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Research paper

Patent infections with *Fasciola hepatica* and paramphistomes (*Calicophoron daubneyi*) in dairy cows and association of fasciolosis with individual milk production and fertility parameters

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ABSTRACT

Infections with the liver fluke *Fasciola hepatica* may result in considerable economic losses in the dairy livestock industry worldwide. Infections have been associated with detrimental impacts on milk production and milk quality as well as reduced fertility. However, most related data rely on examinations on herd level and the rather few studies on individual cow level are based solely on antibodies as measure for *F. hepatica* infections. This entails the risk of including false-positives as anti-*F. hepatica* antibodies persist for months even if the infection is cleared. Therefore, the presented study aimed to overcome this issue by assessing the association between *F. hepatica* infections measured via faecal egg counts (FEC) and milk production as well as fertility parameters in individual dairy cows. In total, 2006 faecal samples from 1166 Black and White dairy cows from 17 small and medium-sized German grassland farms were examined in July and September 2015. The relationship between patent *F. hepatica* infections and the milk production parameters milk yield, milk protein content, milk fat content and somatic cell score (SCS) was assessed in a linear mixed model using test-day records of individual cows. Patent *F. hepatica* infections were found on 35.3% (7/17) of farms with an individual cow prevalence of 10.1% (97/963) in July and 9.1% (95/1036) in September. Patent rumen fluke infections were detected on 17.6% (3/17) farms with an individual cow prevalence of 0.4% (4/963) in July and 0.7% (9/1036) in September. No significant association was found between *F. hepatica* infection status and either SCS as an indicator of udder health or milk production parameters, despite 0.06 and 0.10% lower values for milk protein and fat content in patently infected cows. Linear mixed models and generalized linear mixed models were established to estimate the impact of fasciolosis on the fertility parameters calving to first service (CTFS), calving interval (CI), success in first insemination (SFI) and 56-day nonreturn rate (NRR56). A significantly higher average CTFS of 4.69 days was detected in *F. hepatica* infected cows ($P = 0.025$), but no significant relationship was found for the other fertility parameters.

1. Introduction

Infections with the liver fluke *Fasciola hepatica* are still a major problem in pasture-based dairy farms in Europe (Bennema et al., 2009; Bloemhoff et al., 2015; Selemetas et al., 2015). Based on herd-antibody levels by using bulk tank milk (BTM) samples, prevalences up to 90% are reported for different Western European countries (Bennema et al., 2009; Duscher et al., 2011; McCann et al., 2010; Mezo et al., 2008; Selemetas et al., 2015). In some regions of Germany, up to 74.0% of herds are affected (Bolln et al., 2007; Kuerpick et al., 2012). Several factors, e.g. a regaining importance of pasture usage as well as climatic factors, have led to an increasing exposure of cattle to flukes in Germany as well as in other Western European countries during recent years (Fox et al., 2011; Gordon et al., 2013).

Clinical symptoms as a result of chronic fasciolosis are uncommon in older cattle (Kaplan, 2001). Nevertheless, high economic losses of approximately €299 per infected cow have been estimated, largely arising from reduced milk yield and fertility but additionally due to liver condemnation at abattoirs and a diminished meat production (Knubben-Schweizer et al., 2010; Schweizer et al., 2005). Especially in organic farm production systems, possibilities of anthelmintic treatment are highly restricted. Nevertheless, fasciolosis is also common when flukicides are routinely used (Charlier et al., 2014). Research indicated that older cattle with previous exposure show a lower *F.*

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hepatica burden in comparison to younger, parasite-naive cattle (Kaplan, 2001). Nonetheless, the immune response does not provide adequate protection neither in young nor in older cattle (Cawdery et al., 1977), with the consequence that animals remain susceptible to re-infection each year (Kaplan, 2001). Furthermore, infections with *F. hepatica* may lead to a suppression of immune responses (Molina-Hernández et al., 2015), possibly resulting in a higher susceptibility to other pathogens, especially bacterial infections (Claridge et al., 2012; Flynn et al., 2007). For dairy cattle, somatic cell counts are commonly used for the evaluation of intramammary infections (Schukken et al., 2003). However, so far no association was found between *F. hepatica* infections and somatic cell counts as an indirect expression of udder health (Howell et al., 2015; Mezo et al., 2011).

The impact of fasciolosis or treatment against *F. hepatica* on milk yield and milk composition in dairy cows was estimated in several studies with inconsistent findings. Decreases in the annual average milk yield ranged between 0.7 and 4.2 kg/cow/day in infected herds, as determined by herd antibody levels (Charlier et al., 2007; Howell et al., 2015; Köstenberger et al., 2017; Mezo et al., 2011). Regarding milk quality, an increase in herd *F. hepatica*-antibody levels has been related to a reduction between 0.06 and 0.09% in the annual milk fat content and a decline in the annual milk protein content of 0.05% (Charlier et al., 2007; Köstenberger et al., 2017), while other studies did not find a decline in milk fat content (Howell et al., 2015; Mezo et al., 2011). On an individual cow level, a significant increase of 303 kg in the 305-day milk production was estimated after closantel treatment, while no effect on the average milk protein and fat content was observed (Charlier et al., 2012). To date there is only one study estimating the association between liver fluke infections and dairy cow performance in untreated individual cows, reporting a significant average reduction of 2 kg milk/day in cows with high antibody levels, in comparison to cows with low values (Mezo et al., 2011). However, no associations between individual *F. hepatica*-antibody levels and milk protein content or milk fat content were found (Mezo et al., 2011). Negative effects of fasciolosis on different fertility parameters (e.g. conception rate or inter-calving interval) were described in herds with high antibody-levels compared with those showing low levels (Charlier et al., 2007; López-Díaz et al., 1998). Another study reported an increase in the *F. hepatica*-antibody level accompanied by a significant increase in the mean inter-calving interval per herd, with 4.7 days and longer intervals in herds with the highest antibody levels (Charlier et al., 2007). In contrast, other studies found no significant relationships between the occurrence of fasciolosis in the herd and reproduction performance in dairy cows in different European countries (Howell et al., 2015; Köstenberger et al., 2017; Simsek et al., 2007). Regarding individual dairy cows, a delay in oestrus and higher insemination rates for success to conception in *F. hepatica*-infected compared with non-infected cows were previously found (López-Díaz et al., 1998; Romaniuk, 1977). An increment of 0.75 services per conception and an extension of the service period by 13 days were described as a result of fasciolosis (Schweizer et al., 2005). In contrast, pregnancy rates were not significantly higher in cows treated against flukes compared with an untreated control group (Loyacano et al., 2002). Additionally, no associations between individual *F. hepatica*-antibody levels and calving to conception interval were found on an individual cow level (Mezo et al., 2011). These conflicting results may be due to the fact that all studies were based on measurements of antibody levels. Antibodies to *F. hepatica* increase from 2 weeks after infection and persist for up to 2 years (Santiago and Hillyer, 1988; Ortiz et al., 2000). After treatment with triclabendazol, antibodies were detected for up to 7 months (Castro et al., 2000; Ortiz et al., 2000). Thus, seropositivity does not necessarily reflect current infections. To overcome this limitation, faecal egg counts (FEC) were used in the present study to ensure present infections, accepting that only patent infections are covered by this method. Beside infections with *F. hepatica*, rumen flukes (paramphistomes) have recently been observed as emerging parasites in dairy cattle in several western European countries (Jones et al., 2015; Ploeger et al., 2017; Zintl et al., 2014). In the last decades, rumen flukes were generally considered to be *Paramphistomum* spp., but recent research has shown that most rumen flukes in Europe belong to the species *Calicophoron daubneyi* (Gordon et al., 2013; Zintl et al., 2014). Negative effects of patent rumen fluke infections on mortality and different morbidity parameters, e.g. anaemia, were observed in cattle (Dorny et al., 2011; Millar et al., 2012; Murphy et al., 2008).

