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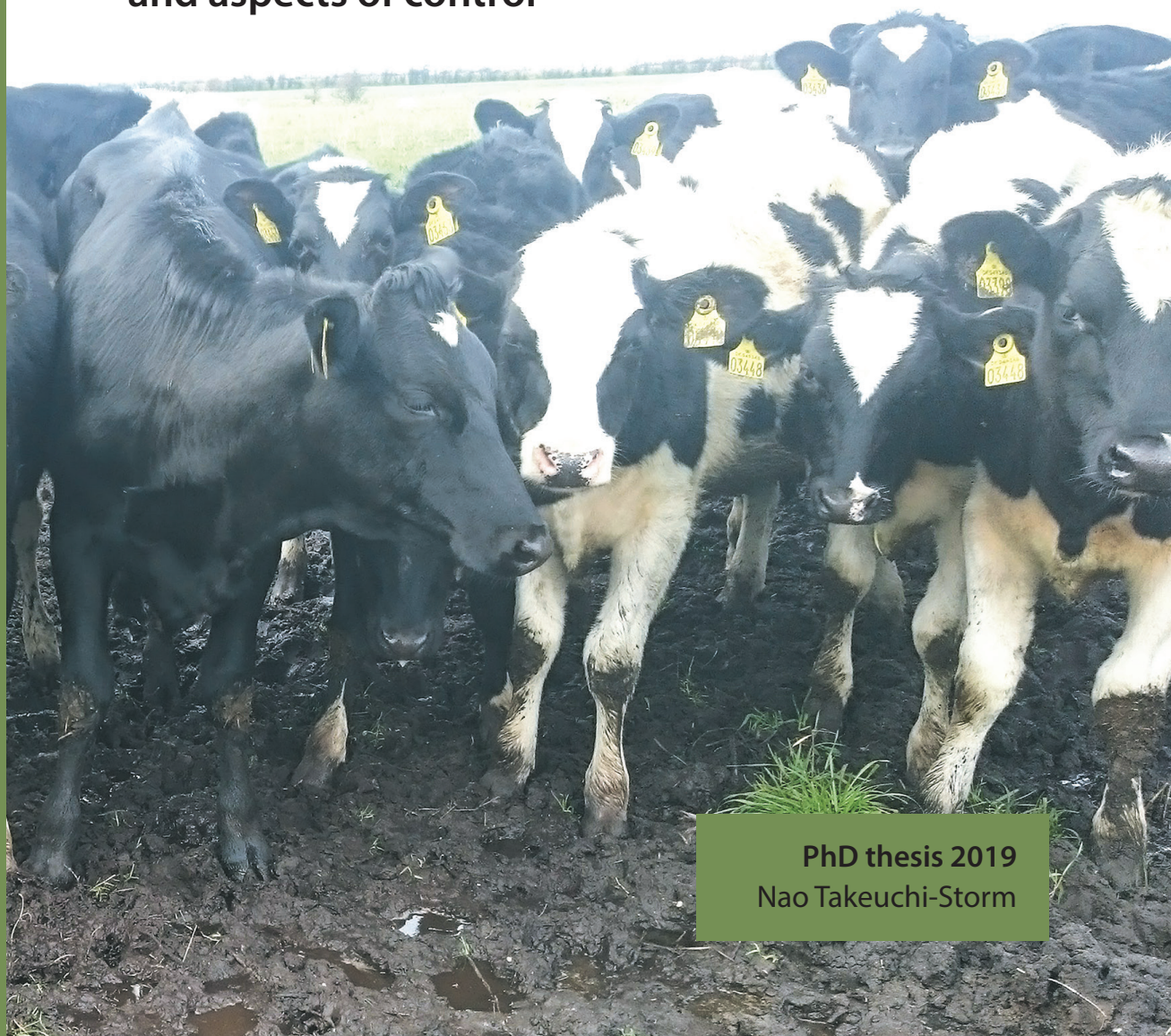
Fasciolosis in Danish dairy cattle: epidemiology, diagnostics, impact and aspects of control



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PhD thesis

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Preface

The work presented in this thesis was conducted from 2015 to 2018 at the Section for Parasitology and Aquatic Pathobiology, Department of Veterinary and Animal Sciences, Faculty of Health and Medical Sciences, University of Copenhagen. Professor Stig M. Thamsborg from Section for Parasitology and Aquatic Pathobiology, University of Copenhagen was the main supervisor, and Associate Professor Matthew Denwood from Section for Animal Welfare and Disease Control, University of Copenhagen and Research Professor Heidi L. Enemark from Norwegian Veterinary Institute were the co-supervisors. Some work of the project were also conducted at Department of Infection Biology, Institute of Infection and Global Health, University of Liverpool in collaboration with Professor Diana Williams, and at Laboratory of Veterinary Parasitology, Faculty of Agriculture, Iwate University in collaboration with Assistant Professor Madoka Ichikawa-Seki.

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Special thank you goes to the collaborators from SEGES (Jaap Boes, Erik Rattenborg, Jørgen Nielsen), Eurofins (Dorte Thanning Lavritsen and Lone Møller Pedersen), ØkologiRådgivning (Erik Andersen), Kirstine Lauridsen and the four farmers who agreed to participate in the longitudinal study. Diana Williams and her team, Madoka Ichikawa-Seki and her team, are also thanked for welcoming me for short research stay with warmth and inspiring discussions on liver flukes.

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I also would like to thank my parents and in-laws for their moral support and their childcare services. Lastly, I cannot thank enough to my husband, Karl Emil, and my two precious sons, Kai and Aki, for their endless love and joy they provide to my everyday life. I certainly could not have achieved this without their existence.

Table of contents

Summary

The common liver fluke, *Fasciola hepatica*, is a parasitic trematode that infects many mammalian host species, especially ruminants. The prevalence of bovine fasciolosis has been increasing in recent years in Denmark, but appropriate guidelines for control under Danish conditions are currently lacking due to the absence of up-to-date local epidemiological data. Therefore, the overall objective of this Ph.D. project was to investigate the current status of bovine fasciolosis using a range of diagnostic measures on Danish dairy farms and to develop practical on-farm recommendations for surveillance and control based on this epidemiological data.

Firstly, the farm-level risk factors for bovine fasciolosis were investigated by two questionnaire surveys (Papers I and III). The first questionnaire included conventional farms without any grazing animals, and therefore the second questionnaire targeted only organic farms, where grazing of all age groups is mandatory. In both studies, "heifers grazing on the wet areas" was found to be highly associated with fasciolosis positive farms, suggesting that heifers constitute a major risk group for fasciolosis. In the first study, "dry cows grazing on wet areas" were also significantly associated with infected farms, suggesting that infection in the cow population should also be considered before developing a specific control strategy on farm.

In Paper I, the farm classification based on liver condemnation data and BTM ELISA was compared. Moderate agreement between the two methods was seen, and discrepancy was likely due to the detection limit of BTM ELISA, low sensitivity and imperfect specificity of liver inspection at slaughter, and the time lag between acquisition of data and BTM. In Paper III, we were able to show that BTM ELISA was highly correlated with the prevalence of fasciolosis within the milking herd and that positive BTM ELISA results can be found on farms with a within-herd prevalence of only 8.8%. This suggests that BTM ELISA is a good herd-level diagnostic method of fasciolosis, and is particularly suitable for monitoring the effect of control programs against *F. hepatica*.

On-farm patterns of infection were investigated on four age cohorts from four farms using three different diagnostic methods (serum antibody ELISA, coproantigen ELISA, and faecal egg count) in Paper II. The study showed that infection was first acquired as heifers on all farms, and that the animals were re-exposed to metacercariae as cows in at least one of the farms. The findings also implied that longevity of *F. hepatica* within the host could be longer than two years, and that determination of whether or not recurrent infection is occurring within the adult herd is not possible

based solely on antibody ELISA. Overwintering infection was not evident in the study, and summer infection of the snails was still the dominant transmission pattern in Denmark. The predominant summer infection and the different properties of the different diagnostic tests meant that antibody ELISA is sufficient to identify infection based on samples collected at housing, while coproantigen ELISA and faecal egg count will likely miss the early stage of infection if samples taken in autumn or early winter.

Finally, the relationships between anti-*F. hepatica* ELISA results and milk production expressed as 305 day energy corrected milk yield (305d ECM) on individual and farm level were investigated in Paper III. The study found that an average reduction of 580.8 kg in 305d ECM was associated with *F. hepatica* ELISA positive farms. At individual animal level, a significant reduction of 919.5kg was found but only for cows in parity three or later. The findings suggest a negative effect on milk production due to *F. hepatica* infection, but further studies are required to confirm a causative relationship and to unravel the mechanisms of this causative pathway, within which age, immunity, and concurrent infections may play a part.

In conclusion, the data and knowledge generated during this Ph.D. indicate that the primary effort to control bovine fasciolosis in Denmark should focus on limiting the risk of infection in heifers, possibly using anthelmintic treatment combined with grazing management. However, the current control measures rely largely on anthelmintics and other options for control is limited if dry (fluke-free) pasture is unavailable on farm. Moreover, further work is required to assess the optimum strategy for use of anthelmintics, including the possible presence and risk of developing anthelmintic resistance within Danish *F. hepatica* populations.

Dansk sammendrag

Den store leverikte, *Fasciola hepatica*, er en parasitisk fladorm (ikter, Trematoda), som inficerer flere pattedyrarter men især drøvtyggere. Forekomsten af fasciolose hos kvæg har været stigende i de senere år i Danmark, men på nuværende tidspunkt mangler relevante rådgivningsværktøjer for kontrol under danske forhold, hvilket blandt andet skyldes mangel på opdaterede epidemiologiske data. Det overordnede formål med denne phd-afhandling var derfor i) at undersøge den nuværende status af fasciolose i danske malkekvæg ved anvendelse af nyere diagnostiske metoder samt ii) at udvikle praktiske retningslinjer for kontrol af fasciolose på besætningsniveau på basis af de samlede epidemiologiske data.

To spørgeskemaundersøgelser blev gennemført for at identificere risikofaktorer for fasciolose i malkekvægsbedrifter. Det første spørgeskema omfattede hovedsageligt konventionelle besætninger, hvoraf flere ikke havde græssende kvæg, og derfor blev det andet spørgeskema alene målrettet økologiske besætninger, hvor græsning af alle aldersgrupper er obligatorisk. Kvier blev påvist som den væsentligste risikogruppe i begge undersøgelser: "kvier, der græsser på våde områder" var således stærkt relateret til fasciolose-positive besætninger. "Goldkøer, der græsser på våde områder" var også en signifikant faktor associeret med fasciolose i den først undersøgelse, hvilket indikerer, at transmission i koflokken også bør overvejes, når en specifik kontrolstrategi skal udvikles i besætningen.

Besætningsklassifikation baseret på to forskellige metoder til besætningsdiagnostik (leverkassation oplyst ved slagtning og antistof-ELISA på tankmælk) blev sammenlignet. Kun en moderat god korrelation blev observeret mellem de to metoder. Sandsynlige årsager til den begrænsede overensstemmelse var: høj detektionsgrænsen for tankmælks-ELISA, kødkontrollens lave sensitivitet og specificitet ved slagtning, og tidsforskydning mellem indsamling af slagtedata og tankmælk. Resultatet af tankmælks-ELISA var stærkt korreleret med forekomsten af fasciolose i besætningerne, og en positiv tankmælks-ELISA betød således, at besætningen havde en forekomst på mindst 8,8 % positive lakterende køer. Tankmælks-ELISA er derfor en god besætningsdiagnostisk metode til påvisning af fasciolose hos malkekvæg og er særligt velegnet til at monitorere forløbet af kontrolprogrammer.

Fasciolosens smittemønster på besætningsniveau blev undersøgt i fire aldersgrupper kvæg fra fire besætninger ved brug af tre forskellige diagnostiske metoder (antistof-ELISA i blod, coproantigen

ELISA og antal æg i gødning ved sedimentation). Undersøgelsen viste, at infektionen i alle fire besætninger først blev etableret i kvierne, og at malkekøerne var eksponerede for metacercariae i mindst en af besætningerne. Resultaterne indikerede også, at *F. hepatica* med stor sandsynlighed kunne leve længere end to år i kvæg. Desuden viste resultaterne, at antistof-ELISA ikke er velegnet som selvstændig analyse til vurdering af, om infektionen forekommer i flokken eller ej. Tidlige infektioner i kvæg (før 1. august) fandt tilsyneladende kun sted i enkelte tilfælde, og sneglenes sommerinfektion var i lighed med 70erne fortsat det dominerende transmissionsmønster i Danmark. Den dominerende sommerinfektion og de diagnostiske metoders forskellige egenskaber betød, at antistof-ELISA i blod kunne identificere infektionen i kvæg allerede ved indbinding, mens diagnostik v.h.a. coproantigen ELISA og antal æg i fæces bør anvendes væsentligt senere i staldperioden.

Yderligere blev sammenhængen mellem anti-*F. hepatica* antistof-niveau og mælkeproduktion (beregnet som 305 dages energikorrigeret mælkeydelse (305d EKM)) undersøgt på individuelt niveau og besætningsniveau. Der var en gennemsnitlig reduktion på 580,8 kg i 305d EKM i *F. hepatica* ELISA-positive besætninger. På individuelt niveau var der en signifikant reduktion på 919,5 kg hos positive køer i tredje eller senere laktationer. Resultaterne tyder på, at mælkeproduktionen er markant reduceret på grund af leverikteinfektion, men flere studier er nødvendige for at undersøge den konkrete årsagssammenhæng og mekanismerne mellem fasciolose og nedsat mælkeproduktion, hvor et komplekst sammenspil mellem alder, immunitet og andre samtidige infektioner menes at have betydning.

De samlede data i den nærværende phd-afhandling viser, at risikoen for infektion i kvier bør begrænses for at kontrollere fasciolose i danske malkekvægsbesætninger. Dette kan finde sted ved anvendelse af ormemiddel, helst kombineret med en strategi for afgræsning. På nuværende tidspunkt afhænger kontrolstrategier for fasciolose i høj grad af effektive ormemidler, og andre kontrolmuligheder er begrænsede, hvis tørre (ikte-frie) arealer ikke er tilgængelige på bedriften. Desuden er der behov for yderligere forskning med henblik på at vurdere effekten af den optimale strategi for anvendelse af ormmidler, herunder belyse forekomst samt risikoen for udvikling af ormemiddelresistens i danske *F. hepatica* populationer.

Abbreviations

Ab-ELISA	Antibody ELISA (on serum or milk)
Ag-ELISA	Copro-antigen ELISA
bp	Base pair
BTM	Bulk tank milk
cox-1	Cytochrome c oxidase subunit 1 - gene
DCD	Danish cattle database
DNA	Deoxyribonucleic acid
ECM	Energy corrected milk yield
ELISA	Enzyme-linked immunosorbent assay
EU	ELISA unit
FEC	Faecal egg count
GAMM	Generalised additive mixed model
ITS-2	Internal transcribed spacer 2
kDa	Kilodalton
LIV-ELISA	In-house anti-*F. hepatica* ELISA at the University of Liverpool
PCR	Polymerase chain reaction
p.i.	Post infection
PP	Percent Positive
S/P%	Sample to positive percentage

1. **Rationale and study objectives**

1.1 Rationale

Fasciola hepatica has special meaning for the history and tradition of Danish parasitology, because prominent Danish parasitologists such as Peter Christian Abildgaard and Japetus Steenstrup in the 18th and 19th century contributed to the discovery of its life cycle (Nansen, 1980; Andrews, 1999). In more practical terms, liver fluke infections caused and still causes considerable problems in Danish livestock production, and more recently several research programs were conducted to combat the disease in the late 1960's to early 1970's (Riising, 1971; Nielsen et al., 1973; Shaka, 1975; Christensen and Nansen, 1976). The Danish research during this time also contributed significantly to the understanding of the biology of the parasite. However, a decrease in prevalence of bovine fasciolosis was seen during 1973-77, presumably due to increased awareness and control efforts following these research activities (Henriksen and Pilegaard-Andersen, 1979). Since the turn of the millennium, an increasing prevalence of bovine fasciolosis has been noted (Ersbøll et al., 2006) and more and more inquiries from veterinarians and consultants have been received to the parasitology section of the University of Copenhagen (S. M. Thamsborg, personal communication, March 20, 2015). This indicates an increasing problem associated with the parasite that had previously been thought to have been under control.

Considering the absence of on-going research in bovine fasciolosis in Denmark, the following research questions were raised:

- What is the current status of fasciolosis in Denmark?
- What are the farm-level risk factors for fasciolosis in Denmark?
- What are the transmission patterns of infection in Denmark, considering the altered trend in the climate and production systems?
- How can the infection be best diagnosed at farm-level and at animal level?
- How can we use different diagnostic methods in a strategic manner?
- How can we best advise farmers to tackle fasciolosis?

These questions formed the basis of this Ph.D. thesis, and led to development of the overall aim of the thesis: **to produce novel data on epidemiology and diagnostics regarding bovine fasciolosis on Danish dairy farms and to use this data to help develop practical on-farm solutions.**

Specific objectives of this thesis were:

1. To identify the key farm-level risk factors associated with bovine fasciolosis in Denmark (Paper I and III)
2. To compare the currently available diagnostic methods for bovine fasciolosis (Paper I and II)
3. To investigate the current patterns of *F. hepatica* infection on selected Danish dairy farms using a range of diagnostic methods, and thereby develop practical and realistic guidelines for on-farm diagnosis and ultimately control of the disease (Paper II)
4. To assess the relationship between antibody levels measured in bulk tank milk (BTM) and within-herd prevalence measured by individual milk samples (Paper III)
5. To evaluate the effect of *F. hepatica* infection on milk production (Paper III)

1.2 Outline of the thesis

The Ph.D. thesis consists of 8 chapters. Chapter 1 describes the background and the aim for the Ph.D. study, and Chapter 2 provides an overview of the aetiology, diagnosis and epidemiology of bovine fasciolosis. Chapter 3 summarises materials and methods, and Chapter 4 presents the results of the studies and discusses these results briefly. Conclusions and perspectives are given in Chapter 5. Additional research outputs produced during the Ph.D. projects are presented in Chapter 6. Chapter 7 contains references and the three manuscripts are presented in Chapter 8.

2. Introduction

2.1 Aetiology of bovine fasciolosis

2.1.1 Taxonomy and morphology

Fasciolosis is a disease caused by digenean trematodes belonging to the genus Fasciola. There are two major species belonging to this genus: *F. hepatica* and *F. gigantica*, and both are considered important aetiological agents of fasciolosis in different parts of the world. *Fasciola hepatica* is predominantly distributed in temperate regions, while *F. gigantica* is found mainly in tropical regions (Andrews, 1999). However, in some regions where the two species co-exist, a hybrid type between the two species has also been described (Itagaki and Tsutsumi, 1998). In Denmark, *F. gigantica* has never been found and this thesis will therefore only discuss *F. hepatica* as the sole aetiological agent for bovine fasciolosis.

Mature *F. hepatica* adult worms are leaf-shaped and measure approximately 30 x 10 mm (Pantelouris, 1965; Valero et al., 2001). The oral sucker is located at the tip of the cone-shaped projection, and the ventral sucker is located at the base of the cone. The tegument is covered with scale-like spines pointing backwards. The spines help maintain the position within organs they reside in and also erode the epithelium and damage blood vessels, making it easier for the parasite to feed. There is a paired intestinal caeca that extend to the posterior end of the body and highly branched lateral diverticula emerge from these caeca. Fasciola is a hermaphrodite; an individual possessing gonads of both sexes (Fairweather et al., 1999; Roberts et al., 2009). The eggs of *F. hepatica* are oval, yellowish brown and measure approximately 130-145 x 70-90 µm. It has an operculum at one end as most other flukes (Andrews, 1999). The metacercariae are approximately 300 µm in diameter (Riising, 1971) with an outer and inner cyst wall for longer survival in the environment. It is white right after encystation, but gradually turns darker and harder (Andrews, 1999).

2.1.2 Life Cycle

The life cycle of *Fasciola hepatica* requires two hosts; the mammalian definitive hosts (mainly herbivores) and the snail intermediate hosts (mainly *Galba s. Lymnaea truncatula* in Europe). Briefly, the life cycle is described as follows: i) unembryonated eggs are released and excreted with

the host faeces; ii) eggs embryonate in the outside environment; iii) miracidia hatch from the eggs and infect the intermediate snail host; iv) the parasites proliferate asexually and develop into sporocysts and redia within the snail host; v) cercariae are released from the host snail; vi) the cercariae lose their tail, encyst, and form metacercariae; vii) metacercariae are ingested by the definitive host and develop into adult worms (Andrews, 1999).

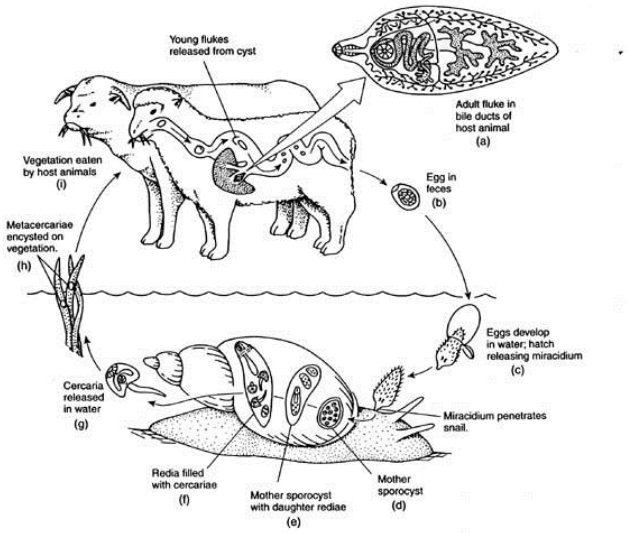

Fig 2.1. The life cycle of *Fasciola hepatica*. (a) Adult worms in the bile ducts of sheep or other mammals; (b) egg; (c) miracidium; (d); mother sporocyst; (e) mother sporocyst with developing rediae; (f) redia with developing cercariae; (g) free-swimming cercaria; (h) metacercariae, encysted on aquatic vegetation; (i) host animal eating vegetation; (j) flukes released from cyst, penetrate the intestinal wall and enter the abdominal cavity and later the liver capsule. Drawing by William Ober (Roberts et al., 2009)

The duration of development from eggs to metacercariae is highly dependent on temperature. The embryonation of eggs and the parasitic development in the snails require a minimum temperature of 10°C. The rate of development increases with temperatures from 10°C to approximately 30°C, but becomes inhibited above this point (Kendall and McCullough, 1951; Rowcliffe and Ollerenshaw, 1960). According to Ollerenshaw (1965), the development from eggs to the metacercariae takes only 5 weeks at 27°C, but 19 weeks at 15°C. In the temperate climate of northern Europe, it takes approximately three months if the eggs are deposited at the start of the grazing season. However, it may be shorter if the eggs are deposited in summer, and it may be extended over the winter if the snails are infected in autumn. The seasonality of infection will be further explained in section 2.4.4.

The definitive hosts are infected by ingestion of metacercariae. The metacercariae excyst in the small intestine and migrate through the small intestinal wall to the abdominal cavity within two hours. The young flukes reach and penetrate the liver capsule within four to six days post infection (p.i.). They wander around the liver parenchyma, feeding on blood and hepatic cells until five to six weeks p.i. Then they reach the bile ducts at about seven weeks p.i., where they mature and reside permanently. Timing of these events are based on experimental infection on sheep and may vary in other host species (Andrews, 1999).

It is possible for migrating immature flukes to reach other organs such as lungs, lymph nodes, skin, eyes, and brain (Boray, 1969; Yi-zhu and Zhi-bang, 2010; Mas-Coma et al., 2014). They may also infect the foetus in pregnant animals and mature in the livers of the foetus. It is a rare event but probably not negligible, as *F. hepatica* was recovered from 0.5% out of 16,776 livers of one to three weeks old calves (Rees et al., 1975). The route of infection is not known but suspected as through the systematic circulation or direct penetration (Mas-Coma et al., 2014).

The pre-patent period vary depending on the host species and the dose of infection (Boray, 1969). In sheep with light infection, the first eggs appeared approximately 56 days p.i, while the egg excretion was delayed to 13-15 weeks p.i. with heavy infections (Boray, 1967). In cattle, the appearance of the eggs can be as early as 56 days (Boray, 1969), but it may also take 12 weeks (Ross, 1968) and is generally one to two weeks longer than in sheep.

2.2. The clinical effect and economic impact due to *Fasciola hepatica*

The clinical presentation of fasciolosis differs depending on the infection dose and the phases of the parasite's life cycle: the acute migratory phase or the chronic biliary phase (Behm and Sanger, 1999). Acute disease is caused by immature flukes migrating through the liver parenchyma and feeding on the hepatic cells and blood. The acute disease develops when a large number of metacercariae are ingested in short period of time and results in a painful abdomen, anaemia, and sudden death. Chronic disease occurs when moderate number of metacercariae are ingested over a long period, causing gradual development of oedema, anaemia, and emaciation in sheep (Ross et al., 1967). Calves can develop clinical fasciolosis due to heavy infections (Behm and Sanger, 1999) but in older cattle, the disease is often subclinical and results in suboptimal performance such as reduced weight gain, milk yield and fertility (Torgerson and Claxton, 1999).

The overall economic loss due to fasciolosis in the cattle industry was estimated as €52 million annually, equivalent to €299 per infected animal in Switzerland (Schweizer et al., 2005). The highest loss was due to reduced milk yield, which comprised 65% of the total loss, followed by extended calving interval (27%) and additional service due to reduced conception rate (6%). Losses associated with meat production, liver contamination, and treatments were minimal (2%) (Schweizer et al., 2005). In contrast, another economic assessment study in Germany estimated that annual losses due to *F. hepatica* infection to €566 per farm (Fanke et al., 2017). The predominant loss was attributed to increased number of artificial inseminations (€10.13 per cow), followed by prolonged calving interval (€9.40 per cow) and reduced milk yield in multiparous cows (€7.95 per cow) in this study.

The variations in cost estimates are not only due to different geographical locations and methodology, but also because of highly variable reports regarding production loss associated with *F. hepatica* infection. For example, with regards to milk yield, it is difficult to show the difference in milk production by experimental infection as it requires a large sample size due to high individual variations (Oakley et al., 1979). The loss of 10% was estimated using Monte Carlo estimation on results from the studies conducted in the 70's (Schweizer et al., 2005). A recent epidemiological survey based on BTM ELISA showed 15% decrease in annual average milk yield in high-yielding dairy herds in England, Wales and Scotland (Howell et al., 2015), while it was only 3% in the Flemish dairy herds (Charlier et al., 2007). The higher loss in UK farms was probably because the UK farms generally had high BTM ELISA levels, possibly indicating higher fluke burdens and consequently greater effect on the productivity in these cattle (Howell et al., 2015).

These recent studies were cross-sectional and do not confirm any causation. However, a randomized, blinded case-control trial was conducted in Belgium using closantel treatment during the dry period. This showed a 3.3% increase in milk yield in the subsequent lactation (approx. 1 kg/day increase) (Charlier et al., 2012), and demonstrated the causative effect of fasciolosis, and that this effect was reversible. Milk yield is known to be influenced by a number of infectious and other diseases. For example in Finland, reduction in 305 day milk yield associated with mastitis was estimated as 1.8-7.4% (1.0-2.5 kg/day) depending on parity and time of the disease (Rajala-Schultz et al., 1999b). Ketosis and lameness have also been shown to reduce the milk yield by 3.0-5.3 kg/day and 1.5-2.8 kg/day, respectively (Rajala-Schultz et al., 1999a), and a 4 kg/day temporary reduction in milk yield has been observed following a severe lungworm outbreak (Holzhauer et al.,

2011). Fasciolosis can cause substantial milk reduction losses without any clinical symptoms, probably due to its long-lasting effect on the liver.

In addition to the direct effect on productivity, *F. hepatica* is likely to cause indirect losses by modulating the host immune responses. Like other helminths, *F. hepatica* triggers a Th2 type response in the host (Brady et al., 1999; Graham-Brown et al., 2018), and it is speculated that *F. hepatica* suppresses pro-inflammatory cytokine productions and thereby increasing the susceptibility to intracellular organisms. Indeed, cattle experimentally infected with *F. hepatica* were more susceptible to *Salmonella dublin* (Aitken et al., 1979). A recent epidemiological study also found a possible association with zoonotic *Escherichia coli* 157 (Howell et al., 2018). Furthermore, the single intradermal comparative cervical tuberculin (SICCT) test response for bovine tuberculosis (BTB) was shown to be reduced by co-infection with *F. hepatica*. This had a tremendous impact on a BTB eradication programme in UK, where increase in incidences and spread of infection to new areas were seen despite a costly eradication program (£108.4 million in 2009) (Claridge et al., 2012).

The production loss estimate due to *F. heptaica* is important when determining if treatment is necessary. Vercruysse and Claerebout (2001) suggested three different thresholds for helminth treatment; a) therapeutic threshold, b) preventive threshold, and c) economic threshold. The therapeutic threshold refers to clinical disease that requires immediate treatment. This was suggested as infection with more than 1000 metacercariae, which rarely occurs for cattle under natural conditions. The preventive threshold is the threshold for preventive treatment for a group of animals to prevent over- or under-treatment. This depends on local climate and management styles, and for example determined by the climate forecasting systems for fasciolosis outbreak. The economic threshold was related to the production loss due to subclinical parasitism. As an economic threshold for fasciolosis, the authors suggested infection with 30 worms or higher, faecal egg counts (FEC) of five eggs per gram or higher, and a herd prevalence of 25% or higher as a starting point, based on the previous experimental studies. Charlier et al. (2008) evaluated these criteria in field conditions and suggested that 10 flukes is more appropriate at the individual level due to the pronounced damage seen in the liver with as few as 10 flukes. Additionally, they suggested that positive findings by coproscopy of four gram faeces by sedimentation-flotation technique were indicative of only heavy infections, and thus could be used to assess if treatment is necessary. In terms of economic threshold at the herd-level, within-herd prevalence of 25% seems to hold for dairy herds based on the recent epidemiological studies. Mezo et al. (2011) showed that milk

production was less by 1.5 kg/day per cow using BTM ELISA with a cut-off that refers to a within-herd prevalence of 25%. Similarly, Charlier et al. (2007) also found a significant reduction between milk production and antibody levels in BTM that equals a within-herd prevalence of 25%. Although BTM is representative for the milking herd only and not strictly the overall within-herd prevalence, significant milk production loss due to *F. hepatica* infection seems to occur when more than 25% of the milk herd is infected.

The relationship between fasciolosis and milk yield at individual and herd level under Danish conditions was investigated in Paper III.

2.3 Diagnosis of *Fasciola hepatica*

The diagnosis of fasciolosis can be challenging due to the parasite's long pre-patent period. Various methods are available with different properties and purposes. Here, the major diagnostic methods currently used are summarised (Table 1). Other methods that are under development (e.g. PCR for coprological examination) and/or used for human infections (e.g. ultrasonography) are outside the scope of this thesis.

2.3.1 Necropsy / liver inspection records

Inspection of the bovine livers for fasciolosis and subsequent condemnation at slaughter is mandatory in Europe according to Regulation (EC) No 854/2004. These recordings can be acquired with permission and used for epidemiological studies at national level (Olsen et al., 2015; Byrne et al., 2016). However, the sensitivity of abattoir inspection is reported to be approximately 65% when compared with a full dissection of the liver (Rapsch et al., 2006; Mazeri et al., 2016), therefore missing many infected livers. Furthermore, large variations in prevalence among abattoirs have been noted in Northern Ireland, possibly due to differences in performance among the abattoirs (Byrne et al., 2016). The specificity of abattoir inspection was shown to be 88% in Scotland, using the Hui & Water model with no gold standard test. The low specificity was due to fibrosis not corresponding to the presence of parasites (Mazeri et al., 2016). A detailed liver examination combined with searching for eggs in the gall bladder can give a much improved sensitivity and specificity (99% and 98% respectively) (Mazeri et al., 2016). In Denmark, the smaller, local abattoirs do not report liver condemnations, so the national database covers around 82% of the total cattle slaughtered (Olsen et al., 2015).

2.3.2 Faecal egg count (FEC) by sedimentation

Trematode eggs are heavier than most nematode eggs, and therefore the sedimentation method is appropriate for detection of eggs in the faeces (Henriksen, 1966; Roepstorff and Nansen, 1998). There are some variations in the method among different laboratories, but it generally requires only simple and cheap apparatus and can be conducted in veterinary clinics. However, egg counting by sedimentation is time-consuming and its sensitivity is low (Charlier et al., 2014). The sensitivity was 69% when examined once, although examination of three samples increased the sensitivity to 92% (Rapsch et al., 2006). Another study showed variable diagnostic sensitivities depending on seasons: 58% in autumn and 81% in summer (Mazeri et al., 2016). It is also known that there is large day to day variations in egg counts in cattle (Boray, 1969; Brockwell et al., 2013), and there may also be diurnal variations; egg output seems highest in the early afternoon (Boray, 1969). The specificity of FEC, however, has been shown to be high (98-99%) (Rapsch et al., 2006; Mazeri et al., 2016). Specificity of FEC is not perfect as the eggs of rumen flukes can be mistaken for the eggs of *F. hepatica* (Gordon et al., 2013; Mazeri et al., 2016). Another limitation of FEC is that it can detect only patent infections, and is useless in case of acute disease. Eggs may be retained in the gall bladder after elimination of the parasites, and therefore false positive results may occur (Flanagan et al., 2011). Finally, sedimentation requires a toxic chemical (malachite green or methylene blue) to stain the organic materials in the faeces to increase the contrast.

2.3.3 Blood biomarkers

The liver fluke infection alters blood composition, and haematological and biochemical analysis can be used for diagnosis. During the acute migratory phase, the liver damage results in a decline in plasma albumin concentrations in sheep (Scott et al., 2005; Matanović et al., 2007). In calves, hypoalbuminaemia is seen later during the infection (biliary phase), possibly due to their ability to eliminate and compensate for the infection during the migratory phase and often accompanied by hypergammaglobulinaemia (Ross et al., 1966; Anderson et al., 1977; Behm and Sanger, 1999). Eosinophilia is typical and occurs soon after infection and lasts also during the biliary phase (Ross et al., 1966; Zhang et al., 2005).

The damage to the liver by *F. hepatica* is measurable by an increase in a range of hepatic enzymes in serum. Glutamate dehydrogenase (GLDH, GDH) and aspartate transaminase (AST) are indicative of the damage to the hepatocytes and the elevated hepatic enzymes are useful in the diagnosis of subacute infections (Ross et al., 1966; Scott et al., 2005). Gamma-glutamyltransferase

(GGT) is present in the bile duct epithelium, and therefore the presence of this enzyme in the blood indicates damage and presence of flukes in the bile duct (Wyckoff and Bradley, 1985; Behm and Sanger, 1999). Elevation in GGT is therefore delayed following the increase in AST and GLDH (Yang et al., 1998). Elevated levels of GGT are seen in natural chronic infection in sheep and cattle (Matanović et al., 2007; Charlier et al., 2008). However, the elevation of GGT is not pathognomonic for *F. hepatica* infection and some authors found a decrease in GGT levels in the chronic stage of experimental *F. hepatica* infections (Ferre et al., 1996; Gonzalo-Orden et al., 2003). Similarly in sheep, not all naturally infected ewes showed elevated GGT (Mooney et al., 2009).

2.3.4 Antibody detection by ELISA (Ab-ELISA)

In contrast to the limitations of faecal egg counts, antibody detection by enzyme-linked immunosorbent assay (ELISA) can detect infection both with higher sensitivity and during the pre-patent period. High levels of antibodies in serum were observed as early as one to four weeks p.i. in experimental infections (Mezo et al., 2010b). The downside of this method, however, is that antibodies may persist post-infection and therefore, the method cannot differentiate current or post infection. Salimi-Bejestani et al. (2005a) noted that experimentally infected calves that were treated with triclabendazole had lasting antibody levels for at least 11 weeks post-treatment. Elevated antibody levels were also seen for at least 16 – 18 weeks post-treatment in both experimentally and naturally infected sheep that were treated with triclabendazole (Sánchez-Andrade et al., 2001; Mezo et al., 2007; Brockwell et al., 2013). Antibody detection is thus not suitable for immediate assessment of treatment success (Novobilsky et al., 2012).

