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Citation for the published paper:
http://dx.doi.org/10.1016/j.vetpar.2014.04.015.

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Standard set statement from the publisher:
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Transmission patterns of *Fasciola hepatica* to ruminants in Sweden

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Keywords: antibodies; eggs; ELISA; epidemiology; *Fasciola hepatica*; *Galba truncatula*; intermediate host; life cycle; overwintering; PCR; rainfall; *Succinea*
Abstract

Transmission patterns of *Fasciola hepatica* were investigated on beef cattle (n=3) and sheep (n=3) farms in Sweden between 2011 and 2012. The dynamics of fluke infection, particularly estimated time of infection, were screened each grazing season by ELISA detection of antibodies in lambs (n=94) and first grazing season calves (n=61). Colostral transfer of *F. hepatica* antibodies from seropositive ewes was detected in sheep up to 11 weeks of age. In sheep, the estimated time of infection differed significantly between herds and years. Typical ‘winter infection’ was observed on two sheep farms in 2012, but the most prevalent transmission pattern was found to be ‘summer infection’, characterised by infection of animals in late summer by *F. hepatica* originating from overwintered and/or spring-excreted eggs. In contrast, beef calves were infected mainly in September-October (‘summer infection’). Furthermore, lymnaeid and succineid snails were collected on the pastures used by these animals both in spring and in the autumn each year. In total, 1726, 588, 130, 93 and 42 specimens of *Galba truncatula*, *Lymnaea palustris*, *L. glabra*, *L. fuscus*, *Radix peregra* and *Succinea putris*, respectively, were collected and identified. These were subsequently examined for the presence of *F. hepatica* DNA by species-specific PCR and the findings compared against mean monthly rainfall and temperature data for each farm. The main intermediate host of the liver fluke was *G. truncatula*, with a prevalence range of *F. hepatica* infection from 0–82%. Only 1 out of 42 terrestrial *S. putris* tested positive for *F. hepatica*, casting doubt on the role of this species in transmission of *F. hepatica* in Sweden. In conclusion, two main peak periods of infection were observed: May-June (from overwintered infected snails = ‘winter infection’) and August-September (from metacercariae developed and produced by snails during summer = ‘summer infection’). The occurrence and frequency of ‘winter infection’ were dependent on local environmental factors such as snail habitat availability or grazing behaviour of animals, rather than on climatic factors.
1. Introduction

_Fasciola hepatica_ (the common liver fluke) is a trematode parasite with a distribution ranging from Scandinavia, Russia and Canada in the north, to Patagonia and New Zealand in the southern hemisphere (Torgerson and Claxton, 1999), which has a significant economic impact on pasture-based ruminant livestock production world-wide. The life cycle of _F. hepatica_ involves lymnaeid freshwater snails as the intermediate host and depends on the development and survival of larval stages both in the snail intermediate host and in the environment. Since the development of the snails and of the free-living external larval stages of the parasite is influenced by environmental factors, seasonal transmission of _F. hepatica_ varies between different climate zones. In Europe, the transmission pattern has been determined in Spain (Luzon-Pena et al., 1994), the United Kingdom (Ollerenshaw, 1971; Ross, 1977), the Netherlands (Gaasenbeek et al., 1992) and Denmark (Shaka and Nansen, 1979). However, information from the Scandinavian Peninsula is lacking.

Temperature and moisture are the two most important environmental factors affecting _F. hepatica_ development (Torgerson and Claxton, 1999). A minimum temperature of 10 °C is required for the successful development of all _F. hepatica_ stages outside the definitive host (Ollerenshaw, 1971). Furthermore, humidity limits both parasite development and intermediate host survival. In a European temperate climate, _F. hepatica_ can overwinter in the external environment as eggs and/or metacercariae, as larvae inside the snail intermediate host (Torgerson and Claxton, 1999), as well as adults in the final host. Ollerenshaw (1959) defined the terms ‘winter infection’ and ‘summer infection’ in relation to the overwintering strategies and timing of the life cycle in the U.K. ‘Summer infection’ is characterised by infection of snails in May-June, with production of metacercariae from August until October. In case of ‘winter infection’, snails are infected with miracidia in late autumn, which overwinter and produce metacercariae that infect the definitive host from May to July. Summer infection seems to be the dominant transmission pattern in north-western Spain (Luzon-Pena et al., 1994), Scotland (Ross, 1977) and Denmark (Shaka and Nansen, 1979). However, according to climate based models based on data from Wales there will be an increase of winter infections in the future (Fox et al., 2011).