The main objective of the presented study was to estimate the association between patent *F. hepatica* infections measured by egg excretion and i) milk production parameters (milk yield, milk protein, milk fat content), ii) somatic cell counts as an expression of udder health, iii) fertility parameters (calving to first service, calving interval, success in first insemination, 56-day nonreturn rate) in individual dairy cows. Additionally, the study aimed to assess patent rumen fluke infections in the individual dairy cows.

2. Material and methods

2.1. Farms and dairy cows

The study population included 1166 cows on 17 dairy farms located in 4 German federal states (Fig. 1). Mean herd size was 72 cows per farm. The selection of farms was based on a “pasture genetics project” aiming at comparisons between different genetic lines of Black and White dairy cattle with regard to endoparasite infections (May et al., 2017). Therefore, requirements for herd selection were i) different

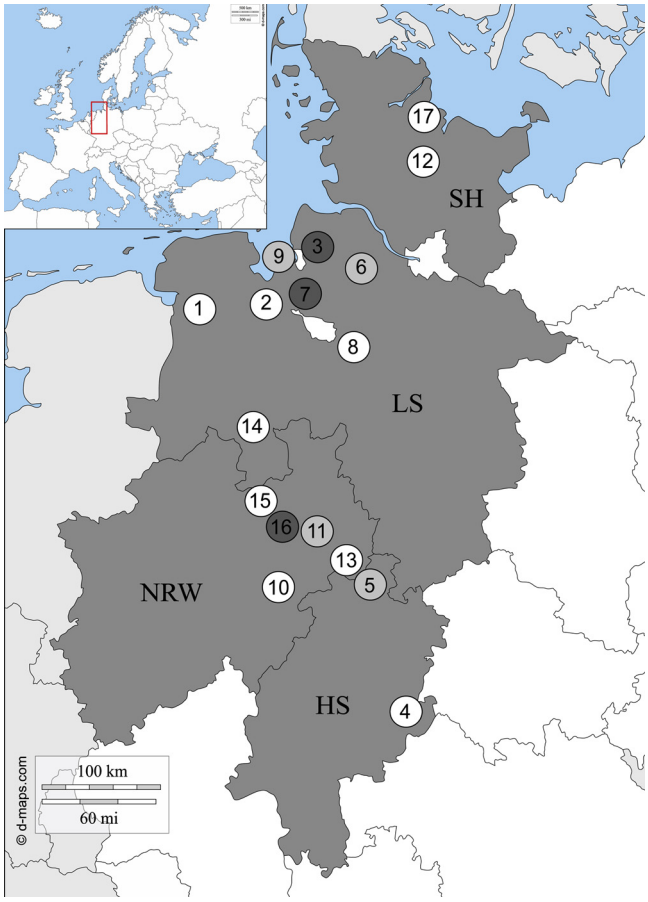

Fig. 1. Geographic distribution of the 17 included dairy farms. Dark grey dots: *F. hepatica* and rumen fluke positive farms; Light grey dots: *F. hepatica* positive but rumen fluke negative farms; White dots: *F. hepatica* and rumen fluke negative farms. German federal states are abbreviated as follows: HE = Hesse, LS = Lower Saxony, NRW = North Rhine-Westphalia, SH = Schleswig-Holstein. The numbers of the farms are corresponding with Table 1.

genetic lines per farm, ii) access to pasture not later than 1st of June and cow grazing > 8 h per day, and iii) no treatments with flukicides or other anthelmintics in the sampling year. The following genetic lines of Black and White dairy cows were represented on the farms: HF-NZ (72 cows) = German Holstein cow (GHC) x New Zealand Holstein sires; HF-GHm (639 cows) = GHC x German Holstein sires with high breeding values for milk yield; HF-GHp (70 cows) = GHC x German Holstein sires selected for pasture conditions and DSN (363 cows) = local Black and White dual-purpose cows of the *Deutsche Schwarzbunte Niederungsrind*, the founder of the current Holstein breed; Crosses (22 cows) = GHC x Jersey, Angler or beef cattle sires.

2.2. Faecal sampling and coproscopical examination

Faecal sampling of lactating dairy cows was conducted during two periods in the year 2015: 963 samples of 16 farms were obtained in July and 1043 samples of 17 farms in September. The interval between both sampling occasions was approximately 8 weeks per farm. Faeces were sampled from each milking cow, resulting in 2006 faecal samples of 1166 dairy cows with repeated sampling of 840 cows. Cows with only one parasitological measurement were dry cows on inaccessible pastures, purchased cows in September (no measurement in July) or cows which were sold or had died. Collected samples were instantly cooled to 4 °C and transported to the Institute for Parasitology, University of Veterinary Medicine Hannover, within 3 h after collection. Samples were processed by the sedimentation technique to determine FEC in 10 g faeces per cow for *F. hepatica* (FEC-FH) and paramphistomes (FEC-P).