Antibody detection in very young animals also requires caution due to colostral transfer of antibodies. Experimental infection of cows resulted in sero-conversion of their offspring following ingestion of colostrum. These calves were sero-negative again within 12 weeks of age i.e. not infected (Mezo et al., 2010b). Lambs of highly seropositive ewes were also moderately seropositive in June/July (up to 11 weeks old) but sero-negative 1.5 months later (Novobilský et al., 2014). Therefore serological diagnosis of fasciolosis should be avoided in animals up to two to three months of age.

There are several different standardized ELISAs for *F. hepatica* diagnosis with slightly different properties, sensitivities, and specificities (Rapsch et al., 2006; Charlier et al., 2008; Mezo et al., 2010a; Kuerpick et al., 2013b; Mazeri et al., 2016). An in-house ELISA based on the complete

adult *F. hepatica* excretory-secretory (ES) products was developed and still used at the University of Liverpool (LIV-ELISA). A standardized ELISA kit based on the complete ES products is also available commercially (Svanovir F. hepatica-Ab, Svanova, Uppsala, Sweden) (Charlier et al., 2014). These ELISAs showed sensitivity and specificity of 72 – 100% and 76 – 99%, respectively, when used on serum (Salimi-Bejestani et al., 2005a; Charlier et al., 2008; Kuerpick et al., 2013b; Mazeri et al., 2016). Cross reactivity with *Dicrocoelium dendriticum* and *Dictyocaulus viviparus* infections in experimental settings have been seen with ELISA using complete ES products (Cornelissen et al., 1999; Mezo et al., 2007). An abattoir study also found some possible false positive ELISA results in cattle infected with rumen flukes (Mazeri et al., 2016), lowering the specificity to 76 – 89%. Another ELISA using the "f2" fraction of ES products are available as a standardised kit from IDEXX (Fasciolosis Verification Test, IDEXX, Hoofddorp, the Netherlands, previously as Pourquier) (Charlier et al., 2014). The reported sensitivity and specificity of this ELISA on serum are 88 – 98% and 84 – 100%, respectively (Reichel, 2002; Molloy et al., 2005; Rapsch et al., 2006; Charlier et al., 2008). Cross reactivity with this ELISA kit has not been evaluated well, but one study found no evidence of cross-reaction with rumen flukes (Molloy et al., 2005). MM3 SERO ELISA (Bio K 211, Bio-X Diagnostics, Jemelle, Belgium) developed by (Mezo et al., 2007) uses the MM3 monoclonal antibody which recognise several *F. hepatica* cathepsins L1 and L2 in a sandwich-ELISA (Muiño et al., 2011). The reported sensitivity and specificity of this test on cow serum was 99% and 100% respectively (Mezo et al., 2010a). Lastly, there are assays based on recombinant cathepsin L1(rCL1). An rCL1 ELISA showed lower sensitivity during the pre-patency period in one study (Kuerpick et al., 2013b), but another recombinant mutant cathepsin L1 developed at the University College Dublin has allegedly sensitivity and specificity of 98% and has been used in some epidemiological studies (Sekiya et al., 2013; Selemetas et al., 2014; Bloemhoff et al., 2015).

An advantage of antibody detection by ELISA is the ability to operate with a range of body fluids such as serum, milk, bulk tank milk (BTM), and muscle transudate ("meat juice") (Charlier et al., 2014). BTM can be easily obtained in countries with routine milk monitoring programs and is a non-invasive, cheap and rapid way to identify infected herds. Strictly speaking BTM represents only the milking herd, but BTM ELISA has been the choice of many bovine fasciolosis studies for herd-oriented research aims, e.g. to assess the approximate herd-level prevalence of fasciolosis, to compare herd-level productivity in relation to fasciolosis, and to assess temporal and seasonal trend in herd-level prevalence (Salimi-Bejestani et al., 2005b; Charlier et al., 2007; Howell et al., 2015;

Munita et al., 2016). However, it is known that farms with low within-herd prevalence are missed using BTM ELISA for herd diagnosis. The detection limit for BTM ELISA varies greatly depending on the kits used. LIV-ELISA detects within-herd prevalence of 25% at a cut-off of 27 per cent positive value (PP) (Salimi-Bejestani et al., 2005b). For IDEXX-ELISA, minimum within-herd prevalence of 60% was first reported to produce positive on BTM (Reichel et al., 2005). But a more recent study using a lower cut-off indicated this was as low as 20% (Duscher et al., 2011). IDEXX's own validation study also indicated this to be low and their current recommended interpretation of BTM is: no or very low prevalence if negative, < 20% prevalence if low positive, 20 – 50% prevalence if moderately positive, and > 50% prevalence if strongly positive (IDEXX Laboratories Inc., 2013). MM3 SERO ELISA detects herds with only 12% infected cows (Mezo et al., 2010a), while rCL1 from Dublin detects within-herd prevalences of approximately 30% with a cut off of 15 PP (Selemetas et al., 2014). We have also investigated the use of IDEXX ELISA on BTM and its relation to within herd prevalence in Paper I, II and III of this Ph.D. thesis.

2.3.5 Antigen detection by ELISA (Ag-ELISA)

A method to detect antigen in serum was developed for human fasciolosis in Cuba (Espino et al., 1990), but the use is still limited in animals. Detection of antigen in faeces, on the other hand, has the advantage that the faecal sampling is less invasive and easier than blood sampling. A commercial ELISA kit using the MM3 monoclonal antibody to capture *F. hepatica* cathepsins in sheep and cattle faeces is available (BIO K 201, Bio-X Diagnostics, Jemelle, Belgium) (Mezo et al., 2004). This ELISA detected infection from five to six weeks p.i. in experimentally infected sheep (Mezo et al., 2004; Flanagan et al., 2011), corresponding to the presence of flukes near the bile ducts during the late migratory phase. It can detect infection with as low as one to two flukes, although false-negatives are seen if the flukes are immature (Mezo et al., 2004). Despite the high sensitivity and specificity reported from experimental infections, field investigations showed varying sensitivities for cattle faeces: 77 – 94% (Charlier et al., 2008; Palmer et al., 2014; Mazeri et al., 2016) . The specificities in these studies, however, were high (93 – 99%). The various sensitivities are partially due to different cut-offs used in different laboratories. No cross reactions with other parasite infections, such as rumen flukes and *D. dendriticum,* have been shown so far using this ELISA (Mezo et al., 2004; Kajugu et al., 2015; Mazeri et al., 2016).

In contrast to the antibody detection that is unable to differentiate current and past infections, antigen detection is a useful indicator of the presence of the parasite (Mezo et al., 2004; Rojas et al.,

2014). Coproantigen was absent from one to three weeks post treatment (Mezo et al., 2004; Flanagan et al., 2011; Brockwell et al., 2013), and therefore Ag-ELISA is considered ideal for detection of anthelmintic resistance. Detection of anthelmintic resistance usually relies on the standardised faecal egg count reduction test (FECRT), but FEC is unsuitable due to its low sensitivity, and therefore Ag-ELISA reduction test is suggested as an alternative (Flanagan et al., 2011; Gordon et al., 2013). Ag-ELISA test is also thought to be highly correlated with the parasite burden (Mezo et al., 2004; Charlier et al., 2008; Brockwell et al., 2013). However, this is still debatable, as some studies were unable to show the expected correlations with fluke burdens (Valero et al., 2009; Martínez-Sernández et al., 2016).

Table 1. Summary of different diagnostic methods for bovine fasciolosis.

Method	Diagnostic stage	Strengths (S) Limitations (L)	Sensitivity (Se) and specificity (Sp)	References
Abattoir inspection	Hepatic stage	S: National wide survey is possible L: Variations between abattoirs, only on dead animals, low burdens may be missed	Se: 63 – 68% Sp: 88%	Rapsch et al. 2006 Mazeri et al. 2016
Detailed necropsy	Hepatic stage	S: High sensitivity and specificity L: Time consuming, only on dead animals	Se: 93 – 99% Sp: 98%	Rapsch et al. 2006 Mazeri et al. 2016
Sedimentation	Patent stage (> 8 – 10 w.p.i.)	S: Cheap, no advanced equipment needed L: Time consuming, no correlation to parasite burden, dyes used to stain the samples are toxic	(5 or 10g faeces) Se: 58 – 69% Sp: 93 – 99%	Rapsch et al. 2006 Charlier et al. 2008 Mazeri et al. 2016
Blood biomarkers	Pre-patent and patent stage	S: May detect subacute phase of infection L: Not pathognomonic/specific	Not applicable	
Ab-ELISA (serum)	Pre-patent stage (2 – 4 w.p.i) and later	S: Detect exposure in early stage of infection L: Possible cross-reactivity, poor correlation to parasite burden, antibodies persist after elimination of parasites (treatment), colostral Ab up to 2 – 3 months old	Depends on kits and seasons	Raichel 2002 Salimi-Bejestani et al. 2005a Molloy et al. 2005 Rapsch et al. 2006 Charlier et al. 2008 Mezo et al. 2010a Kuerpick et al. 2013b Selemetas et al. 2017
Ab-ELISA (milk, bulk tank milk)	Pre-patent stage (2 – 4 w.p.i) and later	S: Non-invasive and easy to sample with milk control scheme, herd-diagnosis by use of BTM L: Same as above, only for lactating herd, minimum within-herd prevalence required to be positive	Depends on kits	Reichel et al. 2005 Salimi-Bejestani et al. 2005b Mezo et al. 2010a
Ag-ELISA (faeces)	Pre-patent stage (4 – 5 w.p.i) and later	S: Indicate presence of the parasite, no cross-reactivity reported so far L: Varying sensitivity and specificity from field studies (different cut-offs used in various laboratories), unknown correlation with parasite burden	Se: 77 – 94% Sp: 93 – 99%	Charlier et al. 2008 Palmer et al. 2014 Mazeri et al. 2016

2.4 Epidemiology of *Fasciola hepatica*

2.4.1 Prevalence of bovine fasciolosis in Denmark

The national-level abattoir record of approx. 1.2 million cattle slaughtered during 1969 to 1971 showed a prevalence of 16.4 – 16.6% in Denmark. The highest prevalence of about 19% was found from Jutland, followed by Zealand (9-10%), Funen (8-11%), and Bornholm and other islands (3 – 4%) (Riising et al., 1973). Henriksen and Pilegaard-Andersen (1979) then described a dramatic decrease in bovine fasciolosis during 1973 – 1977 by reduced numbers of positive faecal samples analysed at the National veterinary laboratory; from > 30% in 1963 to < 10% in 1973 – 1977. The likely causes for this decline according to the authors were intensive research and dissemination activities during early 70's resulting in effective control, and some consecutive dry summers during the mid-70's. The prevalence of bovine fasciolosis based on abattoir recordings of 1.4 million cattle from 2000-2003 showed 2.8% and 0.7% for beef and dairy cattle, respectively (Ersbøll et al., 2006). After a decade, the overall prevalence of bovine fasciolosis had increased to 3.2% – 3.9% in 2011 – 2013. The herd level prevalence increased from 25.6 to 29.3% during 2011 – 2013. The prevalence over the entire three year period was higher in dairy than non-dairy farms (58.0% and 35.22%, respectively) and in organic compared to conventional farms (53.5% and 40.4%, respectively), although differences were not statistically significant on its own (Olsen et al., 2015).

This apparent increase in prevalence in the new millennium in Denmark is in accordance with observations throughout NW-Europe (Pritchard et al., 2005; McCann et al., 2010b; Novobilsky et al., 2015) and could be due to several reasons. The overall global land and ocean surface temperatures of the earth have increased by 0.87 ºC in 2006 – 2015 compared to 1850 – 1900 period (IPCC, 2018). Although the overall global climate change may not be directly translated to the microclimate of certain regions, it could have led to the observed higher transmission rates. Additionally, a series of political decisions in Denmark during 1980 – 2000 could have played a role. For example, "water environmental plan I – III" (1987, 1998, and 2004) were governmental initiatives aimed to secure the aquatic environment from nitrogen and phosphorus leaching. As wet meadows can naturally retain these substances, restoration of wet meadows were initiated (Ministry of Environment and Food of Denmark, 2004). A marginal land scheme (miljøvenlige jordbrugsforanstaltninger (MVJ)) was also initiated to protect the natural areas such as meadows and heathlands by providing economic incentives for the farmers to let their animals graze on these

marginal lands. Overall, these changes may have increased the risk of transmission of fasciolosis, e.g. documented by higher densities of *G. truncatula* in paddocks in the MVJ scheme in marshlands in Tønder, Denmark (Thamsborg et al., 2007). Furthermore, in order to stop the development of anthelmintic resistance, law changes were introduced from 1999 to prohibit preventive treatment without diagnosis and to classify anthelmintics as prescription drugs in Denmark ("Lov nr 1043 af 23/12 1998 om ændring af lov om lægemidler" [Law no. 1043 of 23/12 1998 on the changes of law about medicine]). This may have reduced the use of anthelmintics and consequently resulted in higher prevalence.

The spatial distribution of bovine fasciolosis, however, does not seem to have been altered in the last decade; the highest risks were observed in northern and western Jutland, followed by Zealand and Funen (Ersbøll et al., 2006; Olsen et al., 2015). This most likely reflects the herd densities, but also meteorological and local environmental conditions (Olsen et al., 2015).

2.4.2 *Galba truncatula* as the intermediate host snail

Similar to other vector borne diseases, *F. hepatica* transmission patterns are highly dependent on the population dynamics of the intermediate host species (Mas-Coma et al., 2009). The mud snail, *G. truncatula*, is considered the quantitatively most important intermediate host for *F. hepatica* in Europe (Kendall, 1950; Boray, 1969; Bargues and Mas-Coma, 2005; Novobilsky et al., 2013). However, other European Lymnaeid snails such as *Omphiscola glabra* (Dreyfuss et al., 2003; Dreyfuss et al., 2007), *Lymnaea palustris* (Novobilsky et al., 2013), *L. fuscus* (Novobilsky et al., 2013) and *Radix peregra* (synonym *R. balthica*) (Caron et al., 2007; Relf et al., 2009; Caron et al., 2014; Jones et al., 2015) have also been suggested as potential intermediate hosts of *F. hepatica*.

Galba truncatula is distributed in most European countries including the Faroe islands (Lützen and Bovien, 1934). The snail is highly versatile to different altitudes, and may be found from the coastal zones up to 2600 m altitude in Europe. However, *F. hepatica* infection is typical of lowlands in Europe, due to the temperature requirement of 10 ^{o}C for larval development (Mas-Coma et al., 2001). There is no systematic study to investigate the distribution of *G. truncatula* in Denmark, but it is expected to be ubiquitous all over the country because Denmark is a low-lying country (< 200 m altitude). The snail thrives best in areas that are not excessively wet or dry (i.e. shallow pools and wetlands), and prefers habitats with stagnant waters or shallow watercourses, where the aquatic pH is greater than 5 and calcium ions are above 5 mg / l (Moens, 1991; Dreyfuss et al., 2015). The sunlight is required for the food growth (unicellular algae), and as such temporary puddles in the

soil without herbaceous vegetation and turf (e.g. cattle trampling and passage of heavy machineries) is an ideal habitat for *G. truncatula*. On the contrary, it is unlikely to find *G. truncatula* in salty or acidic water like bogs.

2.4.3 Cattle as the definitive host

Cattle are generally believed to be more resistant to *F. hepatica* infection than sheep, as cattle show some acquired resistance against experimental infections, while sheep do not (Ross, 1965; Ross et al., 1967; Dow et al., 1968; Boray, 1969). The life span of *F. hepatica* may be as long as 11 years in sheep (Pantelouris, 1965), although in cattle 75% of the parasites are eliminated within 21 months post infection (Ross, 1968). The mechanism of resistance in cattle compared to sheep is still unclear, but cattle can form mechanical barriers by developing hepatic fibrosis and bile duct calcification and thickening to prevent further adult worm establishment (Ross et al., 1966; Dow et al., 1968; Boray, 1969). Boray (1969) suggested that clinical disease may be more pronounced in young cattle and resistance develops with age. However, there are reports showing that cattle with chronic natural infection remain as susceptible (Clery et al., 1996) or become susceptible again one year after spontaneous recovery or anthelmintic treatment (Boray, 1969). Epidemiological surveys showed increased prevalence with age (Henriksen and Pilegaard-Andersen, 1979; Gonzalez-Lanza et al., 1989; Innocent et al., 2017), suggesting that cattle is unlikely to produce complete protective immunity against *F. hepatica* (Graham-Brown et al., 2018).

Genetic differences between and within breeds in terms of susceptibility to *F. hepatica* are not well studied in cattle, although several studies in sheep have documented strong genetic components (Boyce et al., 1987; Pleasance et al., 2011). One study showed Jersey calves had more severe symptoms than Hereford calves, although no difference was seen on fluke burden and egg counts (Boray, 1969). In the largest cattle abattoir in Scotland, the lowest prevalence of condemned livers due to fasciolosis was observed in Holstein Friesian (16.4%), while the prevalence was above 25% in other breeds (Mazeri et al., 2017). However, this breed difference could be related to management; prevalence is generally higher in beef than in dairy cattle, probably because beef cattle graze on marginal lands and have extended grazing period. Little genetic resilience to infection in terms of productivity in infected cattle was found in Irish dairy cattle (Twomey et al., 2018), while negative genetic correlation between *F. hepatica* infection and milk yield and protein content were seen in Black and White cattle populations in Germany (May et al., 2017).

2.4.4. Seasonality of infection

Transmission of *F. hepatica* usually follows a seasonal pattern, reflecting the intermediate host vector population dynamics consistent with the local climate. Globally, there are three main types of transmission patterns; year-long transmission (Southern Europe, Mediterranean islands, Cambodia, Northern Bolivian Altiplano etc.), mono-seasonal transmission (south central Asia, extreme latitude areas etc.), and bi-seasonal transmission (Europe, USA, Australia etc.) (Mas-Coma et al., 2018).

Fasciolosis in Denmark follows the bi-seasonal pattern: low transmission in spring and high transmission in autumn. Infection of the intermediate host is termed "summer infection" and "winter infection" in snails according to when the snails are infected with the parasite (Ollerenshaw, 1959; Novobilský et al., 2014). The pasture is contaminated with *F. hepatica* eggs from infected cattle after turn-out in spring. These eggs hatch around 1st of June and infect the snails. The parasites develop in the snail in approximately 60 days and the metacercariae are then deposited on the field from approximately 1st of August (Ollerenshaw and Rowlands, 1959; Riising, 1971; Nielsen et al., 1973). Infections in the definitive hosts acquired from these metacercariae deposited after 1st of august, are therefore due to the so-called "summer infection" in snails. Summer infection results in the definitive hosts showing signs of infection in late summer to autumn. Any overwintered eggs can contribute to this "summer infection" in snails as they constitute an important source of snail infection in spring (Shaka and Nansen, 1979). On the other hand, infections acquired by cattle in the spring are due to either overwintered infected snails or metacercariae. The metacercarial deposition from the overwintered snails (infected in autumn the year before and thus termed "winter infection" in snails) is considered the main cause of early season infection in cattle (Ollerenshaw, 1959; Shaka and Nansen, 1979), because the overwintered metacercariae can lose infectivity quickly during the early part of the grazing season (Shaka, 1975). Although "winter infection" in snails is appreciable in some years, this infection has been considered to play a minor role in the Danish climate compared to "summer infection" (Nielsen et al., 1973; Shaka and Nansen, 1979). However, considering the dependency of the parasite to climate conditions, this pattern of infection could be altered, e.g. due to recent changes in the global climate (Mas-Coma et al., 2009).

2.4.5 Risk factors

Considering the sensitivity of the intermediate host snails to abiotic factors, spatial distribution of *F. hepatica* is expected to reflect the suitable snail habitats defined by the climate and soil factors.

Rainfall, for example, has long been considered as an important factor for *F. hepatica* when temperatures are above 10 ^{o}C and is used for risk calculations in Ireland (Ollerenshaw and Rowlands, 1959). A simpler "Stormont wet day" forecasting system (Ross, 1970) and McIlory computer system has also been developed using the weather data (McIlroy et al., 1990), and forecasting is still available in the UK (Graham-Brown, 2018). Investigations on spatial distribution of *F. hepatica* showed some conflicting results regarding rainfall; higher rainfall was a strong predictor for UK (McCann et al., 2010a; Howell et al., 2018), while rainfall was negatively associated with *F. hepatica* exposure in Belgium. The authors of the latter study suggested that high rainfall "wash away" metacercariae (Bennema et al., 2011). Moreover, rainfall showed only weak associations with *F. hepatica* prevalence in Germany and Sweden (Kuerpick et al., 2013a; Novobilský et al., 2015). The conflicting results regarding rainfall is likely due to different methods used, and inclusion of different factors such as environmental (soil pH and slopes etc.) and herd management factors in these studies. Management factors are shown to influence the spatial distribution of *F. hepatica* (Bennema et al., 2011), and therefore direct comparison of risk factor analyses between countries and regions may be inappropriate and the results are probably not transferrable to other areas (Charlier et al., 2014).

The forecasting systems and spatial distribution maps are still at regional level and local conditions in finer scale is still not taken into account (De Waal et al., 2007; Charlier et al., 2014). It is known that even in the same regions, *F. hepatica* positive and negative herds can exist next to each other (McCann et al., 2010b). *Galba truncatula* prefer to colonise the periphery of a hydrographical network (Dreyfuss et al., 2015), and the presence/absence of suitable snail habitats may differ in these neighbouring farms. By including the number of potential habitats and presence of snails in farms, a prediction model was able to explain 85% of variation in *F. hepatica* distribution between farms in Belgium (Charlier et al., 2011). This suggests that farm-level factors are important in transmission of the parasite and the individual farm conditions need to be considered when developing on-farm control strategies.

2.5 Control of *Fasciola hepatica*

Eradication of bovine fasciolosis is probably an unrealistic option and a control program should therefore aim to reduce the disease and its economic impact. The control methods to be implemented will depend largely on local climatic conditions, local regulations, animal husbandry customs, and the motivation and commitment of the livestock owners (Torgerson and Claxton,

1999). Additionally, the control methods should not rely heavily on anthelmintics due to both cost and the potential for development of anthelmintic resistance following extensive use. Since the first report of triclabendazole resistance (TCBZ-R) in 1995 (Overend and Bowen, 1995), TCBZ-R is a well-documented phenomenon across the world, including in EU countries such as the Netherlands, Ireland, UK and Spain (Moll et al., 2000; Alvarez-Sanchez et al., 2006; Mooney et al., 2009; Gordon et al., 2012; Hanna et al., 2015; Kelley et al., 2016). However, caution is merited in that some of these reports may not reflect true anthelmintic resistance if the studies were based on only FECRT, or if the possibility of under-dosing and quality of anthelmintics were not properly assessed (Hanna et al., 2015). Using Ag-ELISA reduction test, albendazole and closantel resistance have also been reported in Sweden, emphasizing consequences of over-reliance on anthelmintics in parasite control (Novobilsky et al., 2012; Novobilský and Höglund, 2015; Novobilsky et al., 2016).

Despite these problems with anthelmintic resistance, treatment of cattle and sheep with anthelmintics is still the main method for control of *F. hepatica*. There is a range of anthelmintics available with different efficacy and safety. The list of anthelmintics currently available in Denmark for use in livestock is listed in Table 2. So far, anthelmintic efficacy and resistance in Danish *F. hepatica* populations have never been thoroughly investigated.

The use of vaccines and genetically resistant animals may also potentially limit the pasture contamination and therefore reduce the fluke transmission. However, commercial vaccines are not yet available (Spithill et al., 1999; Molina-Hernández et al., 2015) and analysis of potential cattle breeding lines is still in infancy. Other options for non-medicinal control of *F. hepatica* are also still limited. Boray (1969) suggested a pasture rotation system to combat infection on a farm in Australia. This involved treatment of animals before relocation to potentially contaminated pasture and alternate grazing between fluke-free and contaminated pasture. For example, maximum control is obtained if animals are first grazed on fluke-free areas in spring for approximately four months, treated at the end of a four months period and then moved to fluke-infected pasture two weeks post-treatment. Then the animals graze for two months on the contaminated pasture, and are moved back to fluke-free pastures before egg contamination occurs. Animals are re-treated approximately four months later, again, before being moved back to the contaminated pasture. The same author, however, admitted that this recommendation was never adopted in Australia due to difficulty in pasture rotation and costs involved in setting up fences (Boray, 1999). Other pitfalls of this program are that it requires presence of low-risk pastures, and that overwintering eggs have not been taken into account. Although fluke-infected pasture may have a small number of metacercariae if they

were not grazed during spring, it has been shown that eggs can overwinter and give rise to summer infection in snails (Shaka, 1975). Thus, animals can still be highly infected even when grazed only during autumn on contaminated pasture (i.e. pastures grazed during the previous year). In theory, the pasture is fluke-free if no contamination occurred for two years (Shaka and Nansen, 1979), and therefore a three-year rotational system remove most of metacercariae on pasture, although this has not yet been assessed (Prof. Knubben-Schweizer, personal communication, August 03, 2018). However, the potential contamination by local wild animals (e.g. infected deer and hares) also needs to be considered (Walker et al., 2011; Albery et al., 2018).

Fencing off wet areas is often recommended, although the cost-effectiveness of this is unknown (Roberts and Suhardono, 1996) and maybe limited in years of high rainfall and massive propagation of snails in temporary puddles or ponds (Knubben-Schweizer and Torgerson, 2015). Alternative control options such as use of competitive snail species (Rondelaud et al., 2006) or ducks to control snail populations (Hull, 2017), and use of certain plants as molluscicide (Hammond et al., 1994) have been suggested previously, but none of them are widely applied due to logistical reasons. More research describing and assessing the effects of alternative parasite control strategies without use of anthelmintics, e.g. grazing management and biological control, is needed (Thamsborg and Roepstorff, 2003).

Table 2. Summary of anthelmintics available in Denmark with a claimed efficacy against *Fasciola hepatica* in cattle

Substance (Product) **Form**	Indication	Withdrawal period	**Comments**
Albendazole (Valbazen®) **Oral**	Only adult worm	Milk: 4 days Meat: 30 days	Increased dose (10 mg/kg) as compared to nematodes (7.5 mg/kg)
Triclabendazole (Tribex 10%®) **Oral**	Immature larvae (>2 weeks) and adult worm	Milk: not allowed Pregnancy: 41 days before calving (and 84 hours after calving) Meat: 56 days	Requires dispensation – not marketed in DK
(Fasinex® 240, 24%) **Oral**	Same as above	Milk: not allowed Pregnancy: 48 days before calving (and 48 hours after calving) Meat: 52 days	Requires dispensation – not marketed in DK
Closantel (Closamectin® pour-on) **Pour-on**	Immature larvae (>7 weeks) and adult worm	Milk: not allowed for dairy cows, but can be used in the first half of pregnancy in heifers. Meat: 28 days	Primarily for beef cattle. No access to aquatic environment in 14 days after treatment. All animals in a flock should be treated.
Closantel (Santiola Vet®) **S/C Inj.**	Only adult worm	Milk: not allowed for dairy cows and heifers in the last trimester of pregnancy. Meat: 77 days	Primarily for beef cattle. No access to aquatic environment minimum 2 days after treatment. Minimum 11 week break before the second treatment.
Clorsulon (Bimectin plus®) **S/C Inj**	Only adult worm	Milk: not allowed Pregnancy: 60 days before calving Meat: 66 days	No access to aquatic environment in 14 days after treatment.
Oxyclozanide (Distocur®) **Oral**	Only adult worm	Milk: 4,5 days Pregnancy: OK, but needs caution in late pregnancy and when animals are under stress Meat: 13 days	Not for animals on grass (animals should be in stall for min 5 days after treatment).

3. Summary of materials and methods

In this chapter, the relevant data sources, materials and methods (field, laboratory, and statistical) as used in this thesis are presented. An overview of the studies used within this thesis with reference to specific objectives and attached manuscripts is given in Table 3.

3.1 Register data (slaughter data, milk recordings, treatment data)

The Danish Cattle Database (DCD) is managed by SEGES (Danish Agricultural Advisory Service run by the Danish Agriculture and Food Council) and is a large collection of datasets containing a variety of information related to individual cattle and cattle herds. All cattle in Denmark are required to have two ear tags (at least one has to be electronic) and be registered in the Central Husbandry Register (CHR). The CHR is managed by the Ministry of Food, Agriculture and Fisheries (The Danish Veterinary and Food Administration (DVFA), 2018), but the information is shared between DCD and CHR as they run on the shared database. The input to DCD comes from various sources e.g. farmers, veterinarians, slaughter houses, and laboratories, and therefore information such as breed, age at slaughter, type of herd, milking system, milk quality, movements of cattle, disease diagnosis and slaughter date are also available.

The examination of the bovine liver at slaughter is mandatory in EU according to EU meat inspection Regulation (EC) No 854/2004, annex 1, Section IV, chapter 1 A & B). The findings of liver diseases can be entered dichotomously in different categories e.g. liver flukes (or sequelae to liver flukes), acute hepatitis, liver abscess, and chronic hepatitis. All recordings of the meat inspection are transferred to the DCD. The abattoir dataset contains approximately 80% of all registered herds (Olsen et al., 2015).

The Danish milk producers are required to follow milk control scheme laid by DVFA (The Danish Veterinary and Food Administration (DVFA), 2006) in accordance with EU regulation on hygiene of food stuff ((EC) No 853/2004, annex III, Section IX, Chapter I, Part III). This enforces that the bulk tank milk is assessed for somatic cell counts, bacterial counts, and antibiotic residues at least once a week. In addition, the milk producers can register for a voluntary systematic milk recording program, which is organised by the Foundation for Registration and Milk Recordings (RYK). Milk recordings of approx. 90% of all Danish dairy cows are recorded through this voluntary program and the data is available in DCD (Nielsen, 2010). Milk yield expressed as 305 day energy corrected

milk yield (305d ECM), following the guidelines by the International Committee for Animal Recording (ICAR), was used to determine the effect of *F. hepatica* infection on milk production in this thesis.

The Danish farmers are allowed to treat animals only with prescription drugs. The treatments need to be registered in the electronic animal registry (The Danish Veterinary and Food Administration (DVFA), 2017) and this data is also available in DCD.

SEGES extracted the necessary data from DCD and kindly provided them for use in this Ph.D.

3.2 Questionnaire data collection

There were two questionnaire surveys conducted for this thesis. The first questionnaire was developed to collect information about management factors (e.g. grazing management and purchase of animals), as well as control and treatment methods used against fasciolosis. This questionnaire survey was conducted by telephone interview in 2014 for a total of 194 cattle farms. More detailed information can be found in Paper I.

The second questionnaire was developed and conducted specifically for organic dairy farms, in order to reduce problems associated with farms where no animals were grazed. Grazing is mandatory for most age groups for the Danish organic farms following the EU regulation on organic production (Council Regulation (EC) No. 834/2007 of 28 June 2007 on organic production and labelling of organic products and repealing Regulation (EEC) No. 2092/91). Therefore, we aimed to have a more homogenous study population in terms of fasciolosis risk for the second risk factor analysis. The questionnaire also asked for farmers' opinions regarding anthelmintic resistance, which was part of a European project aimed for tackling the parasitological challenges in organic ruminant farms. Additionally, the predicted probabilities for risk of fasciolosis based on the environmental and slaughter data (Olsen et al., 2015) for each farm were included in the risk factor analysis as approximation for environmental factors. This questionnaire survey was carried out also by telephone in 2016 for a total of 218 farms. Detailed information about this second questionnaire is described in Paper II.

3.3 Milk collection

In conjunction with the two questionnaires, bulk tank milk (BTM) samples from the participating farms were collected in 2014 and 2016. The BTM samples were collected as part of milk control

scheme and gathered at Eurofins Steins Laboratorium A/S for routine screening for somatic cell counts etc. The left over BTM was frozen and then shipped to our laboratory for our study in collaboration with SEGES and Eurofins. The milk was centrifuged at 1000 g for 20 minutes and the whey was frozen at -20 ^{o}C until analysis by the IDEXX-ELISA. One aliquot of BTM samples taken in 2016 were air-shipped to the University of Liverpool and were analysed by LIV-ELISA for comparison (Paper III).

The individual milk samples as well as BTM from selected organic farms were also shipped to our laboratory from Eurofins in 2017. This was to study the relationship of anti-*F. hepatica* antibodies in BTM and within-herd prevalence (Paper III). The details are described in Paper III, but the selection of the farms was based on the level of antibodies in BTM in 2016 (negative, low, medium, high), and they had to be part of the voluntary milk recording program. Additionally, only milk producers delivering to the dairy company Arla Foods were selected, because the frequency of BTM delivery was high in these farms, allowing the delivery of individual milk samples and BTM within a few days. A total of seven individual milk samples per farm were selected randomly by Eurofins, and farms with less than four individual milk samples were removed from the dataset.

Finally, monthly BTM from the four farms included in the longitudinal study were also forwarded regularly from Eurofins (every six months or so) (refer to paper II). All milk samples were treated as mentioned above and analysed by IDEXX-ELISA.

3.4 On-farm data collection

Four dairy farms were selected to gather data and in-depth knowledge regarding on-farm fasciolosis transmission using different diagnostic methods. The details are described in Paper II, but briefly, the farms were selected based on high BTM antibody levels and high rates of liver condemnation between 2011 – 2013 (based on information obtained from the 2014 questionnaire survey). The farms were visited seven times during 2015 – 2017 and faecal and blood samples were collected from four age cohorts. Predominantly the same animals were sampled each time, but if the desired animals were no longer available then a replacement animal with similar age was added to the cohort.

The necessary information was collected from the farmers at the time of sampling and on the telephone when necessary. Two meetings were organised with the farmers, their veterinarians and

agricultural advisors to inform about the results and suggestions for control against fasciolosis (winter 2016 and spring 2017).

The farms were also visited on three separate occasions to look for the presence of snails in collaboration with Drs. Anna-Sofie Stensgaard and Mita Sengupta. The farmers provided the necessary maps and described the areas and the use for these areas. Due to limited resources, the snail search was performed non-systematically and based on the likely snail habitats seen on the map described by the farmers.

3.5 Laboratory analysis

3.5.1 Faecal egg counts

Five grams of faecal samples were analysed by sedimentation technique to count the number of trematode eggs. The burden of gastrointestinal nematodes and lungworms were also assessed by modified concentration McMaster technique and Baermann technique simultaneously. The protocols for these methods are taken from Roepstorff and Nansen (1998).

3.5.2 ELISA

The collected serum and milk samples were analysed for detection of antibodies by a commercial ELISA kit (IDEXX *Fasciola* verification test, IDEXX Laboratories, Hoofddorp, the Netherlands) following the manufacturer's instructions. The results of this ELISA test are expressed as the sample to positive percentage (S/P%). This was calculated as the ratio of average net extinction (NE) of the sample and average NE of two positive controls, where NE refers to the difference between the optical densities (OD) measured in the antigen negative and antigen coated wells. The cut-off of 30 S/P% was used in accordance with the manufacturer's instructions. The samples were analysed in duplicate, and the sample was re-analysed if the duplicates differed in OD with > 0.2. Inter-plate variability was also assessed by calculating the average net extinction (OD of positive well – OD of negative well) of positive controls of all plates. The whole plate was re-analysed if the difference in positive control net extinction was > 0.2 from the average net extinction.

The milk sent to the University of Liverpool was analysed by their in-house ELISA (LIV-ELISA) according to Salimi-Bejestani et al. (2005b) in collaboration with Prof. Diana Williams. The result of this ELISA is expressed as per cent positive value (PP), calculated as the ratio of the mean OD of duplicate samples and the mean of the positive control. The cut-off was set at 27 PP.

Two gram of faeces (stored frozen) were analysed for detection of *F. hepatica* coproantigen by a commercial ELISA kit (Bio K210, Bio-X Diagnostics, Rochefort, Belgium). The recommended procedure was optimised by overnight incubation and prolongation of incubation time with tetramethylbenzidine (TMB) from 10 minutes to 20 minutes. The result calculation of this ELISA kit is similar to that of IDEXX Ab-ELISA. The result is expressed as the ratio of the difference between the OD of antigen positive and negative wells of the sample to that of the positive control. The cut-off value was set as 1.89, calculated as the mean value of all negative control (negative faeces from one to three months old calves from a conventional dairy farm) plus three-fold standard deviation of the mean. The samples were analysed in duplicate and the quality of the results was assessed as described above.

