Aus dem Institut für Parasitologie und Tropenveterinärmedizin

des Fachbereichs Veterinärmedizin

der Freien Universität Berlin

Investigations on the current occurrence and anthelmintic resistance situation of *Fasciola hepatica* in German sheep flocks

Inaugural-Dissertation

zur Erlangung des Grades eines

Doktors der Veterinärmedizin

an der

Freien Universität Berlin

vorgelegt von

Alexandra Constanze Kahl

Tierärztin

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List of Abbreviations

°C	Celsius
ABZ	Albendazole
µM	Micromole
µm	Micrometer
ALT	Alanine Aminotransferase
AST	Aspartate Aminotransferase
ATP	Adenosine Triphosphate
ATPase	Adenosine Triphosphatase
BC	Before Christ
bp	Basepair
BSA	Bovine Serum Albumin
BUN	Blood Urea Nitrogen
bw	Bodyweight
C. daubneyi	*Calicophoron daubneyi*
cELISA	Coproantigen Enzyme-Linked Immunosorbent Assay
CI	Confidence Interval
CL	Credibility Limit
CLOR	Clorsulon
CLOS	Closantel
cm	Centimeter
CRT	Coproantigen Reduction Test
CV	Coefficient of Variation
DBIL	Direct Bilirubin
DNA	Deoxyribonucleic Acid
e.g.	Exempli gratia/ for example
ELISA	Enzyme-Linked Immunosorbent Assay
EPG	Eggs Per Gram (faeces)
EVs	Extracellular Vesicles
F. gigantica	*Fasciola gigantica*
F. hepatica	*Fasciola hepatica*
FEC	Faecal Egg Count
FECR	Faecal Egg Count Reduction
FECRT	Faecal Egg Count Reduction Test
FEHT	Fasciola Egg Hatch Test
FMDT	Fasciola Miracidium Development Test
g	Gram
G. truncatula	*Galba truncatula*
GGT	Gamma-glutamyl Transferase
GIN	Gastrointestinal Nematodes
GLDH	Glutamate Dehydrogenase
h	Hours
HCl	Hydrochloric Acid
H. contortus	*Haemonchus contortus*
i.e.	Id est/ that is

IgG	Immunglobulin G
IL	Interleukin
ITT	Indonesian Thin Tail Sheep
kg	Kilogram
LAMP	Loop-Mediated Isothermal Amplification
Lnn. portales	*Lymphonodi Portales*
mAb	Monoclonal Antibody
mg	Milligram
min	Minute
ml	Milliliter
mm	Millimeter
mM	Millimolar
N	Total Sample Size/ Number of Investigated Samples
n	Number of Positive Samples
n.d.	No Data
NADH	Nicotinamide Adenine Dinucleotide
NITROX	Nitroxynil
NaPi	Sodium Phosphate Buffer
ng	Nanogram
nm	Nanometer
no.	Number
NPV	Negative Predictive Value
NTD	Neglected Tropical Disease
O. ovis	*Oestrus ovis*
OD	Optical Density
OH-TCBZ	Hydroxy Triclabendazole
OH-TCBZ.SO	Hydroxy Triclabendazole Sulphoxide
OH-TCBZ.SO2	Hydroxy Triclabendazole Sulphone
OR	Odds Ratio
OXYCLO	Oxyclozanide
p.i.	post infectionem
p.t.	post treatment
PCR	Polymerase Chain Reaction
Pgp	P-glycoprotein
pH	Potential Hydrogen
PPV	Positive Predictive Value
RAFOX	Rafoxanide
spp.	Species
TBIL	Total Bilirubin
TCBZ	Triclabendazole
TCBZ.SO	Triclabendazole Sulphoxide
TCBZ.SO2	Triclabendazole Sulphone
TCBZ-r	Triclabendazole resistant
TCBZ-s	Triclabendazole susceptible
Th	T helper cell
WBC	White Blood Cell Count

Chapter 1: Introduction

The common liver fluke *Fasciola hepatica* is a widespread pathogenic trematode posing a threat to animal welfare and efficient livestock production worldwide (Fairweather et al. 2020; Beesley et al. 2018). The diheteroxenic endoparasite involves freshwater snails as intermediate hosts in its life cycle before infecting the mammalian definitive hosts, mostly ruminants. Clinically, sheep are the most affected host species (Pérez-Caballero et al. 2018). Infected animals may display apathy, anaemia, oedema, and chronic wasting resulting from the serious liver damage. In severe cases, sudden deaths may occur as a result of acute infections (Rojo-Vázquez et al. 2012). Fasciolosis constitutes a financial burden for farmers due to the condemnation of infested livers, reduced productivity in terms of milk and meat yields, and high treatment costs (Charlier et al. 2020).