2.3. Bulk tank milk samples

In September, a bulk-tank milk (BTM) sample was taken from each farm. Samples were transported at a steady temperature of 4 °C to the Institute for Parasitology, University of Veterinary Medicine Hannover. Bulk-tank milk samples were centrifuged at 2000 × *g* for 15 min to separate the cream from the milk. After centrifugation, the fat layer was removed and milk was stored at −20 °C until tested by BTM ELISA (IDEXX Fasciolosis Verification test, Montpellier, France). Results were expressed as sample to positive percentage (S/P%) for each sample. As specified by the manufacturer, samples with S/P% higher than 30% were considered positive for the presence of antibodies against *F. hepatica*.

2.4. Molecular identification of rumen fluke eggs

For rumen fluke species identification, respective eggs were isolated from faecal samples of 5–6 individual cows of the three rumen fluke positive farms. Genomic DNA was extracted by incubating 2–3 eggs per individual cow with 90 µl DirectPCR® Lysis Reagent (Cell) (Peqlab, Germany) and 10 µl proteinase K (Peqlab, Germany) for 16 h at 55 °C, followed by 85 °C for 45 min. A PCR targeting the ITS-2 region and the flanking 5.8S and 28S rDNA sequences was performed, using ITS-2For and ITS-2Rev primers (Itagaki et al., 2003). The reaction was carried out in a volume of 50 µl containing 1 µl DreamTaq DNA Polymerase (5 U/µl) (ThermoFisher Scientific, Germany), 5 µl 10x DreamTaq buffer, 1 µl dNTP mix (10 mM each), 2 µl of each primer (10 µM each), and 10 µl DNA template. Thermocycling conditions were as follows: initial denaturation at 95 °C for 3 min, 40 cycles of 95 °C for 30 s, 53 °C for 1 min, 72 °C for 45 s, and final elongation at 72 °C for 10 min. The PCR products were visualized on a 1% agarose gel and subsequently custom sequenced (Seqlab Sequence Laboratories Göttingen, Germany). Obtained sequences were compared with public sequences deposited in NCBI GenBank.

2.5. Milk production and fertility data

Individual dairy cow data were provided from the National Genetic Evaluation Center (Vereinigte Informationssysteme Tierhaltung, VIT, Verden, Germany). Individual test-day cow milk production data for all 1166 cows included monthly repeated measurements for milk yield (kg milk/cow/day), milk protein content (in %), milk fat content (in %) and somatic cell counts (SCC, cells per ml). Somatic cell counts were **log**-transformed into somatic cell score: SCS = $\log_2$ (SCC/100.000) + 3 (Ali and Shook, 1980). Individual cow fertility data were the most recent available measurement for the interval between calving to first service (CTFS), the interval between two calving events = calving interval (CI), the binary-coded success in first insemination (SFI) and the binary-coded 56-day nonreturn rate (NRR56) of the current lactation. Further variables related to milk production and fertility and included in the analyses were: age, parity, days in milk (DIM), breed or genetic line, calving data, insemination data, insemination bulls according to insemination date.

2.6. Statistical analyses

Statistical analyses were performed using SAS version 9.4 (SAS Institute; Cary, NC, USA) and R version 3.3.4 (R Core Team, 2013). The cows were classified by their *F. hepatica* infection status (FEC-FH ≥ 1 = positive; FEC-FH = 0 = negative) and rumen fluke infection status (FEC-P ≥ 1 = positive; FEC-P = 0 = negative) as assessed with the sedimentation technique. Differences in *F. hepatica* infection status of repeatedly sampled cows between both sampling occasions (July and September) were assessed using a McNemar test. A Chi-squared test was used to estimate differences in *F. hepatica* infection status between primiparous and multiparous cows for July and September separately, since some cows switched lactation number from the first to the second sampling occasion. All cows as of parity five were classified in "parity > 4" due to the low number of cows in higher parities. Differences in *F. hepatica* infection status between parities within sampling occasions were analysed by using the 2-tailed Fisher's exact test. *P*-values ≤0.05 were considered significant for all descriptive statistics and following model analyses.

Linear mixed models (PROC MIXED) were applied to study the association between individual patent *F. hepatica* infection status (independent variable) and milk production parameters (milk yield, milk protein and fat content, SCS) as dependent variables based on individual test-day records (model [1]). Further independent variables, which were included as fixed effects in the multivariable analyses were: Parity number (1, 2, 3, 4, > 4), genetic line (HF-NZ, HF-GHm, HF-GHp, DSN, Crosses) and test-day season (records for milk production parameters before September, records in September and later). For the milk production parameter SCS, the fixed effect" test-day season" was not taken into account, since differences in somatic cells in the milk occur only between summer and winter (Harmon, 1994; Sharma et al., 2011). Days in milk was included as a covariate in model [1]. Farm and cow identity were included as random effects. We compared Akaike information criteria (AIC) for different models by including various definitions of independent variables (e.g., different shapes of lactation curves) and including only statistically significant factors in the models. AIC values were always the best for the final models and included all physiological relevant variables. For milk production data, a dataset was created including the first test-day after each parasitological measurement for each cow. For cows with two parasitological measurements, milk production parameters of two test-days were considered in the model, while one test-day was used for cows having only one FEC. The time span between parasitological measurement and the first following test-day ranged from 0 to 69 days with a mean of 22.7 days and no overlap between the first test-day after parasitological measurement in July and the parasitological measurement in September. Long intervals between parasitological measurement and test-

day date resulted from cows which were dry directly after parasitological measurement. Individual test-day records and parasitological measurements were available for 1030 cows.

To assess the relationship between individual patent *F. hepatica* infection status (independent variable) and fertility parameters CTFS and CI (dependent variables), linear mixed models were defined (model [2]). For the fertility parameters CTFS and CI, the most recent record of both parameters preceding parasitological measurements was used and combined with both measurements for the infection status of each cow. Cows with a CTFS ≥ 200 days were excluded from all analyses regarding this parameter. Furthermore, cows with a CI ≥ 500 days were excluded from all analyses regarding this parameter. Independent variables besides *F. hepatica* infection status were the fixed effects parity number (1, 2, 3, 4, > 4), genetic line (HF-NZ, HF-GHm, HF-GHp, DSN, Crosses) and calving season (Dec-Feb; Mar-May; Jun-Aug; Sep-Nov for the years 2014 and 2015). Farm and cow were included as random effects. Parasitological measurements and records for CTFS were available for 835 cows, while records for CI included 700 cows in the multivariable analysis.