_F. hepatica_ has been reported on the Scandinavian Peninsula, from Norway in the west (Domke et al., 2013) through Sweden (Höglund et al., 2010; Nielsen, 1974; Novobilský et al., 2012a), and to Finland in the east (Sorvetula, 1974). Although little is known about the distribution, epidemiology and impact of _F. hepatica_ on Swedish livestock production, recent reports suggest an increasing trend of fasciolosis (Novobilský et al., 2012b). According to meat inspection data, the average prevalence of _F. hepatica_ in slaughtered cows in Sweden increased from 3% in 2005 up to 9% in
2012. However in southern Sweden, i.e. Skåne with the highest cattle density in Sweden, recovery of flukes in the livers from slaughtered cows reached 24% in 2012 (König and Welling, 2013). This increase may have several explanations, such as climate change (increased rainfall and average temperature), altered cattle management, failure of control strategies, potential resistance to anthelmintics, or a combination of these factors.

Identification of *F. hepatica* by ELISA detection of antibodies in serum is the preferred diagnostic method for early detection of *F. hepatica* (Cornelissen et al., 2001; Reichel, 2002), as increased specific antibody levels can be observed already 2-4 weeks post infection (WPI) in animals originally parasite naïve (Chauvin et al., 1995; Phiri et al., 2006; Salimi-Bejestani et al., 2005). Antibody levels then usually peak between 8 to 12 WPI in ruminants (Martinez et al., 1996; Salimi-Bejestani et al., 2005; Santiago and Hillyer, 1988) and in general remain high for up to 12 months in the absence of treatment (Martinez et al., 1996). Thus, charting antibody dynamics is a useful tool for estimating the time of exposure in *F. hepatica*-naive animals when exposed during their first grazing season, and can replace costly use of sentinel lambs or calves for studies of seasonal transmission of *F. hepatica*.

Effective strategic parasite control programmes for ruminants need to be based on epidemiological data (Stromberg and Averbeck, 1999). Only measures based on the specific appearance of the parasite under specific climate conditions can bring benefits such as decreased treatment costs and maximum reduction of parasite burden. To optimise a strategic control programme for liver fluke infection in cattle and sheep in Sweden, detailed, up-to-date knowledge of the epidemiology and seasonal transmission patterns of *F. hepatica* is needed. The aim of this study was to estimate the time of infection and characterise the transmission pattern of *F. hepatica* in Swedish cattle and sheep and to determine suitable intermediate hosts as well as the dominating pattern of overwintering.

Materials and Methods

1.1. Study areas

The study was performed during 2011 and 2012 on farms where the prevalence of fasciolosis at slaughter exceeded approximately 50%. Three sheep farms (O1, O2 and O3) and three beef cattle farms (B1, B2 and B3) in different areas of Sweden were selected based on abattoir reports on condemned livers, in order to investigate the transmission pattern of *F. hepatica* in domestic ruminants. A summary of all farms, including geographical location and pasture characteristics, is shown in Table 1. Farm O3 was only added to the study in 2012, based on a prior treatment study in 2011 (Novobilský et al., 2012a). On all sheep farms and on beef cattle farm B3, clinical signs of
fasciolosis (e.g. bottle jaw and/or sudden death) have been documented in recent years. All sheep flocks in the study were treated on several occasions during the housing period, either with albendazole (Valbazen) and/or triclabendazole (Fasinex), while the cows and yearlings on farms B2 and B3 were dewormed with closantel (Closamectin) in the winter.

1.2. Local climate data

Monthly climate data for the six farms were obtained from five nearby meteorological stations of the Swedish Meteorological and Hydrological Institute (SMHI, 2012). The distance between farms O2 and B2 was only 20 km, so weather data for both were obtained from the same meteorological station (Örebro). The maximum distance between farm and meteorological station was 45 km. Data on mean rainfall (mm) and mean air temperature (°C) for seven months (April–October) in 2011 and 2012 were obtained from all stations, and compared with 30-year average monthly data (1961-1990).