Several anthelmintics from different drug classes can be applied for the treatment of fasciolosis. However, increasing reports of anthelmintic resistance of *F. hepatica* are of major concern in many parts of the world (Fairweather et al. 2020; Beesley et al. 2018; Pérez-Caballero et al. 2018). In several European countries (e.g. the Netherlands, the United Kingdom, Sweden, and Spain) resistance reports of *F. hepatica* to flukicides have been numerously published in recent years (Fairweather et al. 2020). In particular, resistance to triclabendazole (TCBZ), the only flukicide affecting the highly pathogenic juvenile flukes in the acute stage of infection, hampers prompt and efficient treatment options and is therefore of high importance (Beesley et al. 2023; Cwiklinski et al. 2016; Kelley et al. 2016; Sargison and Scott 2011).

Since data about the susceptibility of *F. hepatica* populations to flukicides in Germany was lacking, the project's main objective was to collect first data regarding the efficacy of TCBZ against *F. hepatica* on German sheep farms within the course of a field study from 2020-2022. For this purpose, anthelmintic efficacy testing by means of a Faecal Egg Count Reduction Test (FECRT) and a Coproantigen Reduction Test (CRT) was performed on sheep farms located in different regions of Germany.

In order to identify sheep farms with current *F. hepatica* infections eligible for the anthelmintic efficacy trial, extensive coproscopic screening examinations of numerous individual sheep flocks were conducted throughout the study to gather data regarding the current occurrence of *F. hepatica* on German sheep farms. As additional findings, the occurrence of Paramphistominae eggs was concurrently recorded.

In conjunction with the field study, a systematic comparison was made between three different coproscopic techniques in order to determine the most suitable method to examine the ovine field samples quantitatively for the presence of trematode eggs.

A further aim of the project was the establishment of the *Fasciola* miracidium development test (FMDT) and its implementation on collected field isolates. The FMDT is an *in vitro* approach to assess the ovicidal effect of albendazole (ABZ) on *F. hepatica* eggs to discriminate between ABZ-susceptible and ABZ-resistant *F. hepatica* isolates (Alvarez et al. 2009).

The results of the efficacy study are intended to gauge the TCBZ resistance situation of *F. hepatica* for the first time in order to release treatment recommendations for a minimised and optimised use of flukicides in Germany. Due to the globally growing resistance problems, a reasonable use of anthelmintics adapted to the susceptibility of German field isolates is of major significance as well as sensitising farmers and clinical veterinarians to the consequences of TCBZ resistance. Data about the current occurrence of the parasite are valuable in order to assess, in which regions of the country fasciolosis constitutes a problem since the development of resistance due to frequent treatments is highly likely in high prevalence regions.

Moreover, this project is supposed to be the base for molecular investigations in the future. By genotypically characterising TCBZ-resistant field isolates found within the study, possible genomic regions for resistance genes might be identified, since the exact resistance mechanism remains to be fully elucidated at the present time.

Chapter 2: Literature review

2.1 Introduction to *Fasciola* spp.

2.1.1 Taxonomy and historical background

Two main classes of parasitic flatworms with veterinary importance are distinguished in the phylum Plathelminthes*:* the class Trematoda and the class Cestoda (Taylor et al. 2016, p. 74). *Fasciola* spp. belong to the class Trematoda, the subclass Digenae, order Plagiorchiida, suborder Echinostomata and family Fasciolidae (Andrews et al. 2021) and are colloquially referred to as “liver flukes”. The subclass Digenea is characterised by an indirect life cycle with dependence on an intermediate host (Taylor et al. 2016, p.74). Two main species are distinguished in the genus *Fasciola*: *F. hepatica*, the “temperate fluke” (Cwiklinski et al. 2016) and *F. gigantica*, the “tropical fluke” (Cwiklinski et al. 2016), of which only *F. hepatica* is of importance in Europe.

The presence of *F. hepatica* eggs in Europe has already been reported in prehistoric faecal material (Dittmar and Teegen 2003). In Germany, Dittmar and Teegen (2003) found *F. hepatica* eggs in an extended cemetery in the federal state Saxony Anhalt. The genesis of this burial site was estimated to reach back to the late Neolithic period to the middle of the third millennium BC. During the course of a large-scale-excavation from 1996 to 1999, the authors examined soil samples originating from the pelvic regions of human and bovine skeletons and found *F. hepatica* eggs in samples of both species. Shells of the intermediate host snail *Galba truncatula* were also found at the excavation site, leading to the assumption that a full infection cycle was ongoing during that period of time.