3.5.3 Snail PCR

The snails collected from October 2017 were stored in ethanol and analysed by PCR to confirm the identification of snail species based on morphology as *G. truncatula* (Mandahl-Barth, 1949; Macan, 1977) and to check for fasciolosis infection status at the University of Liverpool. Briefly, after extraction of DNA from the entire snail using Chelex® method (Caron et al., 2014) and three PCR reactions were conducted for each snail: snail internal transcribed spacer 2 (snail ITS-2 PCR), *F. hepatica* ITS-2 (F hep ITS-2 PCR), and *F. hepatica* cytochrome c oxidase subunit 1(F hep COX-1 PCR). The detailed methods are described in Paper II.

3.6 Statistical analyses

Most statistical analyses were performed using Microsoft Excel (2010) and R (R Core Team, 2017). A range of statistical methods were used and but the most relevant of these are explained briefly below.

For the first risk factor analysis (Paper I), two logistic regression models were built using two different farm classifications as response variable: case and control based on slaughter findings, and BTM ELISA results. A range of management factors from questionnaires were included as potential risk factors, and the final model was selected by stepwise selection based on Akaike Information Criteria (AIC).

Logistic regression was also used for the second risk factor analysis (Paper III). In addition to the questionnaire responses from the farmers on grazing factors, the probability of a particular farm

being positive for fasciolosis (the output from a predictive model by Olsen et al. 2015) was included as an approximation for environmental variation between farms.

For the longitudinal dataset, a generalised additive mixed model (GAMM) was applied. Generalised additive models (GAM) are semi-parametric regression models that allow for a non-linear relationship with unknown functional form between the response and explanatory variables. Using these models, the data determines the shape of the relationship between explanatory and response variables, which makes them particularly suitable for data exploration and analysis of complex temporal patterns (Hastie and Tibshirani, 1986; Yee and Mitchell, 1991; Hemmi et al., 2011). We examined the patterns of diagnostic test results (Ab-ELISA, Ag- ELISA and FEC) in terms of age, season (day of the year), and longer-term temporal trends. As individual animals were repeatedly sampled, GAMM was applied with inclusion of individual animal ID as a random effect to account for within-subject variability. The four farms and three different diagnostic methods were modelled separately (a total of 12 models).

To determine the relationship between antibody levels in BTM ELISA and within-herd prevalence, a mixed effects logistic regression was used (Paper III). The dichotomised outcome of the individual milk samples (positive or negative) was the response variable, farm was used as a random effect, and fixed covariates were breed, and parity (linear and quadratic terms).

To determine the effect of fasciolosis on milk production, average 305d ECM and BTM ELISA values, as well as individual 305d ECM and individual milk Ab-ELISA results were analysed using a generalised linear model and generalised linear mixed model, respectively. The Ab-ELISA results were made into two variables, one with dichotomised results (cut-off at 30 S/P%) and the other reflecting the degree of positivity conditional on the sample being positive. Breed and parity (linear and quadratic terms) were included as covariates, and the interaction between parity and Ab-ELISA results was also assessed. The same analyses were conducted using the results of LIV-ELISA for comparison. Farm demography / management factors were also included as covariates to reduce farm-to-farm variations as much as possible.

Table 3. Overview of objectives, study designs, materials and methods used in this thesis

Objective	Study (Paper no.)	External data source	On-farm data source
1. Identification of risk factors for fasciolosis in Danish dairy farms	Case-control study (Paper I)	Meat inspection data from 2013 to define case and control farms Questionnaire data collected by telephone interviews in 2014 (131 case, 63 control herds).	Ab-ELISA on BTM from 2014
	Cross-sectional study (Paper III)	Questionnaire data collected by telephone interviews in 2016 (218 organic farms) The predicted values for fasciolosis risk for each farm taken from Olsen et al. 2015	Ab-ELISA on BTM from 2016 and 2017, and individual milk
2. Comparison of currently available diagnostic methods	Case-control study (Paper I)	Liver condemnation data (DCD)	Ab-ELISA on BTM from 2014
	Longitudinal study (Paper II)	Farm and animal data (herd size, birth date, calving date etc.)	Ab-ELISA on serum, Ag-ELISA and FEC on faeces from 4 cohorts of animals from 4 dairy farms
			Monthly BTM from 4 dairy farms vs the average serum ELISA values
3. Investigation of the current patterns of infection on Danish dairy farms	Longitudinal study (Paper II)	Farm and animal data (herd size, birth date, calving date etc.)	Ab-ELISA on serum, Ag-ELISA and FEC on faeces from 4 cohorts of animals from 4 dairy farms
4. Relationship between antibody levels in BTM and within-herd prevalence	Cross-sectional study (Paper III)	Farm and animal data such as parity and breed	Ab-ELISA on BTM and individual milk samples from 2017
5. Evaluation of the effect of fasciolosis on milk production	Cross-sectional study (Paper III)	Individual and farm average 305d ECM, farm and animal data such as parity and breed	Ab-ELISA on BTM from 2016 and 2017 and individual milk from 2017

4. Summary of results and discussions

The main results of the current studies and their significance are summarised in this chapter. The specific details of the findings can be found in the attached manuscripts I – III as indicated.

4.1. Heifers (second season grazers) as the main risk group for fasciolosis (Paper I and III)

The first risk factor analysis using 131 case and 63 control farms (where case and control herds were defined by liver condemnation data) found that case farms were associated with heifers grazing on wet pastures, dry cows grazing on wet pastures, increased herd size, breed and concurrent beef cattle production. As there was some discrepancy in identifying *F. hepatica* infected farms using BTM ELISA (section 4.2.1), slightly different results were obtained using BTM ELISA results as the response variable. The final model using BTM ELISA results showed that the odds of being positive were higher with heifers grazing on wet pastures, dry cows grazing on wet pastures, and history of purchase of cows in the previous year. This study included farms where no animals had access to pasture (12 case and 16 control farms), and more than half of the farms did not allow lactating cows to graze (79 case and 41 control).

The second risk factor analysis of 218 organic dairy farms showed that heifers grazing on wet areas and increasing herd size were significantly associated with BTM ELISA positive farms. Additionally, odds of being BTM ELISA positive was higher if the farms applied flukicide treatments. Although statistically non-significant, heifers having access to surface water, absence of other livestock production (beef cattle, sheep and horses etc.), no preventive drainage of wet areas were associated with BTM ELISA positive farms in the final model. No significant association was found between BTM ELISA outcome and the predicted probabilities for fasciolosis based on the environmental data (Olsen et al., 2015).

Overall, the two risk factor studies showed that the heifers were the main risk group for fasciolosis in Danish dairy farms. It is well known that parasite infections such as *Eimeria* spp., *Ostertagia ostertagi*, *Cooperia oncophora*, and *D. viviparus* are substantial threats to young stock on pasture (Höglund et al., 2001). Calves (first season grazers) were not identified as a risk group for fasciolosis, and this probably reflects the typical grazing patterns in Denmark; calves are either housed or put on dry/better pasture whereas heifers are put on marginal lands. This trend was clearly indicated in Paper I, which showed that > 50% of the farms did not have calves on pasture

and 28% allowed calves to graze on dry pasture. In contrast, only 16% of the farms housed the heifers and 70% of the farms put their heifers on wet pasture. As pasture systems require minimum labour and feed costs (White et al., 2002), it is common to raise heifers on pasture in Denmark. Additionally, heifers are the ideal livestock animal type to be put on marginal/natural land as part of governmental incentives to protect the natural areas (Buttenschøn, 2007). These areas are often wet and likely to harbour suitable intermediate host snail habitats, e.g. freshwater meadows, bog, and marshland. Therefore, heifers as a risk group for fasciolosis was an anticipated finding. Indeed, we found that most animals got their primary infection at 1.5 to 2 years of age in the four farms that were involved in the longitudinal study (Paper II). However, only one other study from Sweden has so far found the associations with heifer; length of grazing period for heifers was the only significant factor for *F. hepatica* positive farms (Novobilsky et al., 2015). Overall, it should be emphasized that investigation of management-related risk factors should be on regional level and the results may not be translated to other localities.

In contrast to heifers, cows are usually kept in the stall or put on drier pasture near the milking shed (Marcussen et al., 2008; Kristensen and Sørensen, 2017), and therefore the risk of fasciolosis in cows is expected to be low. However, dry cows grazing on wet areas was also identified as the key risk factor for fasciolosis by the first risk factor study, while this was not the case for the second questionnaire study. The differing result is probably related to the different study populations. The first questionnaire included mostly conventional farms where grazing is limited. Dry cows were the second most common group of animals to be grazed after heifers on these farms, and consequently the risk of exposure to metacercariae was theoretically high for dry cows. Meanwhile, all animals should be on grass for organic farms, and therefore the risk of fasciolosis is potentially equal for all age groups. Heifers were, however, most likely to be put on fluke-contaminated pastures on organic farms. The non-significant association of BTM ELISA positivity and dry cows on wet pasture on organic farms does not necessarily mean that transmission is unlikely to occur in this group. In Paper II, we demonstrated that adult cows were continuously exposed to metacercariae on at least one of the organic farms. It is difficult to determine if the infection was acquired on the paddock for lactating cows or dry cows or both. In any case, it is crucial to establish if transmission is taking place in the adult herd regardless of conventional or organic farm, as the control strategy will differ depending on where transmission occurs.

4.2 Comparison of diagnostics

4.2.1. Herd-level diagnosis (liver condemnation data vs. BTM ELISA) (Paper I)

We classified farms as either case or control farms based on the 2011 – 2013 liver condemnation data, and compared these classifications against the BTM ELISA results. BTM ELISA collected in spring 2014 showed that 74.8 and 12.7% of case and control farms were positive for fasciolosis. The discrepancy between the two diagnostic classifications was probably related to the detection limit of BTM ELISA; it is unable to detect farms with low prevalence, which was shown to be below the within-herd prevalence of 20% with IDEXX ELISA kit used in the study (Duscher et al., 2011). Additionally, there was a time lag between meat inspection data and BTM collection. If all (or most) positive cows were slaughtered by the end of 2013, then BTM taken in 2014 may have shown negative results. Moreover, meat inspection is not perfect and may have produced false positives, as chronic changes may remain without the presence of the worms or even antibodies (Hutchinson et al., 2009; Mazeri et al., 2016). Additionally, meat inspection may have also missed some animals in early or low grade infection (Rapsch et al., 2006; Mazeri et al., 2016), resulting in BTM ELISA positive control farms. However, this was probably unlikely as the control farms were defined as having no liver condemnation for the whole three year period. A more likely scenario is that the *F. hepatica* infection was newly introduced in the farm. Three out of eight control farms that were positive for BTM ELISA had a history of buying animals in 2013. It is also possible that the infection occurred in late 2013, which resulted in an increased BTM ELISA leading to a positive test in 2014. Finally, false positives by BTM ELISA due to cross-reactivity with other parasites (e.g. lungworms or rumen fluke) cannot be excluded (Mazeri et al., 2016), although cross-reactivity has not been shown with the IDEXX ELISA kit (Molloy et al., 2005; Kuerpick et al., 2013b). Overall, there was a good correlation between the BTM ELISA values and apparent within-herd prevalence estimated by the proportion of liver condemnation.

It was apparent from the first questionnaire study that the majority of the farmers (83.5%) knew about the *F. hepatica* status from the abattoir feedback, while only eight (6.1%) case farmers confirmed the *F. hepatica* infection by veterinary professionals. Liver condemnation data could be a good overall indicator for presence of *F. hepatica* on farm, but BTM ELISA is probably more practical, as it has higher sensitivity and specificity than meat inspection, and results can be gathered in a more timely manner (ante-mortem). Additionally, BTM ELISA has been shown to correlate well with sero-prevalence of the milking herd (as confirmed in Paper III), and is therefore

useful for monitoring fasciolosis after introduction of control measure on a farm (Charlier et al., 2007; Mezo et al., 2011).

4.2.2. On-farm diagnosis (Ab-ELISA, Ag-ELISA, FEC, snail sampling, monthly BTM) (Paper II)

Agreement between the three diagnostic methods on-farm (Ab-ELISA, Ag-ELISA and FEC) varied according to the season. Agreement between Ab-ELISA and Ag-ELISA was highest in winter, while that of Ag-ELISA and FEC was highest in spring/summer and Ab-ELISA and FEC was highest in summer. This is due to the fact that these diagnostic methods detect different things; host antibodies or parasite stages (eggs or antigens primarily from late immature and adult worms) and consequently the time of detection differs. Experimentally, Ab-ELISA could detect infection as early as two weeks post infection (Reichel et al., 2005), while Ag-ELISA became positive after 5 – 6 weeks (Brockwell et al., 2013). FEC requires patent infection, i.e. 10 – 12 weeks post infection. Mazeri et al. (2016) also evaluated the effect of seasons on sensitivity and specificity of these diagnostic methods. For Ab-ELISA, the highest sensitivity and specificity was obtained in winter, while the sensitivity for FEC was highest in summer. Sensitivity of Ag-ELISA did not differ between the seasons in their study, but this could have been due to the high cut-off used as the sensitivity was generally low (77%). The results indicate that seasons need to be considered when taking samples for analysis of fasciolosis. Ab-ELISA at housing can detect *F. hepatica* exposure during the grazing season with good confidence, provided that the animals are negative before turn-out or first-season grazers. However, faecal samples taken in autumn or early winter for Ag-ELISA or FEC will not sufficiently identify infected animals at that point.

We also carried out a survey to look for *G. truncatula* on pastures suspected of transmission on the four farms. We confirmed the presence of the intermediate host species *G. truncatula* on the four farms, although only three out of 263 (1.1%) *G. truncatula* collected in 2017 were identified as infected by *F. hepatica* by PCR. The actual prevalence of infection is probably unimportant, as the reported prevalence in *G. truncatula* varies significantly depending on season, location and year (Rondelaud et al., 2004, 2016), and it also depends on PCR methods used (DNA target regions and different set of primers). Still, the presence of snails and the potential habitats are suggested as key indicators for the risk of *F. hepatica* transmission (Charlier et al., 2011). We found that pasture inspection for potential snail habitats and snails were challenging. There was a great variation in the snail survey results depending on the time of visits; some temporal water bodies were dried out in

October 2015, while plenty of snails were found in October 2017 due to more rain that autumn. The inspection of a pasture for snail habitats is time-consuming and requires expert knowledge to identify the species of the snail. Although identification of contaminated pasture and snail habitats are crucial in setting up preventive measures such as fencing-off the areas, we suspect that the method is unlikely to be implemented as a part of routine fasciolosis control as suggested by Knubben-Schweizer and Torgerson (2015) unless a quicker and easier guideline is developed.

Monthly BTM ELISA results corresponded well with the average serum antibody levels of the lactating cows taken closest to the BTM sampling date. Fluctuations were seen with time, but the peaks were seen around January to March in the two farms without treatment (farms C1 and O1) and also on one farm (farm O2) before starting treatment in late 2017. The seasonal fluctuation in BTM results is due to the seasonality of infection, where cattle pick up metacercariae in the second half of the year. Likewise, the rise of monthly BTM ELISA level was also seen from September and peaking in January in 22 predominantly spring-calving Irish dairy herds (Bloemhoff et al., 2015). The seasonal fluctuations in BTM values should be taken into consideration if samples are to be taken repeatedly, e.g. quarterly, from a farm. By the end of the study, BTM ELISA values were negative in the two farms (farms C2 and O2) that started anthelmintic treatment during the study period. The swift changes seen in BTM ELISA values after successful treatment regimen (6 – 12 months) indicate that the BTM ELISA is a useful monitoring tool for farms that initiate control against *F. hepatica*.

4.3. On-farm patterns of infection (Paper II)

The longitudinal study showed that the temporal pattern of infection differed greatly between the four farms. However, one common feature across the four farms was that most animals were exposed to infection as young stock. Elevated Ab-ELISA values were seen at the age of 1.5 to 2 years, except for farm C2, as this farm started control measures from 2015 (anthelmintics to all heifers after housing and most heifers were housed by 1st of August 2015). The common infection pattern on the four farms was therefore that young stock acquired infection and joined the lactating herd, contributing to the high Ab-ELISA values in BTM.

On at least one of the farms (farm O1), recurrent infection seemed to have occurred in the adult herd (lactating cows or dry cows). This was suspected because most older cows over four years of age showed signs of active infection, i.e. elevated Ab-ELISA values, Ag-ELISA values and eggs in the faeces. This was in contrast to farm C1, where some older cows had elevated Ab-ELISA values,

but only a few had elevated Ag-ELISA values and eggs in the faeces. We suspect that adult cows were not exposed to metacercariae on this farm. This was also supported by the fact that all but one cows born in 2012, which were sero-negative at the start of the study, remained uninfected during the entire study period (n=10). Likewise, the source of infection was limited to heifers on farm C2, as lactating animals were permanently housed and dry cows were put on a sandy exercise yard. However, some animals over four years of age were positive for Ab-ELISA, Ag-ELISA and FEC. This suggests that *F. hepatica* may last longer than two years as suggested by Ross (1968), although possible infection through silage or hay cannot be completely dismissed. The possible long persistent infection of *F. hepatica* has an important meaning when assessing for repeated exposure within the lactating herds. Positive Ab-ELISA results in the older cows may not necessarily mean that infection is acquired recently and that the pastures for lactating cows (or dry cows) are contaminated. Interpretation should be complemented with FEC or Ag-ELISA to eliminate the possibility of long-lasting chronic infection. The number of flukes surviving after 24 months is expected to be limited, as 75% of parasites are eliminated by 5th to 21st months of infection and the number of eggs in faeces were low or negative in chronic infection according to Ross (1968). Therefore low positive Ag-ELISA values and low or negative egg counts in faeces are expected in older animals with long lasting infections. However, it is still unclear if Ag-ELISA values correspond to fluke burden in cattle (Martínez-Sernández et al., 2016), thus FEC may be the preferred method of diagnosis.

Finally, we investigated whether the seasonal pattern of infection in cattle in Denmark is still consistent with a prevailing “summer infection” in snails (Nielsen et al., 1973; Shaka and Nansen, 1979). Samples taken in mid-summer (July to August) showed that few animals had sero-converted, but no increase in Ag-ELISA or FEC was seen. This suggests that the infection was acquired a few weeks before the sampling date and unlikely to be due to overwintering infection. It is known that overwintering infection can occur under Danish climate conditions; tracer calves that grazed up to the second week of July in 1971 and 1972 developed fasciolosis (Nielsen et al., 1973). However, overwintering infection seems to occur to limited extent, possibly only in some years after mild winters. A possible increase of fasciolosis incidences due to winter infection in snails (Fox et al., 2011) is not yet evident, at least from our study.

4.4. The relationship between antibody levels in BTM and within-herd prevalence (Paper III)

The results of BTM ELISA by the IDEXX test and within-herd prevalence were highly correlated, and the estimated apparent within-herd prevalence was ≤ 8.8% (95%CI: 4.3 – 14.0) if BTM ELISA negative, > 8.8% and ≤ 28.5% (95%CI: 20.8 – 37.8) if low, > 28.5% and < 74.6% (95%CI: 63.7 – 84.1) if moderate, and ≥ 74.6% if high. This corresponded to the interpretation recommended by the manufacturer to some extent ("negative" as no or low prevalence, "low" as < 20% prevalence, "moderate" as 20 – 50% prevalence, and "high" as > 50% prevalence).

This study demonstrated the usefulness of BTM ELISA in that it can be interpreted quantitatively to estimate the within-herd prevalence. BTM is easily accessible in Denmark and other EU countries, and thus provides a less invasive and cheaper method compared to sub-sampling of individual animal serum to know the level of *F. hepatica* infection on a farm. BTM ELISA may miss farms with low within-herd prevalence as the detection limit has been shown as 20% using this ELISA kit at the same cut-off value (Duscher et al., 2011). However, we demonstrated that the detection limit for BTM ELISA with this kit was as low as 8.8%. This discrepancy may be due to the larger number of farms available in the present study or use of different statistical methods. We assumed that the relationship between the BTM ELISA and within-herd prevalence was sigmoidal (a linear model with logistic link), while some other studies assumed it to be linear. The different results could have also occurred purely due to chance, as the ELISA tests are not perfect and the estimates include some uncertainties.

4.5. Associations between antibody levels and milk production (Paper III)

The association between *F. hepatica* infection and reduction in milk production was assessed both at individual and herd levels. Analyses of BTM ELISA results and milk yield data from 218 organic farms showed that average 305d ECM per farm was 580.8 kg less in BTM ELISA positive farms in a model with farm demography / management factors. This is a difference of approximately 6%, which was comparable to similar studies conducted in Spain and Belgium (3 – 5%), although it was much less than the 15% reduction reported in the UK (Charlier et al., 2007; Mezo et al., 2011; Howell et al., 2015). The greater estimated loss in UK is not related to the difference in ELISA kits used, because similar loss was estimated using LIV-ELISA results on our dataset (485.6 kg, Table 4). The more likely reason is due to higher prevalence and parasite burdens in UK causing greater effect on milk production (Howell et al., 2015).

The model indicated that dichotomised BTM ELISA outcome was significantly negatively associated with milk production, but increasing BTM ELISA values (the degree of positivity) conditional on positivity was not significantly associated with the degree of milk production loss. However, negative estimates for the interaction between the degree of positivity and average lactation was seen (it was significant using LIV-ELISA). Considering that BTM ELISA was shown to be highly correlated with within-herd prevalence (see above), higher milk production loss was expected with higher BTM ELISA values. This was not clearly shown in the present study, probably because there are many farm factors (e.g. average parity) that may play a role for average milk yield and the *F. hepatica* antibody levels in BTM. The present study also included farms that applied flukicide. The questionnaire response indicated that 30% of the positive farms in the dataset applied flukicide in 2015. It is known that the antibody levels can persist for at least three to six months after flukicide treatment (Sánchez-Andrade et al., 2001; Salimi-Bejestani et al., 2005a; Mezo et al., 2007; Brockwell et al., 2013). Therefore cows that eliminated infection and recovered milk production level back to normal could still have high Ab-ELISA levels, contributing to the elevated BTM ELISA values of the ELISA positive farms. Then again, flukicide application was not significant and excluded from the final model, and therefore an effect of flukicide application on milk yield at farm-level was not shown.

Table 4. Estimates from a linear model showing associations between 305 day energy corrected milk yield (305d ECM) and anti-*Fasciola hepatica* antibody ELISA results from BTM samples, as well as variables used to control for farm demographic and management factors using LIV-ELISA (R2=0.291, df=203, P< 0.001) (refer to paper III for the results of the same analysis using IDEXX-ELISA outcome)

Variable	Estimate	95% CI	SE	P value
S/P classification				<0.001
S/P negative	Ref			
S/P positive	-485.6	-754.2; -217.1	136.2	
S/P positive value	-6.16	-18.3; 5.97	6.152	0.317
Average parity (linear)	1451.4	727.2; 2175.5	367.3	<0.001
Average parity (quadratic)	-1549.1	-2646.3; -451.9	556.5	0.005
S/P classification : Average parity	-1496.9	-2454.3; -539.5	485.6	0.002
S/P value : Average parity	-48.4	-91.5; -5.20	21.9	0.027
Herdsize (linear)	0.788	0.281; 1.295	0.257	0.002
Breed				<0.001
Danish Holstein	Ref			
Jersey	-1205.7	-1721.6; -689.7	261.7	
Other	-553.5	-852.8; -254.2	151.8	
Grazing time of cows in summer				0.026
Half day	Ref			
All day	-412.5	-778.6; -46.3.4	185.7	
Prevention by fences				0.011
No	Ref			
Yes	-337.4	-597.6; -77.1	132.0	
Grazing areas for dry cows				0.007
Dry	Ref			
Wet	-438.2	-758.2; -118.3	162.3	
Heifer having access to surface water				0.165
No	Ref			
Yes	187.6	-78.6; 453.7	135.0	
Predicted value	1024.0	-261.1; -2309.1	651.8	0.116

Abbreviations: SE, standard error; 95% CI, 95% confidence interval; Ref, reference; BTM, bulk tank milk

A major weakness of this herd-level investigation is that there are several herd-level factors which may be associated with both milk production and *F. hepatica* infection, meaning that it is extremely difficult to infer causation from any associations found. To address this issue, milk production was also assessed at individual level using a linear mixed effects regression model based on 284 individual-animal milk samples from 55 organic dairy farms. There was a significant reduction in 305d ECM of 919.5kg relating to cows in the third or later parity, but no significant association was seen in cows in the first or second parities. It is possible that older cows have higher fluke burden due to longer exposure and accumulation of flukes, resulting in greater milk yield loss, because the liver flukes may last over two years (Paper II) and complete protective immunity against *F. hepatica* is unlikely to occur (Graham-Brown et al., 2018). It is also possible that the ability to compensate for the effect of *F. hepatica* infection deteriorates with age. Concurrent health problems such as clinical mastitis and lameness occur more frequently in older cattle (Sogstad et al., 2005; Breen et al., 2009), and these disorders could be exacerbating the effect of *F. hepatica* infection. Conversely, cows with *F. hepatica* infection may be more susceptible to such disorders that decrease milk production. Alternatively, increased *F. hepatica* antibody levels in older cows could have been accidental, i.e. as a reaction to other concurrent infections. Increased anti-*O. ostertagia* antibodies were seen in experimentally induced mastitis (Charlier et al., 2006). Older cows produce more natural antibodies due to sensitisation (van Knegsel et al., 2007), so if the older cows were more frequently associated with mastitis and other metabolic disorders, then the increased anti-*F. hepatica* antibodies and reduced milk production could have been a coincidental finding.

Similarly to the farm-level analysis, the model based on individual data showed that the dichotomized Ab-ELISA result at cut-off of 30 S/P% was significantly negatively associated with milk yield, while the degree of positivity conditional on a positive titre was not. Similarly, Charlier et al. (2008) observed that the value of IDEXX ELISA did not reflect the number of parasites in the liver. It seems therefore most reasonable to recommend that the results of Ab-ELISA on individual samples be interpreted qualitatively for *F. hepatica* infection based on a simple threshold determination of positivity, and that a reduction in milk production can be expected to be associated with Ab-ELISA positivity.

5. Conclusions and perspectives

In conclusion, this thesis has achieved the following conclusions with respect to the stated objectives:

1. "Heifers grazing on wet areas" was a key risk factor associated with fasciolosis at farm-level in Denmark (Paper I and III). "Dry cows grazing on wet areas" was also a risk factor for the study population mainly consisting of not-so-extensive farms. Control of fasciolosis should target primarily heifers, but possible transmission within the adult herd should also be considered.
2. For herd-level diagnosis, moderate agreement between liver condemnation data and BTM ELISA results was seen. Due to the different efficiencies and properties of the methods, herd-level diagnosis of *F. hepatica* should be based on positive BTM ELISA result supported by liver condemnation recordings to identify farms in need of improved control (Paper I). Additionally, BTM ELISA decreased 6 – 12 months after introduction of rigorous treatment strategies on two farms, indicating that it is a useful monitoring tool for fasciolosis after commencement of a control program (Paper II). For diagnosis in individual animals, Ab-ELISA, Ag-ELISA and FEC differed in the time of detection. Ab-ELISA is applicable to detect exposure for animals at housing in autumn, while Ag-ELISA and FEC should be applied the following spring (before turn-out) or early summer to increase the chance of detection (Paper II).
3. Animals were first infected as heifers and carried the infection to the lactating herd. On the two farms we followed over time, the transmission was limited to heifers (housed or on non-risk pasture), but continuous exposure to metacercariae seemed to occur on cow paddocks on some of the farms. Our results also suggested that *F. hepatica* may survive longer than two years in cattle. Based on our investigations, we propose that serological samples should be taken before turn-out and at housing for planned second-year grazers to determine if the transmission is taking place in the young stock. Additionally, serological and coprological samples from cows older than third lactation should be taken before turn-out to determine if active transmission is occurring within the cow herd. Due to substantial year-to-year variation, progress of control should be monitored regularly by BTM ELISA, preferably at the same time of year, as BTM ELISA can fluctuate depending on the season. Moreover, we observed that the summer infection in snails was still the dominating transmission pattern in Denmark (Paper II).

4. The results of BTM ELISA using IDEXX kit were highly correlated with within-herd prevalence measured by individual milk samples. Our result indicated that BTM ELISA values were positive when within-herd prevalence was ≥ 8.8% (Paper III). BTM ELISA can be quantitatively interpreted for the within-herd prevalence. Although there was a significant relationship between BTM ELISA positivity and average milk yield, no clear association between quantitative BTM ELISA results (conditional on positivity) and reduced average milk yield per farm was seen.
5. An average reduction of 580.8kg in 305d ECM was found on *F. hepatica* BTM ELISA positive farms relative to those that were negative on BTM ELISA. Furthermore, a significant reduction in 305d ECM was also seen in individual animals with a positive antibody test, although this relationship was limited to cows in their third or later parity (Paper III).

This Ph.D. thesis aimed to generate updated epidemiological data regarding bovine fasciolosis in Danish dairy farms. The results provide clear and practical guidance that can be used to aid control of the parasite on farms similar to those investigated in the various studies comprising this thesis.

The levels of milk production loss shown (Paper III) may justify intensified *F. hepatica* control in Denmark. It is noteworthy that the production loss due to *F. hepatica* infection may not be limited to milk production, although this aspect was not explored in the present study. Other production parameters such as reproduction (puberty onset, calving intervals, and fertility rate), feed efficiency and growth rate may be affected due to *F. hepatica* infection. Furthermore, interactions between *F. hepatica* infection and other disorders are still unknown and need to be explored, not only because it has an important connotation in determining the true effect of *F. hepatica* on production loss but also in terms of controlling other important metabolic and infectious diseases such as mastitis, bovine tuberculosis, and *Salmonella dublin*. Overall, the current evidence suggests that control of bovine fasciolosis is important to limit production losses, although we cannot expect that gains are at similar levels of the analysis, as it is unlikely to completely eradicate *F. hepatica* infection on a farm.

Based on our findings, we suggested practical guidelines for diagnosis and management of fasciolosis (Paper II). We recommended treatment regimen and grazing management directly to the participating farmers as a part of this study. Partly as a consequence of our advice, two farms successfully reduced their BTM ELISA values by the end of the study. However, the options for fasciolosis control involve routine anthelmintic treatment at present. Such control measures are

costly and intensive anthelmintic treatment can lead to development of anthelmintic resistance. The treatment regimen is not sustainable if a farm has no dry pastures and the animals are continuously re-infected. For organic producers, the use of anthelmintics is harder to implement due to the stricter regulations against anthelmintics. Therefore, further research is required to describe and validate the effect of non-medicinal control measures e.g. rotational grazing and systematic and flexible fencing of wet areas. Use of genetically robust breeding lines is still in its infancy but seems promising. More trials are needed to test and assess the effect, feasibility and costs of biological control of snails or competitive snail species or ducks. Additionally, given the current reliance on anthelmintic to control fasciolosis, more work is needed to establish the extent of any potential anthelmintic resistance within *F. hepatica* in Denmark.

Our work only focused on dairy cattle, and the status of *F. hepatica* infection in other livestock species in Denmark is still largely unknown. This is particularly true of sheep as there is no central registry for the sheep population, although it is worth noting that the sheep population of Denmark is relatively small compared to most other European counties. However, wildlife such as deer and hare are known to harbour the parasite, and they have access to fenced areas where cattle are grazing. Therefore, they may be of importance for the transmission of *F. hepatica* between livestock populations. Due to the wide host specificity of *F. hepatica*, it is not likely to be possible to manage the problem without paying attention to fasciolosis in other host species. Consequently, more research in the epidemiology of *F. hepatica* infection in other livestock and wildlife populations, as well as improvement of the detection of infected snails/grazing areas is warranted.

6. Other research outputs

Publications (# indicates popular communication):

- **Takeuchi-Storm, N**., Denwood, M., Hansen, T.V.A., Halasa, T., Rattenborg, E., Boes, J., Enemark, H.L., Thamsborg, S. M. Farm-level risk factors for *Fasciola hepatica* infection in Danish dairy cattle. WAAVP (World Association for Advancement of Veterinary Parasitology) conference, Liverpool, UK. 18 August 2015. Abstract (poster).
- **Takeuchi-Storm, N.,** Denwood, M., Petersen, H. H., Enemark, H, Thamsborg, S. Farm specific transmission patterns of *Fasciola hepatica* in Danish dairy cattle based on different diagnostic methods and monitoring of grazing management. WAAVP, Kuala Lumpur, Malaysia. 7 September 2017. Abstract (oral presentation).
- #**Takeuchi-Storm, N**., Kolthoff, I., Denwood, M., Enemark, H.L., Thamsborg, S.M. (2017). Usynlige omkostninger når kvæg har leverikter (Unseen costs when cattle has liverflukes), KvægNyt. Arhus. 6.
- #Stensgaard, A.S., **Takeuchi-Storm, N**., Sengupta, M.E. (2018). Leverikten: en gammel kending i fremgang (Liver flukes: an old fame in progress), Kaskelot. 219, 24-28
- #**Takeuchi-Storm, N**., Denwood, M., Enemark, H.L., Thamsborg, S.M. (2018). Leverikter i danske malkekvægsbesætninger: vejen til bedre forebyggelse. Resultater fra undersøgelser i perioden 2014 – 2018 (Liver flukes in Danish dairy cattle farms: The way for better prevention. Results from the studies in 2014 – 2018), Kvægposten (Newsletter for cattle veterinarians). September 2018.

Other activities

- **Takeuchi-Storm, N**., Denwood, M., Enemark, H, Thamsborg, S. *Fasciola hepatica* in Danish cattle: epidemiology, diagnostics and control. CPH Cattle: Up-to-date with cattle research, Frederiksberg, Denmark. 12 November 2015. Oral Presentation.
- **Takeuchi-Storm, N.,** Denwood, M., Enemark, H, Thamsborg, S. Liver fluke and cattle on wet areas: focus on management in organic farms, Organic Congress, Vingsted, Denmark. 25 November 2015. Poster.
- Thamsborg, S. & **Takeuchi-Storm, N**. Temaaften Leverikter (Today's theme Liver flukes – for all working with grazing cattle). Information meeting for farmers, consultants and veterinary professionals, Lemvig, Denmark. 4 October, 2016. Oral presentation.
- **Takeuchi-Storm, N.,** Denwood, M., Enemark, H, Thamsborg, S. Relationship between anti-*Fasciola hepatica* antibody levels in bulk tank milk and within-herd prevalence in Danish dairy farms. Danish Society for Parasitology Spring Symposium, Frederiksberg, Denmark. 6 April 2018. Oral presentation.
- **Takeuchi-Storm, N** & Thamsborg, S. Leverikter hos kvæg: Risikofaktorer, diagnostik og praktiske råd omkring forebyggelse (The liver flukes in cattle: Risk factors, diagnostics and practical advice on prevention). LVK meeting for cattle veterinarians, Hobro, Denmark. 12 April 2018. Oral presentation.
- Thamsborg, S. & **Takeuchi-Storm, N**. Leverikter hos kødkvæg: Betydning og praktiske råd omkring forebyggelse (Liver flukes in beef cattle: Relevance and practical advice on prevention). Agrovi Summer meeting for organic and conventional beef producers, Gavnø, Denmark. 21 June 2018. Oral presentation.

- **Takeuchi-Storm, N**. & Thamsborg, S.M. Leverikter hos kvæg: Livcyklus, epidemiologi, klinik, diagnostik og profylakse (Liver flukes in cattle: Life cycle, epidemiology. clinics, diagnostics and prophylaxis). Regional Christmas meeting, Aars and Hovborg, Denmark. 14 – 15 November 2018. Oral presentation.

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8. Publications and manuscripts

8.1 Paper I

Farm-level risk factors for *Fasciola hepatica* infection in Danish dairy cattle as evaluated by two diagnostic methods

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RESEARCH Open Access

Farm-level risk factors for *Fasciola hepatica* infection in Danish dairy cattle as evaluated by two diagnostic methods

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Abstract

Background: The prevalence of bovine fasciolosis in Denmark is increasing but appropriate guidelines for control are currently lacking. In order to help develop a control strategy for liver fluke, a risk factor study of farm management factors was conducted and the utility of bulk tank milk (BTM ELISA) as a tool for diagnosis in Danish dairy cattle farms was assessed.

