Presence and species identity of rumen flukes in cattle and sheep in the Netherlands

H.W. Ploeger [a], L. Ankum [a], L. Moll [b], D.C.K. van Doorn [ab], G. Mitchell [c], P.J. Skuce [c], R.N. Zadoks [cd], M. Holzhauer [b*]

[a] Department of Infectious Diseases and Immunology, Faculty of Veterinary Medicine, Utrecht University, Yalelaan 1, 3584 CL Utrecht, the Netherlands.

[b] GD Animal Health, P.O.Box 9, 7400 AA, Deventer, the Netherlands

[c] Moredun Research Institute, Pentlands Science Park, Bush Loan, Penicuik EH26 0PZ, United Kingdom

[d] Institute of Biodiversity Animal Health and Comparative Medicine, College of Medical Veterinary and Life Sciences, University of Glasgow, Glasgow G61 1QH, United Kingdom

[*]Corresponding author: m.holzhauer@gdanimalhealth.com

Abstract

The purpose of the study was to gain knowledge about the prevalence and identity of rumen flukes (RF) in cattle and sheep in the Netherlands. Routine faecal examinations of diagnostic submissions between May 2009 and September 2014 showed a mean annual herd or flock RF prevalence of 15.8% for cattle and 8.0% for sheep. Prevalence in cattle was higher after 2012 than before, which may reflect a change in detection method as well as an increase in true prevalence. During November and December 2014, an abattoir survey was conducted to allow for scoring of rumen fluke burden and to obtain specimens for molecular species characterization. Over 8 visits to 5 abattoirs in areas deemed to pose a high risk for trematode infection, 116 cows and 41 sheep from 27 herds and 10 flocks were examined. Prevalence of RF was higher in beef cattle than in dairy cattle

and higher in cattle than in sheep. Median fluke burden was >100 specimens per animal for most positive animals.

Using a semi-quantitative RF density score as a gold standard, sensitivity and specificity of a modified quantitative Dorsman egg counting method were estimated at 82.6% and 83.3%, respectively.

Of 14 collected adult rumen flukes, twelve (8 bovine and 4 ovine specimens) were identified as *Calicophoron daubneyi.* The other two, of bovine origin, were identified as *Paramphistomum leydeni*, which was unexpected as in other European countries all recently collected rumen flukes in both cattle and sheep were identified as *C. daubneyi*. The findings implicate that multiple rumen fluke species, intermediate host species and transmission cycles may play a role in rumen fluke infections in the Netherlands.

KEYWORDS: Rumen fluke, *Calicophoron daubneyi*, *Paramphistomum leydeni*, cattle, sheep, abattoir survey, EPG

Introduction

Rumen flukes (RF) are trematodes infecting a number of wild and domesticated ruminants. Their life-cycle shows similarities with the other main trematode parasite in cattle in temperate regions, the liver fluke *Fasciola hepatica.* The exact intermediate host species of RF in the Netherlands is not known, but the mud snail, *Galba truncatula*, is a likely candidate. *G. truncatula* has been shown to act as intermediate host for RF in France (Abrous et al., 1999, 2000) and, more recently, this has also been confirmed in Great Britain (Jones et al., 2015). This snail is also the main intermediate host for *F. hepatica*, suggesting that liver fluke and RF may co-exist in regions with suitable snail habitat. Following ingestion of metacercariae by the final host, the juvenile RF can be found in the small intestine where they attach to the mucosa and grow before they migrate to the rumen (De Waal,

2010). Adult RF live attached to the surface of the rumen and reticulum and have a light to bright red color when fresh, are pear-shaped and about 1.0 cm in length (Taylor et al., 2007). The pre-patent period may be around 70-80 days and the total life-cycle is thought to take at least 3-4 months to complete (Taylor et al., 2007; De Waal, 2010). Recently, renewed interest in this parasite has arisen in West-European temperate regions, related to reported cases in which clinical disease in cattle and sheep was associated with RF infection (Millar et al., 2012; Mason et al., 2012). Watery diarrhoea, severe condition loss, depression and mortality were the described clinical signs in these cases. At necropsy, marked redness of the proximal intestinal mucosa and hemorrhagic duodenitis was seen.