In 1379, *F. hepatica* infections were mentioned in literature for the first time by the French livestock breeder Jean de Brie (Andrews et al. 2021; Rojo-Vázquez et al. 2012; Reinhard 1957), who believed that sheep became sick by the ingestion of certain plants impairing the liver and called it ‘liver rot’ (Andrews et al. 2021). Almost 150 years later, in 1523, the English lawyer Anthony Fitzherbert mentioned flukes in the liver of affected sheep (Andrews et al. 2021; Rojo-Vázquez et al. 2012). In the same century, in 1549, the Italian scientist Fanensi Gabucinus observed worms resembling pumpkin seeds in the liver of sheep and goats (Andrews et al. 2021). The first person illustrating and describing *Fasciola* spp. was the Italian physician Francesco Redi in 1688 after recovering flukes from the liver of a ram (Andrews et al. 2021; Rojo-Vázquez et al. 2012).

2.1.2 Morphology

Fasciola hepatica is a grey-brownish, leaf-shaped fluke with a broader and conical anterior end and a thinner posterior end (Figure 1). The adult stages are approximately 2.5 to 3.5 cm in length and 1.0 cm in width. The anterior end is delimited by obvious shoulders (Taylor et al. 2016, p.78). The fluke's tegument, as for all members of the suborder Echinostomata, is a dynamic syncytical structure covering the surface of the parasites (Brusca et al. 2022, chapter 17) and is coated with spines (Taylor et al. 2016, p.78; Rojo-Vázquez et al. 2012), which are narrowly packed and point posteriorly (Robinson et al. 2021). These spines are important for the parasites to maintain their position in the host's bile ducts and for puncturing the blood vessels for feeding purposes (Robinson et al. 2021). Furthermore, the tegument is a multifunctional, metabolically active layer serving for nutritional absorbance, synthesis and secretion of different substances, osmoregulation, and self-protection against digestive enzymes and immune reactions of the host (Robinson et al. 2021).

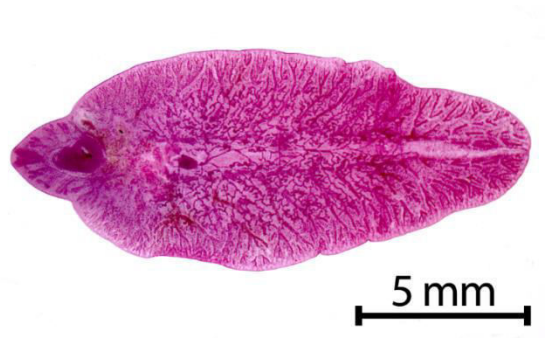

Figure 1: Adult stage of F. hepatica *(lactic acid/carmine -stained)*

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Figure 2: Unembryonated egg of F. hepatica

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The oral and the ventral suckers are located close to one another at the anterior part of the body. The intestinal caeca are laterally branched and extended, reaching the posterior end of the body. *Fasciola hepatica* is hermaphroditic and has branched testes as well as a short uterus and ovaries (Mas-Coma et al. 2019; Taylor et al. 2016, p.78; Hanna 2015). Large parts of the body serve for reproduction purposes (Fairweather et al. 2020), resulting in a high egg excretion rate (Happich and Boray 1969). The oval shaped eggs (shown in Figure 2) are operculated, yellowish-brown coloured and consist of a fertilised ovum surrounded by a huge quantity of yolk granules. They measure approximately 130-145 µm in length and 70-90 µm in width (Andrews et al. 2021) and are unembryonated at the time of shedding (Andrews et al. 2021; Mas-Coma et al. 2019). With an approximate size of up to 7.5 cm in

length and 1.5 cm in width, *F. gigantica* is larger and relatively narrower than *F. hepatica* (Taylor et al. 2016, p.78). The colour of the body is more transparent and the shoulders are not as distinctly visible as in *F. hepatica* (Mas-Coma et al. 2019; Taylor et al. 2016, p. 78). However, due to the resemblance of both species and the fact, that the definitive host species essentially influences the morphology of the flukes and the eggs, an accurate optical differentiation between both *Fasciola* species is not always straightforward (Rojo-Vázquez et al. 2012).