Methods: This case-control study aimed to identify farm-level risk factors for fasciolosis in Danish dairy farms (> 50 animals slaughtered in 2013) using two diagnostic methods: recordings of liver condemnation at slaughter, and farm-level *Fasciola hepatica* antibody levels in BTM. A case farm was defined as having a minimum of 3 incidents of liver condemnation due to liver fluke at slaughter (in any age group) during 2013, and control farms were located within 10 km of at least one case farm and had no history of liver condemnation due to liver fluke during 2011–2013. The selected farmers were interviewed over telephone about grazing and control practices, and BTM from these farms was collected and analysed by ELISA in 2014. The final complete dataset consisting of 131 case and 63 control farms was analysed using logistic regression.

Results: Heifers grazing on wet pastures, dry cows grazing on wet pastures, herd size, breed and concurrent beef cattle production were identified as risk factors associated with being classified as a case farm. With the categorised BTM ELISA result as the response variable, heifers grazing on wet pastures, dry cows grazing on wet pastures, and purchase of cows were identified as risk factors. Within the case and control groups, 74.8 and 12.7% of farms were positive for fasciolosis on BTM ELISA, respectively. The differences are likely to be related to the detection limit of the farm-level prevalence by the BTM ELISA test, time span between slaughter data and BTM, and the relatively low sensitivity of liver inspection at slaughter.

Conclusions: Control of bovine fasciolosis in Denmark should target heifers and dry cows through grazing management and appropriate anthelmintic treatment, and BTM ELISA can be a useful diagnostic tool for fasciolosis in Danish dairy farms.

Keywords: Fasciolosis, Cattle, Liver condemnation, Antibodies, ELISA, Denmark

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Background

Liver fluke infection, or fasciolosis, is a global disease, caused by *Fasciola hepatica* and *F. gigantica*, that affects a wide range of host species including humans. It is classified as a Neglected Tropical Disease by WHO due to the public health impact, particularly in tropical environments [1], but it is also an important animal health disease causing substantial financial losses within livestock production [2]. In cattle, the infection with *F. hepatica* often manifests as a subclinical disease with vague symptoms including reduced productivity [3] apparent as reduction in milk yield, milk fat content, and reproductive performance [4–7]. Additionally, the cost of treatment and penalties for condemnation of infected/fibrotic livers at slaughter may incur substantial economic deficit for the farmers. In Switzerland, the annual loss caused by bovine fasciolosis has been estimated to be €299 per infected cattle and €52 million at the national level, calculated on the mean prevalence of 10.6% in 1.6 million cattle [8].

An increased prevalence of *F. hepatica* has been reported in UK and Sweden, presumably as a result of climate change causing milder winter temperature and increased rainfall, as well as due to government subsidized schemes to utilise wet areas for grazing [9, 10]. Likewise, the farm-level prevalence of *F. hepatica* in Danish cattle farms is steadily increasing based on the national liver condemnation data at slaughter, from 24% in 2003 to 25.6–29.3% between 2011 and 2013 [11, 12]. This is an issue for dairy farmers as there are currently relatively few effective flukicides licensed for use in lactating cows and resistance to these drugs are increasingly reported around the world [13–16]. In order to avoid overuse of anthelmintics, recent research is therefore focused on describing the spatial distribution of and identifying risk factors for fasciolosis [17]. Previously identified risk factors include climate and environmental factors, such as presence of streams, wetland and pastures, and higher rainfall and temperature [18–21]. However, it is also known that farms within a relatively small geographical area may have variable infection levels. This may be due to variations in micro-environment within farms, i.e. presence of suitable snail habitats [19]. Farm management factors are also important for the spatial distribution of *F. hepatica* in temperate climate zones, where only minor climatic and environmental variation exists [22]. Considering that management practices can be highly dependent on local regulations, farming traditions and environment, risk factors and their significance for fasciolosis are likely to vary between countries. This makes it important to quantify risk factors within the highly specific geographical setting in order to propose effective control strategies on a national level. We therefore initiated this follow-up study after Olsen et al. [11] to evaluate the effect of farm management factors on fasciolosis within a Danish setting.

One of the major challenges when designing on-farm control strategies for fasciolosis is the lack of a perfect diagnostic method for *F. hepatica* infection. Although currently not used in Denmark, the enzyme-linked immunosorbent assay (ELISA) test on bulk tank milk (BTM) can be easily obtained as part of a milk control program, and is therefore increasingly being used for farm-level diagnosis, monitoring and identification of risk factors for fasciolosis [18–21, 23]. However, BTM ELISA requires a minimum within-herd prevalence of 20–60% of the lactating animals in order to detect the herd as positive [24–26], which means that farms with low infection levels will not be identified. Alternatively, in countries such as Denmark where registration of individual cattle and meat inspection is mandatory, feedback from abattoirs on liver condemnation is commonly used by farmers and veterinarians as an indicator of the degree of fasciolosis on a farm. It is also possible to analyse this data at the national level to model the spatial distribution and risk factors for infection [11]. However, inspection of the liver at slaughter has been shown to have low sensitivity [27, 28], and factors such as grazing management cannot be extracted from such data.

The aim of this case-control study was to identify farm-level risk factors for fasciolosis in Danish dairy farms using two different approaches; farm classifications based on liver condemnation data and BTM ELISA, respectively. Furthermore, in order to assess the use of BTM ELISA as a diagnostic tool for fasciolosis in Denmark, the agreement between farm-level fasciolosis classifications from the two diagnostic methods was analysed. A secondary aim was to obtain an overview of the extent of Danish farmers' awareness of liver flukes and the use of anthelmintics.

Methods

Selection of farms and questionnaire

The centralised Danish Cattle Database (DCD) managed by SEGES (part of the Danish Agricultural Advisory Service run by the Danish Agriculture and Food Council) contains information related to all Danish individual cattle and farms. It is mandatory to ear-mark individual cattle and register them in DCD, where information regarding the animal's owner, birth, calving date, movement, slaughter date and result of meat inspection etc. is stored digitally. At meat inspection in the abattoirs, each liver is examined for signs of disease including fasciolosis according to Regulation (EC) No 854/2004. The liver is condemned if there are signs of fasciolosis, and the farmer is penalised by approximately €4 per condemned liver. All meat inspection recordings have to be reported to the DCD. However, the data from some of the minor slaughterhouses especially might be incomplete (Poul Møller Hansen, Danish Agriculture and Food Council, personal

communication). The liver condemnation dataset for the present study was extracted from DCD using only the meat inspection code relating to the diagnosis of fasciolosis, with codes relating to non-specific liver lesions being excluded [11].

For selection of fasciolosis positive and negative farms based on liver condemnation data, criteria on herd size and location were also set, in order to avoid hobby farms and minimize variation due to local climate. A case farm was defined as having: (i) at least 50 animals slaughtered in 2013; and (ii) a minimum of three animals (of any age that were also born on the farm) diagnosed with fasciolosis at slaughter in 2013. A control farm was defined as having: (i) at least 50 animals slaughtered in 2013; (ii) no record of liver condemnation due to fasciolosis (in animals of any age) in 2011–2013; and (iii) a location within 10 km from at least one case farm. Within the dairy farms matching these criteria, a total of 145 and 76 farms were randomly selected as case and control, respectively.

Questionnaire surveys were conducted by telephone during summer-autumn 2014 by two veterinary students, during which permission was also sought to access the DCD data for the same farm. The questionnaire contained 18 questions regarding the type of production system, the farmers' knowledge on presence of liver fluke infection in the farm, grazing pattern, anthelmintic treatments and management routines during 2013 (Additional file 1: Table S1). Note that most dairy farms in Denmark operate as all-year calving system (calving occurs throughout the year), and that the flukicides registered for use in dairy cattle in 2013 were limited to albendazole, clorsulon and closantel, while triclabendazole was/is only available after dispensation.

Milk samples and ELISA

All Danish dairy companies are required to send bulk tank milk samples from every herd delivering milk to laboratories for analyses of milk composition, somatic cell counts and antibiotic residues. BTM samples collected as part of the milk control program in the early summer of 2014 were frozen at −20 °C until analysis within 6 months. The full-fat BTM were analysed for *F. hepatica*-specific antibodies using a commercial ELISA kit (Fasciolosis Verification Test, IDEXX, Hoofddorp, the Netherlands) according to the manufacturer's instructions, with two replications for each sample. The antibody levels were expressed as the sample to positive percentage (S/P%) calculated as: S/P% = average net extinction (NE) of the sample / average NE of two positive controls × 100, where NE refers to the difference between the optical densities measured in the antigen negative control well and that of the antigen coated well. An S/P% > 30 was considered positive, while S/P% ≤ 30 was considered negative in accordance with the recommendations from the manufacturer. The sensitivity and specificity of the test for individual milk samples collected from dairy herds were reported as 95% and 98.2%, respectively, relative to sera [26], while Molloy et al. [29] reported sensitivity of 97.7% and specificity of 99.3% relative to faecal egg counts.

Data management and statistical analysis

Data from DCD were extracted using R [30] and subsequently combined with the results of the questionnaire and BTM ELISA using Excel 2010. The complete dataset consisted of 131 case farms (of which 17 were organic) and 63 control farms (of which were 8 organic), after removing 19 farms that did not respond to the questionnaire, 7 farms from which no BTM was available, and one farm that returned an incomplete questionnaire.

For regression analyses, only management factors were selected from the original questionnaire and some related questions were combined in order to avoid confounding and aid interpretability of the results. Additionally, herd size was extracted from DCD farm data as the median of the monthly measured total number of animals in 2013. Therefore 13 explanatory variables were considered for the two logistic regression models using liver condemnation data (case vs control) and BTM ELISA results (positive vs negative) as the response variables. All logistic regression models were implemented in R, and the final model for each response variable was selected using stepwise selection based on AIC [31] using the *MASS* package [32]. The final model fit was assessed using the Hosmer-Lemeshow Goodness of Fit test and by visual inspection of predicted values, and the overall significance of fixed effect terms with multiple levels was assessed by likelihood ratio test using the *lmtest* package [33].

In order to assess the sensitivity of the analyses presented above to imperfect diagnostic test sensitivity and specificity, a third model was constructed based on a more complex classification system incorporating both the dichotomised bulk tank milk test and the liver condemnation results for each animal on the corresponding farm. Briefly, the posterior probability that each farm was positive was directly calculated using Bayes' theorem conditionally on the bulk tank milk test result, number of liver condemnations, number of animals slaughtered, expected within-herd prevalence of liver fluke on an infected farm, and the sensitivity and specificity of the bulk tank and liver inspection tests. These probabilities were then used to re-label each farm as a case or control. To account for uncertainty in the input parameters and classification step, this procedure was repeated for 1000 samples over a distribution of parameter values chosen to reflect their 95% confidence intervals from published studies. Confidence intervals for the coefficients were

calculated using parametric bootstrapping from these 1000 model fits. Full details of this procedure are given in Additional file 2.

Finally, the apparent within-farm prevalence was calculated for case farms by dividing the total number of livers condemned by the number of animals slaughtered in 2013. Correlation between the apparent prevalence and S/P% were analysed by Spearman's rank correlation in R.

Results

The response rate of the questionnaire was 91.4% (202/221), and the non-response rates did not differ significantly between case (9/145, 6.2%) and control groups (10/76, 13.2%) (Chi-square test, $\chi^2 = 2.2452$, $df = 1$, $P = 0.134$). The number of case and control farms for each variable considered for risk factor analysis is summarised in Table 1. It was apparent from the questionnaire that 28 farms (12 case and 16 control farms) did not have any animals on pasture in 2013.

Risk factor analysis

Using the case and control definition as the response variable, the final model based on AIC included five explanatory variables (Table 2). Of these, the significant risk factors were grazing of heifers on wet areas with access to surface water (OR = 7.84, 95% CI: 2.67–25.1), grazing of heifers on wet areas without access to surface water (OR = 3.73, 95% CI: 1.12–12.0), herd size per 100 animals (OR = 1.49, 95% CI: 1.20–1.90), and grazing of dry cows on wet areas (OR = 4.23, 95% CI: 1.31–16.7). Using the BTM ELISA results as the response variable, the final model included three explanatory variables (Table 3). Of these, significant risk factors were grazing of heifers on wet areas with access to surface water (OR = 5.77, 95% CI: 2.10–17.5), grazing of heifers on wet areas without access to surface water (OR = 4.17, 95% CI: 1.41–13.5), and grazing of dry cows on wet areas (OR = 4.75, 95% CI: 1.85–13.5).

Using the Bayesian classification of each farm based on both BTM and slaughter test information, qualitatively similar results were obtained as with the simpler models. The final bootstrapped model based on AIC included three explanatory variables. Of these, significant factors were grazing of heifers on wet areas with access to surface water (OR = 8.82, 95% CI: 2.55–51.61), grazing of heifers on wet areas without access to surface water (OR = 4.76, 95% CI: 1.32–31.77), and grazing of dry cows on wet areas (OR = 3.69, 95% CI: 1.48–12.67) (Additional file 2: Table S3). Beef production on the dairy farm was identified as an additional significant risk factor using the reclassified model, although it was not significant using the bootstrapped model (Additional file 2: Table S3).

Comparison of liver condemnation data and BTM ELISA results

Based on BTM ELISA, 74.8% of the case and 12.7% of the control farms were positive for fasciolosis (Table 4). Distribution of mean S/P% values of all case and control farms are shown in Fig. 1, while Fig. 2 shows the distribution of mean S/P% values against apparent prevalence of case farms. There was a strong correlation between S/P% values and apparent prevalence (Spearman's rho = 0.806, $P < 0.0001$).

All eight control farms that were positive for BTM ELISA had grazing animals in 2013. The proportion of BTM ELISA negative control farms that had animals on pasture was 71% (39/55), and the difference between the two groups was not statistically significant (Fisher's exact test, $P = 0.10$). Of the eight farms, one farm had bought heifers and two had bought cows in 2013. Of the 12 case farms where no animals were on pasture in 2013 (all-in systems), five were positive for BTM ELISA with S/P % varying between 44.0 and 130.6% (low to moderate infection). Four of these farms said they did not buy any calves, heifers, or cows during 2013.

Information regarding liver condemnation and anthelmintic use on the farms

The majority of the farmers (162/194, 83.5%) were able to recall feedback from the abattoirs on liver condemnation. However, 14 case farmers (10.7%) answered that they had no liver condemnation due to liver flukes in 2013, whereas seven control farms (11.1%) answered there was liver condemnation due to liver flukes in 2013. The total number of farmers that had confirmed diagnosis of liver flukes by veterinarians or consultants was eight (6.1%) and one (1.6%) of the case and control farms, respectively.

The number of farms with usage of flukicides in 2013 was 38 (29.0%) case farms and one (1.6%) control farm, while the number that used anthelmintics for gastrointestinal and/or lung-worms was 66 (50.3%) and 18 (28.6%), respectively. Of those who used flukicides ($n = 39$), 36 (92.3%) treated heifers, 11 (28.2%) treated cows, and 11 (28.2%) treated calves. The products used for each group of animals are summarised in Fig. 3. Closamectin pour-on® (closantel and ivermectin, Biovet Aps, Fredensborg, Denmark) was commonly used for heifers and calves, while Valbazen® (albendazole, Orion Pharma Animal Health, Copenhagen, Denmark) was mostly used for cows. The use of Fasinex® (triclabendazole, Novartis, Copenhagen, Denmark) was extremely limited. Most farms (33, 84.6%) treated calves, heifers and/or cows

Table 1 Summary statistics of the questionnaire and slaughter observations, stratified by case and control farms

Farm factors	Case ($n = 131$)	Control ($n = 63$)
Mean herd size ± SD	448.1 ± 266.5	347.2 ± 141.0
Mean number ± SD of animals slaughtered in 2013	107.0 ± 82.5	75.9 ± 28.0
Farm type		
Organic	17	8
Conventional	114	55
Concurrent beef production		
Yes	21	3
No	110	60
Breed		
Danish Holstein	94	48
Cross	18	2
Other	19	13
Management factors		
Grazing of heifers and access to surface water		
Wet pasture + yes	73	15
Wet pasture + no	35	13
Dry pasture + yes	3	5
Dry pasture + no	7	11
Not grazed	13	19
Grazing of calves and access to surface water		
Wet pasture + yes	11	3
Wet pasture + no	17	5
Dry pasture + yes	4	1
Dry pasture + no	35	15
Not grazed	64	39
Grazing of cows		
Wet pasture	5	1
Dry pasture	47	21
Not grazed	79	41
Grazing of dry cows		
Wet pasture	38	4
Dry pasture	45	22
Not grazed	48	37
Period of grazing in 2013 (turn-out in March)		
Before 1st June and > 6 month	67	20
Before 1st June and ≤ 6 months	11	3
After 1st June and < 6 months	8	8
Not grazed	45	32
Any prevention for liver flukes on pasture		
None	82	37
Move animals in late summer	25	8
Other	12	2
Not grazed	12	16
Purchase or grazing of calves with animals from other farms in 2013		
Yes	10	2
No	121	61
Purchase or grazing of heifers with animals from other farms in 2013		
Yes	25	8
No	106	55
Purchase of cows in 2013		
Yes	20	6
No	111	57

Abbreviation: *SD* standard deviation

regularly without the use of supporting individual or herd diagnostics other than liver condemnation data.

Discussion

Risk factor analysis

The present study identified heifers and dry cows grazing on wet areas as high risk groups for fasciolosis using both response variables. Grazing on wet areas is a well-known key risk factor for fasciolosis, but we believe that this is the first time that dry cows have been clearly identified as a risk for a farm being positive. Past prevalence studies of fasciolosis using faecal egg counts showed increasing prevalence with age [34, 35], suggesting that *F. hepatica* infection occurs mainly from the second grazing season for heifers or later for cows. However, grazing of cows was not found to be a risk factor within our data. This most likely reflects the typical management system of a Danish dairy farm, where cows and calves are either not grazed or kept on dry, high ground pastures close to the milking shed, while heifers tend to be grazed further away from the main farm buildings, and left to graze for the entire grazing season (typically April to October) [36, 37], and dry cows are sometimes grazed together with heifers as leading cows (Professor Hanne Hansen, University of Copenhagen, personal communication). Thus, Danish animals are typically first exposed to *F. hepatica* metacercaria as heifers, and in some cases repeatedly exposed as dry cows, and it is therefore important for control measures to target these two groups of animals within a Danish setting. Our results demonstrate the need for conducting tailored risk factor studies that can be interpreted according to specific countries/regions, when developing national guidelines for fasciolosis control and prevention.

In the regression analysis, both models resulted in farms either without grazing or grazing only on dry areas having lower odds of being infected than those with animals grazing on wet areas. This is not surprising,

Table 2 The final multivariable logistic regression model (with risk factors selected using AIC) with case/control classifications based on liver condemnations as the response variable (131 case and 63 control farms)

Variable	Level	Estimate	SE	*P*-value	OR	95% CI
Intercept		-2.400	0.675			
Grazing of heifers (Not grazed, Dry grazing or Wet grazing) combined with access to surface water (No or Yes)				< 0.001		
	Not grazed	Ref			Ref	
	Dry & Yes	-0.368	0.961		0.69	0.09–4.33
	Dry & No	0.218	0.734		1.24	0.29–5.30
	Wet & Yes	2.060	0.568		7.84	2.67–25.1
	Wet & No	1.316	0.580		3.73	1.12–12.0
Herd size (per 100 animals)		0.396	0.001	< 0.001	1.49	1.20–1.90
Grazing of dry cows (Not grazed, Dry grazing or Wet grazing)				0.047		
	Not grazed	Ref			Ref	
	Dry	0.274	0.433		1.31	0.56–3.09
	Wet	1.443	0.637		4.23	1.31–16.7
Breed				0.102		
	DH	Ref			Ref	
	Cross	1.265	0.851		3.54	0.80–25.8
	Other	-0.548	0.472		0.58	0.23–1.47
Beef production				0.113		
	No	Ref			Ref	
	Yes	1.007	0.685		2.74	0.80–12.8

Abbreviations: *SE* standard error, *OR* odds ratio, *95% CI* 95% confidence interval, *Ref* reference

as presence of amphibious snail intermediate hosts is closely linked to wet areas, and moist areas have been identified as a key risk factor in UK and in Belgium [18, 19]. The authors of these studies also showed that the use of streams or ponds as water sources is a risk factor for fasciolosis, although we were not able to investigate this directly due to the design of our questionnaire.

The four other variables that were selected as risk factors for bovine fasciolosis based on model fit were herd size, breed, beef production and purchasing of cows,

Table 3 The final multivariable logistic regression model (with risk factors selected using AIC) with positive/negative classification based on bulk tank ELISA results (106 positive and 88 negative farms)

Variable	Level	Estimate	SE	*P*-value	OR	95% CI
Intercept		-1.555	0.462			
Grazing of heifers (Not grazed, Dry grazing or Wet grazing) combined with access to surface water (No or Yes)				< 0.001		
	Not grazed	Ref			Ref	
	Dry & Yes	0.749	0.870		2.11	0.35–11.6
	Dry & No	-0.218	0.762		0.80	0.17–3.50
	Wet & Yes	1.753	0.536		5.77	2.10–17.5
	Wet & No	1.428	0.570		4.17	1.41–13.5
Grazing of dry cows (Not grazed, Dry grazing or Wet grazing				0.004		
	Not grazed	Ref			Ref	
	Dry	0.489	0.380		1.63	0.78–3.46
	Wet	1.558	0.503		4.75	1.85–13.5
Purchase of cows				0.099		
	No	Ref			Ref	
	Yes	0.81	0.504		2.25	0.86–6.32

Abbreviations: *SE* standard error, *OR* odds ratio, *95% CI* 95% confidence interval, *Ref* reference

Table 4 Number of case and control farms based on liver condemnation results compared to classifications based on the ELISA-test for *Fasciola hepatica*-specific antibodies in bulk tank milk (BTM)

	Case	Control	Total
BTM-ELISA positive	98	8	106
BTM-ELISA negative	33	55	88
Total	131	63	194

although of these only herd size was a significant risk factor in the final model. One potential explanation for the effect of herd size is a recruitment bias in that larger farms with more animals slaughtered will have an increased chance of the required three liver condemnations, although this will have been partly offset by the minimum number of slaughter animals required for the control farms. However, the number of animals slaughtered has also been found to be associated with herd prevalence in Northern Ireland, where a recent survey showed that farms which slaughtered more than 105 animals during three years were all infected with fasciolosis, whereas farms with lower numbers of slaughtered animals had a lower herd-level prevalence [38]. It is therefore likely that some density dependence exists for fasciolosis (as for almost all infectious diseases); however altering herd size is not likely to be a practically relevant solution for the control of fasciolosis.

It is also interesting to note that *F. hepatica* infection was detected by both methods on some farms on which the animals were not grazed. Although most flukes are expelled by 30–50 weeks post-infection [39], *F. hepatica* is known to persist for a long time in cattle; for example Ross [40] observed live flukes 26 months after infection. As the questionnaire only involved data concerning management practice in 2013, it is possible that the presence of *F. hepatica* infection in non-grazing farms was a result of persisting infection acquired prior to 2013. However, other routes of infection, such as metacercariae-contaminated freshly cut grass and hay, should not be disregarded [41, 42]; some nematode parasites have also been shown to develop to infective stages on straw bedding [43]. Transmission by metacercariae-contaminated water is also possible, as it is a common route of transmission for human fasciolosis in the Americas [44, 45].

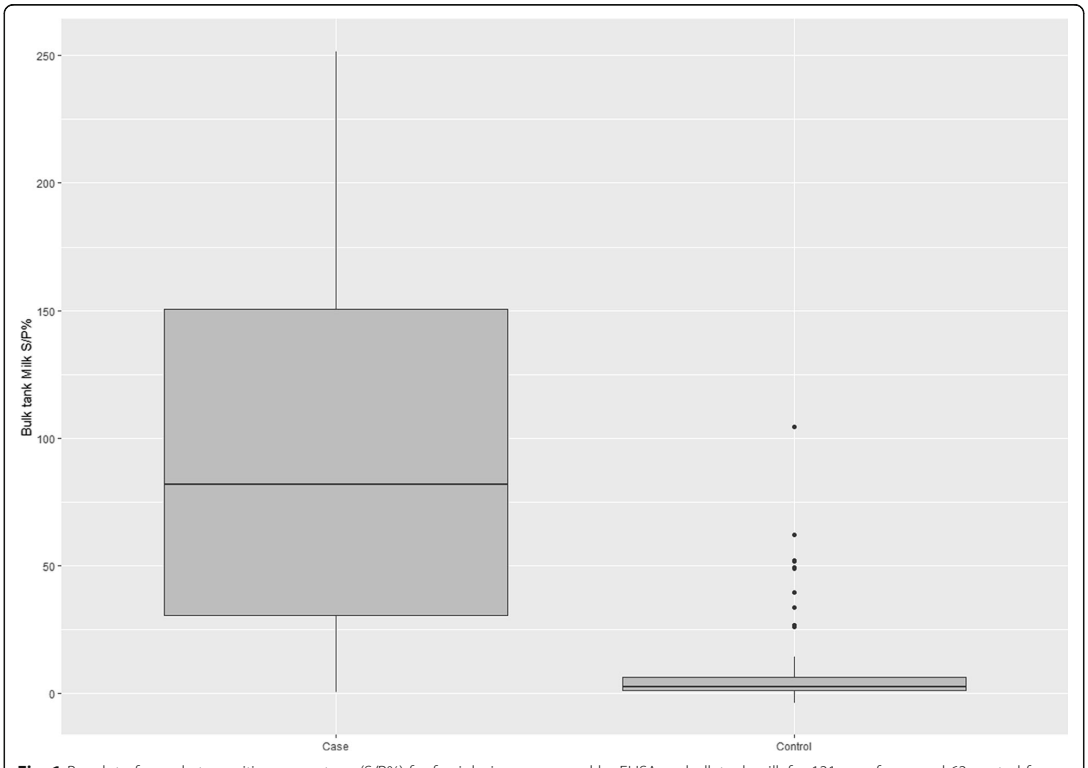

Fig. 1 Boxplot of sample to positive percentage (S/P%) for fasciolosis as measured by ELISA on bulk tank milk for 131 case farms and 63 control farms

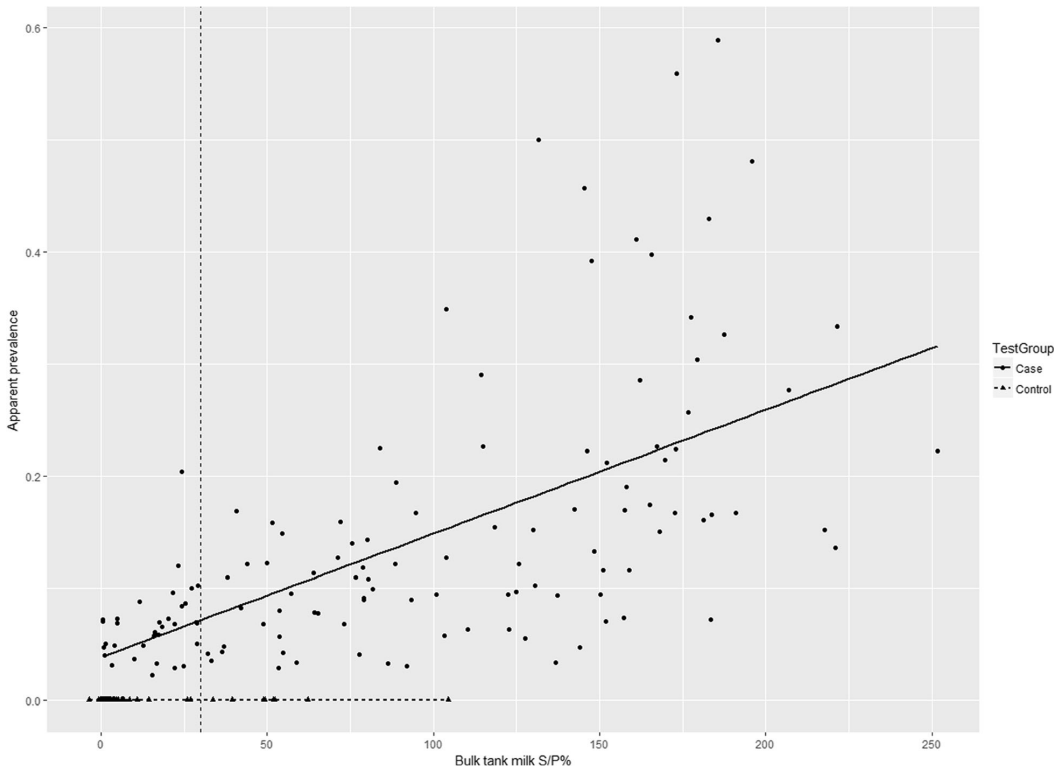

Fig. 2 The relationship between apparent prevalence and sample to positive percentage (S/P%) for fasciolosis as measured by ELISA on bulk tank milk for 131 case farms (*dots*) and 63 control farms (*triangles*). Apparent prevalence is measured by dividing the total number of condemned livers by the number of slaughtered animals in 2013. The dashed line shows the cut-off value for the used commercial ELISA kit (S/P% = 30), and the solid and dotted lines show the lines of best fit for case and control farms, respectively (note that the latter group are defined as apparent prevalence of 0)

One potential criticism of risk factor analyses based on simple classifications is that they do not incorporate diagnostic test sensitivity and specificity when classifying the farms as case or control [27, 46]. In this study, incorporating the relevant diagnostic test characteristics did not result in any of the control farms being reclassified as case farms, indicating that imperfect sensitivity of liver condemnation was not an issue for our dataset. This is likely to be a result of the relatively stringent case definition criteria that we applied (a minimum of three incidents of liver condemnation due to liver flukes out of a minimum of 50 slaughtered animals). However, there were a relatively large number of farms that were re-classified from case farms to control farms based on imperfect specificity (Additional file 2). This highlights the potential difficulties associated with assuming perfect specificity of liver condemnation as a test for liver fluke, but ultimately did not qualitatively affect the inference made from the risk factor study. We also note the relatively large number of additional parameter assumptions that are required in order to account for imperfect diagnostic tests, which has the disadvantage of increased complexity and therefore reduced transparency.

Comparison of liver condemnation data and BTM ELISA results

The comparison of the two diagnostic methods for fasciolosis showed only moderate agreement, which is in line with other previous reports [10, 25, 26, 47]. BTM ELISA requires a minimum level of antibodies in milk for detection and thus farms with low prevalence or intensity amongst lactating cows are likely to be misclassified as negative. Our results are consistent with Duscher et al. [25] in that the highest apparent prevalence for the case farms with negative ELISA result was approximately 20%. There were, however, many farms with positive ELISA results, despite their low apparent prevalence (< 20%). This was probably because the current study used apparent prevalence calculated as the number of positives at slaughter divided by the total

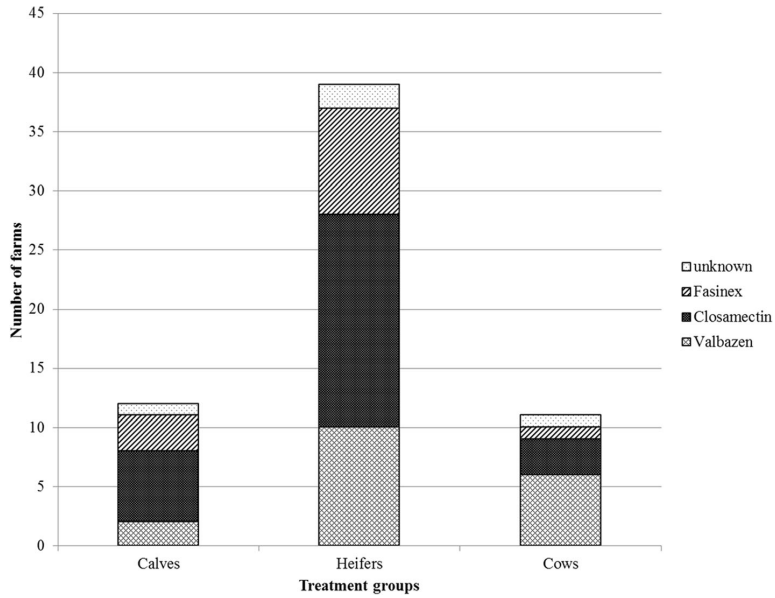

Fig. 3 The different anthelmintic products [Closamectin pour-on® (closantel and ivermectin, Biovet Aps), Valbazen® (albendazole, Orion Pharma Animal Health), Fasinex® (triclabendazole, Novartis)] that were reported for use against liver flukes in different age groups, based on 39 farms that reported giving treatments against liver flukes in 2013

number of slaughtered (all age groups), and therefore it most likely did not accurately reflect the prevalence within the milking herd. Nonetheless, the observed detection limit of BTM ELISA is probably of little concern in terms of using BTM ELISA as a herd health monitoring tool, as a herd prevalence of > 25% is considered the economic threshold (subclinical infections affecting productivity) for anthelmintic treatment against fasciolosis [48]. Continuous monitoring of fasciolosis status by BTM ELISA in Irish dairy farms successfully showed the effect of flukicide treatment [23], and therefore BTM ELISA will be a useful monitoring and decision-support tool for fasciolosis control programs in Denmark. Further studies should investigate how often BTM samples should be obtained for analysis, in order to have a cost-effective monitoring system.

Another possible explanation for case farms to have ELISA negative results could be due to delay in our BTM analysis, as BTM was collected at the end of the housing season in 2013–2014, while the liver condemnation data was only registered until the end of 2013. If most of the positive animals were slaughtered in early 2013, then the farm could have low *F. hepatica* antibody levels in 2014. Finally, inspection of the liver at slaughter may produce false positive results due to chronic pathological changes in animals that eliminated the infection and have low antibody levels, as the liver is condemned based on pathological changes seen in the liver. Mazeri et al. [28] showed the specificity of the routine liver inspection at slaughter as 88% and no parasites were found from some livers classified as having active or historic lesions due to fasciolosis. The exact time required for the recovery of the liver lesions, i.e. no visible lesions, is unknown. However, it perhaps depends on the level of infection and pathological changes may persist even after effective treatment [49].

The control farms were defined as having no livers condemned for a period of 3 years to reduce the risk of false negatives, but eight (12%) control farms showed positive by BTM ELISA. It is possible that these eight farms were truly infected, and that imperfect sensitivity of meat inspection resulted in early and low grade infections being missed [27, 28, 47]. However, a more likely reason for at least three of those farms is that introduced animals were infected, which gave rise to high antibody levels. This conclusion is supported by the fact that no control farms were reclassified as case farms after incorporating the estimated sensitivity of liver condemnation. Another potential explanation is that it is possible for infection to have occurred in the farms for the first time during the last half of 2013; animals slaughtered in 2013 would then show no sign of fasciolosis, but BTM ELISA could show positive a few months later. Finally, false positives due to test cross-reactivity with other

parasite species such as rumen fluke is a possibility [28], although this is quite unlikely with the particular ELISA test kit used [29].

Information regarding liver condemnation and anthelmintic use on the farms

The questionnaire responses demonstrate that most farmers were aware of their fasciolosis status, based mostly on feedback from the abattoirs, although seven control farms recalled liver condemnation that was not recorded in the data. This information could have been provided by small local abattoirs that were not recorded in the national database, but a more likely explanation is that recalled information is unreliable. In addition, farmers and veterinarians would underestimate the extent of fasciolosis in their farms if basing their diagnoses solely on notifications of liver condemnation from abattoirs. Relatively few case farmers were treating against fasciolosis, and there was a general lack of diagnostics to identify the affected group of cattle in which to target interventions and treatments, indicating that the current treatment regimens may be sub-optimal.

Conclusions

Heifers grazing on wet areas as well as dry cows grazing on wet areas were found to be significant risk factors for fasciolosis based on farm classifications using both liver condemnation and BTM ELISA diagnostics. Moderate agreement between the two diagnostic methods was found, which highlights the different properties and target populations of the tests. Overall, our results suggest that assessment of infection status using BTM ELISA supported by liver condemnation recordings will help to identify farms in need of treatment, and that focusing on the management of heifers and dry cows through grazing and appropriate anthelmintic treatment will improve the control of bovine fasciolosis in Denmark.