The main objective of this study was to investigate prevalence and species identity of RF in cattle and sheep in the Netherlands, particularly in known liver fluke areas, where the risk of RF was suspected to be highest.

Materials and Methods

Routine faecal examinations from 2009-2014

At the Dutch Animal Health in Deventer (GD Deventer), a database was kept containing results from routine parasitological examinations on faeces from cattle and sheep submitted for liver fluke diagnosis. Apart from liver fluke eggs, presence of RF eggs was recorded. The database started in May 2009 and submissions up to September 2014 were used in the present study. The database included information on region (farm) of origin of the faeces sample and date of submission. Fig. 1 shows the distribution of all farms having submitted samples during 2009-2014 superimposed on a map showing the risk areas for liver fluke.

From 2009 until December 2012, faecal examination for trematode eggs was based on a simple sedimentation technique (Foreyt, 1989) using 5 g (sheep) to 15 g (cattle) faeces per sample. Faeces were suspended in water and sieved using a 150 µm sieve. After repeated washings with water, the filtrate was allowed to sediment for several minutes. Supernatant was decanted and the sediment

was mixed with an equal volume of methylene blue, after which 4 drops were examined on a microscope slide for presence of trematode eggs. The sensitivity of this technique is unknown, but is expected to be low as the sediment contains much debris, making it difficult to detect low numbers of eggs, and because only a small amount of material is examined. Hereafter, a modified Dorsman technique (Dorsman, 1956), with a detection limit of 5 eggs per gram (EPG), was used (see below). Eggs were identified based on colour and size (Zajac and Conboy, 2006).

Modified quantitative Dorsman technique for detecting fluke eggs

Faeces are weighed in a container (sheep 10 g, cattle 20 g), to which 10 or 20 ml water is added for sheep and cattle, respectively. After homogenising, a 6 ml measuring spoon is filled and brought onto a 160 µm sieve, which is placed on top of a funnel (18 cm Ø) of which the outlet hose was closed with a clamp. The spoon is rinsed clean above the 160 µm sieve with water. With a sprayer, the suspension is flushed through the sieve until the water level rises just above the mesh screen. Then the clamp is loosened and the suspension is captured on a 53 µm sieve. After removing the 160 µm sieve, the substrate on the 53 µm sieve is rinsed thoroughly, leaving behind faecal particles and trematode eggs. The material is then washed off and collected in a container to a volume of 24 ml water. To this volume, 3 drops of methylene blue and 6 ml Cellofas B (carboxymethylcellulose 1.25%) are added. On a shaker (KS-501-D, IKA Labortechnik, Lelystad, the Netherlands), the suspension is thoroughly homogenised for 3 min, after which two 1 ml counting slides (nematode counting slides, www.vetslides.com, Chalex Corporation Wallowa, USA) are filled in a smooth motion with a pipette. Eggs are counted within the grid of the counting chambers. The number of counted eggs on both slides are added and multiplied by 5 to obtain EPG. Eggs are counted to a maximum of 200.

The modified technique was validated for detecting liver fluke eggs in bovine and ovine faeces at GD Deventer (unpublished data). It detected eggs in 95% of low positive samples (<30 EPG in bovine or

