herd status, should be promoted as a decision-support tool for designing a flukicide treatment regimen. It may be possible to use a category 3 or 4 flukicide treatment protocol in the initial years of a control programme. Once levels of *F. hepatica* have significantly reduced, a category 2 protocol could be implemented on a rotational basis, complimented with a continuous monitoring programme. Access to monthly *F. hepatica* results was instrumental in changing the behaviour of farmers in the current study and highlights the benefits of a holistic approach (monitoring and treatment) to yield improved and appropriate *F. hepatica* control.

An unusual finding was the association between a lower likelihood of flukicide treatment in herds where JEX cows were the predominant breed. This may be a function of the low number of such herds in the study ($n = 4$), although six years of data were available for these herds which should improve the power of the findings. This finding may relate to the overall efficiency of JEX compared to HF cows, which are a larger and less efficient animal (Prendiville et al., 2009). If farmers base a treatment programme on animal performance or body condition score alone, the lower maintenance JEX cows (Kristensen et al., 2014) may not have been treated. This finding requires further investigation to establish whether some additional protective factor exists in these crossbred cows.

Weaknesses of the current study include the fact that weather data were obtained from a single centralised weather station. Local weather variations were, therefore, not accounted for. Having said that, all farms were located within a 30 mile radius of the weather station used and should be sufficient for the analysis undertaken. Additionally, the use of farm self-reported land classification has the potential to introduce a degree of variability, as farmer perceptions of 'wet' and 'dry' land will be subjective. Detailed soil maps have been used by Bloemhoff et al. (2014) and Selemetas et al. (2014) for the purposes of assessing the association between soil type and *F. hepatica* infection. These maps, however, are available at a resolution of a 10 km grid which does not account for individual paddock variation within farms. This, coupled to the fact that farmers' own perceptions can be important determinants of the action they take in regard to their farms (Van Duinen et al., 2015), justifies the use of self-reported land-classification in the current study. Finally, due to the longitudinal nature of the study, only a subset of dairy farms in the Munster region of Ireland was incorporated into the study which may introduce bias. Irish dairy farmers are encouraged to engage in discussion groups (Teagasc, 2016), however, and the results presented here highlight the progress that can be made within a farmer group once awareness of a disease and appropriate control measures are improved.

5. Conclusion

This study highlights the importance of examining and comparing both seasonal and annual data with regard to environmental and management practices, as *F. hepatica* outcomes can vary significantly. International models are predicting increases in *F. hepatica* exposure due to global climate change, and the current study highlights the progress that can be made through awareness campaigns and continuous monitoring programmes. Evidence-based control of *F. hepatica* in study herds achieved lower BTM milk seropositivity regardless of weather patterns or changes. Further research is now required to design sustainable flukicide treatment programmes that will minimise the possibility of promoting anthelmintic resistance.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.prevetmed.2016.09.024.

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