Additional files

Additional file 1: Table S1. The questionnaire (mostly related to grazing management and anthelmintic use) as given to 194 farmers for this study. (DOCX 17 kb)

Additional file 2: Text. Description of the method and discussion of additional models developed using a Bayesian re-classification procedure. **Table S2.** The priors used for re-classification of the farms. **Table S3.** The multivariable logistic regression model (with risk factors selected using AIC) for the reclassified model taking into account imperfect diagnostic test characteristics, as well as 1000 samples from bootstrapped fits taking into account uncertainty in the true values of these parameters [50]. (DOCX 42 kb)

Abbreviations

BTM: Bulk tank milk; CI: Confidence interval; DCD: Danish cattle database; ELISA: Enzyme-linked immunosorbant assay; OR: Odds ratio; S/P%: Sample to positive percentage

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Availability of data and materials

The datasets generated and/or analysed during the current study are not publicly available due it containing private information but are available from the corresponding author on reasonable request.

Authors' contributions

TH, ER, JB, HLE and SMT designed the study. TAH performed BTM ELISA. HLE and SMT coordinated telephone interviews. NTS and MD performed data management and statistical analyses. NTS, MD, HLE and SMT interpreted results. NTS wrote the manuscript and all other authors assisted with the revision. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Consent to participate in the study was acquired during the phone interview.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Supplementary material to Paper I

Additional file 1. Table S1. The questionnaire (mostly related to grazing management and anthelmintic use) as given to 194 farmers for this study.

Type of herd and animal, knowledge on liver condemnation	Variable type
Herd type	Nominal (Organic or conventional)
Concurrent beef production	Nominal (yes or no)
Breed	Nominal (Danish Holstein, Jersey, Danish red Holstein, Danish Red Cattle, Cross, other)
Animal sent to slaughter in 2013	Nominal (yes or no)
Liver condemnation in 2013	Nominal (yes, no, unknown)
Diagnosis of liver fluke otherwise (by vet or consultants)	Nominal (yes, no)
Grazing management and anthelmintic use	
Which animals were on pasture in 2013*	Nominal (None#, lactating cows, dry cows, calves, heifers, steer/bulls)
Age of calves when first come out on grass	Scale (age in month)
Turn-out month in 2013	Date (month)
Housing month in 2013	Date (month)
Daily grazing time of cows in 2013	Ordinal (24 h, >6h, <6h)
Pasture type where animals were grazed – for each group (lactating cows, dry cows, heifers, calves, steer/bulls)	Nominal (grass in crop rotation, dry permanent grass, wet permanent grass)
Any prevention for liver flukes on pasture*	Nominal (no, drainage, fencing of waterways, fencing of wet areas, move animals in late summer, other)
Drinking source for calves and heifers on grass*	Nominal (automatic waterbowl (tapwater), water trough, groundwater pump, waterways/pond/lake)
a) Anthelmintic treatment against liver fluke to calves, heifers or cows in 2013	Nominal (yes or no)
b) Anthelmintic product used*	
- for each group (calves, heifers, cows)	Nominal (nothing in this group, Valbazen®, Closamectin pour-on®, Bimectin plus®, Fasinex®, unknown, other)
c) Anthelmintic treatment regimen	
– for each group (calves, heifers, cows)	Nominal (only diseased, prevention/routine)
a) Anthelmintic treatment against gastrointestinal worms (GIN) or lungworm in 2013	Nominal (yes or no)
b) GIN or lungworm*	
- for each group (calves, heifers, cows)	Nominal (gastrointestinal nematodes, lungworms)
17. Grazing condition	
Calves graze with animals from other farms	Nominal (yes or no)
Heifers graze with animals from other farms	Nominal (yes or no)
Cows graze with calves	Nominal (yes or no)
Cows graze with heifers	Nominal (yes or no)
18. Animal purchase in 2013	
Calves	Nominal (yes or no)
Heifers	Nominal (yes or no)
Cows	Nominal (yes or no)

*multiple answers were allowed.
#Proceed straight to question 15 with this answer.

Additional file 2. Text

Background

The dataset presented in the main manuscript uses simple case and control definitions based on observed cases of liver condemnation at slaughter, along with a set of criteria for minimum number of slaughtered animals to try and minimise the number of infected farms for which liver fluke infections were not detected at slaughter. However, there remains a possibility for misclassification despite these precautions. A more complex case/control classification system was therefore applied to the same farms, and the sensitivity of the odds ratios for the same set of risk factors to the change in farm classifications was assessed. This system uses a Bayesian re-classification procedure based on the posterior probability that each farm is truly infected with *F. hepatica* conditional on the observed slaughter data and bulk tank milk (BTM) test data, and a series of parameter values relating to diagnostic test characteristics. Minimum, maximum and most likely values were obtained for each of these priors based on the literature, and are summarised in Table S2.

Methods

Calculation of the posterior

The posterior probability that each farm is a true case was first calculated conditional on the observed BTM data, dichotomised using a cut-off value given by the manufacturer. The probability that each farm is truly positive given a positive BTM result $\mathrm{P}(case \mid b_{pos})$ or a negative BTM result $\mathrm{P}(case \mid b_{neg})$ was calculated according to Bayes' theorem as:

$$\mathrm{P}(case \mid b_{pos}) = \frac{Se_{btm} \cdot \mathrm{P}(case)}{(Se_{btm} \cdot \mathrm{P}(case)) + ((1 - Sp_{btm}) \cdot (1 - \mathrm{P}(case)))}$$

$$\mathrm{P}(case \mid b_{neg}) = \frac{Sp_{btm} \cdot (1 - \mathrm{P}(case))}{(Sp_{btm} \cdot (1 - \mathrm{P}(case))) + ((1 - Se_{btm}) \cdot \mathrm{P}(case))}$$

Where Se_{btm} and Sp_{btm} are sensitivity and specificity of bulk tank test, and P(*case)* denotes the prior probability of each farm being a case. Note that this prior probability should not be based on the expected national farm-level prevalence because the farms were not randomly chosen for inclusion in the study.

The posterior probabilities given above were then used as prior probabilities for a further calculation based on Bayes' theorem in order to take account of the observed slaughter data for each farm. The overall posterior probability of each farm *i* being a true case conditional on the observed BTM and slaughter data is given by:

$$\mathrm{P}(case_i \mid b_i, c_i, s_i) = \frac{\mathrm{P}(c_i \mid s_i, case) \cdot \mathrm{P}(case \mid b_i)}{\mathrm{P}(c_i \mid s_i, case) \cdot \mathrm{P}(case \mid b_i) + \mathrm{P}(c_i \mid s_i, \neg case) \cdot \mathrm{P}(\neg case \mid b_i)}$$

Where b_i is equal to b_{pos} or b_{neg} above depending on the BTM result for farm *i,* and c_i & s_i denote the number of animals with liver condemnations and the total number slaughter for farm *i*, respectively. The probability of observing *c* condemnations out of *s* slaughtered animals was calculated according to the Binomial distribution (conditionally on each farm being a true case and a true control) as follows:

$$\mathrm{P}(c_i \mid s_i, case) = \binom{s_i}{c_i} \cdot {p_i}^{c_i} \cdot (1 - p_i)^{s_i - c_i}$$

$$\mathrm{P}(c_i \mid s_i, \neg case) = \binom{s_i}{c_i} \cdot (1 - Sp_{sl})^{c_i} \cdot {Sp_{sl}}^{s_i - c_i}$$

Where $p_i = Se_{sl} \cdot prev + (1 - Sp_{sl}) \cdot (1 - prev)$ represents the probability of a randomly chosen animal from an infected herd testing positive for liver condemnation, Se_{sl} & Sp_{sl} denote the sensitivity and specificity of liver condemnation as a test for liver fluke, and *prev* denotes the expected within-herd prevalence of liver fluke within a true case herd.

Reclassified model

The procedure given above was used along with the most likely parameter values identified in Table S2 in order to generate posterior probabilities of each farm being a true case conditional on the observed test results for that farm. Each farm was then reclassified as a case or control based on this posterior probability with a threshold of 50%, and the same variable selection procedure as described for the logistic regression model in the main text was applied to this data in order to obtain a final set of risk factors.

Bootstrapped model

A multiple imputation procedure was used in order to account for uncertainty in the estimated diagnostic test parameters taken from the literature. Rather than using the 'most likely' parameter values indicated in Table S2, values for each parameter were randomly chosen from a triangle distribution with given lower limit, upper limit and mode using the *triangle* package in R [51]. Posterior probabilities for each farm were then re-calculated, and the case/control classifications were stochastically re-allocated using these probabilities. The same model with final set of risk factors as identified above was then refitted to the new data. Finally, a parametric bootstrap procedure was used to simulate a single bootstrap dataset, and then the same model refitted to the bootstrapped data set in order to obtain a set of coefficient estimates taking into account uncertainty in the coefficient estimates for the reclassified data for this set of parameter values. This procedure was repeated 1000 times in order to obtain a Monte Carlo approximation of the mean and 95% confidence intervals for each risk factor taking into account uncertainty in both the diagnostic test related parameter values and coefficients estimated from the fitted models.

Results

Reclassified model

Following the reclassification according to the Bayesian posterior probabilities of each farm being a true case, 36 apparent case farms were reclassified as true control farms but all apparent control farms remained as true control farms. Therefore, the more complex classification system yielded a total of 95 true case farms and 99 true control farms, and it can be concluded that the simpler classification system was likely subject to some misclassification bias. Model selection based on AIC suggested to include the following risk factors: grazing of heifers on wet areas with access to surface water (OR = 10.35, 95% CI: 3.30–40.45), grazing of heifers on wet areas without access to surface water (OR = 5.36, 95% CI: 1.60–21.80), grazing of dry cows on wet areas (OR = 3.82, 95% CI: 1.53–10.14), and beef production (OR = 2.98, 95% CI: 1.06–9.58) (Table S2).

Bootstrapped model

The multiple imputation procedure yielded mean coefficient estimates that were qualitatively similar to those obtained from the reclassified model, but wider 95% confidence intervals as a result of including additional uncertainty as regards to the diagnostic test parameters. It is not possible to calculate *P*-values using this procedure, but the lower 95% confidence intervals were above zero for the same coefficients as for the reclassified model except for beef production.

Discussion

The results of the reclassified and bootstrapped models are qualitatively highly consistent with those of the simpler models presented in the main text, except for a change in the model intercept caused by reducing misclassification bias. In particular, heifers grazing on wet areas and dry cows grazing on wet areas can be concluded to be the key risk factors associated with bovine fasciolosis in Denmark. The reclassified model more closely resembles the simpler case vs control model than the BTM model, which is a result of the relatively large contribution of the slaughter data with multiple tests per farm relative to the single BTM test per farm. This highlights one of the difficulties with transparency in interpreting models based on more complex classifications such as these.

The potential strength of the more complex method is the ability to incorporate uncertainties in the true status of animals and farms resulting from imperfect diagnostic test characteristics. However, the method does require the use of a number of assumptions regarding expected on-farm prevalence, sensitivity and specificity that cannot be estimated or verified using our data. Estimates for these parameters were sourced from the literature, but the studies from which these were taken may not necessarily reflect the same conditions as in Denmark. There are also discrepancies between apparently similar parameter values reported by different studies: for example Mazeri et al. [28] estimate the specificity of slaughter inspection for liver fluke to be well below the level of 100% that was assumed by Rapsch et al. [27], and consequently produce quite different estimates for sensitivity of the same test. Methodologically Mazeri et al. [28] is likely to be more robust because of not assuming 100% specificity, but the study was in beef animals in Scotland which cannot be exactly extrapolated to dairy cattle in Denmark. We therefore used estimates mostly from Rapsch et al. [27], but with a compromise to the specificity of the slaughter test to reflect the findings of Mazeri et al. [28]. Alternative parameter values may be more justifiable to other practitioners. Further assumptions are required for the within-farm prevalence on true case farms, which has not been reported in Denmark and is likely also possibly variable between farms. However, ad-hoc sensitivity analysis indicated that the key model inference was robust to moderate changes of these parameter values.

Our efforts to correct for imperfect diagnostic test sensitivity and specificity within the simple case/control definitions have yielded almost entirely the same inference as when simply ignoring the possible misclassification bias. Only the model intercept was altered, but a reduction in the bias

for the intercept is of little value because case control studies such as this cannot be used to estimate prevalence or absolute risks in any case. The major downside of this approach is the over-reliance on externally sourced parameter values in generating the case/control classifications relative to simpler procedures. We therefore conclude that although it is important to consider the potential effect of misclassification when interpreting results from case/control studies, attempting to control explicitly for imperfect diagnostic test sensitivity and specificity does not necessarily lead to more robust inference.

Additional file 2. Table S2. The priors used for re-classification of the farms.

Priors	Most likely estimate (lower and upper bound)	Justification
The prior probability of disease for our population (P($case$))	0.5 (0.4–0.6)	Based on the study selection criteria that intended to recruit approximately equal numbers of cases and controls
Sensitivity of bulk tank test (Se_{btm})	0.86 (0.73–0.99)	As estimated by [24]
Specificity of bulk tank test (Sp_{btm})	0.85 (0.72–0.99)	As estimated by [24]
Sensitivity of slaughter test for an infected animal (Se_{sl})	0.632 (0.556–0.706)	As estimated by [27]
Specificity of slaughter test for a non-infected animal (Sp_{sl})	0.975 (0.95–1.00)	Relaxation of the assumption of 100% made by [27] to account for occasional false positives due to condemnation resulting from liver disease of unrelated etiology
The within-herd prevalence of fluke on an infected farm ($prev$)	0.15 (0.10–0.20)	Crude estimate based on the Danish national slaughter data by [11]

Additional file 2. Table S3. The multivariable logistic regression model (with risk factors selected using AIC) for the reclassified model taking into account imperfect diagnostic test characteristics, as well as 1000 samples from bootstrapped fits taking into account uncertainty in the true values of these parameters.

Variable	Level	Reclassified model			Bootstrapped model	
		Estimate	95% CI	*P*-value	Estimate	95% CI
Intercept		-2.166	-3.433 – -1.187		-1.881	-3.734 – -0.637
Grazing of heifers (Not grazed, Dry grazing or Wet grazing) combined with access to surface water (No or Yes)	Not grazed	Ref		<0.001	Ref	
	Dry & Yes	-0.128	-3.209 – 2.032		-0.378	-16.58– 2.023
	Dry & No	0.078	-1.766 – 1.826		-0.183	-16.51 – 1.904
	Wet & Yes	2.337	1.194 – 3.700		2.177	0.936 – 3.944
	Wet & No	1.678	0.467 – 3.082		1.559	0.279 – 3.458
Grazing of dry cows (Not grazed, Dry grazing or Wet grazing)				0.014		
	Not grazed	Ref			Ref	
	Dry	0.429	-0.352 – 1.215		0.352	-0.545 – 1.282
	Wet	1.340	0.427 – 2.317		1.305	0.391 – 2.539
Beef production				0.038		
	No		Ref		Ref	
	Yes	1.092	0.061 – 2.260		1.094	-0.092 – 2.753

Abbreviations: 95% CI, 95% confidence interval; Ref, reference

8.2 Paper II

Patterns of *Fasciola hepatica* infection in Danish dairy cattle: implications for on-farm control of the parasite based on different diagnostic methods

Takeuchi-Storm, N.; Denwood, M.; Petersen, H.H.; Enemark, H.L.; Stensgaard, AS; Sengupta, M.E.; Beesley,N.J.; Hodgkinson, J.; Williams, D.; Thamsborg, S.M. Patterns of *Fasciola hepatica* infection in Danish dairy cattle: implications for on-farm control of the parasite based on different diagnostic methods. Parasit. Vectors. (in press)

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RESEARCH Open Access

Patterns of *Fasciola hepatica* infection in Danish dairy cattle: implications for on-farm control of the parasite based on different diagnostic methods

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Abstract

Background: Bovine fasciolosis is an economically important livestock disease in Europe, and represents a particular challenge for organic farms, where cattle are grazed extensively and the use of anthelmintic is limited. A two-year longitudinal study was conducted on two conventional and two organic Danish dairy farms to examine the current temporal trend of *F. hepatica* infection on-farm, and to gather data of practical relevance for parasite control. Data were collected both at the herd and individual level using currently available diagnostic methods: a commercial serum antibody ELISA, a commercial copro-antigen ELISA, faecal egg counts, and monthly bulk tank milk (BTM) ELISA. The temporal patterns (animal age, farm-level temporal trends and seasonality) in the animal-level test results were analysed by generalised additive mixed models (GAMM).

Results: Patterns of infection differed substantially between the farms, due to different grazing management and anthelmintic use. However, animals were first infected at the age of 1.5–2 years (heifers), and most at-risk animals sero-converted in autumn, suggesting that summer infections in snails prevail in Denmark. Our results also suggest that the lifespan of the parasite could be over 2 years, as several cows showed signs of low grade infection even after several years of continuous indoor housing without access to freshly-cut grass. The serum antibody ELISA was able to detect infection first, whereas both copro-antigen ELISA and faecal egg counts tended to increase in the same animals at a later point. Decreasing BTM antibody levels were seen on the two farms that started anthelmintic treatment during the study.

Conclusions: While important differences between farms and over time were seen due to varying grazing management, anthelmintic treatment and climatic conditions, the young stock was consistently seen as the main high-risk group and at least one farm also had suspected transmission (re-infection) within the lactating herd. Careful interpretation of test results is necessary for older cows as they can show persistent infections several years after exposure has stopped. Rigorous treatment regimens can reduce BTM ELISA values, but further research is needed to develop a non-medicinal approach for sustainable management of bovine fasciolosis.

Keywords: *Fasciola hepatica*, Transmission, Dairy, Diagnostic, ELISA, Denmark

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Background

The trematode *Fasciola hepatica* raises substantial concerns for the cattle industry due to reduced productivity, increased susceptibility to other diseases and interaction with diagnostic tests for bovine tuberculosis [1–4]. Despite efforts to develop a vaccine against the parasite, control of bovine fasciolosis still relies largely on preventive measures such as drainage, avoiding or fencing off snail habitats, and anthelmintic treatment [3, 5]. Increasing anthelmintic resistance [2, 6] further emphasise the importance of responsible and efficient use of anthelmintics, i.e. in combination with grazing management.

To successfully control fasciolosis on a dairy farm, it is crucial to identify which pasture is the source of infection. This is most often achieved by taking samples from representative groups of animals in different age groups grazing identified pastures, and analysing them either by faecal egg counts or by ELISA to detect antibodies in serum or milk, depending on which age group is tested [5]. However, careful interpretation of the results is needed, because *F. hepatica* infection is known to be seasonal, has a long prepatent period and each diagnostic test provides different information about the infection. Copro-antigen ELISA is a relatively new diagnostic technique that can detect infections at least five weeks after uptake [7, 8]. Yet, sensitivity and specificity vary depending on field conditions [9–12], and interpretation of copro-antigen ELISA results from the field is still unclear. Additionally, pasture can be examined for potential snail habitats (wet areas) to identify the source of infection [5], because transmission is unlikely to occur if snail habitats are absent on the pasture in question. In fact, presence of the intermediate host snails, *Galba truncatula*, has been described as the most significant factor in predicting the herd-level exposure for *F. hepatica* [13]. Identification of snail habitats and intermediate host snails, is thus an important part of on-farm fasciolosis control, although the procedure can be time-consuming and requires specialized taxonomical skills or molecular tools to correctly identify the *G. truncatula* snails [3].

Recent studies have suggested an altered transmission pattern of *F. hepatica*, both spatially and temporally, as a consequence of changing climatic conditions [14]. Extended geographical distribution and increased prevalence have already been observed in recent years in some parts of Europe, attributed to altered temperature and rainfall patterns [15–18]. Concerns over future impacts of climate change on the seasonality have also been raised; increased outbreaks due to winter infection and decreased summer infection are to be expected in bi-seasonal transmission areas [19]. Despite the increasing concerns, only a few studies have investigated the temporal patterns of *F. hepatica* infection in animals on individual farms in recent years [20, 21]. Transmission patterns were extensively studied in 1970s in Denmark, showing that winter infection occurred in some years, but the major part of the total fluke population could be ascribed to summer infection of the snails [22, 23]. Since then, no studies have been conducted in Denmark to assess if the transmission patterns have changed. In addition, change in transmission patterns may be attributed to a recent shift in production systems, i.e. an increase in organic production. In 2017, the number of organic cattle in Denmark was approx. 200,000, corresponding to ten times more than in 1995 [24]. Compared to Sweden, where all cattle have to graze regardless of whether they are organic or conventional [16], only organic farms are obliged to graze all stock in Denmark, and conventional farms with zero-grazing are not uncommon [25]. The parasitic challenge is greater in farms with outdoor access [26, 27] and the prevalence of *F. hepatica* is higher in organic than conventional farms in Denmark [17]. Additionally, the withdrawal period for veterinary medicines including anthelmintics are twice as long for organic farms [28] and minimum use of veterinary medicines is an important concept for organic producers [29]. Integrated control, e.g. by grazing management is therefore desirable [26], and updated knowledge about on-farm *F. hepatica* transmission is crucial for development of such control strategy. Moreover, a pragmatic approach is required for implementation of on-farm control strategies. Questions such as "can cattle get re-infected?", "how long do liver flukes live in cattle?" and "how long do the antibodies last after treatment?" are often asked by the cattle producers and veterinary practitioners, but are insufficiently addressed in the current literature.

The aim of this longitudinal observational study was to explore the temporal patterns of infection on four Danish dairy farms (conventional and organic) in terms of age groups, individual and herd-level diagnostic methods, and seasonality, including relative importance of summer and winter infection of snails. Each farm was examined extensively including grazing and treatment strategies to elucidate the similarities and differences of the transmission of *F. hepatica* due to varying farm-specific management. Ultimately, we aimed to generate data that can be translated into suggestions for improved practical and realistic guidelines for diagnosis and control of fasciolosis.

Methods

Farm selection and background

Potentially suitable study farms were identified from our previous study [25] in conjunction with SEGES (part of the Danish Agricultural Advisory Service run by the Danish Agriculture and Food Council) and Økologisk

landsforening (National Organic Association) based on likely farmer compliance and interest in participating in the study for the entire planned study period of 2015–2017. From these, two conventional (C1 and C2) and two organic (O1 and O2) farms were selected based on known infection status as judged by bulk tank milk (BTM) ELISA values and high levels of liver condemnation at slaughter during the period 2011–2014 (Table 1). Farm C1 was located on the Island of Zealand, while Farm C2, O1 and O2 were located within 30 km of one another in South Jutland (Fig. 1). Danish organic rules abide by Council Regulation (EC) No. 834/2007 of 28 June 2007 on organic production and labelling of organic products and repealing Regulation (EEC) No. 2092/91. All selected farms had all-year calving, and automatic milking systems were used on farms O2 and C2. Further farm-specific details are given below together with a simple schematic plot and Gantt chart for each farm (Figs. 2 and 3). The participating farmers were regularly updated with our findings and consultation meetings were held twice (halfway and end of the study period) together with their consultants and veterinary practitioners.

Farm C1

Calves are turned out when they are 5–9 months-old on a pasture away from the stall (Fig. 2 C1-D). Animals of 9 to 12 months of age are all grazed with larger heifers on pasture, which is located along a fjord with seawater (Fig. 2 C1-C). This pasture is shared with two beef farms with no previous anthelmintic treatment for liver fluke. The heifers are divided into five different groups based on age, and every five weeks during the grazing season, one group at a time is housed for insemination and given ectoparasitic treatments (Noromectin® pour-on, Biovet ApS, Denmark and Butox® 7.5% Pour-on, MSD Animal Health A/S, Denmark). This means that some heifers graze only for five weeks, while some others may graze for the whole grazing season. The longest grazing period for the heifers during the study period was mid-May to mid-November. Dry cows graze from 6 weeks before calving on a dry, high-lying (high elevation) pasture near the farm house (Fig. 2 C1-B). The area is available for grazing from May to November. Milking cows are housed in a deep litter stall with access to pasture 24 hours a day and all year around. The pastures for milking cows are found on terrain that has a slight slope towards a drainage canal that cannot be accessed by the animals (Fig. 2 C1-A). The milking cows are furthermore prevented from having access to a fenced-off waterhole in this paddock. No treatments for liver fluke were given during the study period, but triclabendazole (Tribex 10%® ScanVet Animal Health A/S, Denmark) was given at housing to all young animals that grazed on the fjord pasture (Fig. 2 C1-C) in 2017. Animals are mostly Danish Holsteins (DH) with some cross-breeds.

Farm C2

Heifers are the only grazing animals on this farm (i.e. all milking cows are housed year round). The pasture for heifers is on wet, low ground with a central peat bog (Fig. 2 C2-A). Dry cows utilise a sandy exercise yard

Table 1 Summary of data used as inclusion criteria for the 4 farms in the study

Farm	Year	No. of heifers	No. of cows	Total no. of cattle	Liver condemnation (%)	BTM ELISA value (S/P%)[a]
C1	2011	72.5	176.5	314	6.2	–
	2012	65.5	184.5	303	21.3	–
	2013	65	187.5	315	30.0	–
	2014	63	183.5	312	18.6	179.3
C2	2011	103	135	292	8.3	–
	2012	98.5	145	300	11.9	–
	2013	105.5	144	314	16.1	–
	2014	111	149	331	19.4	181.2
O1	2011	145	172	367	2.6	–
	2012	141	168	362	7.6	–
	2013	124	174.5	354	33.3	–
	2014	172	183	425	23.3	221.4
O2	2011	90.5	113.5	251	18.1	–
	2012	97.5	124	275	32.6	–
	2013	111	131.5	282	27.7	–
	2014	113	133.5	285	38.1	206.9

[a]by IDEXX ELISA test (cut-off is 30 and ≥ 150S/P% is considered high)

f1.1 f1.2 **Fig. 1** Map of Denmark, showing the regions and locations of the four farms that participated in the study

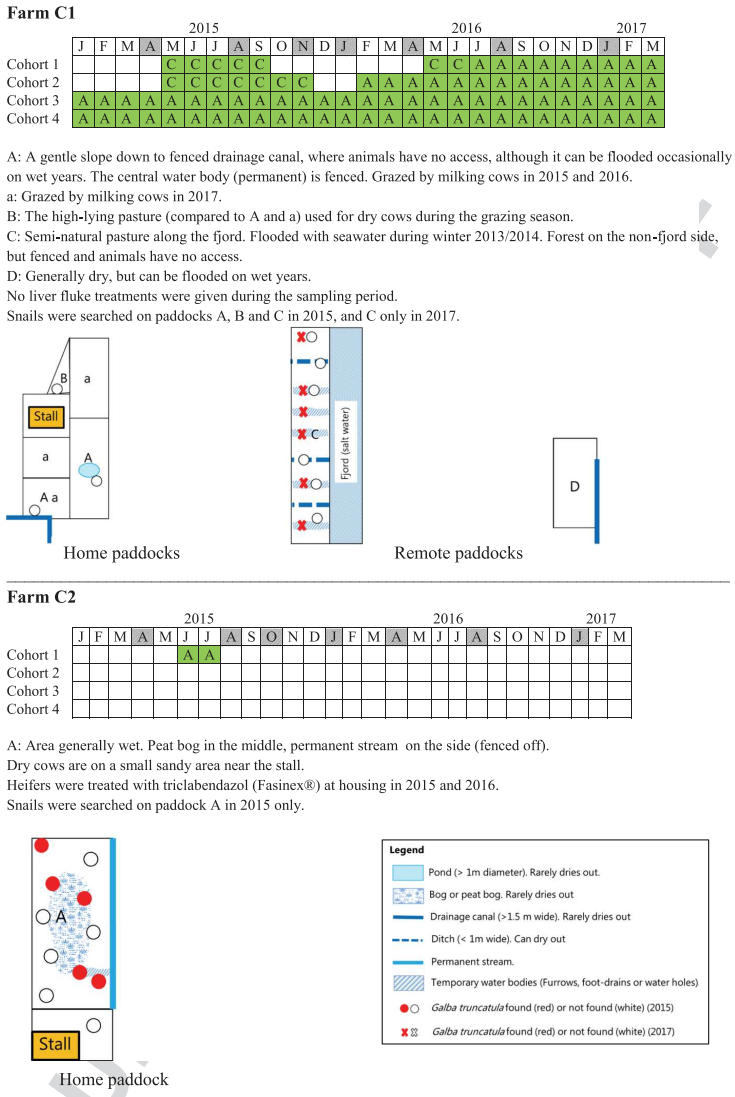
near the stall, which is not considered to be suitable snail habitat because it is consistently dry. Before commencement of our study, the farmer treated only some of the heifers with triclabendazole (Fasinex240®, Elanco, Denmark) every November. However, this management changed part-way through the study (in 2015) so that all heifers in the first and second trimester were routinely treated with triclabendazole following housing. The grazing period for heifers is typically early June to mid-October, although 90% of animals were housed in late July 2015 due to low feed availability. Most animals are DH and the rest are cross-breeds.

Farm O1

The calves are turned out when they are 4 months-old on a dry permanent pasture with access to a stall close to the farm house (Fig. 3 O1-C). They have access to feed *ad libitum*. Young heifers are grazed in two separate areas away from the farm house (Fig. 3 O1-D and E). Heifers to be inseminated graze together with dry cows close to the farm house on a wet pasture (Fig. 3 O1-B). They are fed once a day in the stall. Once pregnant, heifers are moved to a pasture on reclaimed marshland (freshwater meadows) (Fig. 3 O1-F). During winter, this pasture is grazed by sheep who are treated twice yearly with triclabendazole. Milking cows graze around the farm house rotationally, and some of these pastures can be very wet depending on weather and season (Fig. 3. O1-A). Albendazole (Valbazen®, Orion Pharma Animal Health A/S, Denmark; unknown dosage) was applied to a few selected heifers due to sub-optimal weight gain during 2015. Six treated animals were included at the first sampling, but high copro-antigen levels and faecal egg counts were observed in these animals. The treatment dosage was therefore assumed to be targeted against nematodes rather than liver fluke. The grazing period during our study was early April to late November. Most animals on the farm are cross-breeds mainly with DH, and the rest are Danish red and DH.

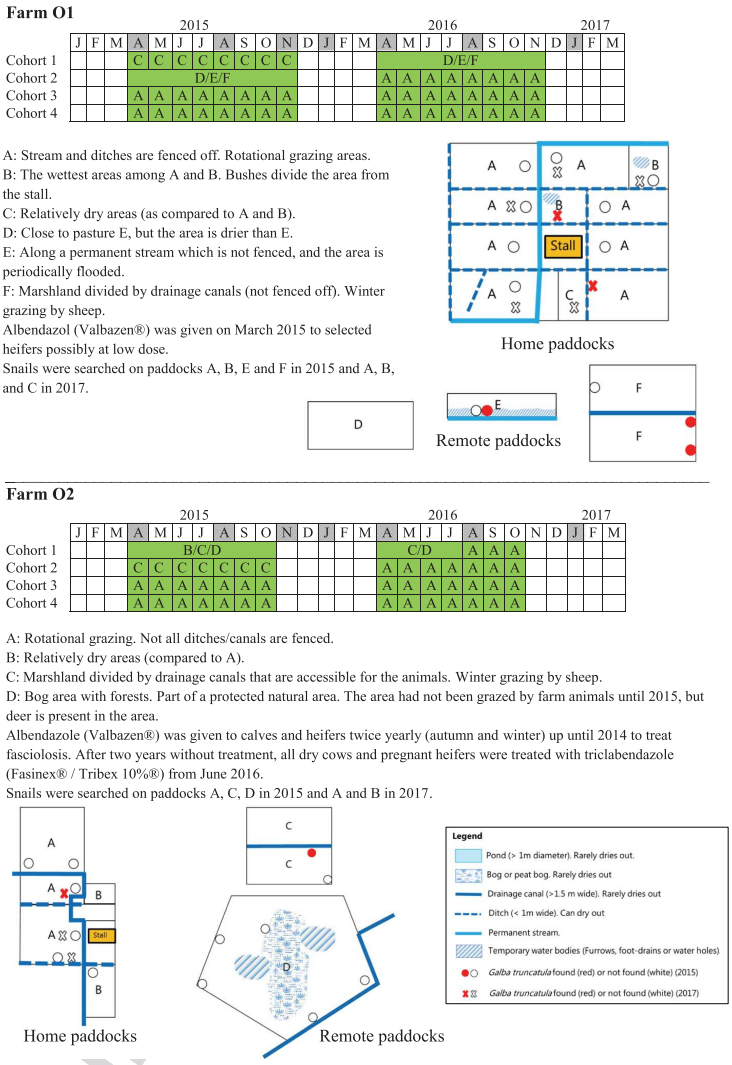
Farm O2

Calves are turned out on pasture near the farm house at 4 months-old (Fig. 3 O2-B). Older calves and heifers are grazed in two separate areas (Fig. 3 O2-C and D). One



f2.1 **Fig. 2** Schematic map and Gantt chart of grazing periods (grey shaded, time of sampling; green shaded, grazing; pasture areas are indicated by
f2.2 capital letters), pasture characteristics (refer to the common map legend) and treatment against *Fasciola hepatica* on farms C1 and C2, 2015–2017
f2.3

area is a reclaimed marshland (freshwater meadow), where sheep graze during winter (Fig. 3 O2-C) and are treated as above (O1). The other area is a bog, which is part of a protected natural area with forests and in which red deer (*Cervus elephus*) and roe deer (*Capreolus capreolus*) are regularly observed (Fig. 3 O2-D). This bog area was granted to the farmer for grazing from 2015; the area had not been grazed by farm animals previously. Milking cows graze around the farm house in pastures which are rotated between years (Fig. 3 O2-A). Some of these pastures are low-lying and consistently wet. Albendazole was given to calves and heifers twice yearly (autumn and winter) up until 2014 to treat fasciolosis. After two years of no treatment and following the results of the first four sampling events,



f3.1 **Fig. 3** Schematic map and Gantt chart of grazing periods (grey shaded, time of sampling; green shaded, grazing; pasture areas are indicated by
f3.2 capital letters), pasture characteristics (refer to the common map legend) and treatment against *Fasciola hepatica* on farms O1 and
f3.3 O2, 2015–2017
f3.4

the farmer started treating all dry cows and pregnant heifers with triclabendazole from June 2016. The grazing period during the study was mid-April to mid-October. Most animals on the farm are DH and the rest are cross-breeds.

On-farm animal sampling and other data sources

Each farm was visited seven times at the following time points: turn-out (spring) 2015, summer 2015, housing (autumn) 2015, winter 2015/2016, turn-out (spring) 2016, summer 2016, and winter 2016/2017 (Figs. 2 and 3). At the first sampling event, animals were enrolled into the study within four age groups as follows: calves with a first grazing season in 2015, heifers that had first grazed in 2014, primiparous cows, and multiparous cows. These animals were selected randomly at farm O1, but on the other farms animals were selected

for convenience by the farmers. At each time point, blood and faecal samples were collected from each cohort of animals. If an enrolled animal was slaughtered during the study, it was replaced by another animal within the same age group at the next sampling time point. In total, 229 individual animals were sampled, equating to 1078 faecal samples that were analysed by serum ELISA, 1170 by copro-antigen ELISA, and 1172 by sedimentation, respectively (Table 2). Of these, 39 animals (12 and 27 animals from farms C2 and O2, respectively) were treated with triclabendazole during the study period. Blood samples from primiparous and multiparous cows were not taken on the first visit due to logistical difficulties. The summer samples from calves and heifers from O2 were not taken due to lack of safe handling facilities on pasture.

Blood samples were centrifuged at 1450 g for 10 min within 24 h of collection and serum was stored at -20 °C until analysis. Additionally, BTM collected as part of the mandatory milk control scheme laid by the Ministry of Food, Agriculture and Fisheries in accordance with EU regulation on hygiene of food stuff (EC No. 853/2004) was stored frozen once a month at a commercial laboratory and periodically (every 6 to 12 months) forwarded by courier to our laboratory. BTM were centrifuged at 1000g for 20 min to separate the fat and the whey was kept at -20 °C until analysis.

In addition to the on-farm data, register data regarding birth date, calving dates, lactation number, liver condemnation at slaughter of each animal present on the study herds during 2014–2016 was extracted from the Danish Cattle Database (DCD). Furthermore, treatment history over the period 2014–2016 for the relevant farms was extracted from the Danish centralised register for sales of veterinary medicines (VetStat, The Danish Veterinary and Food Administration (DVFA), Ministry of Environment and Food) and from the farmer's own paper-based records where necessary. Monthly climate data for the period 2015–2017 as well as the 30-year average air temperature and precipitation records over the period 1960–1990 were obtained from the online archive of the Danish Meteorological Institute [30].

