Transmission patterns of *Fasciola hepatica* to ruminants in Sweden

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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, 138, 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 (Sorvettula, 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

142 The presence of *F. hepatica* larvae in snails was determined by PCR amplification of a 112-bp species-
143 specific region of ITS-2 (Bazsalovicsová et al., 2010; Kráľová-Hromadová et al., 2008). Snails were
144 defrosted and DNA from whole snail soft tissues was extracted using the DNeasy Blood and Tissue Kit
145 (Qiagen, Germany). After a lysis step with proteinase K and overnight incubation, snail samples were
146 pooled, with a maximum of 10 individuals per pooled sample, by mixing 20 µL of each sample to
147 achieve 200 µL pooled lysate. DNA was then extracted according to the manufacturer's instructions.
148 The primers used for PCR amplification were those designed by Kraľová-Hromadová et al. (2008):
149 Forward: FH-ITS2-SPEC-F 5'-CTTATGATTTCTGGGATAATT-3', Reverse: FH-ITS2-SPEC-R 5'-
150 CCGTCGCTATATGAAAA-3'. The PCR mixture contained: 1 µL DNA, 10 mM Tris-HCl pH 8.3, 50 mM KCl,
151 2 mM $MgCl_2$, 4 µg bovine serum albumin (BSA; New England Biolabs, UK), 0.8 µM of each primer, 0.2
152 mM dNTP, and 0.3 U Taq polymerase (AmpliTaq Gold, Applied Biosystems, USA). Amplifications were
153 generated in a 2720 Thermal Cycler (Applied Biosystems, USA) as follows: 40 cycles of 45 sec at 95 °C,
154 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
155 products were separated on 1.5% agarose gel. Whenever *F. hepatica*-positive pooled samples were
156 identified, all individual samples were re-tested using the same PCR protocol. The specificity and the
157 sensitivity of the PCR method have already been tested in previous study (Novobilský et al., 2013).

158 1.5. Collection of serum samples and ELISA

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

165 Sera were examined by indirect enzyme-linked immunosorbent assay (ELISA) using a native *F.*
166 *hepatica* excretory-secretory antigen (E/S Ag) as described by Novobilský et al. (2012a; 2007). In
167 brief, sera were diluted 1:50 in buffer (0.05% Tween 20, 5% non-fat milk in phosphate-buffered
168 saline, pH=7.2), and each serum sample was tested in duplicate. The same positive, weakly positive
169 and negative reference samples were included in all assays. For the sheep ELISA, peroxidase-labelled
170 anti-sheep IgG produced in donkey (Sigma-Aldrich, Sweden) at 1:3000 dilution was used as the
171 secondary antibody. For the bovine ELISA, horseradish peroxidase-conjugated anti-bovine IgG
172 antibodies (Svanova Biotech, Sweden) were used at 1:10 000 dilution. The ELISA results were
173 expressed as percentage of the mean optical density ratio (ODR) of the positive control as: % of
174 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.

208

209 2.2. Snail species identified and screening of *F. hepatica* in snails

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

217

218 A total of 1726, 588, 138, 130, 93 and 42 specimens of *G. truncatula*, *L. palustris*, *L. glabra*, *L. fuscus*,
219 *R. peregra* and *S. putris*, respectively, were examined by PCR. The species most frequently infected
220 with *F. hepatica* was *G. truncatula*, with infection levels ranging from 0% to 82% (Figure 1;
221 Supplementary Table 1). The overall prevalence in *G. truncatula* on all six farms was almost 13%, with
222 224 of the 1726 snails diagnosed/confirmed as infected. Except for one snail on farm B2, *F. hepatica*-
223 positive snails were detected only in autumn 2011, whereas the prevalence of *F. hepatica* in
224 *G. truncatula* was even higher in spring 2012 than in autumn 2012 on farms O2 and B2. No *F.*
225 *hepatica*-positive *G. truncatula* were found on farms B3 or O3 during the entire study period.
226 Furthermore, only one terrestrial snail (*S. putris*) from farm O2 tested positive for *F. hepatica*.