In geographic regions, where both *Fasciola* spp. co-exist, the same definitive host can be infected by both species at the same time leading to the opportunity of cross-breeding and the development of hybrids (Cwiklinski et al. 2016; Mas-Coma et al. 2009b). Intermediate forms have for example been identified in areas of Japan, Vietnam, and Korea (Cwiklinski et al. 2016) and Egypt (Khalifa et al. 2013).

2.1.3 Life cycle

The life cycle of *F. hepatica* is diheteroxenic including a vertebrate as a definitive host and a lymnaeid snail as an intermediate host (Figure 3). The adult fluke stages inhabit the biliary system of the definitive host, where they produce eggs either via self-fertilisation or cross-fertilisation (Hanna 2015), which are shed with the bile fluid into the definitive host's duodenum and are excreted with the faeces into the environment (Andrews et al. 2021; Howell and Williams 2020; Mas-Coma et al. 2019). An earlier study from 1960 investigated different factors influencing the development and hatching of *F. hepatica* eggs (Rowcliffe and Ollerenshaw 1960). The authors revealed inter alia that the trematode eggs must be liberated from the faeces for a complete development and hatching process but can stay viable at moist conditions for up to several months when not separated from the faeces (Rowcliffe and Ollerenshaw 1960). Under dry conditions, the eggs rapidly desiccate (Howell and Williams 2020; Rowcliffe and Ollerenshaw 1960). The development inside the egg is highly dependent on temperature (Beesley et al. 2018; Rowcliffe and Ollerenshaw 1960). At 10 °C environmental temperature, the development process takes about six months, whereas at 30 °C the development takes only about eight days (Andrews et al. 2021; Rowcliffe and Ollerenshaw 1960). However, at temperatures exceeding 30 °C, the development is then again suppressed, and no development occurs at temperatures above 37 °C. A 100% mortality is reached after 24 days at 37 °C (Rowcliffe and Ollerenshaw 1960). At temperatures lower than 10 °C, *F. hepatica* eggs may still remain viable for a long time span. In laboratory experiments, *F. hepatica* eggs were kept refrigerated at 4-5 °C after removal from the gall bladder of infected animals. These eggs survived for at least two years and were still infective to snails after incubation (Boray 1969). However, the eggs seem to be

sensitive to temperatures below freezing, since in the experiments from Boray (1969) the eggs lost their viability when kept at -15 °C for 24 hours. Additional criteria influencing the embryonation inside the egg are moisture, oxygen tension, and pH. A surface film of moisture around the egg is required as well as aerobic conditions and a pH range of 4.2-9.0 (Andrews et al. 2021; Rowcliffe and Ollerenshaw 1960).

If the climatic conditions in the environment favour the development, the miracidium develops inside the egg and emerges from the eggshell after stimulation with increased light and temperature (Andrews et al. 2021; Mas-Coma et al. 2019; Beesley et al. 2018). The miracidium is approximately 130 µm long and has an epidermis with a ciliated surface (Andrews et al. 2021). Immediately after hatching, the liberated larval stage starts to swim rapidly using its cilia until it locates a suitable intermediate host. The time span in which the miracidium needs to find a snail before it dies is relatively short (reports differ from three (Taylor et al. 2016, p.78; Skuce and Zadoks 2013) up to 24 hours (Bogitsh et al. 2019, p. 163; Yadav 2015)). The miracidium of *F. hepatica* is phototropic (Andrews et al. 2021; Mas-Coma and Bargues 1997), meaning that the larva reacts to light stimuli and moves toward the source of light. It is presumed, that this characteristic is an adaption for locating the intermediate host, since the habitat of the main intermediate host snail species, *G. truncatula*, is along the edge of small ponds, ditches, and marshy land and not at dark localisations such as the bottom of ponds (Andrews et al. 2021). Furthermore, the miracidia also react to chemoattractant molecules exuded from the snails, so that the larva can locate the molluscs by chemotaxis (Neuhaus 1953). Specific interactions involving carbohydrates in the contact zones between the miracidium and the molluscs are required for recognition and these interactions play a decisive role in the trematode-snail interactions (Georgieva et al. 2019). After penetration of the snail, the miracodermis is shed before the asexual multiplication within the snail starts. The miracidium contains germ cells (pluripotent stem cells), which grow and multiply by mitosis to form germ balls (Bogitsh et al. 2019, p. 163). The germ balls transform into membrane-enclosed entities constituting the next larval generation called sporocysts (Bogitsh et al. 2019, p. 163). These elliptical saccular sporocyst of 150-500 µm in length produce mother rediae which in turn produce cercariogenous daughter rediae (Mas Coma and Bargues 1997). The mature rediae are about 1-3 mm in length (Andrews et al. 2021) and show a primitive digestive system (Mas Coma and Bargues 1997). The final stages developing inside the intermediate host are called cercaria with an approximate size of 0.25-0.35 mm, a rounded body, and a thin unbranched tail of 0.5 mm (Yadav 2015). The developmental process from the infection of the snail to the cercariae takes about six to eight weeks (Cwiklinski and Dalton 2022; Hodgkinson et al. 2018; Taylor et al. 2016, p.78; Mas Coma and Bargues 1997), but the process may be slowed when