Generalized linear mixed models (SAS GLIMMIX) were applied to identify associations between individual patent *F. hepatica* infection status (independent variable) and the binary-defined fertility parameters SFI and NRR56 (dependent variables) (model [3]). In this model, the fixed effect of first insemination season (12 classes for the 12 months in 2015) and the age of cows (in months) as a covariate were included in the analysis. Fixed effects and the covariate 'age', which were taken into account in models [2] and [3], are known as influential factors on fertility parameters in dairy cattle (Dohoo et al., 2001; Jaeger et al., 2016; Sun and Su, 2010). For both fertility parameters SFI and NRR56, observations of the year 2015 were used exclusively, in order to minimize the time span between both parameters and present *F. hepatica* infections. Farm, cow and insemination bull were considered as random effects. For model [3], parasitological measurements and records for SFI and NRR56 were available for 711 cows.

For model validation, diagnostic plots of residuals of all final models were visually inspected. Quantile-quantile plots of residuals from linear mixed models are shown in Supplementary Fig. 1, comparing their distribution to the standard normal distribution. For binomial generalized linear mixed models, distribution and densities of residuals were checked and the models were found to be valid.

3. Results

3.1. Patent F. hepatica infections

The coproscopically determined *F. hepatica* prevalence on herd level was 37.5% in July (6/16) and 35.3% in September (7/17). Regarding the 16 repeatedly sampled farms, a similar *F. hepatica* herd-level prevalence was found in July (37.5%, 6/16) and September (31.3%, 5/16) (McNemar test = 0; DF = 1; P = 1). In 4 of the 5 repeatedly positive farms, prevalence dropped in September, while an increase in the prevalence of 20.4% was observed for one farm in September (Table 1).

On individual level, 11.9% (139/1166) of the included cows were excreting *F. hepatica* eggs on at least one sampling date. Of these, 10.1% (97/963) were positive in July and 9.1% (95/1036) in September. FEC-FH values ranged from 0 to 26 eggs/10 g faeces in July with a mean of 0.5 (SE = 2.3), and from 0 to 89 eggs/10 g faeces in September with a mean of 0.7 (SE = 4.5) eggs/10 g faeces (Table 1). For the *F. hepatica* positive samples, mean FEC-FH was 5.2 (SE = 5.6) eggs/10 g faeces in July and 7.5 (SE = 13.2) eggs/10 g faeces in September. Of the 840 cows with repeated measurements, 6.3% (53/840) were positive for *F. hepatica* at both sampling occasions, with no significant difference between July and September (McNemar test = 0.907; DF = 1; P = 0.341). In July, no significant difference in *F. hepatica* infection status between primiparous cows (8.3% positive) and multiparous cows (10.7% positive) was observed (Chi squared test; χ^2 = 1.14; DF = 1; P = 0.290). In September, the prevalence for *F. hepatica* was significantly higher in multiparous cows (10.3% positive) compared with primiparous cows (5.9%) (Chi squared test; χ^2 = 4.71; DF = 1; P = 0.030). The percentage of cows showing egg excretion within different parity categories in July and September is presented in Fig. 2. In September, the percentage of patently infected cows in parity > 4 was significantly higher than in parity 1 (Fisher's exact test, P = 0.040).

3.2. Rumen fluke infections and molecular species identification

Rumen fluke eggs were detected in 12.5% (2/16) of herds in July and 17.6% (3/17) in September. In all positive herds, *F. hepatica* eggs were detected as well.

On individual level, 0.9% (11/1166) of the included cows excreted rumen fluke eggs on at least one sampling date. In the sampling month July individual prevalence was 0.4% (4/963) and in September 0.7% (9/1036). FEC-P values of all observations ranged from 0 to 9 eggs/10 g faeces with a mean of 0.02 (SE = 0.3) eggs/10 g faeces. For all the rumen fluke positive samples (n = 13), mean FEC-P was 3.08 (SE = 2.7) eggs/10 g faeces. Only 0.2% (2/840) of the cows with repeated measurements showed rumen fluke egg excretion at both sampling occasions, and no significant difference in rumen fluke prevalence was observed between the sampling months (McNemar test = 2.3; DF = 1; P = 0.131). At the first sampling occasion in July, egg excretion was found in cows as of parity 2, while in September egg excretion was distributed over all parities up to parity 7. In all but one cow, co-infections with *F. hepatica* were identified. Rumen fluke species identification was feasible for 2 farms (farm 7 and 16). The sequenced ITS-2 PCR product showed 100% identity with *C. daubneyi* (GenBank accession number KP201674).

3.3. F. hepatica BTM ELISA results

Positive BTM ELISA results (i.e. values for S/P % > 30%) were observed for all six farms showing patent *F. hepatica* infections in September (Table 1), even for farm no. 9 with the lowest within-herd prevalence according to fecal samples (3.6%). For the farm showing a low patent within-herd prevalence only in July (1.4%, farm no. 6; Table 1), the BTM ELISA was negative in September (S/P % value ≤30%). Negative BTM ELISA results were also obtained for all farms with a negative result in the coproscopical examination.

3.4. Milk production and fertility data

Table 2 presents descriptive statistics for test-day cow milk production data and fertility data for all cows, *F. hepatica* positive and negative cows. For milk production parameter analysis, up to 1683 test-day records of 1030 cows were used in the multivariable analysis. Overall the averaged milk production parameters were 21.4 kg (range, 2.4–47.6 kg) per cow/day milk yield, 3.5% (range, 2.42–6.32%) milk protein content, 4.2% (range, 1.94–8.51%) milk fat content, and 3.2 (range, 0.01–9.64) SCS.

Regarding fertility parameters, 1441 observations of 835 cows were available for CTFS and 1206 observations of 700 cows for CI. The average CTFS was 88.3 (range, 19–198) and the average CI 383.3 (range, 286–500). The fertility parameters SFI and NRR56 included each 1200 observations of 711 dairy cows. The average probability was 53% for SFI and 66% for NRR56.

3.5. Relationship between F. hepatica infection status and milk production parameters in individual cows

Results of linear mixed model [1] for milk production parameters are presented in Table 3. The association between *F. hepatica* infection status and milk yield was not statistically significant (P = 0.091), showing a slightly lower average daily milk production in *F. hepatica*

Table 1
Patent *F. hepatica*-infections on sampled farms and BTM ELISA results (available for September only).