Diagnostic tests

Faecal egg count (FEC) by sedimentation

Five-gram faecal samples were examined by sedimentation technique for presence of trematode eggs [31]. According to Rapsch et al. [32], this technique has a sensitivity and a specificity of 69% and 98%, respectively, when 10 g faecal samples are analysed.

Serum and bulk tank milk ELISA

Anti-*F. hepatica* antibody levels were assessed in individual serum samples and monthly BTM by a commercial ELISA kit (IDEXX *Fasciola* verification test®, IDEXX Laboratories, Hoofddorp, the Netherlands) in duplicate according to the manufacturer's instructions. The results were expressed as sample to positive ratio (S/P%), and it was considered positive if the average of the duplicates was S/P% > 30 (following the manufacturer's recommendations).

Table 2 Summary of the number of animals sampled at each time point

		Spring 2015	Summer 2015	Autumn 2015	Winter 2015/16	Spring 2016	Summer 2016	Winter 2016/17
C1	Cohort 1	11	11	11	11	11	11	8
	Cohort 2	11	11	11	11	11	11	10
	Cohort 3	11[a]	11	11	11	11	11	9
	Cohort 4	11[a]	11	11	11	11	11	9
C2	Cohort 1	11	11	10	10	13	13	13
	Cohort 2	11	11	11	11	12	10	8
	Cohort 3	11[a]	10	10	10	9	9	6
	Cohort 4	13[a]	12	13	12	8	7	6
O1	Cohort 1	11	11	11	11	12	12	11
	Cohort 2	11	11	11	12	11	10	9
	Cohort 3	11[a]	11	10	10	10	10	8
	Cohort 4	11[a]	11	11	11	11	10	8
O2	Cohort 1	11	2[b]	11	11	11	8	8
	Cohort 2	11	8[b]	12	12	12	14	13
	Cohort 3	11[a]	9	10	10	10	11	8
	Cohort 4	11[a]	13	12	12	12	8	8

[a]Blood samples from primiparous and multiparous cows were not taken on the first visit due to logistic reasons

[b]The summer samples from calves and heifers from O2 were not taken due to lack of safe handling facilities on pasture

The reported sensitivity and specificity of this commercial test using bovine sera are 88–98%, and 84–98%, respectively [12, 32, 33].

Copro-antigen ELISA

Two grams of faecal samples were frozen at -20 °C until analysis by a commercial ELISA kit (Bio K210, Bio-X Diagnostics, Rochefort, Belgium). The procedure followed manufacturer's instructions with the following modifications. The dilution buffer was added to defrosted faecal samples and kept at 5 °C overnight [10]. Incubation with tetramethylbenzidine (TMB) chromagen was extended from 10 to 30 min in order to improve the discrimination between positive and negative samples. Each sample was tested in duplicates and faecal samples from five, 1–3 month-old in-door reared calves from a conventional Danish dairy farm were pooled and included as negative faeces control in each plate. The ELISA results were expressed as ELISA unit (EU). The sample was considered positive if the average EU of duplicates was equal to or above the custom cut-off value (1.89 EU) calculated as the mean EU of all negative faeces controls plus 3-fold standard deviation of the mean. The reported sensitivity and specificity of this test are 77–87% and 99%, respectively [10, 11].

Analysis of longitudinal data

ELISA results were calculated in Microsoft Excel 2010 (Version 14.0) and all diagnostic test data were then imported into R [34] and merged with data from DCD using the animals' unique identification numbers. Graphic visualisations of the raw data for each animal along with monthly trends in BTM results were made using the *ggplot2* package [35]. Samples taken after anthelmintic treatment were omitted from the dataset used for drawing graphs. Correlations between the average serum antibody levels of all milking cows and the antibody levels in the BTM taken closest to the sampling date (within 1–24 days) were quantified by Pearson's correlation coefficient.

The associations between observed individual-animal results and the age of the animals, seasonality, and longer-term temporal trends at the time of sampling were estimated using a generalised additive mixed model (GAMM) implemented using the *mgcv* package [36]. This statistical modelling method allows smoothed spline functions to be fit to linear predictors without imposing any predetermined form on the relationship, and therefore allows the relationship between the diagnostic test result and each of the linear predictors given above to be estimated in a multivariable model that also accounts for the other, highly correlated, predictor variables. Serum ELISA and coproantigen ELISA were log-transformed and used as a linear response variable. A quasi-Poisson distribution was used to model the response variable of FEC (count per 5 g) in order to allow for the over-dispersion that was assumed to be present based on previous experience with FEC data. This quasi-Poisson distribution was used instead of a Poisson model using an observation-level random effect because the latter model failed to converge for two farms, and in place of the more commonly used negative binomial distribution that is not implemented for the GAMM function. Each combination of diagnostic test and farm was modelled independently. Seasonality was incorporated in the model using a standard sine wave method with period set to 365 days and linear transformations of phase and amplitude estimated as linear effects. Longer-term temporal effects were estimated using a smoothing spline based on the sampling date. The effect of animal age was estimated using a smoothing spline based on the age of the animal at the time of sampling. Individual animal ID was included as a random effect in order to control for repeated sampling within animals. Finally, a dichotomous variable reflecting recent treatment record (treated within 180 days from the sampling date or not) was also included as a fixed effect for farms C2 and O2. Model fit was assessed by inspecting residual versus fitted plots and quantile-quantile plots of residuals. In addition, predictions for all animals with more than 3 samples were selected and the residuals for these observations were plotted against the age of the animal to check for any residual temporal autocorrelation. Final model results were visualised by estimating the predicted hypothetical values (and associated 95% confidence intervals) for each of varying animal age, season, and date given fixed values for the other predictors.

Changes in test values post treatment

The treatment response measured by diagnostic test results were summarised graphically for those pre- and post- treatment samples that were available. Number of days since treatment was calculated and changes in test values were then visually assessed for each diagnostic method.

Comparison of diagnostic test results

Pairwise agreement among the three diagnostic tests was assessed by Cohen's kappa using the *irr* package [37, 38]. In addition, agreement between liver condemnation results at slaughter and the results of any diagnostic tests that were taken within 60 days of slaughtering was also assessed using Cohen's kappa. The interpretation of the Kappa values were following: "very good" (> 0.8); "good" (> 0.6 and ≤ 0.8), "moderate" (> 0.4 and ≤ 0.6), "fair" (< 0.2 and ≤ 0.4), and poor (≤ 0.2) [39].

Snail surveys and detection of *Fasciola hepatica* in snails

Farms were visited in June and October 2015 and again in October 2017 to search for *G. truncatula* snails on pastures where *F. hepatica* transmission was suspected to take place. Due to the large size of the areas that were used for grazing, snail sampling was done in a qualitative manner, by screening all surface water bodies present at the time of sampling. Permanent water bodies were searched by scooping and by visual inspection of the moist/muddy zones at water body edges. The more transient surface water bodies (i.e. moist areas and furrows) were inspected visually and snails were picked with tweezers. All retrieved snails were kept alive in plastic containers with water, and transported back to the laboratory, where they were identified to species level based on morphological characteristics [40, 41]. The *G. truncatula* snails collected in 2015 were furthermore subjected to light-induced shedding for cercarial parasite stages, and finally crushed and dissected to search for patent and pre-patent stages of *F. hepatica* in the snail tissue. Due to the low parasite infection rate typically observed in snails [42], the snails collected in 2017 were also subjected to PCR analyses to assess the presence of *F. hepatica* DNA and confirm the morphological identification of the snails following a protocol described in Graham-Brown et al., University of Liverpool (manuscript in preparation). Briefly, DNA was extracted from the entire snail using Chelex® method as described by Caron et al. [43]. The supernatant containing DNA was diluted 10 times with Tris-EDTA and stored at -20 °C until PCR.

A total of three PCR reactions were conducted for each snail. The first PCR targeted amplification of snail internal transcribed spacer 2 (snail ITS2) to confirm snail identify as *G. truncatula* [44], and also to act as an internal positive control, since snails are known to contain PCR inhibitors. Then the second and the third PCRs were used to determine *F. hepatica* infection status by targeting *F. hepatica* ITS2 (F hep ITS2), and *F. hepatica* cytochrome *c* oxidase subunit 1 (F hep *cox*1), respectively. F hep ITS2 was as described by Novobilsky et al. [45] and Caron et al. [46], and F hep *cox*1 was as described by Cucher et al. [47], with addition of 4 μg bovine serum albumin (BSA) in PCR mix and an increase of the PCR cycles to 40. The total volume of each PCR reaction was 25 μl, consisting of 4 μl 1:10 diluted template snail DNA, 12.5 μl Biomix^{TM} Red (Bioline, London, UK), 1μl (4 μg) BSA, 2 μl of 10 μM primer pairs in case of snail ITS2 PCR and F hep ITS2 PCR, and the rest made up with double distilled water. For F hep *cox*1 PCR, 0.5 μl of 5 μM primer pairs was used instead. Negative controls (double distilled water) and the following positive controls were included in each PCR reaction: 4 μl 1:10 diluted DNA extracted from *G. truncatula* infected with *F. hepatica*, 4 μl 1:10 diluted DNA extracted from non-infected *G .truncatula*, and 4 μl (0.1ng) of DNA extracted from adult *F. hepatica* tissue.

To confirm the snail identity based on shell morphology, representative samples of snail ITS2 PCR products were sequenced, i.e. four snails identified as *G. truncatula*, one snail identified as *Succinea putris* and one snail identified as *Radix balthica*. A snail was considered positive for *F. hepatica* infection if both F hep ITS2 and F hep *cox*1 PCRs amplified a product of the expected size (approximately 112 bp and 405 bp, respectively). All snails that had amplified a product for the F hep *cox*1 PCR were sequenced. The PCR products were purified using QIAquick PCR purification kit (Qiagen, Manchester, UK) and sequencing was performed by Source Bioscience (Nottingham, UK). Sequences were aligned using *Staden* package (preGAP4 version 1.6 and GAP4 version 11.2) and run through NCBI Nucleotide BLAST (http://blast.ncbi.nlm.nih.gov/) and compared with sequences available on GenBank.

Results

Analysis of longitudinal data

Summary of climate data

Climate data for the whole of Denmark for the study period as well as the 30-year average (1931–1960) are shown in Fig. 4. In general, the maximum air temperature exceeded 10 °C from April to October between 2015 and 2017 (Fig. 4a-c), although also in March 2015. The air temperature was highest in July and August 2015/2017, although air temperatures were high (*c*.20 °C) throughout June to September in 2016. The maximum and minimum temperatures during both winter periods (Nov 2015 - Mar 2016 and Dec 2016 - Mar 2017) were higher than the 30-year average. As for precipitation, 2016 was comparable to the 30-year average, while precipitation was very low in October 2015 followed by above average rainfall in November and December 2015 (Fig. 4d). Above average precipitation was also seen during June to October 2017 (Fig. 4f).

Graphs of overall individual animal data

The raw data are plotted against the age of the animals according to diagnostic methods and farms in Fig. 5. On farm C1, most animals born during 2013 and 2014 seroconverted between the ages of 1.5 to 2 years (Fig. 5 C1-a). Animals over 4 years of age were also mostly seropositive, while a group of animals born in 2012 were seronegative throughout the study period. Copro-antigen values and FEC were positive from 2 years of age (Fig. 5 C1-b, c). On farm C2, no young animals seroconverted during the study period (Fig. 5 C2-a). Some animals born before 2012 had high serum and copro-antigen ELISA values, but only

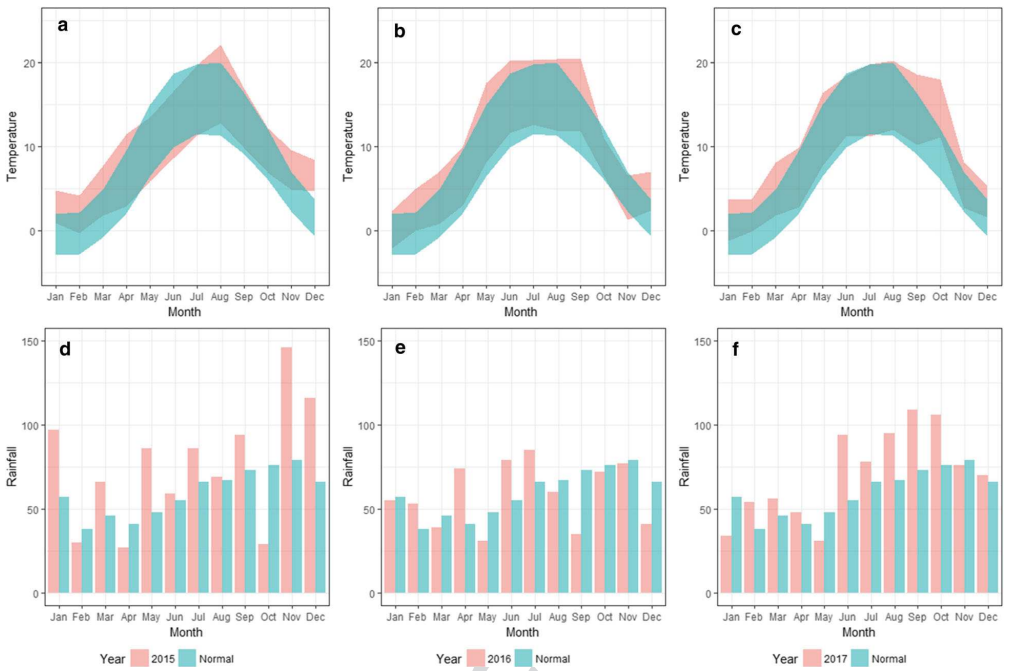


Fig. 4 Danish climate data for the four farms for the study period (2015–2017: red) and 30 year average (1961–1990: blue). The climate in Denmark is a mixture of oceanic and continental temperate. The mean day highest and lowest temperatures of each month are shown above, while the total monthly precipitations are shown below

a small number of animals excreted liver fluke eggs (Fig. 5 C2-b, c). On farm O1, most young animals seroconverted by the age of 2 years (Fig. 5 O1-a). The older animals on this farm were all seropositive throughout the study period, and copro-antigen ELISA test from these animals were also positive, although the actual test values were variable (Fig. 5 O1-b). Copro-antigen values and FEC were positive from around 2 years of age, and high egg excretions were seen in both young and older animals (Fig. 5 O1-b, c). On farm O2, not all young animals seroconverted and the age at which young animals seroconverted was variable (Fig. 5 O2-a). High copro-antigen values and FEC were seen in animals younger than 2 years as well as in older animals (Fig. 5 O2-b, c).

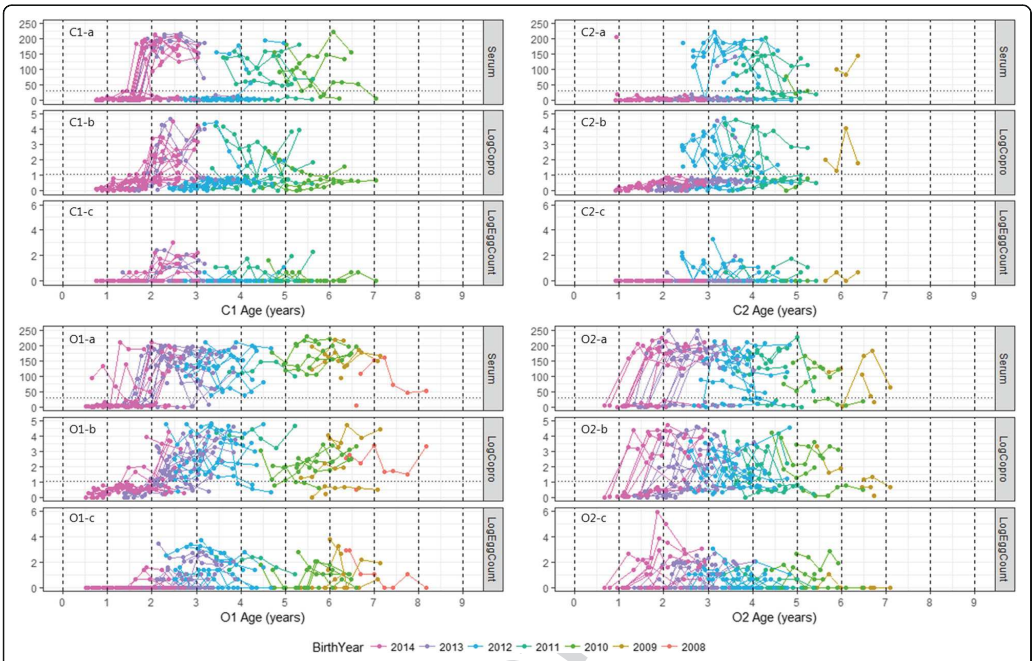
Overall, the infection seemed to first occur when the animals were between 1–2 years of age on all four farms. The summary of raw data over the seven sampling days according to the farms and age cohorts is provided in Additional file 1: Figure S1. It should be noted that any post-treatment data have been excluded from Fig. 5 and Additional file 1.

Monthly BTM data

Antibody levels measured in BTM showed fluctuations during the study period (Fig. 6). On farms C2 and O2 a general decrease in BTM antibody levels was seen. The decrease was seen from the end of 2015 in C2 and from the end of 2016 in O2 (Fig. 6-b, d). BTM antibody levels corresponded well with the average serum antibody levels of milking cows. Pearson's product moment correlation was $r_{(22)} = 0.86$ (95% CI: 0.70–0.94) and statistically significant ($P < 0.0001$).

GAMM analysis

The results of the separate GAMM models for each combination of farm and test type are shown in Fig. 7. Based on the model results, the animals' age had a greater impact on the expected test results than either seasonality or the longer-term temporal effect associated with the farm as a whole on all four farms (Fig. 7a, d, g). In general, test values were low in very young animals, but peaked at the age of 2–4 years, and slowly declined as the animals got older, except for farm C2, where test values continued to increase with age. Differences in



f5.1 **Fig. 5** The summary of *F. hepatica* diagnostic test results according to farms and age during the study period (from spring 2015 to winter 2017).
f5.2 Colour indicates animals that were born in the same year. Coproantigen ELISA values are log-transformed (after adding a fixed constant of 1), and
f5.3 the cut-off defined as 1.89 (1.061 after transformation). Faecal egg counts in 5 g faeces were also log-transformed (after adding a fixed constant
f5.4 of 1) for the benefit of visualisation. Any post-treatment data are excluded
f5.5

expected diagnostic test results through different seasons (time of year) were not substantial, but a small peak was observed later in the year for serum ELISA values (Fig. 7b), while peaks of copro-antigen ELISA and FEC occurred at the beginning of the year (Fig. 7e, h). Long-term temporal effects differed between the farms; farms C1 and O1 were relatively stable, while farms C2 and O2 (those using routine anthelmintics) showed a reduction at the end of the study period (Fig. 7c, f, i).

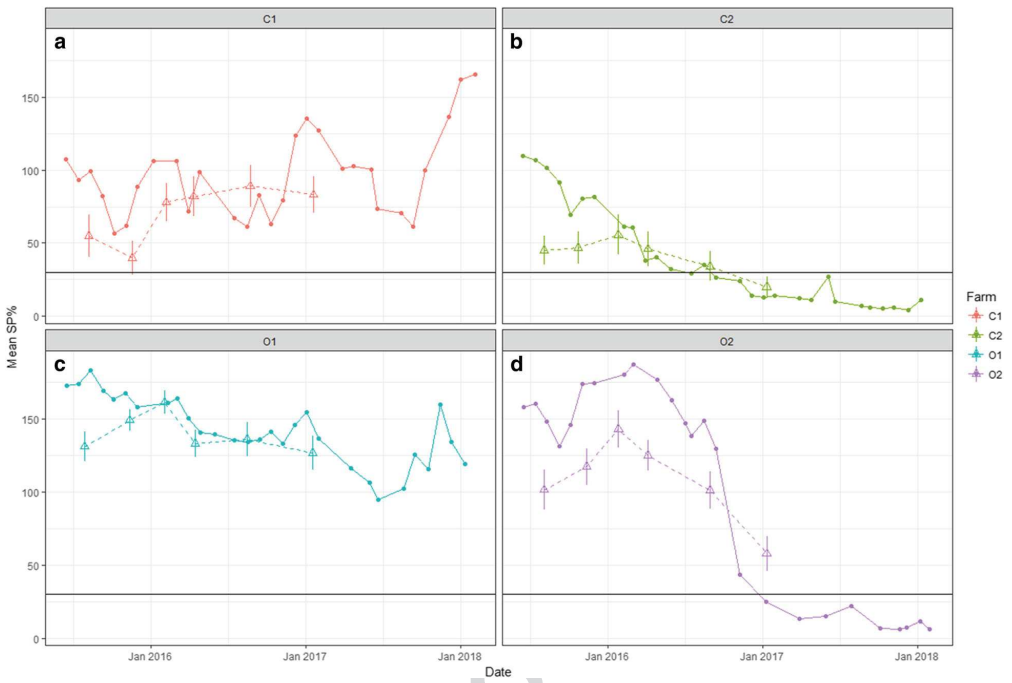
Approximate normality of residuals was observed in residual plots from all models. A small degree of residual temporal autocorrelation was observed in a small number of young animals from C1 and two animals from C2 on the copro-antigen models. Re-running the models without these observations gave qualitatively the same results, so the original models including all available data were retained.

Changes in test values post treatment

Pre- and post-treatment data were available from 24 animals from O2 and 6 animals from C2. Of these, 17 animals (15 from O2 and 2 from C2) had high serum antibody levels pre-treatment and 12 of those converted to negative status within 195 days post-treatment (Additional file 2: Figure S2). Out of the remaining five animals, two animals were still highly positive (174.9 and 142.2 S/P%) at last sampling at 20 and 36 days post-treatment. Three others showed decreased, low antibody levels at last sampling (63.5, 43.8 and 65.7 S/P%), which were at 32, 132 and 148 days post-treatment, respectively. The copro-antigen ELISA results of 13 animals were positive just before treatment and all of these animals reverted to negative status at 20 to 85 days post-treatment, except for one animal. A sample collected 195 days post-treatment from this animal was nevertheless negative. The FEC of 12 animals were positive immediately before treatment and egg excretion was detected in only one of these animals 195 days post-treatment. In the other animals, *F. hepatica* eggs were not detected at 20 to 85 days post-treatment sampling.

Comparison of diagnostic test results

The pairwise agreement between diagnostic methods at seven sampling times is summarised in Table 3. Good agreement was seen between serum ELISA and copro-antigen T3



f6.1 **Fig. 6** Monthly anti-*F. hepatica* antibody levels in bulk tank milk (BTM) (solid line) and average serum antibody levels of milking cows during the
f6.2 study period (triangle points with dashed line, error bars showing standard error of the mean) in the four farms
f6.3

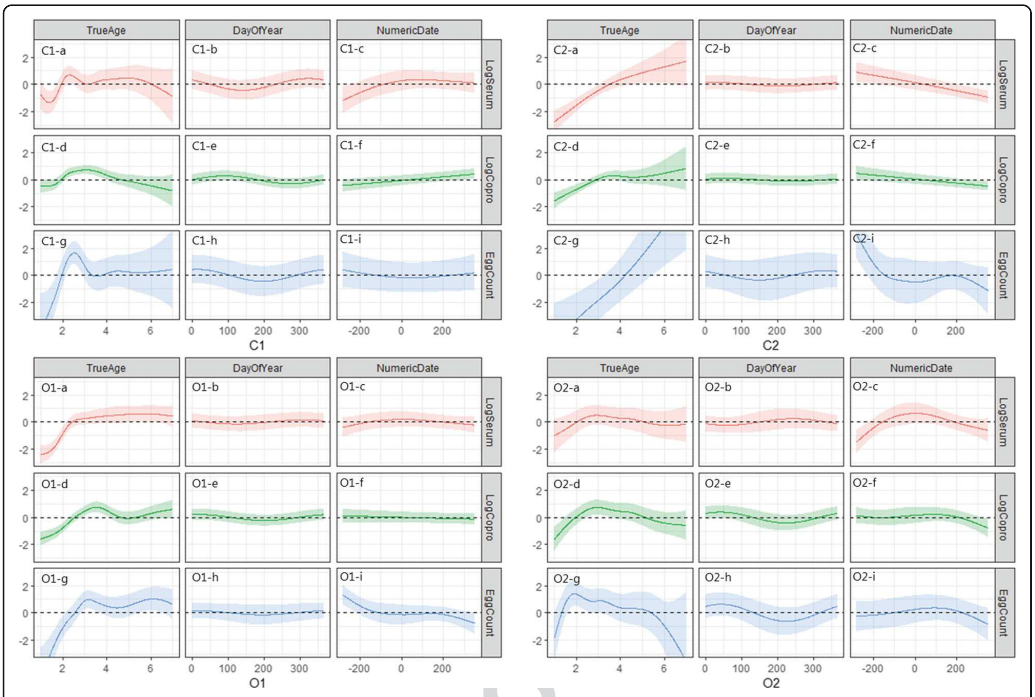
ELISA and moderate to good agreement was seen between copro-antigen ELISA and FEC. Only fair to moderate agreement was seen between serum ELISA and FEC. Agreement between serum ELISA and copro-antigen ELISA was highest in winter, while agreement between serum ELISA and FEC was highest during summer. The highest agreement between copro-antigen ELISA and FEC was seen during spring/summer period.

There were 45 animals in the study that were slaughtered within 60 days after sampling and for which meat inspection data was therefore available. Of those, 10 animals were identified as liver fluke positive based on liver inspection at slaughter. The summary of the diagnostic tests results is shown in Additional file 3: Figure S3 and their pairwise Cohen's kappa are summarised in Table 4.

Snail surveys and detection of *Fasciola hepatica* in snails

In total, 301 *G. truncatula* snails were found on pastures used for grazing on the 4 farms. Other freshwater snails identified were *Lymnaea stagnalis* (Linnaeus, 1758), *Lymnaea palustris* (O.F. Müller, 1774), *Bythinia tentaculata* (Linnaeus, 1758), *Radix balthica* and number of specimens belonging to the genera *Planorbis*, *Succinea* and *Anisus* that could not be identified to species level based on shell morphology. In brief, the snails were found in typical habits such as riparian areas (along ditches), dense rush and water puddles created by heavy trampling, but a large number of snails were also observed within drinking troughs that were in use (Additional file 4: Table S1).

In June 2015, no *G. truncatula* was observed on two paddocks of farm C1: the home paddock for the milking cows and the pasture along a fjord for heifers (Fig. 2 C1-A/a, B and C). On farm C2, ten *G. truncatula* were found in June and another 13 specimens in October on the paddock used for heifers (Fig. 2 C2-A). On farm O1, three separate areas used for grazing were searched for snails (Fig. 3 O1-A, B, E, F). One *G. truncatula* was found on one of the two paddocks for young heifers (Fig. 3 O1-E) and another five were found on a marshland paddock grazed by larger heifers (Fig. 3 O1-F). On a similar, near-by marshland paddock used for grazing by farm O2, nine *G. truncatula* were found in October 2015, whereas no snails were observed on the home paddocks, and a more remote bog area also used for



f7.1 **Fig. 7** Results of generalised additive mixed models (GAMM) showing the relative effects of animal age, season of sampling, and date of the
f7.2 sampling (for longer-term temporal trends) within the studied farms. Each combination of farm and diagnostic test was modelled independently.
f7.3 The estimates are shown using solid lines and shaded areas indicate 95% confidence intervals. The y-axis is on the natural logarithm scale
f7.4

grazing (Fig. 3 O2-A, B, C, D). Shedding and dissection of the 38 collected *G. truncatula* in 2015 did not reveal any infection with *F. hepatica* or any other trematode parasites.

In October 2017, a total of 298 snails (263 *G. truncatula*, 33 *R. balthica*, one *S. putris*, and one terrestrial snail, which was not further identified) were retrieved from farms C1, O1, and O2 (farm C2 was not visited). A total of 246 *G. truncatula* were found in the fjord paddock on farm C1 (Fig. 2 C1-C; Additional file 4: Table S1). On farm O1, ten *G. truncatula* were obtained from the paddock for dry cows/in-heat heifers (Fig. 3 O1-B) and one additional *G. truncatula* from a home paddock for milking cows (a part of rotational grazing; Fig. 3 O1-A). On farm O2, six *G. truncatula* were detected in a ditch where milking cows were grazed (Fig. 3 O2-A), and 33 wandering snails (*R. balthica*, as identified by PCR) were collected from a water trough on another paddock grazed by milking cows (a part of rotational grazing; Fig. 3 O2-A). PCR products from the four snails morphologically identified as *G. truncatula* (344–411 bp) were 99–100% identical (E-values 0 or $2 \times e^{-178}$) to *G. truncatula* sequences on GenBank (KT781267 and KF887031.1). Likewise, the identities of *S. putris* (168 bp) and *R. balthica* (356 bp) were verified by comparison with sequences from GenBank demonstrating 99% (MF148322.1, E-value: $1 \times e^{-76}$) and 100% (LT623580.1, E-value: 0), respectively. The newly-generated sequences were deposited in the GenBank database under accession numbers MH561918-MH561923 (Additional file 4: Table S1).

t3.1 **Table 3** Agreement between diagnostic tests (Cohen's kappa) at each sampling time. Total number of observations is given in
t3.2 brackets, and any samples taken within 180 days of treatment are excluded

t3.3		Spring 2015	Summer 2015	Autumn 2015	Winter 2015/16	Spring 2016	Summer 2016	Winter 2016/17
t3.4	Serum ELISA *vs* copro-antigen ELISA	0.794 (84)	0.747 (150)	0.571 (167)	0.830 (176)	0.807 (175)	0.721 (155)	0.811 (126)
t3.5	Serum ELISA *vs* FEC	0.476 (87)	0.525 (150)	0.357 (167)	0.414 (175)	0.540 (175)	0.610 (155)	0.538 (126)
t3.6	Copro-antigen ELISA *vs* FEC	0.710 (174)	0.641 (157)	0.520 (168)	0.539 (175)	0.662 (175)	0.771 (156)	0.574 (126)

t4.1 **Table 4** Cohen's kappa statistics of pairwise comparisons of
t4.2 diagnostic tests for *F. hepatica* infection in animals (n=45) that
t4.3 were slaughtered 7 to 60 days after the last sampling date
t4.4 during the study period

t4.5		Test positives	Cohen's kappa pairwise comparison		
t4.6 t4.7			Serum ELISA[a]	Copro-antigen ELISA	FEC
t4.8	Liver inspection	22.2% (10/45)	0.287	0.245	0.267
t4.9	Serum ELISA[a]	41.9% (18/43)	–	0.751	0.538
t4.10	Copro-antigen ELISA	28.9% (13/45)	–	–	0.762
t4.11	FEC	20% (9/45)	–	–	–

t4.12 [a]2 missing values (n=43)

Three out of 263 *G. truncatula* (1.1%) were found to be infected with *F. hepatica* by PCR. All the *F. hepatica* positive snails were *G. truncatula*: one from farm C1 found within dense rush, one from farm C1 found in a drinking trough, and one from farm O1 found within sparse rush (Additional file 4: Table S1). The sequences (348–353 bp) were 99–100% identical to *F. hepatica* sequence on GenBank (AF216697.1, E-values ranged from 0 to $2 \times e^{-177}$). The sequences were deposited in GenBank under the accession numbers MH5619124-MH561926 (Additional file 4: Table S1).

Discussion

Our study used intensive data collection from a number of different sources to investigate issues relevant to the control of *F. hepatica* on four Danish dairy farms. Each farm recruited for this study had critically different grazing management styles and the farmers had different attitudes towards *F. hepatica* control. However, despite these differences, there was a similar association between animal age and *F. hepatica* diagnosis across the four farms; infection tended to be acquired as young stock, although not necessarily in the first grazing season. This finding is consistent with our previous risk factor analysis, which showed heifers grazing on wet areas as a risk group and a predictor of farm status [25]. This was likely to be a reflection of the typical Danish practice, where younger calves and cows (with the exception of dry cows) are grazed close to the main farm buildings on relatively dry, high grounds, while heifers (and sometimes dry cows) are placed on marginal lands and allowed to graze freely for the entire grazing season [48, 49]. Indeed, many of the primiparous cows were already infected at the start of the study except for those from farm C1. This particular group of animals on farm C1 grazed on the same heifer paddock near the fjord (Fig. 2 C1-C) in 2014 without being infected. We speculate that flooding with seawater that occurred during winter 2013/2014 wiped out the snail population in that area, and animals consequently escaped liver fluke infection in this particular grazing season.

Most animals were infected before calving and carried the infection as they moved into the lactating herd. In older animals, interpretation of the diagnostic test results is a challenge, as it is unknown how long the parasite can live and how long the antibodies persist after elimination of the parasite. Ross [50] observed that most parasites were lost between 5th and 21st months after infection, while the remaining parasites could live at least 26 months. Based on our results, the longevity of the parasites could be longer than 26 months, as the multiparous cows from farm C2 that had no access to outdoor areas (except for dry cows in a sandy yard) or freshly-cut grass, were still seropositive at 4 years of age and over. Lasting antibodies after elimination of the parasites is a possible scenario, but antibody levels declined within 195 days post-treatment in the present study and similar findings have been seen in other studies [51, 52]. Additionally, copro-antigen ELISA values were above the cut-off and liver fluke eggs were present in the faeces in some of these older animals, indicating active infection.

If the parasites can persist for longer than two years, then positive results from cows in their third or higher lactation can either be a result of persistent infection or re-infection, which occurred most likely during the dry period. Dry cows are frequently grazed on marginal land together with heifers, and indeed our previous risk factor analysis showed odds of farm infection status was approximately four times higher if dry cows grazed on wet areas [25]. On farm C1, some multiparous animals over four years of age had low to moderate serum antibody levels and elevated copro-antigen ELISA levels, suggestive of potential reinfection on the pasture used for the lactating herd. However, the cohort of primiparous cows on farm C1 that were uninfected at the start of the study remained uninfected for the entire study period (Additional file 1: Figure S1). This suggests that the pasture used for the lactating cows constituted a minimal risk and that multiparous cows were probably carrying the parasites for years from the initial infection. In contrast, it seems that milking cows were continuously exposed to *F. hepatica* metacercariae on farm O1 because multiparous cows over four years of age showed continuously high serum ELISA values (> 100 S/P%) and many of their copro-antigen ELISA values were well above the cut-off. In addition, while most multiparous cows on farm C1 did not excrete any *F. hepatica* eggs in the faeces, egg excretion was observed in a number of multiparous cows on farm O1. This coincides with the findings of Ross [50] that egg laying capacity of the fluke was maximal at 3–8 months post-infection, but reduced to low or negative faecal egg counts during the chronic

phase of infection (> 10 months post-infection). Mezo et al. [53] also documented that animals infected with low fluke burden showed low antibody responses during the chronic phase of *F. hepatica* infection. Although Knubben-Schweizer et al. [5] recommended serological testing of the oldest animals in the herd to determine infection in the milking cows, our conclusion is that the assessment of whether the lactating herd is re-infected by *F. hepatica* or not, is difficult solely based on serum ELISA dichotomised into positive or negative results. The serum ELISA could be high with continuous exposure, while long-lasting infection may have low to moderate serum ELISA levels. However, to document/confirm re-infection within the milking herd, it should be complemented with either copro-antigen ELISA or faecal egg counts.

The temporal patterns of infection differed greatly among the four farms over the study period. This is likely to be due in part to different grazing management, but also relate to the introduction of regular treatments against *F. hepatica* on two of the farms in 2016, which was, of course, influenced by our consultations with the farmers on the findings during the study period. BTM ELISA showed a good correlation with average serum antibody levels of milking cows, and the overall progression of the disease was clearly seen from the BTM ELISA results (Fig. 6). The two organic farms had high infection levels shown by BTM ELISA compared to the two conventional farms at the start of the study. During the study, two farms initiated *F. hepatica* control by treatment (heifers at housing on farm C2 and dry cows and heifers pre-calving on farm O2) and grazing management, resulting in decreased level of *F. hepatica* infection at the end of the study. This was also reflected in the decreasing longer-term temporal trend estimated by the GAMM on farms C2 and O2 (Fig. 7). Farm C1 also started treatment of heifers pre-calving in 2017, but BTM ELISA showed an increased level of infection from late 2017 (Fig. 6). This was unexpected, based on the assumption that re-infection was unlikely to occur on the permanent paddock for the lactating cows. However, the second half of 2017 was wetter than normal (20–30% more rain) (Fig. 1), and therefore transmission of *F. hepatica* on the lactating cow paddock (Fig. 2 C1-A/a) may have occurred in 2017.