227

228 2.3. Local climate data and correlation to infection levels on farms

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

239

240 3. Discussion

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

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

260 Accepting colostral transfer, we concluded that all the lambs in 2011 were infected with
261 metacercariae at the beginning of August at the earliest. On sheep farms O1 and O2, higher
262 prevalences were in general observed in 2012, when between 70–80% were seropositive already in
263 June 2012. Owing to the high infection levels in the June collection and the fact that no animal were
264 tested as negative in August 2012, we estimate that these lambs were infected approximately in late
265 May-early June. At the same time, we cannot exclude colostral transfer of antibodies as the cause of
266 seropositivity in June. The typical dynamics of antibodies during early *F. hepatica* infection in
267 ruminants is characterised by antibody response first appearing between 2–4 weeks post-infection,
268 which then progressive increases until 10–12 weeks (Cornelissen et al., 2001; Novobilsky et al., 2007;
269 Phiri et al., 2006). The occurrence of a few lambs (e.g. 12100 on O1; 9225 and 9226 on O2;
270 Supplementary Table 2) on farms O1 and O2 that had lower titres in the second collection in August
271 2012 than in June 2012, suggests that these lambs both received maternal *F. hepatica* antibodies and
272 were infected later than the rest of the lambs in the same flock. Therefore, we conclude that a
273 combination of post-infection and passive transfer of colostral antibodies are the most likely
274 explanations for the high prevalence observed in June on farms O1 and O2 in 2012. This observation

275 further confirms that lambs on those two farms were first infected between May and June, by
276 overwintered *F. hepatica* in snails or eventually as metacercariae.

277 In cattle, colostral transfer of antibodies was never detected, but it should be borne in mind that for
278 logistic reasons early summer blood collection was feasible on only one cattle farm. Nevertheless,
279 compared with the antibody dynamics in sheep, the humoral immune response in the calves tested
280 was delayed in both years. This suggests that calves on farms B1 and B3 were originally exposed to
281 metacercariae between September and late October, which is somewhat later than in sheep. This
282 delayed infection time in cattle might be explained in several different ways. First, pasture
283 management may play a considerable role in *Fasciola* transmission in dairy cattle (Bennema et al.,
284 2011; Charlier et al., 2011). For instance, the length of the grazing period, mowing of pastures and
285 herd size are key factors that can increase or decrease exposure risk to animals. At the same time,
286 these factors generally could explain the elevated risk or prevalence in the herd but not the
287 differences between infection time points. Furthermore, differences in the grazing behaviour of
288 cattle and sheep should be considered. The feeding style of sheep is more selective for preferred
289 legumes than is that of beef cattle. It has also been suggested that sheep use manipulative non-biting
290 jaw movements to maintain their foraging preferences and to avoid non-preferred items (Bremm et
291 al., 2012). Finally, the late infection of cattle in the study might simply consist of habitat differences
292 between sheep and cattle farms, e.g. less suitable conditions for surviving metacercariae or snails on
293 the cattle farms. However, all of these explanations remain very speculative until more evidence is
294 available.

295 It is evident that the time-point of the first infection on sheep farms O1 and O2 differed between
296 2011 and 2012. Similarly, the infection dynamics on farms O1 and O2 differed from those on O3 in
297 2012. Based on the Ollerenshaw (1959) definition, we can conclude that most lambs and calves,
298 except those on farms O1 and O2 in 2012, were infected with metacercariae originating from spring-
299 infected snails, which result in the so-called 'summer infection'. On the other hand, early infected
300 lambs on farms O1 an O2 in 2012 corresponded to typical 'winter infection'. This is further supported
301 by the high prevalence of *F. hepatica*-positive *G. truncatula* in spring 2012. These snails were
302 collected during the first half of May, when the mean temperature was below 6 °C (SMHI, 2012;
303 Table 2). Based on the information in Ollerenshaw (1971), development, hatching and subsequent
304 snail infection by miracidia from overwintered eggs is impossible to complete within one month
305 under these low temperature. Therefore, overwintering of *G. truncatula* infected with *F. hepatica* in
306 late autumn 2011, and subsequent production of metacercariae in May and June 2012, is the most
307 likely explanation. At the same time, overwintering of metacercariae on pasture from the previous
308 season must be considered as another source of 'winter infection'. As has been reported from other

309 countries, metacercariae produced during autumn can survive the winter months on pasture,
310 although their viability decreases rapidly from April to June. Thus, the role of overwintered
311 metacercariae has been suggested to be small (Luzon-Pena et al., 1994; Ollerenshaw, 1971; Shaka
312 and Nansen, 1979). This implies that 'winter infections' depend on infections of snails in autumn and
313 survival of *F. hepatica*-infected snails during winter and spring months.