1.3. Collection and identification of snails

To screen for *F. hepatica* infection in snails, lymnaeid and succineid snails were collected in May and September each year from the pastures used by the livestock. Due to permanent snow cover in winter 2011/2012, animal turn-out was somewhat delayed in spring 2012. Collection of snails was always performed by the same persons in the same quadrants in different local habitats during each visit, following Malone et al. (1984). Snail hotspots were selected by pre-screening certain wet areas in the different pastures. Collected snails were placed in 0.1 L plastic containers and transferred immediately to the laboratory. Within 24 hours, snails were identified according to their shell morphology and then snap-frozen (-20 °C) for further examination.

Snail identification was first based on conchological species characteristics, such as shell shape, formation of whorls and sutures, aperture shape and aperture size (Beran, 2002; Gloer, 2002; Jackiewicz, 2000). Furthermore, two typical snails per morphotype were confirmed by molecular identification. PCR amplification and sequencing of the internal transcribed spacer 2 (ITS-2) region of the snail’s ribosomal rDNA were carried out according to Bargues et al. (2001) with a few modifications (Novobilský et al., 2013). The ITS-2 sequences of snail isolates have been deposited in GenBank (KF887031, KF887032, KF887033, KF887034, KF887035, KF887036, KF887037, KF887038, KF887039, KF887040, KC248371, KC248373, and KC905167). These were aligned with sequences already available in GenBank (accessed in July 2013) using Clustal W2 (EMBL-EBI) and the basic local alignment search (BLAST) tool (BLAST, 2013).

1.4. Detection of *F. hepatica* in snails
The presence of *F. hepatica* larvae in snails was determined by PCR amplification of a 112-bp species-specific region of ITS-2 (Bazsalovicsová et al., 2010; Kráľová-Hromadová et al., 2008). Snails were defrosted and DNA from whole snail soft tissues was extracted using the DNeasy Blood and Tissue Kit (Qiagen, Germany). After a lysis step with proteinase K and overnight incubation, snail samples were pooled, with a maximum of 10 individuals per pooled sample, by mixing 20 µL of each sample to achieve 200 µL pooled lysate. DNA was then extracted according to the manufacturer’s instructions. The primers used for PCR amplification were those designed by Kráľová-Hromadová et al. (2008): Forward: FH-ITS2-SPEC-F 5’-CTTATGATTTCTGGGATAATT-3’, Reverse: FH-ITS2-SPEC-R 5’-CCGTCGCTATATGAAAA-3’. The PCR mixture contained: 1 µL DNA, 10 mM Tris-HCl pH 8.3, 50 mM KCl, 2 mM MgCl₂, 4 µg bovine serum albumin (BSA; New England Biolabs, UK), 0.8 µM of each primer, 0.2 mM dNTP, and 0.3 U Taq polymerase (AmpliTaq Gold, Applied Biosystems, USA). Amplifications were generated in a 2720 Thermal Cycler (Applied Biosystems, USA) as follows: 40 cycles of 45 sec at 95 °C, 1 min at 55°C and 1 min at 72 °C, preceded by 10 min at 95°C and followed by 5 min at 72 °C. The PCR products were separated on 1.5% agarose gel. Whenever *F. hepatica*-positive pooled samples were identified, all individual samples were re-tested using the same PCR protocol. The specificity and the sensitivity of the PCR method have already been tested in previous study (Novobilský et al., 2013).

1.5. Collection of serum samples and ELISA

To estimate when the animals were infected during grazing, blood samples were taken from first grazing season lambs and calves born in spring 2011 or 2012. In both years, blood samples were collected up to three times from the same 15-20 individuals in the herds on each farm. For ethical and practical reasons, blood samples were collected only twice and once per year on farms B1 and B2, respectively. All blood samples were centrifuged at 1125 g for 15 min, and the sera were then collected and stored at -20 °C until use.