temperatures are lower (Taylor et al. 2016, p. 78; Mas Coma and Bargues 1997). Hodgkinson et al. (2018) showed that snails infected with only one miracidium may generate more than 3000 cercariae. This high reproduction capability is not only pivotal for the biology of *F. hepatica*, but also a crucial aspect with regards to the development and spread of drug resistance, since emergence of numerous individuals of several generations in a short period facilitates the spread of resistance alleles through a parasite population (Wolstenholme et al. 2004), so that the potential of clonal amplification of drug resistant *F. hepatica* isolates at this part of the life cycle is particularly high within the snail (Hodgkinson et al. 2018). Finally, the cercariae are shed from the snail as an active and motile form, which attach to stalks of plants, forming the encysted and infectious metacercaria (Andrews et al. 2021; Taylor et al. 2016, p. 78) by secreting a four-layered cyst covering produced by cystogenous glands (Yadav 2015). Encystment may also occur on the water surface (Andrews et al. 2021). The infectious metacercariae may survive for up to one year if climate conditions are suitable (Howell and Williams 2020). The metacercarial cyst is only moderately resistant and not able to withstand desiccation under dry conditions (Yadav 2015).

After ingestion of the plant, the metacercariae excyst in the host's small intestine, a process stimulated by high concentrations of carbon dioxide, reducing conditions, temperatures around 39 °C, and the presence of bile (Dixon 1966). The excysted juvenile flukes are dependent on endogenous glycogen stores (Cwiklinski and Dalton 2022). They penetrate the intestinal mucosa and move into the peritoneal cavity from where they migrate towards the liver in about 24 hours (Sargison 2008). It takes four to six days for the immature flukes to penetrate the Glisson's capsule of the liver before they start migrating through the liver parenchyma for five to six weeks causing massive haemorrhagic tracks and fibrosis. The juvenile flukes feed on blood and hepatic cells and grow considerably during their migration (Cwiklinski and Dalton 2022). About seven weeks post infection (p.i.), the young flukes reach the bile system of the host, where they settle and grow into sexually mature flukes starting to produce eggs, which then again are shed with the bile fluid and are excreted with the faeces (Andrews et al. 2021). Various data regarding the daily egg output per fluke were reported in the literature: Adult flukes may produce up to 2000–2500 eggs per fluke per day according to Rojo-Vázquez et al. (2012) or even 5000 eggs per fluke per day according to Sargison (2008). An older publication by Happich and Boray (1969) indicated a maximal egg production of 50,000 eggs (average: 20,000-25,000 eggs in light or medium infection intensities) per fluke per day. In their experiments investigating the egg-producing capacity of *F. hepatica* in sheep, the authors observed that the egg-producing capacity successively diminished with the number of flukes concurrently parasitising in the liver in heavy infections

(most likely due to overcrowding), resulting in a non-proportional relationship between fluke burden and faecal egg shedding (Happich and Boray 1969).

Infections of the definitive hosts usually take place during autumn under European conditions and therefore, the highest adult fluke burdens usually appear in winter and spring (Martínez-Pérez et al. 2012; Charlier et al. 2008). Especially the first half of the autumn in temperate regions provides favourable conditions for the transmission of *F. hepatica* (mild temperatures coinciding with high rainfall). The climatic conditions during the winter season are not suitable for the development of the eggs and the snails in most temperate regions of the world, but a proportion of the infectious metacercariae are able to survive until spring, so that animals may also become infected during the grazing period in spring (Rojo-Vázquez et al. 2012).