104 <75 EPG in ovine faeces) and in 100% of high positive samples (>30 EPG in bovine or >75 EPG in
105 ovine faeces).
106
107 *Abattoir survey*
108 Four abattoirs in the western part and one near the GD Deventer laboratory in the eastern part of
109 the Netherlands were visited during November – December 2014. Abattoirs were relatively small
110 (<100 cows slaughtered per day), mainly slaughtering cattle and sheep from within their own region.
111 The abattoirs were selected based on their location within regions thought to pose a high risk for
112 liver fluke and RF infections (high groundwater levels and presence of wet pastures with many
113 ditches creating suitable habitat for *G. truncatula*) and their small slaughter capacity. The latter
114 allowed easier tracking of carcass and organs during the process of slaughtering.
115
116 Overall, 116 cows from 27 herds and 41 sheep from 10 flocks were inspected. Of the inspected cows,
117 origin and type (dairy vs. suckling beef cows) were recorded.
118
119 On the slaughter line, the liver was inspected macroscopically for indications of liver fluke infection
120 (migration tracks, thickened bile ducts, adhesions and haemorrhages). After opening and removing
121 the content, the rumen was visually inspected for the presence of adult RF. The number of RF
122 present was estimated as a "fluke density score" from 0-3 (0= no flukes visible, 1= 1-100 flukes, 2=
123 100-500 flukes, 3 >500 flukes). The reticulum was opened and inspected, but here RF were not
124 counted systematically, as numbers present were substantially lower and tended to follow the
125 numbers found in the rumen. In total, 80 adult RF specimens from 23 cows were collected and
126 stored in 70% ethanol. In addition, four RF specimens were collected from the only two sheep that
127 were found positive for RF.
128

Rectal faeces samples were collected from 23 cattle and 2 sheep in which RF were observed and from 12 cattle without visible presence of RF in the rumen or reticulum. Samples were stored in a cool box and, within 4 hours, transported to the GD laboratory for further processing. At the GD laboratory the faeces samples were examined for rumen and liver fluke eggs using the modified quantitative Dorsman technique described above.

Identification of collected rumen flukes

Collected RF specimens were sent to the Moredun Research Institute, Penicuik, UK, for species identification. DNA was extracted from individual adult RF using the Qiagen DNEasy Blood and Tissue kit (QIAGEN, Germany) as specified by the manufacturer. Amplification of ITS-2 rDNA, plus partial flanking 5.8S and 28S region was achieved using the generic trematode primers ITS-2 F: 5'-TGTGTCGATGAAGAGCAG-3' and ITS-2 R: 5'-TGGTTAGTTTCTTTTCCTCCGA-3' as described by Itagaki et al. (2003) and Rinaldi et al. (2005). PCR was conducted with a total reaction volume of 25 µl, containing 10x Buffer (Invitrogen, USA), 12.5 pmol of each primer (Eurofins, Germany), 0.2 mM of each dNTP (Invitrogen, USA), 2 mM MgCl2 (Invitrogen, USA), 2.5 U platinum Taq polymerase (Invitrogen, USA) and 1 µl of template DNA, under the following conditions: 95°C for 10 min; 35 cycles of 94°C for 1 min; 53°C for 1.5 min; 72°C for 1 mins; and 72°C 10 mins. PCR products of ~440 bp were purified using QIAquick PCR Purification Kit (QIAGEN, Germany) and sent to Eurofins MWG Operon (Germany) for direct nucleotide sequencing. The quality of the sequences was assessed using Lasergene 10 core suite Software SeqMan Pro (DNASTAR, USA) and compared to reference sequences in GenBank using BLASTn at the European Bioinformatics Institute website (http://www.ebi.ac.uk/).

152 Statistical analysis

153

154 *Routine faecal examinations from 2009-2014*

155 Veterinary practitioners or farmers submitted one or more samples from a herd. Results were
156 analysed per submssion or per herd or flock, rather than per individual sample. If at least one faeces
157 sample in such submissions was found positive for RF eggs, the entire herd or flock was considered
158 positive. Sometimes samples were submitted within a short interval after a preceding submission for
159 confirmation or additional check. In these cases, re-submissions were recorded as if submitted at the
160 same time as the preceding submission. Herd prevalence was estimated as the number of positive
161 submissions divided by the total number of submissions in a certain time period. Difference in
162 prevalence of RF between sheep and cattle was tested with a chi-square test. Statistical significance
163 was defined at $P< 0.05$. To test presence of co-infection with liver fluke as a risk factor, the relative
164 risk (RR) with a 95% confidence interval was calculated.