314 The egg of the parasite is the key overwintering stage for 'summer infection' (Ollerenshaw, 1971;
315 Ross, 1977; Shaka and Nansen, 1979). Although it is difficult to demonstrate directly, our data for
316 farm O3 confirm that this is also possible under the prevailing climate conditions in Sweden. On farm
317 O3, all of the animals were dewormed with triclabendazole during winter 2011/2012, with 100%
318 efficacy as documented in a previous study (Novobilský et al., 2012a). Therefore, no contamination
319 with fresh fluke eggs from the ewes was expected after turn-out in 2012. From the antibody
320 dynamics in the sheep in the same flock, it was evident that *F. hepatica*-naïve lambs were infected no
321 earlier than the beginning of August 2012. Considering that it takes approximately two months to
322 complete larval development in *G. truncatula*, and since survival of overwintered metacercariae or
323 metacercariae emerging from overwintered snails is minimal during June and July (Ollerenshaw,
324 1971), infection of snails with overwintered eggs is the most likely explanation for the recurrence of
325 the parasite in lambs on farm O3.

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

331 From our results, it is evident that *G. truncatula* acts as the main intermediate host of *F. hepatica* in
332 Sweden. On the other hand, we have also previously demonstrated that other species, such as *L.*
333 *palustris* and *L. fuscus*, are susceptible to *F. hepatica* but with a high level of age resistance
334 (Novobilský et al., 2013). The finding of the single specimen of *L. palustris* (Novobilský et al., 2013)
335 and of *S. putris* naturally infected with *F. hepatica* probably results from the fact that these two
336 species share a habitat with *G. truncatula*. Naturally infected *Succinea* sp. has been reported
337 previously in Ireland (Relf et al., 2009). However, further investigation is required before adding
338 *Succinea putris* to the list of *F. hepatica*-susceptible snail species, especially as the development and
339 infection characteristics have not been confirmed experimentally. An obvious weakness with PCR of
340 *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 turn-out. 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*.