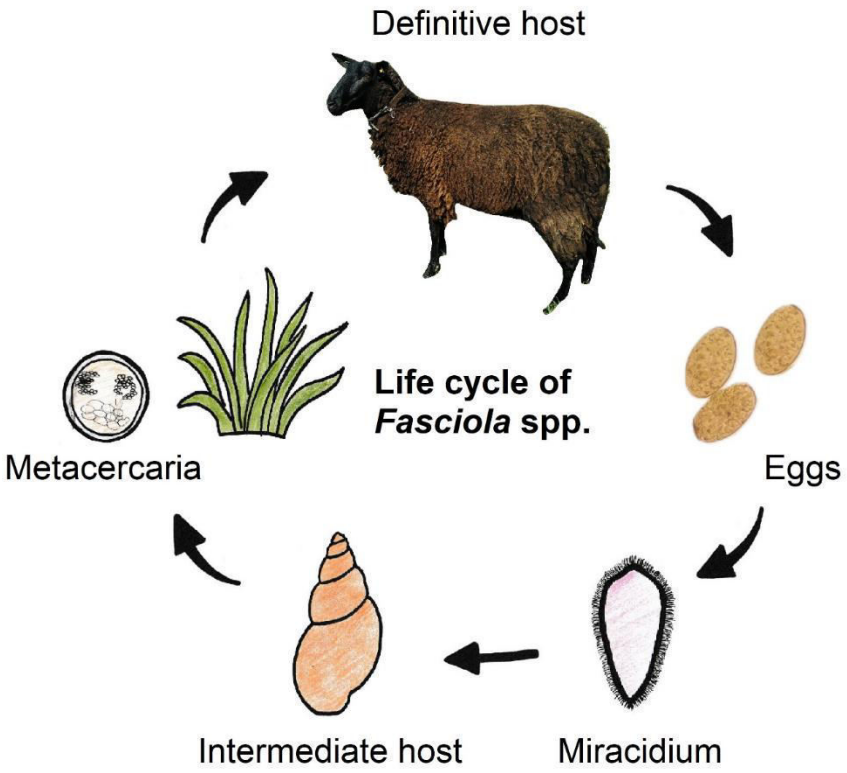

Figure 3: Life cycle of Fasciola *spp. Parasite eggs are shed with the definitive host's faeces. Following a development period in the environment, the miracidium hatches and actively penetrates the intermediate host snail. After an asexual multiplication period inside the intermediate host, the snail gives rise to a huge quantity of cercariae, which subsequently encyst on plants or the water surface forming the infectious metacercariae.*

2.1.4 Distribution

2.1.4.1 Geographical distribution

Fasciola spp. are diheteroxenic parasites involving mud snails as intermediate hosts in their life cycle and a significant proportion of their development takes part in the environment including intermediate hosts. Hence, the natural occurrence is substantially limited to areas, where the weather conditions are favourable for the existence of the snail intermediate host species since the habitat of the intermediate host and the definitive host must coincide (Dixon 1964) and be suitable for the development of the free living *Fasciola* spp. stages outside the intermediate and definitive hosts (Knubben-Schweizer et al. 2021; Charlier et al. 2014; Garcia-Rodriguez et al. 1985). Therefore, climatic factors such as air temperature, rainfall, and evapotranspiration play a decisive role in the spatial distribution of *Fasciola* spp. (Mas-Coma et al. 2019; Beesley et al. 2018). High humidity and moderate air temperatures are required for the development and multiplication of the intermediate hosts and *Fasciola* spp. stages (Mas Coma and Bargues 1997). Therefore, a seasonal transmission of fasciolosis is typical in almost any endemic regions due to dynamics of the intermediate host populations according to local climatic conditions (Mas-Coma et al. 2018). Mas-Coma et al. (2018) differentiated between three ways of transmission seasonality depending on geographical latitude and altitude: A permanent, year-long transmission is observed in regions with consistently favourable climatic conditions for the larval development without considerable fluctuation of the average monthly temperatures and where the intermediate hosts are adapted to permanent water bodies, for example in southern Europe (Mas-Coma et al. 2018). Monoseasonal transmission is present in extreme latitudes with only one period throughout the year allowing the multiplication and development of the lymnaeid vector since the remaining part of the year is either too hot, or too cold or too dry outside the rainy season (Mas-Coma et al. 2018). In contrast, biseasonal transmission occurs in most parts of Europe, the USA, and Australia with a low transmission in spring and a high transmission in autumn (Mas-Coma et al. 2018). This biseasonal transmission in Europe is traced back to the activity periods of the intermediate host snails in spring and autumn (Mas-Coma et al. 2019). Since the relationship between the intermediate host snails and the climatic conditions resemble the one seen in arthropods involved in the transmission of infections, Mas-Coma et al. (2019) suggested that using the term ‘vector’ for the molluscs transmitting fasciolosis is suitable, although snails are not true ‘vectors’ in the proper sense as they do not inoculate the parasite into the definitive host (Mas-Coma et al. 2019).