Sera were examined by indirect enzyme-linked immunosorbent assay (ELISA) using a native *F. hepatica* excretory-secretory antigen (E/S Ag) as described by Novobilský et al. (2012a; 2007). In brief, sera were diluted 1:50 in buffer (0.05% Tween 20, 5% non-fat milk in phosphate-buffered saline, pH=7.2), and each serum sample was tested in duplicate. The same positive, weakly positive and negative reference samples were included in all assays. For the sheep ELISA, peroxidase-labelled anti-sheep IgG produced in donkey (Sigma-Aldrich, Sweden) at 1:3000 dilution was used as the secondary antibody. For the bovine ELISA, horseradish peroxidase-conjugated anti-bovine IgG antibodies (Svanova Biotech, Sweden) were used at 1:10 000 dilution. The ELISA results were expressed as percentage of the mean optical density ratio (ODR) of the positive control as: % of positivity = (mean OD of tested sample (n=2)/mean OD of the positive control) x 100. The sheep
ELISA was validated by applying sera from different sheep with known infection status. The cut-off in the ELISA was 10% of positivity, in agreement with a prior study (Novobilský et al., 2012a).

1.6. Statistical analysis

Statistical analyses were performed using GraphPad Prism version 5.02 (GraphPad Software, USA). To validate the bovine ELISA, 60 reference F. hepatica-negative bovine sera and 30 F. hepatica-positive reference bovine sera were analysed. The cut-off value for cattle was determined by receiver operating characteristic (ROC) curve analysis. Pearson correlation analysis was used to evaluate the relationship between local climate data and F. hepatica prevalence both in G. truncatula and in animals. First, temperature and rainfall in the months of snail collection were tested against F. hepatica prevalence in G. truncatula for the respective period (May, September). Further, the total rainfall and temperature values for the entire vegetation period (April-October) in each year were tested against F. hepatica prevalence in G. truncatula and animals in autumn 2011 and 2012. $P < 0.05$ was considered significant.

2. Results

2.1. Screening of antibodies in lambs and calves

The cut-off value for bovine ELISA calculated by ROC analysis was 15% of positivity. The specificity of E/S Ag was tested against Dicrocoelium dendriticum (n=6), Dictyocaulus viviparus (n=30) and Haemonchus contortus (n=40) positive sera and no cross-reactions were observed. In 2011, a few lambs from the June collection were weakly seropositive on farms O1 and O2, but the same lambs then tested negative in the following blood collection in August. On the other hand, four other lambs on O2 seroconverted in August and then remained positive (Supplementary Table 2, lambs 1002, 1009, 1017, 1090) in October 2011. On farm O1 in 2011, seroconversion in lambs was first observed in September (Figure 2). In contrast, calves seroconverted in November on both farms B1 and B3 in 2011 (Figure 3). In 2012, most lambs on farms O1 and O3 were already seropositive in June, and their antibody levels increased further during the course of the grazing season. On farm O3, three weakly F. hepatica-positive lambs were observed during the first two collections (June, July), but these lambs tested negative at the following collection in August. Again, seroconversion in lambs on O3 was first observed in August (Figure 2). In comparison to antibody dynamics in lambs, no weak positive calves were detected in the first blood collection in July 2011 and 2012. In the cattle sera analysed in 2012, the course of antibodies was similar to that observed in 2011, and most calves did not seroconvert until November. However, two calves on farm B3 had already seroconverted in September (Figure 3).

For a detailed presentation of the serological data for individual animals, see Supplementary Table 2.
2.2. Snail species identified and screening of *F. hepatica* in snails

During all four visits to all farms, five species of the family Lymnaeidae and one species of the family Succineidae were identified. The most abundant species was *Galba truncatula* (GenBank: KF887031- KF887036), followed by *Succinea putris* (GenBank: KF887038) and *Lymnaea palustris* (GenBank KC248373, KC905167) (Table 1). These three species shared a habitat of temporary wet areas with muddy soil, with or without herbage. In contrast, *L. fuscus* (KC248371), *L. glabra* (KF887037) and *Radix peregra* (KF887039, KF887040) were found on only two farms, in deeper permanent water bodies (streams, small ponds, lake).