Farm	Farm size (no. of cows)	July 2015			September 2015			BTM ELISA result[b]
		No. of tested samples	Positive samples (no. / %)	Mean FEC-FH (eggs/10 g faeces)	No. of tested samples	Positive samples (no. / %)	Mean FEC-FH (eggs/ 10 g faeces)	
1	215	164	0 / 0	0.0	197	0 / 0	0.0	–
2	80	54	0 / 0	0.0	54	0 / 0	0.0	–
3*	70	n.d.	n.d.	n.d.	50	3 / 6.0	0.1	+ + +
4	65	55	0 / 0	0.0	54	0 / 0	0.0	–
5	82	59	24 / 40.7	1.7	76	27 / 35.5	1.2	+ + +
6	105	74	1 / 1.4	0.0	60	0 / 0	0.0	–
7*	90	70	43 / 61.4	2.4	81	36 / 44.4	2.9	+ + +
8	70	68	0 / 0	0.0	54	0 / 0	0.0	–
9	57	56	6 / 10.7	0.3	56	2 / 3.6	0.1	+ +
10	79	76	0 / 0	0.0	75	0 / 0	0.0	–
11	71	60	8 / 13.3	1.2	67	8 / 11.9	0.7	+ + +
12	77	76	0/ 0	0.0	68	0 / 0	0.0	–
13	48	44	0 /0	0.0	45	0 / 0	0.0	–
14	16	15	0 / 0	0.0	15	0 / 0	0.0	–
15	32	26	0 / 0	0.0	31	0 / 0	0.0	–
16*	31	30	15 / 50.0	4.8	27	19 / 70.4	12.3	+ + +
17	40	36	0 / 0	0.0	33	0 / 0	0.0	–
Mean	**72.2**	**963**[a]	**97 / 10.1**	**0.5**[c]	**1043**[a]	**95 / 9.1**	**0.7**[c]	**n.d.**

FEC-FH = faecal egg count (eggs/10 g faeces); n.d. = no determined.
* Farms also positive for rumen flukes.
[a] Total number.
[b] Expressed as sample to positive percentage (S/P%): – = negative (% S/P ≤ 30%), + = mild positive (30 < % S/P ≤ 80), + + = positive (80 < % S/P < 150), + + + = strong positive (% S/P ≥ 150).
[c] Mean was based on all individual samples in July and September 2015.

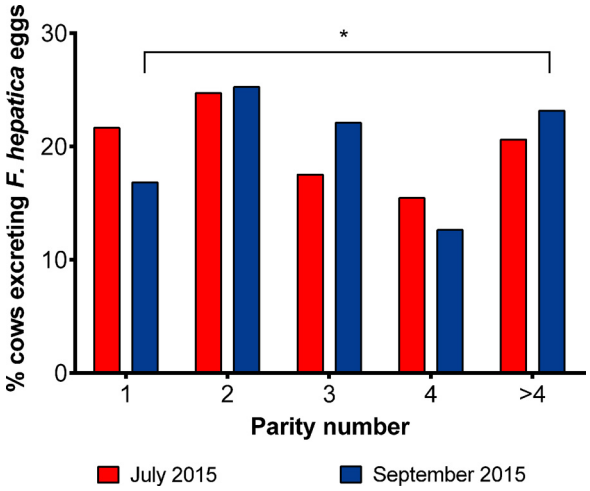

Fig. 2. Percentage of cows excreting *F. hepatica* eggs within parity categories in July and September 2015. The asterisk indicates statistical significance.

negative (21.22 kg/cow/day) compared with *F. hepatica* positive (22.05 kg/cow/day) cows (Supplementary Table 1). All other fixed effects were significantly associated with the average daily milk production in model [1] (Supplementary Table 1). A non-significant difference of 0.06% higher milk protein content in non-infected compared with infected cows was observed ($P = 0.079$). Similarly, milk fat content was 0.10% higher for non-infected cows, but again no statistical significance was found ($P = 0.102$), whereas the other fixed effects were significantly associated with both milk quality parameters (Table 3; Supplementary Table 1). Regarding SCS, non-infected cows showed a higher mean SCS (3.26) compared with infected cows (mean SCS: 3.10), but the effect of *F. hepatica* infection status on SCS was not significant ($P = 0.274$; Supplementary Table 1). Further potential confounders (parity, DIM) tested in the present analysis were statistically significant except of genetic line for SCS.

3.6. Relationship between F. hepatica infection status and fertility parameters in individual cows

Full model results, results for least-squares means and the test of significance for fixed effects (sum of squares type III) as included in the linear mixed model [2] are given in Table 4 and Supplementary Table 2. *F. hepatica* infected cows showed a significantly ($P = 0.025$) higher average CTFS of 4.69 days compared with non-infected cows (Supplementary Table 2). The genetic line effect was identified as an influential factor on the fertility parameter CI but not on CTFS. No association was found between *F. hepatica* infection status and CI ($P = 0.502$; Table 4; Supplementary Table 2). Results for the association between *F. hepatica* infection status and fertility parameters SFI and NRR56 are shown in Table 4 and Supplementary Table 2. Infected cows showed similar results for SFI (59.5%) compared with non-infected cows (58.9%, $P = 0.956$). Furthermore, no significant difference in probability for NRR56 was found between *F. hepatica* negative cows (73.9%) compared with *F. hepatica* positive cows (78.2%, $P = 0.594$).

4. Discussion

In the presented study, we used FECs as an indicator of current *F. hepatica* infections to investigate the impact of fasciolosis on individual dairy cow performance. Regarding milk production parameters, no associations with infection status were identified. This contrasts the only other available study on untreated individual cows, which detected a significant average reduction of 2 kg milk/cow/day for cows showing high anti-*F. hepatica* antibody levels (Mezo et al., 2011). On a herd level, the association between anti-*F. hepatica* antibodies in BTM and milk production has been addressed in several studies (Charlier et al., 2007; Howell et al., 2015; Köstenberger et al., 2017; Kuerpick et al., 2012; Mezo et al., 2011). Although total number of included herds, model analyses and correction for influential factors on milk yield varied in these studies, significant reductions in total milk yield between 0.7 and 4.2 kg milk/cow/day in herds showing high BTM antibody levels compared with those with low antibody levels were

Table 2
Descriptive statistics for milk production parameters[a] and fertility parameters in individual dairy cows.