165

166 *Abattoir survey*

167 For analysis purposes, visual presence or absence of RF in the rumen was considered the “gold
168 standard”. Difference in presence of liver fluke between cattle with or without RF in the rumen was
169 tested with the chi-square test. Statistical significance was defined at $P< 0.05$.

170

171 **Results**

172

173 *Routine faecal examinations from 2009-2014*

174 Over the selected period, 3753 faecal samples from cattle or sheep, distributed over 1894
175 submissions, were sent to the GD laboratory for liver fluke diagnosis (Table 1). Most submissions
176 were in 2013, after a period of increased incidence of liver fluke disease (autumn 2012).

177 Fig.2 shows the annual percentage of cattle and sheep herds found positive for RF eggs. The annual
178 average herd prevalence was 15.8% for the cattle herds and 8.0% for sheep herds, the difference
179 being significant ($p<0.01$). The incidence of RF positive submissions varied between quarters within
180 years, but there was no clear seasonal pattern over the years nor a noticeable correlation between
181 RF prevalence in the two host species.
182 Table 2 presents the number of cattle and sheep herds found positive or negative for either or both
183 liver fluke and RF eggs. Although all submissions had been submitted for liver fluke diagnosis, about
184 half of all herds tested negative for liver fluke eggs. For both cattle herds and sheep flocks, a
185 significant association was found between presence of liver fluke and RF eggs. For cattle the RR was
186 3.1 ($P<0.001$, 95%CI: 2.01 – 4.59), for sheep the RR was 46.2 ($P<0.001$, 95%CI: 6.3 – 339.7).
187
188 *Abattoir survey - cattle*
189 The examined 116 cattle originated from 27 different herds. In twenty-seven cows (23.3%) from 23
190 herds RF were found, giving a herd prevalence of 85.2%. The majority of RF were seen in the cranial
191 and ventral sac and around the passage between rumen and reticulum. The frequency of RF density
192 scores is given in Table 3.
193
194 Of the dairy cattle inspected, 17.8% (15/84 animals) were positive for RF. Of the suckling beef cows,
195 42.7% (9/21 animals) were positive for RF, which was significantly higher ($\chi^2 = 5.95$, $P<0.05$). No
196 correction was made for clustering by herd as, in most herds, just one animal was found to be RF
197 infected. The type (dairy or suckling beef) of 11 cattle, including 3 that were RF positive, could not be
198 determined at the abattoir.
199 Table 4 shows the mean and median RF EPG for each RF density score in the rumen. There is a clear
200 increase in EPG with increasing RF burden, although up to burdens of 100-500, RF faecal
201 examination may occasionally turn up negative. Sensitivity and specificity of the modified Dorsman

technique for detecting RF eggs were estimated to be 82.6% (95%CI: 74.3-90.9%) and 83.3% (95%CI: 77.5-89.1%), respectively.

Of 81 cattle livers examined, 19 showed signs of (previous) liver fluke infection and in 4 (21.1%) of these cows RF were found. Of the other 62 cows without signs of liver fluke infection, 9 (17.0%) had RF (NS). There was also no association between presence of RF in the rumen and *F. hepatica* eggs in the faeces.

Abattoir survey - sheep

Two of the 41 (4.9%) examined sheep, from two different flocks, had low numbers of RF (density score 1). The two RF infected sheep showed no RF eggs in their faeces.

Species differentiation

Fourteen RF specimens, collected from 6 different animals, were identified. Eight of 10 bovine specimens, from three cows, and all four ovine specimens, from two sheep, were identified as *C. daubneyi.* The ITS-2 region of these 12 specimens matched the *C. daubneyi* reference sequence for 99.8-100% (Genbank KP201674; Chryssafidis et al., 2015). The two remaining bovine RF specimens, from one cow, were identified as *Paramphistomum leydeni*. These had a 99.8 and 100% identity match to the *P. leydeni* reference sequence, respectively (Genbank KJ995527; Sanchis and Sanabria, unpublished).