A total of 1726, 588, 138, 130, 93 and 42 specimens of *G. truncatula*, *L. palustris*, *L. glabra*, *L. fuscus*, *R. peregra* and *S. putris*, respectively, were examined by PCR. The species most frequently infected with *F. hepatica* was *G. truncatula*, with infection levels ranging from 0% to 82% (Figure 1; Supplementary Table 1). The overall prevalence in *G. truncatula* on all six farms was almost 13%, with 224 of the 1726 snails diagnosed/confirmed as infected. Except for one snail on farm B2, *F. hepatica*-positive snails were detected only in autumn 2011, whereas the prevalence of *F. hepatica* in *G. truncatula* was even higher in spring 2012 than in autumn 2012 on farms O2 and B2. No *F. hepatica*-positive *G. truncatula* were found on farms B3 or O3 during the entire study period. Furthermore, only one terrestrial snail (*S. putris*) from farm O2 tested positive for *F. hepatica*.

2.3. Local climate data and correlation to infection levels on farms

The highest precipitation was observed on farms O1 and O3, located on the Swedish west coast (Table 2). The lowest precipitation was in the south, in Kristianstad (farm B3). Except for June, temperatures in 2012 were generally lower than in 2011. No correlation between *G. truncatula* positivity and rainfall or temperature was observed during May and September in 2011 and 2012. Similarly, no correlation was documented in 2011 between total rainfall and temperature for the entire vegetation period and the level infection in *G. truncatula* and animals. In 2012, however, an apparent positive but not significant correlation was observed between rainfall and *F. hepatica* prevalence both in *G. truncatula* (R=0.684; *P*=0.203) and in animals (R=0.720; *P*=0.170). Furthermore, strong but not significant negative correlations were obtained between temperature and prevalence in snails (R=-0.866; *P*=0.057) and in animals (R=-0.838; *P*=0.076) in 2012.

3. Discussion
Based on the fact that antibodies against \textit{F. hepatica} can be detected by ELISA between 2-4 weeks after ingestion of metacercariae (Chauvin et al., 1995; Phiri et al., 2006; Salimi-Bejestani et al., 2005), it was possible to estimate the time-point of infection in first season grazing animals that were originally \textit{F. hepatica}-naïve.

The finding of several weakly seropositive animals on farm O2 (Supplementary Table 2, 2011, farm O2, lambs 1021, 1022, 1025, 1057) in 2011 that turned seronegative 1.5 months later suggests that these sheep received maternal antibodies through intake of colostrum. This was supported by the identification of three weakly positive lambs (12134, 12139, 12140) on farm O3 in 2012 (Figure 2, Supplementary Table 2) in June and July. It was confirmed that these lambs were the offspring of two highly seropositive ewes (>150% in autumn 2011) (Novobilsky et al., 2012a). Additional support for colostral transfer of \textit{F. hepatica} antibodies in sheep comes from the fact that two weakly positive animals (farm O3, lambs 12139, 12140) investigated around turn-out in 2012 were siblings, which showed decreasing antibody levels later in the grazing season. Furthermore, all other siblings from the same mothers on farm O3 in 2012 had an almost identical percentage of positive values in June (Supplementary Table 2). Colostral transfer of \textit{F. hepatica} antibodies has been described earlier from dairy calves and it has been suggested that maternal antibodies are detectable until 12 weeks after birth (Mezo et al., 2010). Based on our results, we conclude that colostral \textit{F. hepatica} antibodies can be detected in lambs until at least 11 weeks after birth. Thus, serological diagnosis of fasciolosis is not recommended for diagnosis of active infection in lambs during their first two months on pasture.