Variable	Total records				Negative *F. hepatica* infection status (FEC-FH = 0 eggs/10 g faeces)				Positive *F. hepatica* infection status (FEC-FH ≥ 1 eggs/10 g faeces)			
	n	Mean	SD	Range	*n*	Mean	SD	Range	*n*	Mean	SD	Range
Average milk yield (kg/cow/day)	1683	21.36	7.12	2.40–47.60	1522	21.52	7.09	2.40–47.60	161	19.83	7.22	3.00–39.00
Average milk protein content (%)	1682	3.51	0.43	2.42–6.32	1521	3.51	0.43	2.42–6.32	161	3.50	0.40	2.52–5.49
Average milk fat content (%)	1682	4.18	0.70	1.94–8.51	1521	4.20	0.71	1.94–8.51	161	4.07	0.58	2.28–6.46
Average somatic cell score	1681	3.18	1.66	0.01–9.64	1520	3.16	1.67	0.01–9.64	161	3.36	1.58	0.01–7.74
CTFS[b] (days)	1441	88.34	36.59	19.00–198.00	1319	88.81	36.55	19.00–198.00	122	83.26	36.39	26.00–188.00
CI[c](days)	1206	383.29	45.96	286.00–500.00	1115	383.81	45.26	286.00–500.00	91	376.92	53.61	304.00–500.00
SFI[d]	1200	0.53	0.50	0.00–1.00	1117	0.53	0.50	0.00–1.00	83	0.46	0.50	0.00–1.00
NRR56[d]	1200	0.66	0.47	0.00–1.00	1117	0.67	0.47	0.00–1.00	83	0.61	0.49	0.00–1.00

SD, standard deviation; *n*, number of records; FEC-FH, faecal egg count in 10 g faeces.
[a] records included each first test-day record after parasitological measurement.
[b] cows with a CTFS ≥ 200 days were excluded.
[c] cows with a CI ≥ 500 days excluded.
[d] only observations in the year 2015 were included.

Table 3
Fixed-effect parameter estimates with corresponding confidence limits (95% CI)[1] as well as *P*-values for fixed effects and covariance parameter estimates[2] for the milk production parameters milk yield (kg/cow/day), protein content (%), fat content (%) and somatic cell score from the multivariable linear mixed model [1].

	Milk yield		Protein content		Fat content		Somatic cell score	
	Estimate (95% CI)[1]	*P*-value	Estimate (95% CI)[1]	*P*-value	Estimate (95% CI)[1]	*P*-value	Estimate (95% CI)[1]	*P*-value
Intercept	29.65 (27.31; 31.99)	< 0.001	3.20 (3.07; 3.33)	< 0.001	4.07 (3.81; 4.34)	< 0.001	2.73 (2.11; 3.34)	< 0.001
F. hepatica status								
negative	−0.83 (−1.80; 0.13)	0.091	0.06 (-0.01; 0.12)	0.079	0.10 (-0.02; 0.23)	0.102	0.15 (-0.12; 0.43)	0.266
positive	Baseline		Baseline		Baseline		Baseline	
Parity								
1	−3.77 (−4.65; −2.90)	< 0.001	0.03 (-0.02; 0.09)	0.262	−0.00 (-0.11; 0.11)	0.975	−1.25 (-1.52; -0.97)	< 0.001
2	−1.89 (−2.79; −0.99)	< 0.001	0.12 (0.07; 0.18)	< 0.001	0.11 (-0.00; 0.22)	0.055	−0.72 (-0.99; -0.44)	< 0.001
3	0.34 (−0.59; 1.28)	0.472	0.09 (0.03; 0.15)	0.003	−0.05 (-0.17; 0.07)	0.423	−0.58 (-0.86; -0.28)	< 0.001
4	0.51 (−0.51; 1.52)	0.328	0.08 (0.01; 0.14)	0.017	0.07 (-0.05; 0.20)	0.256	−0.45 (-0.76; -0.13)	0.006
> 4	Baseline		Baseline		Baseline		Baseline	
Genetic line								
DSN	−1.58 (−3.27; 0.12)	0.069	−0.02 (-0.12; 0.08)	0.750	−0.24 (-0.45; -0.04)	0.020	0.54 (0.04; 1.05)	0.035
Crosses	0.44 (−1.82; 2.70)	0.701	−0.03 (-0.17; 0.12)	0.715	−0.14 (-0.42; 0.15)	0.345	0.44 (-0.27; 1.45)	0.229
HF-GHm	0.64 (−0.56; 1.84)	0.296	−0.17 (-0.25; -0.09)	$< 10^{-4}$	−0.34 (-0.49; -0.19)	$< 10^{-4}$	0.20 (-0.18; 0.58)	0.306
HF-GHp	0.92 (−0.63; 2.48)	0.245	−0.18 (-0.28; -0.08)	< 0.001	−0.35 (-0.55; -0.16)	< 0.001	−0.15 (-0.64; 0.35)	0.562
HF-NZ	Baseline		Baseline		Baseline			
Test-day season								
< September	1.00 (0.58; 1.43)	< 0.001	−0.22 (-0.25; -0.19)	< 0.001	−0.20 (-0.25; -0.14)	< 0.001	n.d.	n.d.
≥ September	Baseline		Baseline		Baseline		n.d.	n.d.
Days in milk	−0.038 (−0.039; −0.036)	< 0.001	0.002 (0.002; 0.002)	< 0.001	0.002 (0.002; 0.002)	< 0.001	0.004 (0.003; 0.005)	< 0.001
Random effects	Estimate[2]		Estimate[2]		Estimate[2]		Estimate[2]	
Farm	7.56		0.008		0.049		0.237	
Cow	11.66		0.042		0.155		1.418	
Residual	12.06		0.057		0.238		0.803	

F. hepatica status: binary defined FEC-FH (FEC-FH = 0 classified as '*F. hepatica* negative'; FEC-FH ≥1 classified as '*F. hepatica* positive'). n.d. = not examined for somatic cell score.

Table 4
Fixed-effect parameter estimates with corresponding confidence limits (95% CI)[1] as well as *P*-values for fixed effects and covariance parameter estimates[2] for the fertility parameters calving to first service (CTFS) and calving interval (CI) from the multivariable linear mixed model [2] and for success in first insemination (SFI) and 56-day nonreturn rate (NRR56) for fixed effect classes included in generalized linear mixed model [3][3].