Fasciola hepatica is globally distributed (Andrews et al. 2021; Knubben-Schweizer et al. 2021; Fairweather et al. 2020; Howell and Williams 2020; Mas-Coma et al. 2019; Beesley et al. 2018; Cwiklinski et al. 2016; Rojo-Vázquez et al. 2012; Mas-Coma and Bargues 1997), occurring in temperate climate zones in more than 70 countries on every continent (Howell and Williams 2020; Mas-Coma et al. 2019; Mas Coma and Bargues 1997) except antarctica (Fairweather et al. 2020). In contrast, the occurrence of *F. gigantica* is mostly limited to the tropical and subtropical regions in Asia and Africa (Andrews et al. 2021; Fairweather et al. 2020; Mas-Coma et al. 2019; Cwiklinski et al. 2016; Rojo-Vázquez et al. 2012; Bargues et al. 2001). In regions, where climatic conditions are favourable for both species, *F. hepatica* and *F. gigantica* may coexist and hybridise (Andrews et al. 2021). In Europe, *F. gigantica* occurs only sporadically with smaller endemic areas in the southern regions of the continent, e.g., Turkey, the near east and some southern states of the old USSR (Bargues et al. 2001).

In Germany, *F. hepatica* is the only occurring species. Most studies regarding the distribution of *F. hepatica* in Germany focus on the occurrence in dairy cattle, showing that the parasite is distributed in many different regions of the country (Forstmaier et al. 2021; May et al. 2019; Fanke et al. 2017; Kuerpick et al. 2013). A recent study from Alstedt et al. (2022) estimated the prevalence of *F. hepatica* in small ruminants comparing the occurrence of the common liver fluke in the south and the north of the country, reporting a higher *F. hepatica* prevalence in the southern federal state Bavaria compared to the northern federal state Lower Saxony.

2.1.4.2 Host species of *Fasciola* spp.

2.1.4.2.1 Definitive host species

Fasciola hepatica is not specific for a certain definitive host species. Principally any mammal that ingests the infectious metacercariae could become infected (Skuce and Zadoks 2013). However, there are different levels of susceptibility between species and the pathological impact differs considerably from host to host species (Boray 1969). Sheep and cattle represent the main reservoir for *F. hepatica* (Mezo et al. 2007). In terms of clinical impacts, sheep seem to be even more sensitive than bovines (Boray 1969; Dixon 1964) and count as the most susceptible animal species among ruminants (Pérez-Caballero et al. 2018). In sheep, the natural lifespan of *F. hepatica* may be up to eleven years (Rojo-Vázquez et al. 2012), whereas in cattle, most flukes are eliminated within nine to twelve months (Mas-Coma and Bargues 1997; Boray 1969). Apart from domestic ruminants like sheep, cattle, and goats, also horses, donkeys, mules as well as wild animals (e.g. deer, wild boars, rabbits, hares, and nutrias) may serve as definitive hosts (Mas-Coma et al. 2019; Beesley et al. 2018; Taylor et al. 2016, p. 78; Mas Coma and Bargues 1997), although not every species participates equally in the transmission of the parasitosis (Valero and Mas-Coma 2000). For

example, an older study from Ross et al. (1967) describes the occurrence of natural and experimental *F. hepatica* infections in pigs, but the authors found that this species possesses considerable natural resistance to the parasite compared to other species. Not only in mammals, but also in birds *F. hepatica* infections have been observed (Soares et al. 2007; Vaughan et al. 1997).

Due to the different geographical distribution, *F. gigantica* also infects definitive host species in tropical areas such as buffaloes and camelids as well as wild herbivorous animals (larger antelopes, deer, giraffes, and zebras) (Taylor et al. 2016, p. 78; Rojo-Vázquez et al. 2012).

Fasciolosis is also a food- or waterborne zoonotic disease causing major human health problems in many parts of the world (Andrews et al. 2021; Fairweather 2009; Mas-Coma et al. 2009b). The increasing public health importance in South, Central and North America, Europe, Africa and Asia initiated scientific interest in human fasciolosis (Mas-Coma et al. 2009b). Andrews et al. (2021) specifically mentioned Bolivia, Peru, Ecuador, Egypt, Iran, China, Vietnam and at a lower level also Portugal and Spain as countries with a high number of human infections. Humans become infected after ingesting aquatic plants carrying attached metacercariae or by drinking contaminated water (Saba et al. 2004). In humans, the infection can be asymptomatic without pathognomonic symptoms (Mas-Coma and Bargues 1997). Human fascioliosis is considered a Neglected Tropical Disease (NTD) among the group of food-borne trematodiases by the World Health Organisation (Savioli et al. 2013). In most cases, human fasciolosis is a rural disease and infections in urban spaces occur only sporadically (Mas-Coma et al. 2018).