Accepting colostral transfer, we concluded that all the lambs in 2011 were infected with metacercariae at the beginning of August at the earliest. On sheep farms O1 and O2, higher prevalences were in general observed in 2012, when between 70–80% were seropositive already in June 2012. Owing to the high infection levels in the June collection and the fact that no animal were tested as negative in August 2012, we estimate that these lambs were infected approximately in late May-early June. At the same time, we cannot exclude colostral transfer of antibodies as the cause of seropositivity in June. The typical dynamics of antibodies during early \textit{F. hepatica} infection in ruminants is characterised by antibody response first appearing between 2–4 weeks post-infection, which then progressive increases until 10–12 weeks (Cornelissen et al., 2001; Novobilsky et al., 2007; Phiri et al., 2006). The occurrence of a few lambs (e.g. 12100 on O1; 9225 and 9226 on O2; Supplementary Table 2) on farms O1 and O2 that had lower titres in the second collection in August 2012 than in June 2012, suggests that these lambs both received maternal \textit{F. hepatica} antibodies and were infected later than the rest of the lambs in the same flock. Therefore, we conclude that a combination of post-infection and passive transfer of colostral antibodies are the most likely explanations for the high prevalence observed in June on farms O1 and O2 in 2012. This observation
further confirms that lambs on those two farms were first infected between May and June, by overwintered *F. hepatica* in snails or eventually as metacercariae.

In cattle, colostral transfer of antibodies was never detected, but it should be borne in mind that for logistic reasons early summer blood collection was feasible on only one cattle farm. Nevertheless, compared with the antibody dynamics in sheep, the humoral immune response in the calves tested was delayed in both years. This suggests that calves on farms B1 and B3 were originally exposed to metacercariae between September and late October, which is somewhat later than in sheep. This delayed infection time in cattle might be explained in several different ways. First, pasture management may play a considerable role in *Fasciola* transmission in dairy cattle (Bennema et al., 2011; Charlier et al., 2011). For instance, the length of the grazing period, mowing of pastures and herd size are key factors that can increase or decrease exposure risk to animals. At the same time, these factors generally could explain the elevated risk or prevalence in the herd but not the differences between infection time points. Furthermore, differences in the grazing behaviour of cattle and sheep should be considered. The feeding style of sheep is more selective for preferred legumes than that of beef cattle. It has also been suggested that sheep use manipulative non-biting jaw movements to maintain their foraging preferences and to avoid non-preferred items (Bremm et al., 2012). Finally, the late infection of cattle in the study might simply consist of habitat differences between sheep and cattle farms, e.g. less suitable conditions for surviving metacercariae or snails on the cattle farms. However, all of these explanations remain very speculative until more evidence is available.

It is evident that the time-point of the first infection on sheep farms O1 and O2 differed between 2011 and 2012. Similarly, the infection dynamics on farms O1 and O2 differed from those on O3 in 2012. Based on the Ollerenshaw (1959) definition, we can conclude that most lambs and calves, except those on farms O1 and O2 in 2012, were infected with metacercariae originating from spring-infected snails, which result in the so-called ‘summer infection’. On the other hand, early infected lambs on farms O1 an O2 in 2012 corresponded to typical ‘winter infection’. This is further supported by the high prevalence of *F. hepatica*-positive *G. truncatula* in spring 2012. These snails were collected during the first half of May, when the mean temperature was below 6 °C (SMHI, 2012; Table 2). Based on the information in Ollerenshaw (1971), development, hatching and subsequent snail infection by miracidia from overwintered eggs is impossible to complete within one month under these low temperature. Therefore, overwintering of *G. truncatula* infected with *F. hepatica* in late autumn 2011, and subsequent production of metacercariae in May and June 2012, is the most likely explanation. At the same time, overwintering of metacercariae on pasture from the previous season must be considered as another source of ‘winter infection’. As has been reported from other
countries, metacercariae produced during autumn can survive the winter months on pasture, although their viability decreases rapidly from April to June. Thus, the role of overwintered metacercariae has been suggested to be small (Luzon-Pena et al., 1994; Ollerenshaw, 1971; Shaka and Nansen, 1979). This implies that ‘winter infections’ depend on infections of snails in autumn and survival of *F. hepatica*-infected snails during winter and spring months.