	CTFS		CI		SFI		NRR56	
	Estimate (95% CI)[1]	P-value	Estimate (95% CI)[1]	P-value	Estimate (95% CI)[1]	P-value	Estimate (95% CI)[1]	P-value
Intercept	83.98 (68.22; 99.75)	< 0.001	411.95 (392.59; 431.30)	< 0.001	4.25 (1.63; 6.88)	0.002	4.41 (1.87; 6.95)	0.001
F. hepatica status								
Negative	−4.69 (-8.80; -0.59)	0.025	−1.45 (-5.69; 2.79)	0.502	0.02 (-0.84; 0.89)	0.956	−0.23 (-1.10; 0.63)	0.594
Positive	Baseline		Baseline		Baseline		Baseline	
Parity								
1	−7.10 (−13.85; −0.36)	0.039	93.80 (4.32; 183.28)	0.040				
2	−0.02 (−6.58; 6.54)	0.995	−32.74 (-41.73; -23.75)	< 0.001				
3	5.89 (−0.68; 12.46)	0.079	−25.76 (-33.90; -17.61)	< 0.001				
4	1.98 (−4.54; 8.49)	0.551	−37.33 (-45.74; -28.93)	< 0.001				
> 4	Baseline		Baseline					
Genetic line								
DSN	−3.99 (-17.31; 9.33)	0.557	−4.77 (-22.23; 12.69)	0.592				
Crosses	−3.90 (−21.66; 13.85)	0.666	−21.94 (-51.44; 7.56)	0.145				
HF-GHm	2.73 (−5.95; 11.42)	0.537	9.00 (-5.88; 23.88)	0.235				
HF-GHp	−3.21 (−14.67; 8.26)	0.583	12.33 (-6.63; 31.28)	0.202				
HF-NZ	Baseline		Baseline					
Calving season								
Jun-Aug 2014	17.79 (11.58; 24.00)	< 0.001	−16.46 (-24.06; -8.87)	< 0.001				
Sep-Nov 2014	5.03 (-1.08; 11.14)	0.107	10.90 (4.24; 17.56)	0.001				
Dec 2014-Feb 2015	9.39 (1.56; 17.22)	0.019	−6.02 (-16.33; 4.29)	0.252				
Mar-May 2015	8.02 (0.56; 15.49)	0.035	−18.16 (-27.12; -9.19)	< 0.001				
Jun-Aug2015	24.34 (17.98; 30.69)	< 0.001	−4.10 (-11.32; 3.13)	0.266				
Sep-Nov 2015	Baseline		Baseline					
Insemination season								
January					−5.41 (-7.88; -2.95)	< 0.001	−3.43 (-5.78; -1.07)	0.004
February					−3.21 (-5.72; -0.71)	0.012	−2.39 (-4.81; 0.03)	0.053
March					−3.74 (-6.22; -1.25)	0.003	−2.51 (-4.91; -0.11)	0.040
April					−3.59 (-6.09; -1.09)	0.005	−2.89 (-5.31; −0.48)	0.019
May					−3.42 (-5.90; -0.94)	0.007	−2.53 (−4.90; −0.13)	0.039
June					−3.05 (-5.55; -0.56)	0.016	−1.87 (−4.29; 0.56)	0.131
July					−3.48 (−5.98; −0.97)	0.007	−2.59 (−5.02; −0.17)	0.036
August					−3.75 (−6.24; −1.25)	0.003	−2.51 (-4.93; -0.09)	0.042
September					−3.60 (−6.13; −1.08)	0.005	−2.95 (−5.38; −0.51)	0.018
October					−4.05 (−6.57; −1.52)	0.002	−3.11 (−5.54; −0.67)	0.012
November					−3.41 (−5.88; −0.95)	0.007	−3.27 (−5.64; −0.91)	0.007
December					Baseline		Baseline	
Age (in months)					−0.01 (-0.02; −0.00)	0.035	−0.01 (−0.02; −0.00)	0.008
Random effects	Estimate[2]		Estimate[2]		Estimate[2]		Estimate[2]	
Farm	308.00		132.09		0.3319		0.2325	
Cow	974.91		1978.65		0.3486		0.3492	
Insemination bull					0.2395		0.2389	
Residual	87.48		59.51					

noticed in all these studies. However, *F. hepatica* antibody detection does not allow differentiation between a current infection and a previous exposure to the parasite, which does not always have detrimental effects on productivity (Mezo et al., 2011). Moreover, cross-reactivity of *F. hepatica* excretory-secretory (ES) products, which are used as antigen in the ES ELISA, with antibodies against *D. viviparus* or *D. dendriticum* has been described (Cornelissen et al., 1999; Mezo et al., 2007). FECs in turn have the limitation that prepatent infections remain undetected. Thus, only cows with access to pasture before June were included (except for farm 3 with sampling only in September) in the present study, to allow cows to reach patency at the first sampling in July. Nevertheless, it cannot be entirely excluded that some cows were still prepatently infected in July, and thus considered as negatives in the analysis. Moreover, we used FEC as a binary definition in all model analyses, since correlation between FEC and fluke burden is known to be low (McCaughey and Hatch, 1964). For the analysis of model [1], we considered the first-test day after parasitological measurement since negative effects on milk production parameters might not be apparent in the prepatent period for newly or re-infected cows before the first sampling occasion. The inclusion of only one or two observations for milk production parameters might lead to reduced statistical power to estimate the long-term effect of an infection on individual cow performance. However, variations in immunity and infection pressure between individual cows hamper the identification of the optimal time point or time span covering the strongest influence of patent infections on production. In the presented study, we detected no significant difference in the least-square means for milk yield between *F. hepatica*-positive cows (22.1 kg/cow/day) and *F. hepatica*-negative cows (21.2 kg/cow/day). This finding may be the result of genetic variation in resilience to *F. hepatica* infections among individual cows as described in studies on nematode infections in cattle or sheep (Bishop, 2012; Bisset and Morris, 1996). Resilience is defined as the ability to maintain the current level of productivity in the face of infection (Bishop, 2012). Hence, the underlying hypothesis here is that some cows might be more resilient against *F. hepatica* with the ability to maintain performance during infection. Moreover, genotypic-specific differences in the ability of *F. hepatica* to complete tissue migration and liver establishment in the final host have been hypothesized (Zintl et al., 2015). Thus, maintenance of performance despite a current infection due to "low-pathogen *F. hepatica* genotypes" might be a further explanation in this regard, but has not yet been examined.

Associations between *F. hepatica* infection status and milk protein as

well as fat content were not significant in our analysis, which is in accordance with antibody-based studies in individual dairy cows (Charlier et al., 2012; Mezo et al., 2011). Additionally, no relationship between *F. hepatica* antibody levels and milk quality parameters was observed on herd level via multivariable analysis by Howell et al. (2015). In contrast, significantly lower values of 0.05 to 0.09% for protein and fat content due to *F. hepatica* infection were reported by Charlier et al. (2007) using multivariable analysis and by Köstenberger et al. (2017) using *t*-test. Albeit the relationship between *F. hepatica* infection status and milk quality parameters was not significant in our study, the tendency of lower protein and fat contents (0.06 to 0.10%) is in accordance with the above mentioned studies.