Discussion

Overall, RF prevalence in cattle herds in the Netherlands was estimated at 15.8% from the 2009-2014 dataset. This RF herd prevalence probably overestimates the true prevalence in the Netherlands because samples were preferentially submitted or collected from areas deemed to pose

228 a high risk of trematode infection. As in other countries, RF prevalence may have increased in the
229 Netherlands. For cattle, the highest proportions of faeces samples found positive for RF eggs were
230 observed in the more recent years (2012-2014). An explanation may be that there has been a trend
231 to raise groundwater levels in several areas for nature conservation purposes, a process that has
232 contributed to the emergence of liver fluke in England (Pritchard et al., 2005). On the other hand,
233 the trematode egg detection technique was also changed at the end of 2012, which probably
234 resulted in improved detection of RF eggs. If the prevalence in cattle had indeed increased, one
235 would have expected a similar trend in sheep (Zintl et al., 2014; Toolan et al., 2015). However, the
236 sheep data did not suggest a trend towards increased prevalence. Overall, for sheep, an 8.0% flock
237 prevalence was estimated from the faecal examination database. This was lower than for cattle,
238 which was supported in the abattoir survey with 2 of 41 sheep (4.9%) and 27 of 116 cattle (23.3%)
239 found RF positive.
240
241 In the abattoir survey at least one cow was found RF positive in 23 of 27 herds, which amounts to a
242 herd prevalence of 85.2%. This much higher prevalence figure compared to the one from the 2009-
243 2014 dataset can probably be explained by the fact that the abattoir survey was conducted in
244 purposively selected, small scale abattoirs in areas of high fluke risk, which were not representative
245 of the general cattle population in the Netherlands nor of the overall cattle population from which
246 faeces samples are submitted to check for liver fluke eggs (Fig. 1). The data from the abattoir survey
247 indicated that not all cows in a RF-positive herd will show visible presence of RF, as 116 cows from
248 27 herds were examined of which 27 cows from 23 herds were found infected. RF infection was
249 more often found in suckling beef cows, which may be explained by differences in areas grazed by
250 dairy versus suckling cows. The latter are normally grazed on fields with a higher ground-water level
251 and in the flood plains of rivers. These areas, in particular, contain suitable habitats for the RF
252 intermediate hosts (González-Warleta et al., 2013).
253

There were no associations between RF and liver fluke infection in the abattoir survey. Given that both fluke infections may be more prevalent in similar areas and with both known to share the same intermediate host species, this might be unexpected. However, the number of examined cattle may have been too small to find an association. In the 2009-2014 egg count database, RF eggs were more often found if liver fluke eggs were present, which is in line with Toolan et al. (2015) and Sargison et al. (2016).

Most RF specimens from both host species were identified as *C. daubneyi*. Conversely, two specimens derived from one cow were identified as *P. leydeni*, a distinction that was not obvious visually. In several studies on cattle and sheep from the British isles, all RF were identified as *C. daubneyi* (Gordon et al., 2013; Zintl et al., 2014; Toolan et al., 2015). *P. leydeni* was only found in fallow deer or red deer in contact with fallow deer (O'Toole et al., 2014). It has also been reported in reindeer in Finland (Nikander and Saari, 2007). Here, we report the presence of *P. leydeni* in cattle. Unlike *C. daubneyi*¸ which uses the mud snail *G. truncatula* as intermediate host, *P. leydeni* has been found to use freshwater planorbid snails as intermediate host (Samnaliev et al., 1984; Abrous et al., 2000; Jones et al, 2015), hinting at the possible role of wildlife as reservoir host for some RF. Interestingly, *P. leydeni* is named after the Dutch city of Leiden, which is located in an area of high fluke risk and where it was first described almost a century ago (K.E. Näsmark (1937) as cited by O'Toole et al., 2014). The *P. leydeni* specimens found in this study originated from the region east of Leiden roughly within the triangle formed by Rotterdam, Amsterdam and Utrecht. Most deer within this region are roe deer (*Capreolus capreolus*). Fallow deer (*Dama dama*) are common in the Netherlands, but are mostly restricted to specific areas elsewhere.