The egg of the parasite is the key overwintering stage for ‘summer infection’ (Ollerenshaw, 1971; Ross, 1977; Shaka and Nansen, 1979). Although it is difficult to demonstrate directly, our data for farm O3 confirm that this is also possible under the prevailing climate conditions in Sweden. On farm O3, all of the animals were dewormed with triclabendazole during winter 2011/2012, with 100% efficacy as documented in a previous study (Novobilský et al., 2012a). Therefore, no contamination with fresh fluke eggs from the ewes was expected after turn-out in 2012. From the antibody dynamics in the sheep in the same flock, it was evident that *F. hepatica*-naïve lambs were infected no earlier than the beginning of August 2012. Considering that it takes approximately two months to complete larval development in *G. truncatula*, and since survival of overwintered metacercariae or metacercariae emerging from overwintered snails is minimal during June and July (Ollerenshaw, 1971), infection of snails with overwintered eggs is the most likely explanation for the recurrence of the parasite in lambs on farm O3.

Although all flocks, except those on farm B1, were dewormed with albendazole, triclabendazole and/or closantel, no effect on *F. hepatica* incidence was observed on *F. hepatica* naïve animals. However, it needs to be pointed that deworming with closantel on cattle farms started for the first time in winter 2011–2012 in Sweden. High prevalence in sheep and cattle herds despite annual deworming further emphasizes the epidemiological importance of overwintering *F. hepatica* stages.

From our results, it is evident that *G. truncatula* acts as the main intermediate host of *F. hepatica* in Sweden. On the other hand, we have also previously demonstrated that other species, such as *L. palustris* and *L. fuscus*, are susceptible to *F. hepatica* but with a high level of age resistance (Novobilský et al., 2013). The finding of the single specimen of *L. palustris* (Novobilský et al., 2013) and of *S. putris* naturally infected with *F. hepatica* probably results from the fact that these two species share a habitat with *G. truncatula*. Naturally infected *Succinea* sp. has been reported previously in Ireland (Relf et al., 2009). However, further investigation is required before adding *Succinea putris* to the list of *F. hepatica*-susceptible snail species, especially as the development and infection characteristics have not been confirmed experimentally. An obvious weakness with PCR of *F. hepatica* in snails is that only the presence of DNA can be detected, rather than active infections.
Overall, these snail species seem to play only a minor role in transmission of *F. hepatica* in Sweden, as the overall prevalence of both was very low.

The prevalence of *F. hepatica* in *G. truncatula* in general varied extensively between sites, seasons and years. The main reason for the lower spring prevalence of *F. hepatica* in *G. truncatula* in 2011 might be that April and May were dry in that year (Table 2) and that the total number of snails collected was lower than in 2012 (Supplementary Table 1). The one and only *F. hepatica*-positive *G. truncatula* found in spring 2011 was collected on farm B2 at the end of May, one month after turnout. Thus, early infection from spring-hatched miracidia cannot be completely excluded. Furthermore, as few overwintering *F. hepatica*-positive snails were found in spring 2011, this explains the absence of ‘winter infection’ on all six farms in 2011.

At Weybridge (UK), *G. truncatula* population growth peaked between August and September during 1962-1966 (Ollerenshaw, 1971). The percentage of infected snails reached its highest values at the same time. In contrast, no difference in the prevalence of *G. truncatula* between different sampling months has been reported in similar studies in Switzerland and France (Abrous et al., 1999; Schweizer et al., 2007). In our study, *F. hepatica* prevalence was consistently significantly higher in autumn 2011 than in spring, whereas extreme variations in infection levels were shown in 2012. The high proportion of overwintered infected snails found on farms O1, O2 and B2 indicates that the pastures grazed by livestock on these farms possess optimal conditions for snail survival. This implies that overwintering in snails is influenced not only by rainfall and humidity, but also by other unspecified factors.

Interestingly, few or no *F. hepatica*-positive snails of *G. truncatula* were found on the cattle farms B1 and B3, where the prevalence in calves varied between 53 and 81%. In contrast, positive snails were found on beef cattle farm B2, where the pasture size and ecotype were similar to those on sheep farm O2. The chances of finding positive snails on spacious pastures are influenced by the identification of snail hotspots. In addition, the proportion of infected snails is positively associated with snail population density in different habitats (Ross, 1977; Smith, 1981). From a diagnostic point of view, it is important to highlight that examination of snails cannot substitute for other methods, such as coproscopy or serology in animals, to estimate level of infection in the herd.