According to the GAMM, seasonality did not seem to be as strongly associated with the test values compared to age and the longer-term temporal trends within the farms. Considering the relatively long-lasting nature of infection, it is not unexpected that test values are relatively stable between seasons after accounting for the effects of age and longer-term temporal trends. However, modest fluctuations according to seasons were seen, and generally speaking, the peak occurred first for serum ELISA in autumn, followed by copro-antigen ELISA and FEC (Fig. 5). Agreement between serum ELISA and copro-antigen ELISA was also highest in winter, while agreement between serum ELISA and FEC was highest in summer. This reflects the fact that the three diagnostic tests differ in the time of detection; serum ELISA can detect infection within 2–4 weeks post infection [54], while copro-antigen ELISA values rises 6–8 weeks post-infection in cattle [55]. This has an important connotation for the timing of sampling in order to diagnose *F. hepatica* on a farm. Serum ELISA can be used to test for *F. hepatica* exposure at housing in the autumn, whereas under-diagnosis is likely to occur if copro-antigen ELISA or FEC is used at that time of the year.

It has been speculated that as a result of climate change, release of metacercariae in spring from overwintered snails, may become more significant as a source of infection for grazing animals [5]. There was little indication of this in our study. Some animals sero-converted by summer in 2015, but as our sampling time was end July to August, the infection could have been acquired either early in the grazing season (winter infection) or just before the summer sampling (summer infection). However, no increase in copro-antigen ELISA values or egg excretion was observed from these animals, suggesting that infection occurred mid-summer and therefore that summer infection is still the most relevant to consider for cattle in Denmark.

Three out of 263 *G. truncatula* (1.1%) were found to be infected with *F. hepatica* by PCR. Prevalences of *F. hepatica* in *G. truncatula* reported from previous studies differ substantially from 0.5% to 82% [21, 56, 57], although large studies conducted in France found around 5–12.5% of the snails infected [58, 59]. Significant differences in *F. hepatica* prevalence in snails have been found to be associated with differences in seasons, locations, and year of the study [18, 57, 59]. Likewise, we found great inter-annual variation in the snail survey results; many snails were found in October 2017 compared to June-October 2015. As evident from Fig. 4, many temporary water bodies especially on the fields around the farms were dried out due to scarce rainfall in October 2015, while potential habitats were expanded in October 2017 due to high rainfall. This highlights the effect of climatic factors on snail habitats and also the importance of frequent samplings/observations to avoid false negative findings. Nevertheless, we confirmed over time the presence of snails on many of the pastures where we suspected transmission took place. It is perhaps noteworthy that a positive snail was recovered from a drinking trough. It is known that floating metacercariae can form on the surface of the water after cercariae exit the snail [60], and thus transmission through infected drinking water is possible. These authors

considered the transmission route by the floating metacercariae to be unimportant, as dispersal and survival of floating metacercariae was low in running water under both laboratory and field conditions. Yet the authors mentioned that metacercariae could float for over three months on the surface of stagnant water, and therefore the presence of an infected snail in a water trough without a pump or a tap could become a source of *F. hepatica* infection. Overall, this study demonstrated some of the difficulties related to detection of snails and snail habitats. Unless a clear, quick and easy guideline is developed, precise identification of snail habitats (as transmission sites) is unlikely to be accepted as part of practical control programs. Recent developments in environmental DNA (eDNA) based methods to detect *G. truncatula* and *F. hepatica* directly in the environment [61], as well as the use of drone imagery to delineate potential snail habitats [62] could provide a future avenue, given that these methods become sufficiently easy and cheap.

Based on our results we suggest improved practical guidelines for diagnosis and management of fasciolosis on dairy farms with grazing stock. First, it is important to determine whether transmission is taking place in the young stock only (e.g. farm C2) or both in young stock and older cows (e.g. farm O1). This pattern of infection is again related to whether they graze contaminated pastures or not. We therefore recommend that identification of contaminated pasture is assessed by taking representative serological samples from planned second-year grazers and from cows older than third lactation (or the oldest cows) before turn-out. In addition, faecal samples from cows should preferably be analysed by copro-antigen ELISA or FEC to confirm active infection. Positive samples suggest that the pasture used to graze this cohort of animals during the previous summer was contaminated with metacercariae. The procedure should be repeated at housing for young stock, if they were negative at turn-out, to determine if they have picked up infection over the summer grazing period. If animals are grazed on different pastures, representative samples from each group should be taken. Once age groups at risk is clarified and fluke risk paddocks are identified, medicinal and non-medicinal control can be tailored and applied depending on the farmer's motivation and capabilities as suggested by Knubben-Schweizer et al. [5]. However, as demonstrated in farms O1 and O2, some farms have very limited options for non-medicinal control as the majority of pastures have extensive wet areas suitable for the intermediate host snails. Efficacious treatment with triclabendazole during the dry period was a challenge to farmers due to restrictions related to expected calving, particularly on organic farms with long withdrawal periods. After implementation of the control program, progress can be monitored by BTM ELISA, preferably in spring when the antibody levels are highest. The detailed diagnosis of individual animals may need to be repeated in order to reduce the impact of year-to-year variation within the same farm.

Conclusions

This longitudinal study on four dairy farms in Denmark showed that the patterns of *F. hepatica* infection varied considerably between farms due to different grazing management (e.g. snail habitats) and anthelmintic strategies employed. Careful interpretation was required based on the grazing history of the animals in the context of precipitation (climate), as year-to-year variation was also evident. However, some commonalities were seen despite these differences; in particular heifers were the main risk group for *F. hepatica* infection on all the farms. On two farms old cows had persistent infections derived from initial infection as heifers, while lactating cows were continuously exposed (most likely as dry cows) to metacercariae on one of the other farms. We conclude that the adoption of a stringent treatment schedule of pre-calving heifers when there is no transmission in the lactating cow herd (housed or on non-risk pasture) can lead to lower BTM ELISA values, indicative of reduced exposure to *F. hepatica*. If there is transmission in the lactating cow herd, consistent dry cow treatments can reduce the prevalence. However, such an intensive treatment program may not readily be accepted by organic producers, and further studies are required to demonstrate if non-medicinal approach (e.g. genetically robust breeding lines, a more precise spatiotemporal delineation of pasture risk areas and/or biological control of snails) in a longer perspective can limit the requirement of anthelmintic treatments.

Additional files

Additional file 1: Figure S1. Summary of raw data according to farms, cohort groups, and *F. hepatica* diagnostic test results. (Cohort 1 is the youngest group, and cohort 4 is the oldest animals). Serum ELISA values are not log-transformed and the cut-off is defined as 30. Coproantigen ELISA values are log-transformed (after adding a fixed constant of 1) and cut-off defined as 1.89 (1.061 after transformation). Faecal egg counts in 5 g faeces were also log-transformed (after adding a fixed constant of 1) for the benefit of visualisation. Any post-treatment data were excluded. The samples from same animals are connected with solid lines and the pink shows the average value of each sampling point within the cohorts. (TIFF 2812 kb)

Additional file 2: Figure S2. The antibody response after treatment in 17 animals. Day 0 is the day of treatment. (TIFF 723 kb)

Additional file 3: Figure S3. The results of diagnostic tests for *F. hepatica* infection in animals ($n = 43$, as two serum samples were missing) that were slaughtered 7 to 60 days after the last sampling date during the study period (1, liver condemnation; 0, no liver condemnation). (TIFF 2812 kb)

Additional file 4: Table S1. Summary of snails collected in 2017 that were analysed by PCR for snail species and *F. hepatica* infection status. (XLSX 11 kb)

Abbreviations

bp: base pair; BTM: Bulk tank milk; *cox*1: cytochrome *c* oxidase subunit 1; DCD: Danish cattle database; DH: Danish Holsten; DNA: Deoxyribonucleic acid; ELISA: Enzyme-linked immunosorbant assay; EU: ELISA unit; FEC: Faecal egg count; GAMM: generalised additive mixed model; ITS-2: internal transcribed spacer 2; PCR: polymerase chain reaction; S/P%: Sample to positive percentage

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Availability of data and materials

Data supporting the conclusions of this article are included within the article and its additional files. The datasets used and/or analysed during the study are available from the corresponding author upon reasonable request. Sequences obtained during the present study are available in the GenBank database with accession numbers MH561918-MH561923.

Authors' contributions

NTS, MD, HLE and SMT designed the study. NTS and HHP performed the diagnostic analysis. NTS, SMT, ASS, and MES performed the snail survey. NTS, NB, JH, and DW were involved in PCRs of the snails. NTS and MD performed data management and statistical analyses. NTS, MD, HLE and SMT interpreted results. NTS wrote the manuscript and all the co-authors assisted with the revision. All authors read and approved the final manuscript.

Ethics approval

This study was approved by the Danish Animal Experimentation Inspectorate (License number 2015-15-0201-00760, license holder: SMT) and consent to participate in the study was acquired from the four farmers.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Publisher's Note

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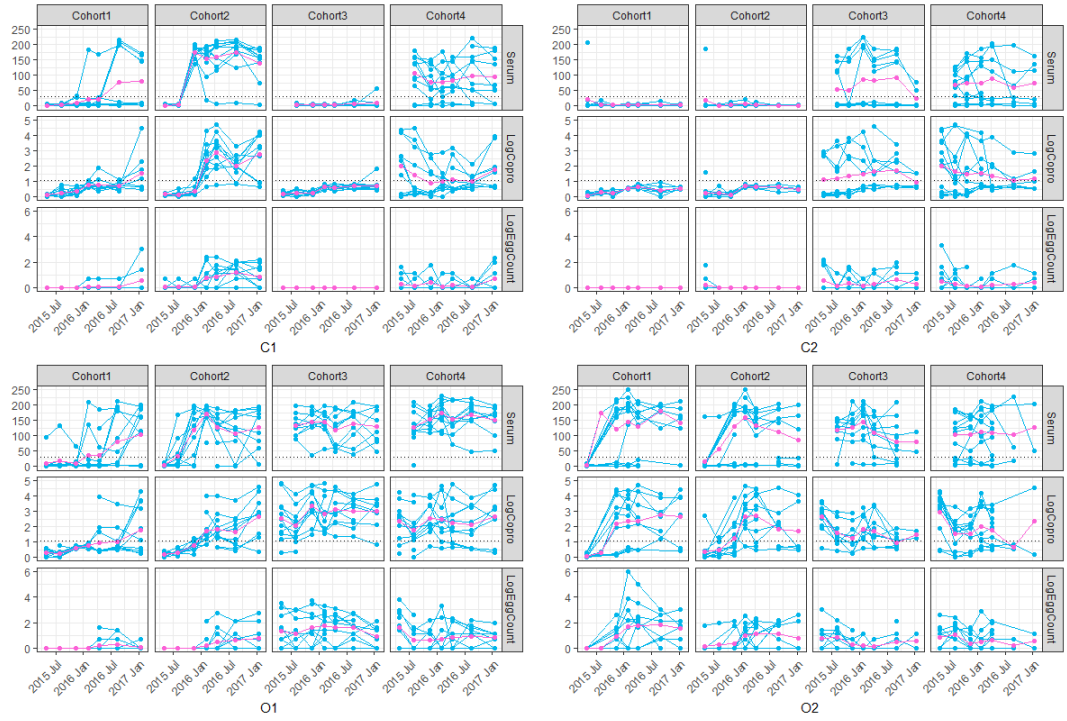
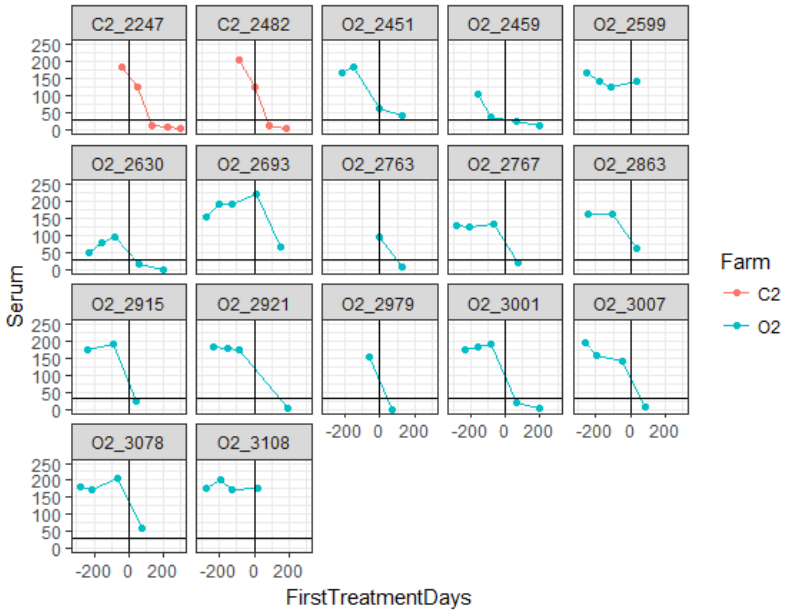
Supplementary material to Paper II

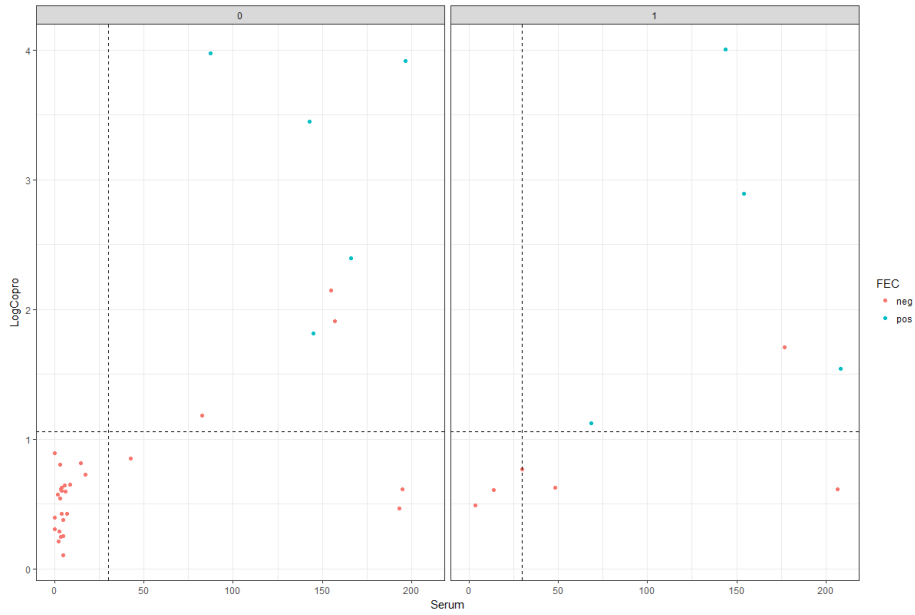
Additional file 1. Figure S1. Summary of raw data according to farms, cohort groups, and *F. hepatica* diagnostic test results. (Cohort 1 is the youngest group, and cohort 4 is the oldest animals). Serum ELISA values are not log-transformed and the cut-off is defined as 30. Coproantigen ELISA values are log-transformed (after adding a fixed constant of 1) and cut-off defined as 1.89 (1.061 after transformation). Faecal egg counts in 5 g faeces were also log-transformed (after adding a fixed constant of 1) for the benefit of visualisation. Any post-treatment data were excluded . The samples from same animals are connected with solid lines and the pink shows the average value of each sampling point withn the cohors.



Additional file 2. **Figure S3**. The antibody response after treatment in 17 animals. Day 0 is the day of treatment.



Additional file 3. Figure S3. The results of diagnostic tests for *F. hepatica* infection in animals (n = 43, as two serum samples were missing) that were slaughtered 7 to 60 days after the last sampling date during the study period (1, liver condemnation; 0, no liver condemnation).



Additional file 4. Table S1. Summary of snails collected in 2017 that were analysed by PCR for snail species and *F. hepatica* infection status.

Farm	Collection site number	Collection date	Description of habitats	Numbers	*G. truncatula* positive (accession no.)	*F. hepatica* positive (accession no.)
C1	1 (Fig.2 C1-C)	03-Oct-17	Riparian area (ditch)	44	44	0
C1	2 (Fig.2 C1-C)	03-Oct-17	Dense rush	20	20	1 (MH561925)
C1	3 (Fig.2 C1-C)	03-Oct-17	Riparian area (ditch)	48	48	0
C1	4 (Fig.2 C1-C)	03-Oct-17	Riparian area (heavily trampled areas near water stream)	4	3	0
C1	5 (Fig.2 C1-C)	03-Oct-17	A water puddle created due to heavy trampling	6#	5 (MH561918)	0
C1	6 (Fig.2 C1-C)	03-Oct-17	Water trough	126	126 (MH561921)	1 (MH561926)
O1	2 (Fig.2 O1-B)	23-Oct-17	Sparse rush	10	10 (MH561919)	1 (MH561924)
O1	5 (Fig.2 O1-A)	23-Oct-17	A water puddle created due to heavy trampling	1	1	0
O2	2 (Fig.2 O2-A)	23-Oct-17	Riparian area (ditch)	6	6 (MH561920)	0
O2	3 (Fig.2 O2-A)	23-Oct-17	Water trough	33*	0	0

One *Succinea putris* (GenBank acc. no. MH561922) was also identified

* These snails were identified as *Radix balthica* (GenBank acc. no. MH561923)

8.3 Paper III

Association between milk yield and milk anti-*Fasciola hepatica* antibody levels, and the utility of bulk tank milk samples for assessing within-herd prevalence

Takeuchi-Storm, N., Thamsborg, S.M., Enemark, H.L., Denwood, M.. Association between milk yield and milk anti-Fasciola hepatica antibody levels, and the utility of bulk tank milk samples for assessing within-herd prevalence (manuscript in preparation)

Association between milk yield and milk anti-*Fasciola hepatica* antibody levels, and the utility of bulk tank milk samples for assessing within-herd prevalence

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Abstract

Fasciola hepatica is an important disease of livestock that is responsible for substantial economic losses worldwide. Herd-level diagnosis of fasciolosis using anti-*Fasciola* antibody ELISA applied to bulk tank milk (BTM) represents a cheap and convenient tool for estimating the infection level in dairy cattle, but the utility of this test with regards to management of fasciolosis is currently unknown. In this study, we estimated the association between 305 day energy corrected milk yield (305d ECM) and *F. hepatica* infection in Danish organic farms, using tests applied both at individual and herd level. Additionally, BTM ELISA results were used to evaluate the relationship with animal-level prevalence and to determine the key risk factors associated with the presence of *F. hepatica* antibodies at farm-level. Telephone questionnaire interview results and BTM samples from 218 farms were collected in spring 2016. The corresponding farm-level production data covering the period 2014-2017 were subsequently collected from the Danish national cattle registry. Additionally, 284 individual milk samples (4-7 per herd) along with BTM samples were collected from a subset of the same herds (n=55) in spring 2017. All samples were analysed by IDEXX ELISA. Generalised linear mixed models were used to estimate the association between milk production and ELISA value at both individual and farm levels, as well as to assess the relationship between within-herd prevalence and BTM ELISA. Significant negative associations between ELISA positivity and milk yield were found at both farm and individual level: BTM positivity was associated with a reduction of 580.5 kg in average 305d ECM, and individual-level ELISA positivity was associated with a 919.5 kg reduction in milk yield in *F. hepatica* positive cows in their third or later lactations. However, no clear association was found between milk yield and the degree of ELISA positivity at either individual or farm level. Despite this, there was a significant correlation between quantitative BTM ELISA results and apparent within-herd prevalence based on individual milk results. Heifers on wet areas were confirmed to be associated with *F. hepatica*

infected farms. We conclude that dichotomised BTM ELISA results are a useful predictor of reduced milk yield, and that this estimated difference in milk yield is consistent when examined at individual level based on the same dichotomised test result. We also determined that the degree of BTM ELISA positivity can be used to estimate the prevalence within the milking herd, which represents a cheap and useful diagnostic tool for monitoring the long-term success of control strategies for *F. hepatica* infections on a dairy farm.

Keywords: *Fasciola hepatica*, Bulk tank milk ELISA, individual milk ELISA, in-herd prevalence

Introduction

The common liver fluke, *Fasciola hepatica*, has a worldwide distribution and is an important parasitic trematode of grazing livestock. In cattle, the disease is most often subclinical and the full impact of the disease is difficult to assess. Older literature, including experimental studies, has estimated that milk production is reduced by approximately 10% in dairy cows with fasciolosis (Schweizer et al., 2005). Likewise, more recent observational studies also found associations between *F. hepatica* infections and a reduced milk production of between 3 – 15% (Charlier et al., 2007; Mezo et al., 2011; Howell et al., 2015). The variation in these estimates is likely due to several factors, including geographical differences in intensity of infection, genetics, farm-level management, and nutritional and health status of the animals (Torgerson and Claxton, 1999; Dorny et al., 2011; Mezo et al., 2011). The different diagnostic methods employed to identify *F. hepatica* infection could also have influenced the estimates via imperfect classification of disease status. The traditional faecal egg counting by sedimentation has low sensitivity (Rapsch et al., 2006), and positive result may frequently indicate heavy infection (Charlier et al., 2008). In contrast, antibody detection by ELISA has been shown to be more sensitive than faecal egg counts (Rapsch et al., 2006; Charlier et al., 2008; Mazeri et al., 2016), but it only indicates past exposure to metacercariae as antibodies can last for three to six months after anthelmintic treatment (Castro et al., 2000; Salimi-Bejestani et al., 2005b; Mezo et al., 2007). ELISA on milk samples instead of serum is a non-invasive and convenient diagnostic method especially if milk samples are routinely collected for monitoring of the productivity (Charlier et al., 2014). In dairy herds, bulk tank milk (BTM) can be used to obtain a herd-level diagnosis because the antibody levels in BTM have been shown to be highly correlated with the within-herd seroprevalence in milking herds (Salimi-Bejestani et al., 2005a; Mezo et al., 2010). Although it may not be possible to translate antibody levels in BTM directly to overall within-herd prevalence, it may reflect the level of infection within the milking herd and therefore the effect on milk productivity. However, it is also known that between-farm variability is high, and a number of factors can affect both milk production (Mezo et al., 2011) and *F. hepatica* infection. Associations between milk production and *F. hepatica* infection should

therefore be interpreted in the context of regional differences, the diagnostic method used to classify *F. hepatica* status, and potential for confounding between herd-level factors of both milk yield and *F. hepatica* exposure. The latter represents one of the main limitations in estimating the association between production and parasite infections at farm level: several general farm management factors affecting milk yield (Mezo et al., 2011) are also likely to be correlated with *F. hepatica* infection. For example, animals within extensive farming systems generally have lower milk yield relative to those in intensive systems, and might also be expected to have higher exposure to *F. hepatica* because cattle are infected by ingestion of metacercariae while at grass. Indeed, the prevalence of bovine fasciolosis at farm-level based on liver condemnations at slaughter has previously been shown to be higher in organic farms, where grazing is mandatory for all age groups, than conventional farms (53.5% vs. 40.4%) in Denmark (Olsen et al., 2015). Similarly, a previous case-control study of 194 Danish farms identified several farm-level management factors that were associated with *F. hepatica* infection (Takeuchi-Storm et al., 2017). When conducting association studies at farm level, it is therefore important to collect and incorporate information regarding farm-level management factors, so that these factors can be controlled for as far as possible by including them as covariates in a multivariable model. Alternatively, comparison of productivity between individual *F. hepatica* negative and *F. hepatica* positive animals from the same farm reduces the impact of these intrinsic farm-level factors and may therefore give more robust estimates.

It is important to be able to estimate an approximate within-herd prevalence, as this will determine the likely impact of a control program in terms of limiting production loss (Vercruysse and Claerebout, 2001). Although high correlation between BTM ELISA and within-herd prevalence has previously been demonstrated, the results of BTM ELISA are often interpreted qualitatively as dichotomised negative/positive status based on a threshold of the minimum within-herd prevalence detection level. Furthermore, the currently available BTM ELISA assays have varying detection levels: IDEXX (previously Pourquier) ELISA (Fasciolosis Verification Test, IDEXX, Hoofddorp, the Netherlands) has been shown to detect a within-herd prevalence of 20% based on milk samples (Duscher et al., 2011), while MM3-SERO ELISA detected farms with a seroprevalence of 12% (Mezo et al., 2010), and an in-house antibody ELISA, based on excretory-secretory (ES) antigens developed at the University of Liverpool (LIV-ELISA), have been shown to detect a within-herd seroprevalence of 25% (Salimi-Bejestani et al., 2005a). However, it would be more useful if BTM ELISA results could be interpreted more quantitatively to estimate the value of the within-herd prevalence beyond a simple positive/negative result. According to the IDEXX ELISA instructions, BTM test results can be interpreted to the prevalence of infection within the farms as follows: "negative" as no or low prevalence, "low" as < 20% prevalence, "moderate" as 20 – 50%

prevalence, and "high" as > 50% prevalence. However, to our knowledge this has not yet been validated in the field.

The overall aims of this study were to estimate the association between milk yield and *F. hepatica* infection in Danish dairy farms, and to evaluate the extent to which BTM ELISA can be used to assess the within-herd prevalence of infection. This was achieved via three separate but related analyses: first to assess the herd-level association between average milk yield and anti-*F. hepatica* antibody level in BTM milk; second to assess the individual-level association between milk yield and anti-*F. hepatica* antibody level in individual milk samples; and third to evaluate the extent to which BTM ELISA results can be interpreted as reflecting the within-herd prevalence as determined by individual milk samples. In addition, BTM ELISA results were used to determine the key risk factors associated with *F. hepatica* infection on Danish organic dairy farms.

Materials and methods

Farm selection

In order to minimise management related differences between farms this study included only organic dairy farms in Denmark. According to the EU regulation on organic production, grazing throughout the entire grazing season is mandatory for all animals from organics farms (Council Regulation (EC) No. 834/2007 and (EEC) No. 2092/91), so some level of risk for *F. hepatica* is possible for all included herds. Organic dairy farms constitute approximately 10% of all dairy farms and all milk deliveries in Denmark (The Danish Agriculture & Food Council, 2017).

On-farm sampling for the study was conducted in two phases. For the first phase, all registered organic farms with a minimum of 100 animals in total (n=351) were identified as candidates for inclusion in the study during spring 2016. BTM from these farms was collected and the farmers were contacted by telephone to take part in a questionnaire survey to record farm-level management factors. The BTM samples were analysed by IDEXX ELISA (Fasciolosis Verification test, IDEXX, Hoofddorp, the Netherlands). The results of this test are expressed as the sample to positive percentage (S/P%) which is a difference in optical density between antigen coated and antigen non-coated wells relative to that of positive control on the same plate. The farms were classified as "high" ($150 \leq$ S/P%), "medium" ($80 <$ S/P% < 150), "low" ($30 <$ S/P% ≤ 80) and "negative" (S/P% ≤ 30) according to manufacturer's instructions. For the second phase, farms that were registered in the voluntary milk performance control scheme and delivered milk to the dairy company Arla Foods Ltd. were selected for the study in order to ensure the availability of milk production records and avoid delays in the collection of milk. Within these, 20 farms from each BTM ELISA category were randomly selected for BTM sampling as well as individual-level milk samples from a target of 7

randomly selected animals in the spring of 2017. Due to logistical issues it was not always possible to obtain 7 individual milk samples: any farms with fewer than 4 individual milk samples were excluded from this dataset. Data from a total of 71 farms formed the 2017 dataset.

Collection of register data

All cattle in Denmark are electronically ear tagged and registered in the Central Husbandry Register (CHR), which is managed by the Ministry of Food, Agriculture and Fisheries. Corresponding animal-level information provided by veterinarians, farmers, and abattoirs is stored in the Danish Cattle Database (DCD), which is managed by SEGES Landbrug og Fødevare F.m.b.A. A national milk control scheme is conducted pursuant to EU regulations on food hygiene (EC No 853/2004). Milk producers representing approximately 90% of Danish milking cattle are also voluntarily registered in a milk recording program to measure milk production and quality from individual animals. The 305 day energy corrected milk yield (305d ECM) combines mean yield, protein and fat contents in one measure and is calculated according to the guidelines of the International Committee for Animal Recording (ICAR) as standard information in DCD. The data required for this study representing herd size, 305d ECM, breed and parity was extracted for the relevant farms covering the period between 2014 and 2017 from DCD in collaboration with SEGES. Because milk yields from multiple lactations were available for the same animals, an attempt was made to use the most relevant milk recording data based on the assumption that infection occurred before the previous autumn, i.e. at least approximately four to six months before the spring 2017 sampling date. We therefore selected the most relevant lactation for each animal in the following order of preference: 1) a lactation within which the sampling date occurred over half way through the lactation period, 2) a lactation that had ended before the sampling date but for which the difference between the sampling date and the middle point of that lactation was less than 365 days, and 3) a lactation within which the sampling date occurred during the first half of that lactation.

Collection of milk samples and ELISA

All milk samples included in this study were acquired through Eurofins Steins Laboratorium A/S (Vejen, Denmark), where the routine milk analyses (e.g. somatic cell counts) were performed. For the 2017 data, any individual milk samples taken more than four days before or after the corresponding BTM collection date were removed. The milk samples were frozen at -20 °C and shipped to our laboratory. For BTM samples collected in 2016, one aliquot was tested by IDEXX ELISA at our laboratory in autumn 2016 and the other was sent to the University of Liverpool and tested by an in-house ELISA test (LIV-ELISA) in winter 2016/2017 for comparison.

The milk samples were defrosted and centrifuged at 1000 *g* for 20 minutes, after which the fat was removed, and the remaining whey was divided into two cryo-tubes and frozen at -20 °C until analysis. For ELISA, the samples were defrosted overnight in the refrigerator and analysed in duplicate by IDEXX ELISA according to manufacturer's instructions. The arithmetic mean of the two replicates was used for this study. If optical densities in duplicate values differed by 0.2, then the sample was repeated. To minimise the inter-plate variation, the whole plate that exceeded more than 0.2 in positive control mean net extinction was re-tested. The sensitivity and specificity of this particular test on milk have been reported as: 95% and 98.2% relative to sera (Reichel et al., 2005), and 97.7% and 99.3% relative to faecal egg counts (Molloy et al., 2005).

The method used for LIV-ELISA is described by (Salimi-Bejestani et al., 2005b). The results of this test are expressed as percent positivity (PP) of positive control and a PP of 27 or above is considered positive. The sensitivity and specificity of this test on milk have been reported as 92% and 88% relative to sera (Salimi-Bejestani et al., 2007).

Questionnaire survey

The questionnaire consisted of 25 questions regarding various farm-level demographical data (e.g. herd size, size of grazing and crop production areas etc.) and management practices (e.g. type of grazing areas, grazing periods and duration of the grazing etc.). It also contained questions concerning gastrointestinal nematodes and farmers' opinions about anthelmintic resistance, because this aspect of the data collection also formed part of an EU project (Practices for Organic Parasite Control "PrOPara") aiming to determine the status quo of helminth control practices in organic ruminant farms across EU (Additional file 1). The telephone interviews were conducted by three individuals using the same script in spring 2016. Out of 30 variables in the initial questionnaire, 12 were removed because the main category comprised > 90% of responses (Additional file 2).

An additional farm-level proxy for environmental risk factors was also collected in the form of the model output from a previous spatial analysis of liver fluke infections in Denmark (Olsen et al., 2015). This represents the overall estimated probability that a given farm will send a positive animal for slaughter based on environmental variables such as presence of streams and grasslands (CORINE database), and animal movement data along with meat inspection data from approximately 1.5 million cattle during 2011 to 2013 in Denmark (Olsen et al., 2015).

Statistical analysis

Three separate statistical models were used to address the various different aspects of this study. Firstly, a linear model was used with farm-level average 305d ECM as the response, along with explanatory variables reflecting the 2016 BTM ELISA results, predominant breed, average parity,

questionnaire responses, and farm-level proxy for environmental risk factors. In order to determine the utility of both qualitative and quantitative interpretations of ELISA results, this information was recoded into two variables: S/P classification, a simple dichotomous classification using a cut-off at 30 S/P%, and S/P positive value (degree of positivity), a continuous variable reflecting the S/P% conditional on a positive dichotomous classification. The pair of effects to be estimated could take one of the following two sets of values within the model:

- Underlying S/P% ≤ 30%:
    1. S/P classification: the reference category (negative result)
    2. S/P positive value: variable recoded to zero, thus having no effect within the model
- Underlying S/P% > 30%:
    1. S/P classification: effect to be estimated (positive result)
    2. S/P positive value: effect to be estimated (variable reflecting the S/P% value; adjusted to have mean of 0)

Average 305d ECM was calculated using data from all available individual-animal 305d ECM from a lactation period corresponding to the day of BTM sampling, and the number of available individuals from which the average was calculated was used as a weighting variable in the regression. The average parity was calculated from the same individuals, and was fit using both linear and quadratic terms within the model. The herd size was also fit using both linear and quadratic terms. Interaction terms between BTM ELISA variables and average parity were also included. Due to the large number of potential risk factors from the questionnaire, these were selected for inclusion using forwards & backwards stepwise selection based on AIC. The final dataset comprised complete recordings from 218 of the farms sampled in 2016. The same analysis was also conducted using the results of LIV-ELISA. For the purposes of identifying risk factors for parasitism, a separate logistic regression model was conducted using the same data but using the dichotomised BTM result as the response and ignoring milk yield information.

The second analysis comprised individual-level analysis of milk yield and milk sample ELISA data from the 2017 dataset. Individual-level 305d ECM was used as the response in a mixed effects linear regression, along with explanatory fixed effects of individual-level milk ELISA, breed, parity group (classified as 1st lactation, 2nd lactation, & 3rd or older), and a random effect of farm. Individual milk sample ELISA values were transformed into two variables in the same way as described for the BTM ELISA values. Interaction terms between lactation group and ELISA positivity, as well as between lactation group and degree of ELISA positivity were tested in the model using AIC. Complete data were available from a total of 284 lactating cows from 55 farms.

The final analysis related to the relationship between BTM ELISA and apparent within-herd prevalence. This was analysed using a mixed effects logistic regression, where the outcome was the dichotomized individual-animal milk ELISA results, and fixed effects of BTM ELISA S/P%, lactation number and breed as well as a random effect of farm were fitted as explanatory variables. Using the fitted model, the apparent within-herd prevalence corresponding to BTM ELISA S/P% values between 0-250 were predicted at 0.1 intervals. Corresponding 95% confidence intervals for these predicted values were estimated using a parametric bootstrap procedure.

In addition to these main analyses, visual assessment along with Spearman's correlation coefficients were calculated between IDEXX ELISA and LIV-ELISA results from the 2016 dataset, and between BTM ELISA results from 2016 and 2017. ELISA test results were entered in Microsoft Excel (2010) and all other data management and statistical analysis was performed using R (R Core Team, 2017). Mixed effects models and parametric bootstrapping was done using the *lme4* package (Bates et al., 2015), and all linear (mixed) models were checked for residual normality and homoscedasticity by visually inspecting a plot of residuals against fitted values as well as a quantile-quantile plot of residuals. Graphs were made in R using the *ggplot2* package (Wickham, 2009).

Results

Overall descriptions of the datasets used in the study are given in Table 1. From the 351 farms identified for inclusion in the 2016 dataset, BTM was not available from three farms and questionnaire responses were not available from 96 farms for various reasons including organic status under conversion and unwillingness to participate. The overall prevalence of fasciolosis in the final 2016 dataset was 49.5% (108/218). Using LIV-ELISA the prevalence was 48.6% (106/218), and the results of the two different ELISAs were strongly correlated (r_s=0.80, P<0.0001, Figure 1). As shown in Figure 2, BTM ELISA results from 2017 were highly correlated to those from 2016 (r_s=0.88, P<0.0001). Of the farms where the ELISA results differed in 2016 and 2017, only one farm had changed status by more than one adjacent category (from moderate to negative).

Table 1. Descriptive statistics of the 218 organic dairy farms used to estimate the effect of Fasciola hepatica infection on average 305 day energy corrected milk yield per farm.