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References

406 Abrous, M., Rondelaud, D., Dreyfuss, G., Cabaret, J., 1999. Infection of *Lymnaea truncatula* and
407 *Lymnaea glabra* by *Fasciola hepatica* and *Paramphistomum daubneyi* in farms of central
408 France. Vet. Res. 30, 113–118.
409 Bargues, M.D., Vigo, M., Horák, P., Dvořák, J., Patzner, R.A., Pointier, J.P., Jackiewicz, M., Meier-
410 Brook, C., Mas-Coma, S., 2001. European Lymnaeidae (Mollusca: Gastropoda), intermediate
411 hosts of trematodiases, based on nuclear ribosomal DNA ITS-2 sequences. Infect. Genet.
412 Evol. 1, 85–107.
413 Bazsalovicsová, E., Kraľová-Hromadová, I., Špakulová, M., Reblanová, M., Oberhauserová, K., 2010.
414 Determination of ribosomal internal transcribed spacer 2 (ITS2) interspecific markers in
415 *Fasciola hepatica*, *Fascioloides magna*, *Dicrocoelium dendriticum* and *Paramphistomum cervi*
416 (Trematoda), parasites of wild and domestic ruminants. Helminthol. 47, 76–82.
417 Bennema, S.C., Ducheyne, E., Vercruysse, J., Claerebout, E., Hendrickx, G., Charlier, J., 2011. Relative
418 importance of management, meteorological and environmental factors in the spatial
419 distribution of *Fasciola hepatica* in dairy cattle in a temperate climate zone. Int. J. Parasitol.
420 41, 225–233.
421 Beran, L., 2002. Aquatic molluscs of the Czech Republic – distribution and its changes, habitats,
422 dispersal, threat and protection, Red List. Sborník přírodovědného klubu Uherské Hradiště.
423 (in Czech)
424 BLAST, 2013. http://blast.ncbi.nlm.nih.gov/
425 Bremm, C., Laca, E.A., Fonseca, L., Mezzalira, J.C., Elejalde, D.A.G., Gonda, H.L., Carvalho, P.C.D.,
426 2012. Foraging behaviour of beef heifers and ewes in natural grasslands with distinct
427 proportions of tussocks. Appl. Anim. Behav. Sci. 141, 108–116.
428 Charlier, J., Bennema, S.C., Caron, Y., Counotte, M., Ducheyne, E., Hendrickx, G., Vercruysse, J., 2011.
429 Towards assessing fine-scale indicators for the spatial transmission risk of *Fasciola hepatica*
430 in cattle. Geospatial Health 5, 239–245.
431 Chauvin, A., Bouvet, G., Boulard, C., 1995. Humoral and cellular immune-responses to *Fasciola*
432 *hepatica* experimental primary and secondary infection in sheep. Int. J. Parasitol. 25, 1227–
433 1241.
434 Cornelissen, J.B.W.J., Gaasenbeek, C.P.H., Borgsteede, F.H.M., Holland, W.G., Harmsen, M.M.,
435 Boersma, W.J.A., 2001. Early immunodiagnosis of fasciolosis in ruminants using recombinant
436 *Fasciola hepatica* cathepsin L-like protease. Int. J. Parasitol. 31, 728–737.
437 Domke, A.V., Chartier, C., Gjerde, B., Leine, N., Vatn, S., Stuen, S., 2013. Prevalence of gastrointestinal
438 helminths, lungworms and liver fluke in sheep and goats in Norway. Vet. Parasitol. 194, 40–
439 48.
440 Fox, N.J., White, P.C., McClean, C.J., Marion, G., Evans, A., Hutchings, M.R., 2011. Predicting impacts
441 of climate change on *Fasciola hepatica* risk. Plos One 6, e16126.
442 Gaasenbeek, C.P.H., Over, H.J., Noorman, N., Deleeuw, W.A., 1992. An epidemiologic study of
443 *Fasciola hepatica* in the Netherlands. Vet. Quart. 14, 140–144.
444 Gloer, P., 2002. Die Süßwassergastropoden Nord- und Mitteleuropas. Conchbooks, Hackenheim, 327
445 p. (in German)
446 Höglund, J., Dahlström, F., Engström, A., Hessle, A., Jakubek, E.B., Schnieder, T., Strube, C.,
447 Sollenberg, S., 2010. Antibodies to major pasture borne helminth infections in bulk-tank milk
448 samples from organic and nearby conventional dairy herds in south-central Sweden. Vet.
449 Parasitol. 171, 293–299.
450 Jackiewicz, M., 2000. Błotniarki Europy (Gastropoda: Pulmonata: Lymnaeidae). Wydawnictwo
451 Kontekst, Poznan. (in Polish)
452 Kráľová-Hromadová, I., Špakulová, M., Horáčková, E., Turčeková, L., Novobilský, A., Beck, R., Koudela,
453 B., Marinculić, A., Rajský, D., Pybus, M., 2008. Sequence analysis of ribosomal and
454 mitochondrial genes of the giant liver fluke *Fascioloides magna* (Trematoda : Fasciolidae):
455 Intraspecific variation and differentiation from *Fasciola hepatica*. J. Parasitol. 94, 58–67.

Kuerpick, B., Conraths, F.J., Staubach, C., Schnieder, T., Strube, C., 2013. Seroprevalence and GIS-supported risk factor analysis of *Fasciola hepatica* infections in dairy herds in Germany. Parasitol. 140, 1051–1060.

König, U., Welling, V., 2013. Stora Leverflundror. In: Svenska Djurhälsovården, Uppsala, Sweden.

Luzon-Pena, M., Rojo-Vazquez, F.A., Gomez-Bautista, M., 1994. The overwintering of eggs, intramolluscal stages and metacercariae of *Fasciola hepatica* under the temperatures of a Mediterranean area (Madrid, Spain). Vet. Parasitol. 55, 143–148.

Malone, J.B., Loyacano, A.F., Hugh-Jones, M.E., Corkum, K.C., 1984. A 3-year study on seasonal transmission and control of *Fasciola hepatica* of cattle in Louisiana. Prev. Vet. Med. 3, 131–141.

Martinez, A., Martinez-Cruz, M.S., Martinez, F.J., Gutierrez, P.N., Hernandez, S., 1996. Detection of antibodies to *Fasciola hepatica* excretory-secretory antigens in experimentally infected goats by enzyme immunosorbent assay. Vet. Parasitol. 62, 247–252.

Mezo, M., Gonzalez-Warleta, M., Castro-Hermida, J.A., Carro, C., Ubeira, F.M., 2010. Kinetics of anti-*Fasciola* IgG antibodies in serum and milk from dairy cows during lactation, and in serum from calves after feeding colostrum from infected dams. Vet. Parasitol. 168, 36–44.