Rainfall, temperature and their interaction are considered to be the most important predictors of *F. hepatica* infection in the United Kingdom (Fox et al. 2011; McCann et al., 2010). From the data in our study it is evident that the vegetation period of 2012 was generally colder than in 2011. However, the local average temperature on farms in 2012 was negatively correlated (not significantly) with *F. hepatica* prevalence both in *G. truncatula* and in young ruminants. In contrast, the most affected
farm O1 (prevalence 100% and 82% in lambs and G. truncatula, respectively) had the highest rainfall of all herds examined in 2012. Interestingly, *F. hepatica* positive overwintered snails were observed just in the colder year 2012, after the long winter 2011–2012. This contradicts the observation (Fox et al., 2011) that higher temperature increases the risk of *F. hepatica* overwintering and transmission. Climate factors as dominant predictors of *F. hepatica* risk have already been questioned in Germany (Kuerpick et al., 2013). For instance, pasture management factors have been shown to be one of the most important risk predictors in Belgium (Bennema et al. 2011). The amount of suitable snail habitat also plays a significant role in transmission (Charlier et al. 2011). Although the number of farms examined in our study is too low to reach conclusions about transmission predictors, it is still evident that factors other than rainfall and air temperature are crucial for *F. hepatica* transmission.

In conclusion, we observed both ‘winter infection’ and ‘summer infection’ in Sweden. Since typical ‘winter infection’ was observed only on sheep farms O1 and O2 during 2012, we believe that ‘summer infection’ is the preferred strategy for *F. hepatica* overwintering in Sweden. This coincides with previous studies in other countries in north-west Europe (Ross, 1977; Shaka and Nansen, 1979). Ollerenshaw (1959) speculated that ‘winter infection’ would be more important in regions where the summer is too short for parasite development. Our data provide a more complicated picture, and indicate that the occurrence of ‘winter infection’ is dependent not only on climate factors that influence snail density in autumn and parasite prevalence, but also on other environmental factors that might locally influence transmission of *F. hepatica*.

### Acknowledgements

This study was supported by the Swedish Foundation for Agricultural Research (contract no. H1050003) and the 7th Framework Programme of the European Union (GLOWORM, Project FP7-KBBE-2012-288975). We are grateful for the help of all of the farmers and veterinarians involved in the study and for cooperation from the Swedish Animal Health Service (Ulrika König, Virpi Welling and Helen B. Averpil). We also thank Lenka Novobilská for help with DNA isolation and PCR examination of snails, and Luboš Beran (Agency for Nature Conservation and Landscape Protection of the Czech Republic, Kokořínsko Protected Landscape Area Administration, Mělník, Czech Republic) and Ted von Proschwitz (Gothenburg Natural History Museum, Sweden) for help with snail identification.

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Table legends

**Table 1.** Summary of the locations studied, including snail collections.

* Number of cows/ewes
n.s. not sequenced

**Table 2.** Local climate data: Average monthly rainfall and temperatures recorded at five meteorological stations for the period April-October 2011 and 2012. The 30-year average (1961-1990) is included for each station.

**Supplementary Table 1.** Numbers of *Galba truncatula* collected from the six farms during 2011-2012 and prevalence of *Fasciola hepatica* infection in *G. truncatula* as determined by PCR.

**Supplementary Table 2.** Individual values of the antibody positivity index in lambs and calves on farms O1, O2, O3, B1 and B3 in 2011 and 2012.

**Figures:**

**Figure 1.** Prevalence of *Fasciola hepatica* infection in *Galba truncatula* in Sweden, as determined by PCR, on farms O1, O2, B1 and B2 in spring (S) and autumn (A) 2011 and 2012.

**Figure 2.** Dynamics of *Fasciola hepatica* antibodies in lambs on sheep farms O1, O2 and O3 during 2011 and 2012. Dashed line is cut-off limit (cut-off=10% of positivity).

**Figure 3.** Dynamics of *Fasciola hepatica* antibodies in first grazing season calves on beef cattle farms B1 and B3 in Sweden during 2011 and 2012. Blue dots are negative animals, red dots are positive animals. Dashed line is cut-off limit (cut-off=15% of positivity).