	Mean	Range	SD	N
A) 2016 BTM dataset (N=218)				
Average 305d ECM	9166	6265; 12355	1126	--
Average parity	2.4	1.6; 3.5	0.29	--
No. milk recordings	109.0	14; 474	65.6	--
Herd size	329.3	98; 1307	189.4	--
BREED				
Danish Holstein	--	--	--	140
Jersey	--	--	--	24
Other	--	--	--	69
B) 2017 BTM dataset (N=71)				
Average 305d ECM	9239	6733; 12882	1232.1	--
Average parity	2.4	1.7; 4.0	0.38	--
No. milk recordings	115.3	2; 309	69.4	--
Herd size	414.0	137; 1237	220.5	--
BREED				
Danish Holstein	--	--	--	43
Other	--	--	--	28
C) 2017 individual cows (N=284, 55 farms)				
Average 305d ECM	9057	5042; 16250	1832	--
Average parity	2.1	1; 10	1.48	--
Number of samples per farm	5.4	4; 7	1.04	--
BREED				
Danish Holstein	--	--	--	185
Other	--	--	--	99

Figure 1. Relationship between the results of two BTM ELISA methods (a commercial ELISA by IDEXX and an in-house ELISA at the University of Liverpool, n=218). The blue line shows the line of best fit, and grey areas indicate 95% confidence intervals. The dashed lines show the cut-off values for each methods (27 PP for LIV-ELISA and 30 S/P% for IDEXX-ELISA).

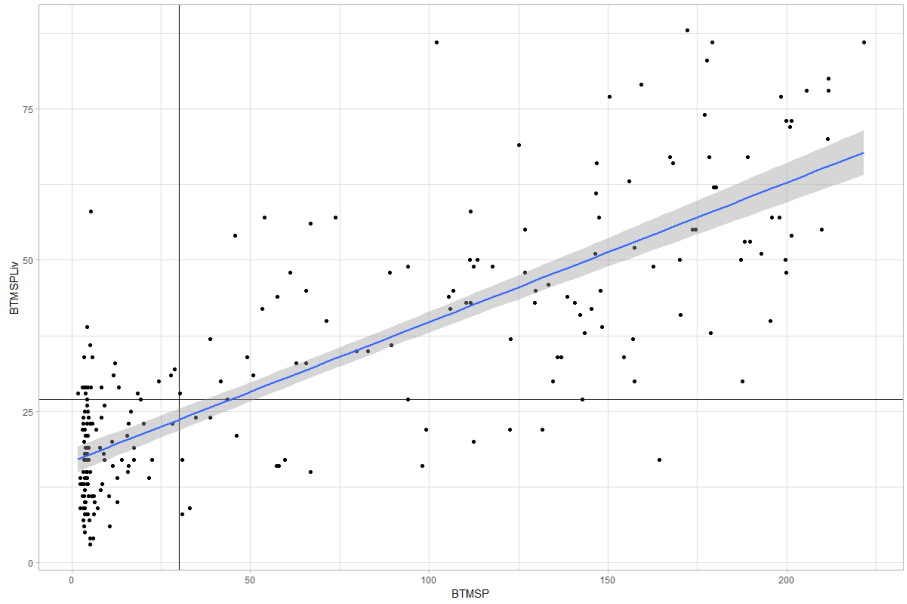
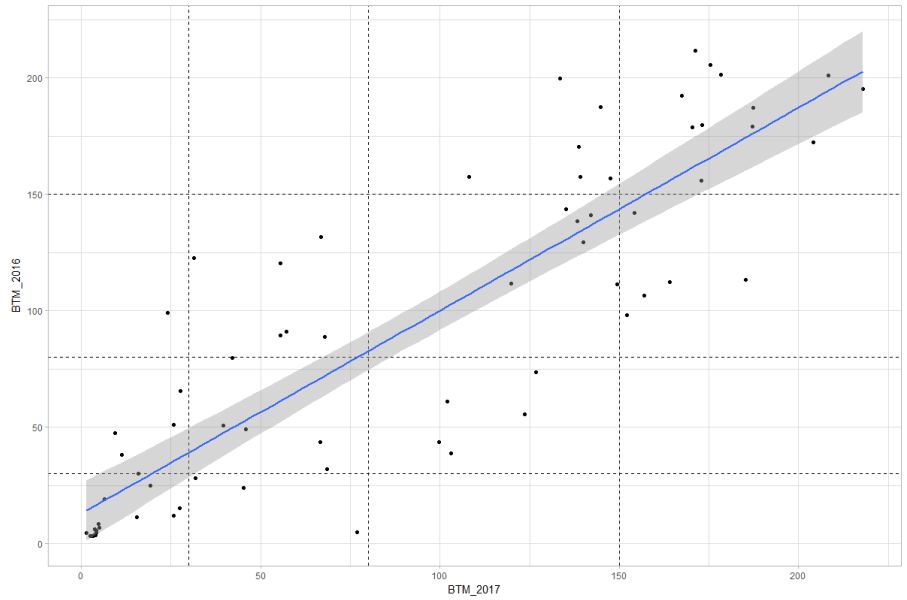


Figure 2. Regression plot showing correlation of bulk tank milk ELISA results collected in spring 2016 and spring 2017 from 71 Danish organic farms. The blue line shows the line of best fit, and grey areas indicate 95% confidence intervals. The dashed lines indicates the cut-off value for categorizing ELISA results (30, 80 and 150 S/P%).



Relationship between ELISA results and 305d ECM at farm level

A significant association between farm-average 305d ECM and BTM ELISA results was found in the 2016 data (Table 2); a 580.5 kg (CI: 200.8-750.7) reduction in average milk yield was associated with BTM positive farms compared to negative farms. Breed and average parity in linear and quadratic forms were also highly associated with average 305d ECM. The interaction term between BTM ELISA classification and average parity was significantly associated with 305d ECM; higher reduction was seen in positive farms with higher average parity. The degree of positivity and the interaction term between the degree of positivity and parity was not statistically significant. Qualitatively similar estimates were confirmed using the LIV-ELISA results; a 485.6 kg (CI: 217.1; 754.2) lower average milk yield per farm was significantly associated with BTM *F. hepatica* positive farms. The degree of positivity was non- significant but the interaction between the degree of positivity and average parity was significant in this model (48.4 kg, CI: 5.2; 91.5, P=0.027, data not shown).

Eight farm-level demography/management variables were associated with 305d ECM and included in the final linear regression model (Table 2) and of these, only five were significantly associated with higher 305d ECM: farms with Danish Holstein (compared to Jersey and other breeds), cows grazing half day during summer (compared to all day), larger herd size, no prevention measures/no fencing off wet areas, and dry cows grazing on dry pasture. The final farm-level logistic regression model with dichotomised BTM ELISA as the outcome included seven explanatory variables (Table 3). Odds of being BTM ELISA positive was higher in farms with application of flukicide treatments, heifers grazing wet areas, heifers having access to surface water, larger herd size, absence of other type of livestock (beef cattle, sheep and horse etc.) and no preventive drainage of wet areas. However, the only statistically significant variables were herd size and heifers grazing on wet areas. The predicted values for fasciolosis risk on farms (output of spatial risk analysis from Olsen et al. 2015) were not statistically significant in either model. None of the BTM ELISA negative farms applied flukicide treatment.

Relationship between ELISA results and 305d ECM at individual level

The analysis of individual milk samples from the 2017 data showed that 98 animals out of 284 animals (34.5%) were positive for *F. hepatica* by ELISA. The mixed linear regression model showed that 305d ECM was higher in animals with higher lactation numbers, and animals in 3rd or higher lactations that were ELISA positive had 919.5kg lower 305d ECM than ELISA negative animals in 3rd or higher lactation. The degree of positivity was not significantly associated with milk yield (Table 4).

Table 2. Estimates from a linear model showing associations between 305 day energy corrected milk yield (305d ECM) and anti-*Fasciola hepatica* antibody ELISA results from BTM samples, as well as variables used to control for farm demographic and management factors (R^2=0.330, df=200, P< 0.001)

Variable	Estimate	95% CI	SE	P value
S/P classification				<0.001
S/P negative	Ref			
S/P positive	-580.8	-847; -314.3	135.1	
S/P positive value	1.352	-1.98; 4.69	1.692	0.425
Average parity (linear)	1503.4	877.1; 2129.7	317.6	<0.001
Average parity (quadratic)	-1958.9	-3001; -916.4	528.7	<0.001
S/P classification : Average parity	-1676.1	-2608.9; -743.2	473.1	<0.001
S/P positive value : Average parity	-12.6	-26.4; 1.26	7.01	0.073
Herdsize (linear)	1.748	0.702; 2.795	0.531	<0.001
Herdsize (quadratic)	-1.55	-3.17; 0.071	0.823	0.059
Breed				<0.001
Danish Holstein	Ref			
Jersey	-912.0	-139.2; -431.6	243.6	
Other	-486.1	-779.9; -193.2	148.6	
Grazing time of cows in summer				0.003
Half day	Ref			
All day	-535.8	-893.2; -178.4	181.3	
Prevention by fences				0.023
No	Ref			
Yes	-296.4	-552.4; -40.4	129.8	
Grazing areas for dry cows				0.030
Dry	Ref			
Wet	-342.8	-654.7; -31.0	158.2	
Age of calf at turn-out in months				0.078
≥4	Ref			
<4	-302.2	-640.0; 35.6	171.3	
Heifer having access to surface water				0.062
No	Ref			
Yes	252.2	-14.5; 519.0	135.3	
Other Animals				0.160
No	Ref			
Yes	282.7	-113.9; 679.4	201.2	
Predicted value	1064.3	-213.5; 2342.1	648.0	0.101

Abbreviations: SE, standard error; 95% CI, 95% confidence interval; Ref, reference; BTM, bulk tank milk

Table 3. Estimates from a logistic regression model showing risk factors associated with anti-Fasciola hepatica BTM ELISA infection status.

Variable*	Estimate	SE	*P*-value	OR	95% CI
Herdsize (linear)	0.004	0.001	0.002	1.00	1.00; 1.00
Herdsize (quadratic)	-0.004	0.002	0.069	1.00	0.99; 1.00
Grazing areas for heifers					
Dry	Ref		<0.001	Ref	
Wet	1.184	0.347		3.27	1.67; 6.55
Heifers having access to surface water			0.164		
No	Ref			Ref	
Yes	0.463	0.331		1.59	0.83; 3.04
Other Animal			0.507		
No	Ref			Ref	
Yes	-0.308	0.465		0.74	0.29; 1.83
Prevention by drainage			0.395		
No	Ref			Ref	
Yes	-0.301	0.354		0.74	0.37; 1.48
Predicted value	0.365	1.542	0.813	1.44	0.07; 30.3

*Treatment against liver flukes was a significant risk factor, but it was omitted from the final model for estimate calculations as it was quasi-separated with the outcome.

Abbreviations: SE, standard error; 95% CI, 95% confidence interval; Ref, reference; BTM, bulk tank milk

Table 4. Estimates from a linear mixed model showing associations between 305 day energy corrected milk yield (305d ECM) and anti-*Fasciola hepatica* antibody ELISA results in individual milk samples taken from 284 cows on 55 farms in 2017.

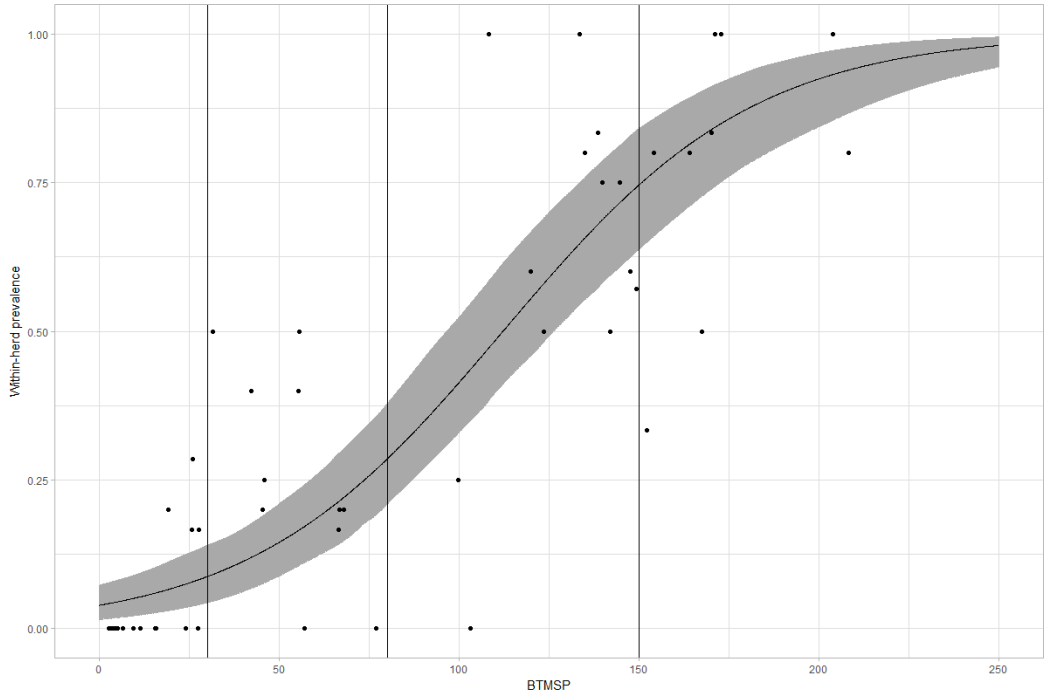
Variable	Estimate	95% CI	SE	P value
S/P classification				0.713
S/P negative	Ref			
S/P positive	90.8	-388.3; 569.9	247.0	
S/P positive value	-1.287	-6.586; 4.006	2.730	0.638
Parity				<0.001
Parity 1	Ref			
Parity 2	1051.4	622.8; 1482.3	221.1	
Parity 3+	2256.1	1836.2; 2675.7	216.3	
Parity : S/P classification interaction				<0.001
Parity 1 : S/P positive	Ref			
Parity 2 : S/P positive	334.8	-490.2; 1155.6	423.4	
Parity 3+ : S/P positive	-919.5	-1629.3; -209.7	365.8	
Breed				0.5123
Danish Holstein	Ref			
Other	158.6	-313.7; 634.1	238.3	

Abbreviations: SE, standard error; 95% CI, 95% confidence interval; Ref, reference

BTM ELISA results and within-herd prevalence

The mixed effects logistic regression with dichotomised individual milk ELISA values from the 2017 data as the response did not show a statistically significant association with breed or parity (*P*= 0.06, *P*= 0.105, respectively). These variables were therefore removed, leaving only BTM ELISA value as a fixed effect and farm as a random effect within the final model. Based on the coefficient obtained from this model, the estimated mean apparent within-herd prevalence was ≤ 8.8% (95%CI: 4.3-14.0) if BTM ELISA was negative, > 8.8% (95%CI: 4.3-14.0) and ≤ 28.5% (95%CI: 20.8 – 37.8) if low, > 28.5% (95%CI: 20.8 – 37.8) and < 74.6% (95%CI: 63.7 – 84.1) if moderate, and ≥ 74.6% (95%CI: 63.7 – 84.1) if high (Figure 3).

Figure 3. Association between bulk tank milk (BTM) ELISA values and within-herd prevalence determined from the coefficient estimates produced by a mixed effects logistic regression model (using 284 individual milk samples from 55 farms). The black line shows the line of best fit, and grey areas indicate 95% confidence intervals for the mean relationship between ELISA value and within-herd prevalence. The vertical line indicates the cut-off value for categorizing ELISA results (30, 80 and 150 S/P%) and the solid dots show the raw prevalence observations at farm level.



Discussion

A significant reduction in average 305d ECM corresponding to 580.8 kg was associated with BTM ELISA positive farms in this study (Table 2), which equates to an approximately 6% reduction in overall milk production per cow. This study used 305d ECM to include the effect on the fat and protein contents and included potential confounders in the model, and therefore the results may not be directly comparable with other studies. However, our results are similar to findings in Belgium and Spain where reductions of 3% and 5% in milk yield were observed (Charlier et al., 2007; Mezo et al., 2011). Higher reduction (15%) was seen in UK where the parasite is more prevalent and burdens are generally higher (Howell et al., 2015). To put this loss into perspective, milk yield reduction due to clinical mastitis was estimated by systematic review to be 375 kg (5%) on average (Seegers et al., 2003). Average total loss during one lactation period due to ketosis was 353.4 kg per cow in fourth or more lactations in Finland (Rajala-Schultz et al., 1999). Therefore, liver fluke infection seems to cause similar or possibly higher milk production loss than mastitis and ketosis. This is probably due to the fluke causing long lasting effect over the entire lactation period, despite the absence of obvious clinical symptoms.

Nevertheless, the farm-level results should be interpreted with caution, because the model can only conclusively demonstrate correlation and does not prove causation of *F. hepatica* infection on milk production. One of the difficulties in dealing with herd-level production is that substantial variation is observed among farms (Mezo et al., 2011). Although this study tried to minimise this variation by selecting only organic farms, it is apparent that many factors other than *F. hepatica* infection status cause variation in 305d ECM (Table 2). For example, our model demonstrated that if the lactating cows were on grass all day rather than half day during summer, the average milk yield per farm was reduced by 535.8 kg: this is most likely a proxy for extensively vs. intensively managed herds. Despite the efforts to account for farm-level demographic and management factors using questionnaire data, these data are not perfect, and it is therefore likely that some exogenous farm-level demographic and management factors that are related to both production and parasitism remain unaccounted for. We therefore included a second study relating individual-animal milk ELISA values to milk yields based on animals sampled from the same farms. This comparison within farms should be more robust to unexplained farm-level demographic and management factors due to the use of a farm-level random effect. Analysis of the individual-animal dataset also showed a significant reduction in 305d ECM of 919.5kg in infected older cows (3rd or higher lactations), although no associations between 305d ECM and ELISA results were seen in younger

cows (Table 4). This was also reflected in the herd-level data; the ELISA positive farms with higher average parity had a significantly lower average 305d ECM relative to ELISA positive farms with lower average parity (Table 2; interaction between average parity and S/P classification). One of the possible reasons for this finding could be that the older cows have accumulated higher fluke burdens with longer exposure, as complete protective immunity against *F. hepatica* is unlikely (Graham-Brown et al., 2018). In addition, our previous epidemiological study also indicated that flukes may last longer than two years, suggesting the long-lasting effect on the liver (Takeuchi-Storm et al., 2018). Chronic infection induces hepatic changes, which may take one to two years to reverse after elimination of the parasites, or even longer (three to four years) in case of severe fibrosis (Rahko, 1974). Charlier et al. (2012) found that the highest increase in milk yield was seen in first lactation cows after closantel treatment against liver flukes. This superficially seems to contradict our finding, but may make sense when considering that recovery from fluke infection may be faster in young animals with less extensive hepatic changes compared to the older cows with chronic infection. It is also possible that the effect from the liver fluke infection is indirect i.e. due to concurrent infections. Other diseases such as clinical mastitis and lameness are also shown to be associated with increasing age (Sogstad et al., 2005; Breen et al., 2009). The liver fluke infection may be exacerbating the effect of these disorders that reduce milk yield, or older animals with concurrent infections could be more susceptible to liver fluke infections. Moreover, the older animals have greater milk-producing capacity. The magnitude of milk production loss may be thus greater upon parasitic challenges as seen in nematode infections (Sanchez et al., 2004; Blanco-Penedo et al., 2012).

However, these explanations are all speculative and the present study does not conclusively prove causation despite the corroborating evidence from both datasets. Older cows are more efficient in producing general immune response due to sensitisation (van Knegsel et al., 2007). Milk antibodies against *O. ostertagi* were elevated in cows with experimentally induced mastitis (Charlier et al., 2006). These studies indicate that the elevated anti-*F. hepatica* antibody levels in older cows could be due to non-specific immune response, as a result of concurrent infection that may reduce milk yield. Additionally, animals were in different stages of lactation at the time of sampling and multiple lactations were available for some animals. Although we selected the 305d ECM estimate from the most appropriate lactation event based on biological assumptions, we do not know when the animals acquired infection in our study: this could have biased the results. Ultimately, the interactions between milk production, parasitic disorders and other concurrent infections as well as the effect of parity are likely to be complex and therefore require further investigation.

The model also showed that the dichotomized ELISA result at cut-off of 30 S/P% was significantly associated with the milk reduction, but the degree of positivity above 30 S/P% was not statistically significant at both farm and individual level. This means that the most appropriate interpretation of ELISA is based on a qualitative assessment of negative vs. positive. This is partly consistent with the previous finding by Charlier et al. (2008), showing that IDEXX ELISA was unable to differentiate cows with high and low fluke burdens, while LIV-ELISA could. We therefore used within-herd prevalence (based on a dichotomised within-animal test result) rather than quantitative individual milk ELISA results (i.e. average S/P% of individual milk samples) to assess associations with BTM antibody levels. The model showed that the values of BTM ELISA were highly correlated with within herd-prevalence in accordance with previous studies (Salimi-Bejestani et al., 2005a; Mezo et al., 2010). The economic threshold for *F. hepatica* infection at herd-level is suggested as a within-herd prevalence of 25% (Vercruysse and Claerebout, 2001). Using LIV-ELISA with the cut-off that is able to detect herds with 25% within-herd sero-prevalence (Salimi-Bejestani et al., 2005a), a significant milk yield reduction was shown in UK (Howell et al., 2015). Using the MM3-SERO ELISA assay, which was able to detect a 12% within-herd prevalence (Mezo et al., 2010), Mezo et al. (2011) found a negative association between fasciolosis and milk yield loss only when the cut-off was increased to a level that corresponds to >25% within-herd prevalence. The IDEXX ELISA used in the present study was previously shown to identify farms with 20% infected cows at the manufacturer's recommended cut-off (Duscher et al., 2011). However, based on our results, the minimum within-herd prevalence was 8.8% for a farm to be positive by BTM ELISA, and decrease in average 305d ECM was seen above this cut-off. This means that the economic threshold for *F. hepatica* infection could be lower than 25%.

Despite our expectation that milk yield reduction would increase with increasing within-herd prevalence, no clear difference in milk production according to the degree of ELISA positivity was seen. The absence of associations with BTM ELISA positivity and milk yield in our study could be due to lack of statistical power. Many farm factors affect both *F. hepatica* antibody levels and milk yield (e.g. average parity) and therefore the underlying associations may be overwhelmed by the factors that were not controlled in the model. The use of flukicides on some of the BTM ELISA positive farms could perhaps result in the farms with high anti-*F. hepatica* antibody levels without any production loss, because the antibody levels can persist three to six months after treatment against liver flukes (Castro et al., 2000; Salimi-Bejestani et al., 2005b; Mezo et al., 2007). However, this factor was not statistically significant and excluded from the model, probably because the farms that use anthelmintics had variable infection levels and the effect on milk yield could also be variable. Despite the missing link between increasing within-herd prevalence and greater milk yield

loss, the present study demonstrated the usefulness of BTM ELISA for quantitative estimation of the within-herd prevalence.

A number of factors related to farm demography/management were tested for inclusion in both the milk yield and *F. hepatica* infection models at herd-level. Of these, herd size was the only significant factor associated with both milk yield and *F. hepatica* infection (Table 2 and 3). However, a number of related variables were identified by both models: both longer grazing time and wet grazing for dry cows were associated with lower milk yield, and wet grazing areas for heifers was associated with *F. hepatica* antibodies. Both sets of variables probably partly reflect the difference between more intensive and extensive farms. The identification of heifers as a risk group for fasciolosis is in accordance with our previous case-control study including both conventional and organic farms (Takeuchi-Storm et al., 2017). It is common to raise heifers on pasture in Denmark, and relative to other age groups heifers are more frequently used to graze marginal/natural land to protect natural areas due to governmental incentive (Buttenschøn, 2007; Takeuchi-Storm et al., 2017). Therefore, it was not surprising that heifers on wet pasture were the most influential contributing factor for *F. hepatica* infection. The predicted probability for *F. hepatica* infection status of farms based on herd and spatial factors from a previous Danish study (Olsen et al., 2015) was not significantly associated with either milk yield or *F. hepatica* infection. This variable was calculated based on the outcome of fasciolosis spatial distribution analysis calculated from the liver condemnation data (at least one liver condemnation at slaughter) and environmental factors associated with cattle farms. The estimated effect of this predicted value is positive as expected, although this positive effect is not statistically significant in our risk factor analysis; this is most likely due to inadequate power to detect a significant effect, but may also be due to differences in herd classification between the two studies. Our outcome was based on ELISA results, which detects a farm with certain within-herd prevalence, while the previous study by Olsen et al. 2015 was based on liver condemnation and a farm with only one infected animal as also classified as infected.

Conclusion

This study showed a significantly reduced 305d ECM of 580.8 kg in *F. hepatica* infected organic Danish dairy herds as determined by BTM IDEXX ELISA. A significant decrease in 305d ECM corresponding to 919.5kg at the individual animal level was also observed in infected cows in 3rd or later lactations, although no significant milk production loss was seen in younger infected cows. Further research is required to validate these findings, and establish causative pathways for the biological mechanisms leading to reduced production in older cows. We also demonstrated that

interpretation of individual milk ELISA results is most appropriately qualitative rather than quantitative. In accordance with our previous study, heifers grazing on wet areas was identified as the main risk factor for fasciolosis. Additionally, we found that BTM ELISA values were highly correlated to within-herd prevalence using the individual milk sample results to estimate the within-herd prevalence. The minimum detection within-herd prevalence in the current study was low (8.8%), suggesting that the economic threshold could be lower than 25%.

List of abbreviations

BTM: Bulk tank milk; CI: Confidence interval; DCD: Danish cattle database; ELISA: Enzyme-linked immunosorbant assay; OR: Odds ratio; S/P%: Sample to positive percentage.

Declarations

Farmers consented to milk samples and herd information being used for research as part of milk control and supply contracts. Consent from SEGES to use data extracted from DCD for this project was acquired on 19th January 2017.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

NTS, MD, HLE and SMT designed the study. NTS performed BTM ELISA. NTS and MD managed data and performed statistical analyses. NTS, MD, HLE and SMT interpreted results. NTS wrote the manuscript and all the other authors assisted with the revision. All authors read and approved the final manuscript.

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Additional file 1. Table1. Original questionnaire form

Cattle Questionnaire PrOPara

To be completed by the interviewer of the respective country

Germany		Lithuania	
Sweden		Denmark	
Netherlands		Switzerland	

1 Is the farm organic (cattle systems)?

Please mark the farm system with "1"

Conventional	
Organic	
In-conversion	

If organic, for how many years since starting conversion?

2 Please indicate the approximate number of animals (if of economic importance)

	Approx. Number		Approx. Number
Dairy cows (milk)		Beef Cows (meat)	
Dairy (growing cattle) 1yr+		Beef growing cattle 1yr+	
Dairy (young stock) <1yr		Beef (young stock) <1yr	

	Approx. Number		Approx. Number
Breeding sheep/Goats		Other (*specify*)	
Growing lambs/kids		Other (*specify*)	

3 Please indicate the approximate area of varying grassland and crop types

	Approx. Area (ha)
Permanent pasture	
Arable cropland and permanent crops	
Temporary leys	
Other (specify)	
Total area	

Additional short term grazing, e.g. mountain grazing? *1=Yes, 2=No, 3=Don't know*

4 Please indicate the typical number of hours grazed daily in summer and winter

Please mark the appropriate answers with "1", only one mark per column and season

		Dairy cows	Beef cows	Young stock (dairy/beef) 1st grazing year	Growing cattle (dairy/beef) 2nd grazing year
Summer	12 - 24 hrs				
	1 - 12 hrs				
	Housed				
Winter	12 - 24 hrs				
	1 - 12 hrs				
	Housed				

5 Use of specific grazing routines/pasture management to control gastrointestinal worms / flukes

a) Worms	Heard of	Tried on-farm	Currently utilised
	1=Yes, 2=No, 3=Don't know		
Reduced stocking rates			
Young stock use lower risk areas (see definition)			
Other (specify)			

b) Fluke	Heard of	Tried on-farm	Currently utilised
	1=Yes, 2=No, 3=Don't know		
Pasture drainage			
Fencing of streams and ponds			
Pasture rotation from infectious to non-infectious during grazing season*			
Other (specify)			

** The option pasture rotation means move cattle from wet (and therefore potentially infective) pastures to dry pastures during the grazing season.*

Is fluke a diagnosed problem (e.g. lab/slaughterhouse) on your farm?

1=Yes, 2=No, 3=don't know

6 Please list the 3 main methods used for monitoring worms and flukes

Please mark up to 3 boxes per column with "1"	Gastro-intestinal worms	Liver flukes
Warnings via media, e.g. nematodirus linked to spring temperature		
Veterinary advice		
Based on previous experience		
Analysis of faecal samples		
Slaughterhouse feedback		
Diarrhoea		
Poor/dull fur/hair quality		
Anaemia		
Loss of weight/body condition/milk yield		
Antibodies in milk		
Other (specify)		

7 Do you typically (in the last 5 years) use commercial anthelmintics (dewormers) for the hereafter mentioned groups to control worms and flukes?

Please mark the appropriate answers with "1"

	Gastro-intestinal worms				**Liver flukes**			
	Dairy cows	Beef cows	Young stock (dairy/beef) 1st grazing year	Growing cattle (dairy/beef) 2nd grazing year	Dairy cows	Beef cows	Young stock (dairy/beef) 1st grazing year	Growing cattle (dairy/beef) 2nd grazing year
No treatments used for this group								
Animals are usually treated individually								
Animals are usually treated as a group								
Typical number of treatments per year								

8 If you use commercial anthelmintics (dewormers) when are they usually applied?

Please mark the appropriate answers with "1"

	Gastro-intestinal worms				**Liver flukes**			
	Dairy cows	Beef cows	Young stock (dairy/beef) 1st grazing year	Growing cattle (dairy/beef) 2nd grazing year	Dairy cows	Beef cows	Young stock (dairy/beef) 1st grazing year	Growing cattle (dairy/beef) 2nd grazing year
Before calving/dry cows								
After calving								
At start of grazing season (around turnout)								
At start or during housing period								
Before transferring to clean (low-risk) pasture								
At weaning								
Other (please specify)								

9 Frequent deworming of the whole herd may lead to worms or flukes that are resistant to commercial anthelmintics; against this background, indicate if you agree or disagree with the following statements:

	Please mark with a "1" (Only one per question/row)	Disagree strongly	Disagree	Not sure	Agree	Strongly agree
a	Anthelmintic resistance is an increasing problem					
b	To prevent further anthelmintic resistance farmers may have to accept reduced production through less treatments					
c	Industry will develop improved treatments/vaccines before resistance becomes a problem					
d	I would accept alternative control methods that may incur greater costs; e.g. monitoring, products or new equipment, analysis of faecal samples					
e	I would accept alternative control methods that may incur greater on farm labour input; e.g. sample collection, animal monitoring					
f	Increased focus on monitoring and treating individual animals is a feasible worm control strategy					

10 Have you had anthelmintic resistance confirmed on your farm for worms?

1=Yes, 2=No, 3=Don't know

Gastrointestinal worms	

If yes, to which active component?

			Yes=1
Worms	Benzimidazole	(White drenches)	
	Levamisole	(Clear drenches)	
	Macrocyclic lactones	(Ivermectin/Moxidectin)	

11 Have you had anthelmintic resistance confirmed on your farm for flukes ?

1=Yes, 2=No, 3=Don't know

Liver flukes	

If yes, to which active component?

			Yes=1
Liver fluke	Triclabendazole	(White, liver fluke)	
	Albendazole	(White, liver fluke)	
	Closantel	(Liver fluke)	
	Clorsulon	(Liver fluke)	

12 What is the breed of the milking cows?

Danish Holstein	
Jersey	
Danish Red Holstein (Rødbroget)	
RDM (red Danish milkbreed)	
Cross	
Other	

13 Do you graze lactating cows?
if yes, on wet (meadows, marsh) or dry areas (arable land, grassland, forest)?
And do they have access to natural waterway/surface water or drink from communal/ground water?

Not grazed	
Grazed - dry areas – access to surface water (water from river or lake)	
Grazed - dry areas - access to communal water / ground water	
Grazed – wet areas - access to surface water (water from river or lake)	
Grazed – wet areas – access to communal water / ground water	

14 Do you graze dry cows?
if yes, on wet (meadows, marsh) or dry areas (arable land, grassland, forest)?
And do they have access to natural waterway/surface water or drink from communal/ground water?

Not grazed	
Grazed - dry areas – access to surface water (water from river or lake)	
Grazed - dry areas - access to communal water / ground water	
Grazed – wet areas - access to surface water (water from river or lake)	
Grazed – wet areas – access to communal water / ground water	

15 Do you graze heifers (age group from 1 year-old up to calving)?
if yes, on wet (meadows, marsh) or dry areas (arable land, grassland, forest)?
And do they have access to natural waterway/surface water or drink from communal/ground water?

Not grazed	
Grazed - dry areas – access to surface water (water from river or lake)	
Grazed - dry areas - access to communal water / ground water	
Grazed – wet areas - access to surface water (water from river or lake)	
Grazed – wet areas – access to communal water / ground water	

16 Do you graze cavles (under 1 year old)?
if yes, on wet (meadows, marsh) or dry areas (arable land, grassland, forest)?
And do they have access to natural waterway/surface water or drink from communal/ground water?

Not grazed	
Grazed - dry areas – access to surface water (water from river or lake)	
Grazed - dry areas - access to communal water / ground water	
Grazed – wet areas - access to surface water (water from river or lake)	
Grazed – wet areas – access to communal water / ground water	

17 What is the age (in month) when calves come out on grass for the first time

in month

18 When did cows turned out in 2015? (month)

month

19 When did cows housed in 2015? (month)

month

20 Do lactating cows graze with heifers and/or calves

Yes
No

21 Do dry cows graze with heifers and/or calves

Yes
No

22 Did you buy any animals (incl. cows, heifers, calves) in the last three years?

	cows	heifers	calves
Yes			
No			

23 Do you have any shared grazing with other farms

Yes
No

24 Did any of the pasture used for grazing in 2015 also used to graze other types of animals other than cattle (e.g. sheep)?
If yes, what?

Yes — if yes, species
No

25 Did you treat cows, heifers, and/or cavles against liver flukes in 2015?

Yes
No

If yes, which anthelmintics were used?

	cows	heifers	calves
No treatment in this group			
Valbazen (albendazole) oral, white drench			
Closamectin pour-on (closantel+ivermectin) on skin, clear			
Bimectin plus (clorsulon+ivermectin) injection, clear			
Fasinex (triclabendazol) oral, only dispensation			
Cannot remember			
Other			

26 Further comments

Additional file 2. Table2. The number of farms for each explanatory variable used in the risk factor analysis (n=218). The farms were categorized according to anti-F. hepatica antibody levels in bulk tank milk measured by IDEXX-ELISA

	Negative (n=110)	**Positive (n=108)**
Mean herd size ± SD	296.0±172.6	363.1±200.3
Mean Predicted Value ±SD	0.69±0.09	0.71±0.11
Breed		
Danish Holstein	69	66
Jersey	16	6
Mixed	25	36
Daily grazing time for cows		
Half day	88	82
All day	22	26
Grazing areas for dry cows		
Dry	93	76
Wet	17	32
Grazing areas for heifers		
Dry	57	24
Wet	53	84
Grazing areas for calves		
Dry	100	100
Wet	10	8
Dry cows having access to surface water		
No	94	81
Yes	16	27
Heifers having access to surface water		
No	63	36
Yes	47	72
Prevention by drainage		
No	81	84
Yes	29	24
Prevention by fencing around wet areas		
No	67	67
Yes	43	41
Prevention by moving to dry areas during grazing season		
No	64	47
Yes	46	61
Any treatment for liver flukes during 2015		
No	110	75
Yes	0	33
Age of calf at turn-out		
≥4	89	89
<4	21	19
Turn-out month in 2015		
April	92	92
May	18	16
Housing month in 2015		

September/October	71	75
November/December	39	33
Dry cows grazing with calves or heifers		
No	58	53
Yes	52	55
Purchase of heifers during 2013-2015		
No	98	99
Yes	12	9
Other livestock production on the farm (e.g. beef cattle, sheep, horse, deer etc.)		
No	96	97
Yes	14	11
Any grazing areas shared with other animal species (e.g. winter grazing with sheep)		
No	97	91
Yes	13	17