In the dairy cattle livestock industry, udder health and diseases related to mastitis play a decisive role. Previous research documented no significant influence of *F. hepatica* infections on milk somatic cell count or bacterial status in dairy cows (Howell et al., 2015; Mezo et al., 2011), which is in accordance with our results. Providing that somatic cell counts are a good indicator for udder health, patent infections with *F. hepatica* do not appear to trigger mastitis or increase susceptibility to udder infections. In contrast, a trend towards higher SCS values was identified for non-infected cows in the current study. One explanation for this observation addresses the specific immune response in the course of *F. hepatica* infections, characterized by activation of macrophages (Adams et al., 2014; Donnelly et al., 2005, 2008), which are the dominant cells in low SCS milk. In addition, apoptotic effects on other immune cells (Guasconi et al., 2012; Serradell et al., 2007) might result in a decreased migration of neutrophilic granulocytes, the dominant leukocyte population during mastitis, to the mammary gland (Sordillo and Streicher, 2002).

Regarding fertility parameters, no significant relationship was found between *F. hepatica* infection status and SFI or NRR56 in the current study. Regarding individual dairy cows, higher insemination rates for success to conception in *F. hepatica*-infected compared with non-infected cows were previously noticed (López-Díaz et al., 1998; Romaniuk, 1977). In contrast, pregnancy rates were not significantly higher in cows treated for flukes compared with an untreated control group (Loyacano et al., 2002). However, a significant relationship between patent *F. hepatica* infections and CTFS was observed in our study, while CI was unaffected. Both parameters are influenced by a number of environmental factors, e.g. voluntary waiting period or quality of heat detection, which were covered in our analysis in the fixed farm effect. The CI as the time between the birth of a calf and the birth of the subsequent calf by the same cow represents a long period in contrast to the CTFS, which covers the beginning of lactation as a time with the highest susceptibility to stress. Several reports have shown that physiological stress after calving was accompanied by a higher susceptibility to non-infectious (Butler and Smith, 1989) and infectious diseases (Mallard et al., 1998). Hence, we can hypothesize that CTFS might be influenced more strongly by *F. hepatica* infections than CI. However, other studies did not find a relationship between the length of calving to conception interval and *F. hepatica* antibody levels in individual dairy cattle (Mezo et al., 2011). Regarding CI, a significant increase in the mean CI of 4.7 days was found in dairy herds showing the highest antibody levels (Charlier et al., 2007), which is in contrast to our findings in individual dairy cows using FECs. However, most studies on herd level did not detect any association between fertility and *F. hepatica* antibody titres (Howell et al., 2015; Köstenberger et al., 2017; Mezo et al., 2011; Simsek et al., 2007). These observations were often explained by the fact that only high fluke burdens, which are uncommon in naturally infected animals, lead to an alteration of sex hormone metabolism in the liver (Mezo et al., 2011; Vercruysse and Clearebout, 2011). Thus, the lack of an effect on three of the four analysed fertility parameters in positive animals in our study might be due to low fluke burdens. However, no detailed information is currently available regarding the question whether reproductive performance may be reduced due to altered sex hormone metabolism (Dargie, 1987; López-Díaz et al., 1998) or due to a worse general health status induced by parasite infections (Dorny et al., 2011), resulting in indirect effects on fertility (Rickard et al., 1992). Significant weight loss and lower body condition score values were observed in *F. hepatica* infected cattle under two years of age (Dorny et al., 2011; Kaplan, 2001). Hence, energy deficiency might lead to negative effects on reproductive performance, especially in younger cattle. Collection of continuous measurements for both, FEC and antibody levels per month over a longer time period might be an interesting approach to study the effect of *F. hepatica* on fertility in young vs. older cows.

Coproscopical examinations also revealed the presence of rumen flukes in the study cattle. For these parasites, growing prevalences have been reported during the last years in several Western European countries, such as Belgium, France, Great Britain, Ireland, Portugal, Spain and The Netherlands (Arias et al., 2011; Chauvin, 2012; Foster et al., 2008; Maltrait et al., 2015; Murphy et al., 2008; Ploeger et al., 2017). Molecular analysis revealed the species *C. daubneyi* in the present study, which is in accordance with recent findings in Ireland, The Netherlands and the UK (Gordon et al., 2013; Ploeger et al., 2017; Zintl et al., 2014). However, molecular analyses were not successful for all infected cows. Thus, the presence of other rumen fluke species in the studied cattle herds cannot be excluded. Previously, higher rumen fluke prevalences were reported in cattle below 2 years of age (Díaz et al., 2006), while in our study rumen flukes were observed in all parities. Moreover, all but one of the cows infected with rumen flukes were co-infected with *F. hepatica* in the current study. The high co-infection rate might be due to the fact that *C. daubneyi* shares a common intermediate host (*Galba truncatula*) with *F. hepatica* (Martínez-Ibeas et al., 2013). Associations between clinical paramphistomosis and considerably lower body condition score as well as higher morbidity and mortality in cattle have been reported (Dorny et al., 2011; Mason et al., 2012; Millar et al., 2012). Unfortunately, calculations to estimate the relationship between rumen fluke infected cows or co-infected (rumen and liver fluke) cows with milk production or fertility parameters could not be conducted on our dataset since the prevalence of rumen flukes was too low (0.9%).

5. Conclusions

The findings of the present study indicate that patent infections with *F. hepatica* might not result in lower milk production, maybe due to low fluke burdens in adult cattle resulting in only low energy deficiency and consequently maintenance of performance. Furthermore, no significant relationship was detected between *F. hepatica* infection status and milk quality parameters or somatic cell score, albeit infected cows yielded a 0.06 to 0.10% lower protein and fat content compared with non-infected cows. CI seems to be unaffected during patent infections with *F. hepatica*, while CTFS was significantly increased by 4.7 days in infected cows. As CTFS coincides with the beginning of lactation, a period characterized by the highest susceptibility to stress and disease in dairy cows, it might be more vulnerable to *F. hepatica* infections than the fertility parameter CI. Significant relationships between *F. hepatica* infection status and insemination parameters SFI and NRR56 were not detected in individual dairy cows.

Conflict of interest

The authors have no conflict of interest to declare.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.vetpar.2019.01.012.

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