No recent information was available on the RF species present in the Netherlands. The Fauna Europaea (2013) mentioned *Paramphistomum cervi, P. hiberniae,* and *P. leydeni*. *Paramphistomum* species are also mentioned in reports from Ireland (Murphy et al., 2008), Scotland (Mason et al.

2012) and Great Britain (Foster et al., 2008; Millar et al., 2012). The Fauna Europaea did not mention *C. daubneyi* as a species present in the Netherlands. Similarly, the Dutch species register (www.nederlandsesoorten.nl/) did not list *C. daubneyi* as a species present in the Netherlands. This species has been found recently and, based on molecular identification, confirmed in Belgium (Malrait et al., 2015), Spain (Ferrerras et al., 2014), Great Britain (Gordon et al., 2013), the Republic of Ireland (Zintl et al., 2014; Toolan et al., 2015) and North Portugal and North-West Spain (Arias et al., 2011). This report is the first to confirm the presence of *C. daubneyi* in the Netherlands. One may speculate if this species has emerged recently or that it has been confused with *P. cervi* for a very long time.

In conclusion, results indicate that rumen flukes are present in many cattle herds in known liver fluke areas in the Netherlands. Next to *Calicophoron daubneyi* in cattle and sheep, *Paramphistomum leydeni* was identified in cattle.

Acknowledgements

We are grateful to Coen Hegeman for his skilful assistance with all faecal examinations, Anouk Veldhuis for helping out with validating the improved Dorsman technique, and all five abattoirs for their hospitality and collaboration.

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Table 1. Annual number of submissions, faecal samples and herds or flocks submitting samples during 2009-2014.

Year	Cattle			Sheep		
	Submissions	Samples	Herds	Submissions	Samples	Flocks
2009[1]	29	59	26	32	65	24
2010	121	285	109	46	103	38
2011	74	156	72	57	118	48
2012	173	434	152	168	252	132
2013	403	908	323	345	583	246
2014[2]	270	561	238	176	229	141
Total	**1070**	**2403**	**730**	**824**	**1350**	**489**

[1] Submissions in 2009 were from May onwards.

[2] Submissions in 2014 were until September.

Table 2. Number of cattle herds or sheep flocks found positive or negative for either or both liver fluke and rumen fluke eggs over the period 2009-2014.

Eggs from	Cattle herds		Sheep flocks	
	Number	%	Number	%
Liver fluke only	224	30.7	203	41.5
Rumen fluke only	42	5.8	1	0.2
Liver fluke and rumen fluke	73	10.0	38	7.8
Negative	391	53.6	247	50.5

Table 3. Frequency distribution of rumen fluke density scores in 116 cattle investigated in an abattoir survey in The Netherlands (November – December 2014).

Fluke density score	Number of animals	Percentage of animals
0 (no flukes visible)	89	76.7%
1 (1-100 flukes)	9	7.8%
2 (100-500 flukes)	11	9.5%
3 (> 500 flukes)	7	6.0%

Table 4: Rumen fluke egg counts in relation to the density score of flukes in the rumen.

Fluke density score	No. of samples	EPG				
		mean	95%CI	min	max	median
0 (no flukes visible)	12	0.8	0.3-1.4	0	5	0
1 (1-100 flukes)	8	20.0	8.5-31.5	0	95	5
2 (100-500 flukes)	9	51.7	31.7-71.7	0	175	20
3 (>500 flukes)	6	>371	208.3-533.4	40	>1000	197.5