Nielsen, K., 1974. Fascioliasis - a general presentation. Nordisk Veterinärmedicin 26.

Novobilský, A., Averpil, H.B., Höglund, J., 2012a. The field evaluation of albendazole and triclabendazole efficacy against *Fasciola hepatica* by coproantigen ELISA in naturally infected sheep. Vet. Parasitol. 190, 272–276.

Novobilský, A., Christensson, D., König, U., 2012b. Stora leverflundran i fokus runt mötesbordet. Svensk Veterinärtidning 14, 26–29. (in swedish)

Novobilský, A., Kašný, M., Beran, L., Rondelaud, D., Höglund, J., 2013. *Lymnaea palustris* and *Lymnaea fuscus* are potential but uncommon intermediate hosts of *Fasciola hepatica* in Sweden. Parasit. Vect. 6, 251.

Novobilský, A., Kašný, M., Mikeš, L., Kovaříčík, K., Koudela, B., 2007. Humoral immune responses during experimental infection with *Fascioloides magna* and *Fasciola hepatica* in goats and comparison of their excretory/secretory products. Parasitol. Res. 101, 357–364.

Ollerenshaw, C.B., 1959. The ecology of the liver fluke (*Fasciola hepatica*). Vet. Rec. 71, 957–963; 963–965 pp.

Ollerenshaw, C.B., 1971. Some observations on the epidemiology of fascioliasis in relation to the timing of molluscicide applications in the control of the disease. Vet. Rec. 88, 152–164.

Phiri, I.K., Phiri, A.M., Harrison, L.J.S., 2006. Serum antibody isotype responses of *Fasciola*-infected sheep and cattle to excretory and secretory products of *Fasciola* species. Vet. Parasitol. 141, 234–242.

Reichel, M.P., 2002. Performance characteristics of an enzyme-linked immunosorbent assay for the detection of liver fluke (*Fasciola hepatica*) infection in sheep and cattle. Vet. Parasitol. 107, 65–72.

Relf, V., Good, B., McCarthy, E., de Waal, T., 2009. Evidence of *Fasciola hepatica* infection in *Radix peregra* and a mollusc of the family Succineidae in Ireland. Vet. Parasitol. 163, 152–155.

Ross, J.G., 1977. 5-year study of epidemiology of fascioliasis in N, E and W of Scotland. Brit. Vet. J. 133, 263–272.

Salimi-Bejestani, M.R., McGarry, J.W., Felstead, S., Ortiz, P., Akca, A., Williams, D.J., 2005. Development of an antibody-detection ELISA for *Fasciola hepatica* and its evaluation against a commercially available test. Res. Vet. Sci. 78, 177–181.

Santiago, N., Hillyer, G.V., 1988. Antibody profiles by EITB and ELISA of cattle and sheep infected with *Fasciola hepatica*. J. Parasitol. 74, 810–818.

Schweizer, G., Meli, M.L., Torgerson, P.R., Lutz, H., Deplazes, P., Braun, U., 2007. Prevalence of *Fasciola hepatica* in the intermediate host *Lymnaea truncatula* detected by real time TaqMan PCR in populations from 70 Swiss farms with cattle husbandry. Vet. Parasitol. 150, 164–169.

507 Shaka, S., Nansen, P., 1979. Epidemiology of fascioliasis in Denmark - studies on the seasonal
508 availability of metacercariae and the parasite stages overwintering on pasture. Vet. Parasitol.
509 5, 145–154.
510 SMHI, 2012. http://www.smhi.se/klimatdata/meteorologi/ars-och-manadsstatistik-2.1240
511 Smith, G., 1981. A 3-year study of *Lymnaea truncatula* habitats, disease foci of fascioliasis. Brit. Vet. J.
512 137, 398–410.
513 Sorvettula, O., 1974. Fascioliasis - occurence and epidemiology in Finland. Nordisk Veterinärmedicin
514 26, 39–41.
515 Stromberg, B.E., Averbeck, G.A., 1999. The role of parasite epidemiology in the management of
516 grazing cattle. Int. J. Parasitol. 29, 33–39; discussion 49–50.
517 Torgerson, P., Claxton, J., 1999. Epidemiology and Control, In: Dalton, J.P. (Ed.) Fasciolosis. CABI Pub,
518 Wallingford, pp. 113–149.
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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).