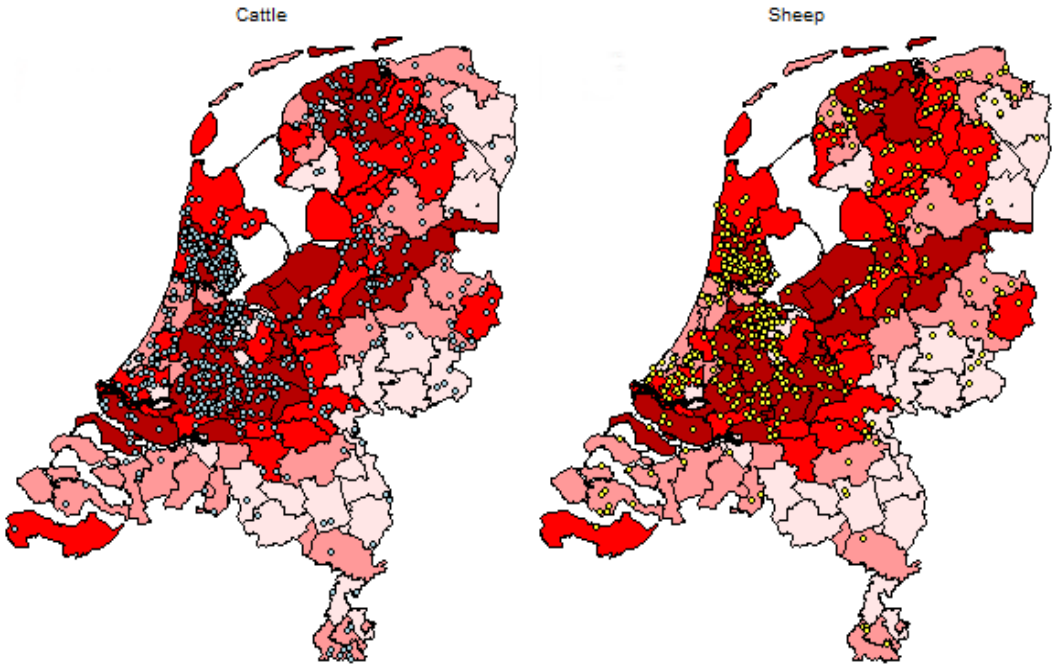
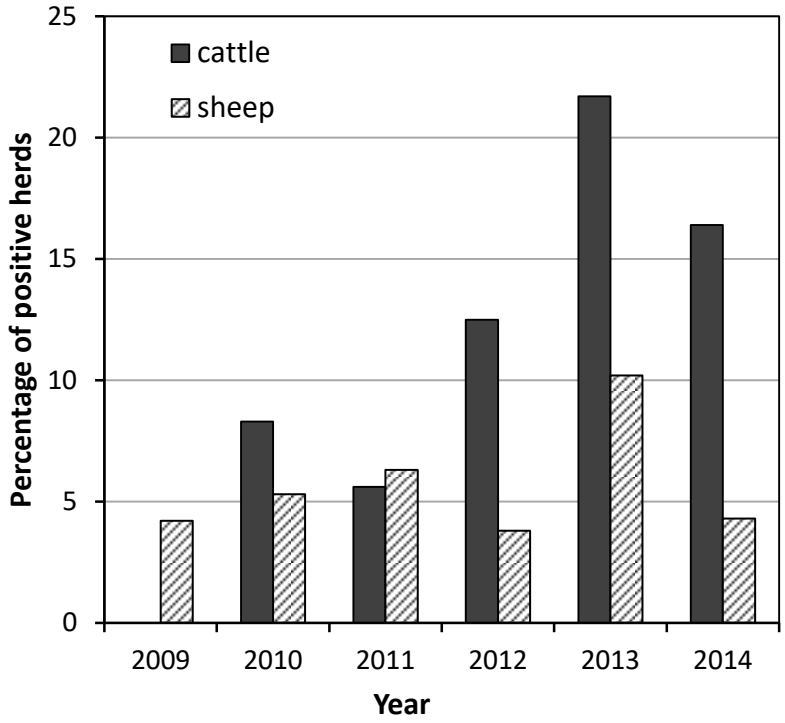


Fig. 1: Distribution of farms (dots) submitting faeces samples for liver fluke diagnosis during 2009-2014. The colour of the areas indicates prevalence of liver fluke, with increasing intensity representing increasing prevalence.



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410 **Fig. 2**. Percentage of cattle herds and sheep flocks tested positive for rumen fluke eggs in the faeces
411 during 2009-2014 (N= 730 cattle herds and 489 sheep flocks).

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