2.1.4.2.2 Intermediate host species

For the asexual reproduction part of its lifecycle, *F. hepatica* is reliant on the occurrence of amphibious and aquatic mud snails belonging to the family Lymnaeidae serving as intermediate hosts (Caminade et al. 2015; Mas-Coma and Bargues 1997). The principal snail species in Europe involved in the lifecycle of the parasite is the lymnaeid snail *G. truncatula* (Andrews et al. 2021; Mas-Coma et al. 2019; Beesley et al. 2018; Hodgkinson et al. 2018; Charlier et al. 2014; Caron et al. 2007; Bargues and Mas-Coma 2005; Bargues et al. 2003; Bargues et al. 2001), also known as *Lymnaea truncatula* (Dreyfuss et al. 2021; Mahulu et al. 2019). Other European lymnaeid snail species involved in the transmission of *F. hepatica* under natural conditions are *Omphiscola glabra* (Bargues and Mas-Coma 2005; Dreyfuss et al. 2003), *Stagnicola (Lymnaea) palustris* (Novobilský et al. 2013; Bargues and Mas-Coma 2005), *Succinidea* spp. (Relf et al. 2009) and *Radix* spp. (Caron et al. 2014; Relf et al. 2009). Very recently, *F. hepatica* DNA has been detected in *Lymnaea stagnalis* in Turkey (Ünlü et al. 2023) and in *Peregriana peregra* on the island of Corsica (France) (Alba et al. 2023).

With regards to the principal intermediate host species, *G. truncatula*, it has been proven that this species also serves as intermediate host for the rumen fluke *Calicophoron daubneyi*, another trematode species infecting ruminants that frequently occurs in Central Europe. Co-infections with both trematode species are also possible in one individual mollusc (Jones et al. 2015).

Fasciola gigantica is mainly transmitted by snail species of the genus *Radix*. Known principal intermediate hosts for *F. gigantica* worldwide are *Radix natalensis, Radix auricularis, Radix rufescens, Radix rubiginosa, Radix swinhoei* as well *as Fossaria cubensis* and *Austropeplea ollula* (Mas-Coma et al. 2001; Mas-Coma and Bargues 1997).

2.1.5 Economical aspects of fasciolosis

Among other helminth infections, fasciolosis is a major constraint on efficient ruminant livestock production worldwide (Charlier et al. 2020). The worldwide financial losses in animal production due to liver fluke infections were estimated to exceed 3.2 US$ billion per annum (Mehmood et al. 2017). In Europe, the annual economic burden to the ruminant livestock industry were estimated to be € 635 million for *F. hepatica* infections (Charlier et al. 2020). A study from Switzerland (Schweizer et al. 2005) estimated that the median financial loss due to bovine fasciolosis was approximately € 52 million per year and reported a median loss of € 299 per infected cow. The economic damages result not only from treatment costs (Charlier et al. 2020), but also from reduced animal productivity, loss of condemned livers and interference with other diseases (Charlier et al. 2014; Robles-Pérez et al. 2013). Infected livestock show poor growth rates, decreased milk yields, reduced wool quality and quantity as well as a reduced fertility (Rojo-Vázquez et al. 2012; Schweizer et al. 2005). Not only the reduced yields of meat, milk, and wool, but also the higher death rate of infected animals (mostly sheep) is of major economic relevance for the farmers.

2.1.6 Pathogenesis of fasciolosis

2.1.6.1 Organ damage

The pathological impact of *F. hepatica* infections on the definitive host depends on different factors: host species, total number of ingested metacercariae, duration of the infection and nutritional condition of the host (Rojo-Vázquez et al. 2012). The penetration of the intestinal wall and the migration across the peritoneal cavity of the immature flukes does normally not lead to evident lesions or only minor haemorrhagic damages on the peritoneum when the flukes attach temporarily to the peritoneal tissue. Only in severe or repeated infections, acute and exudative or chronic and proliferative peritonitis may occur (Rojo-Vázquez et al. 2012).

During their extensive migration through the liver parenchyma, the juvenile flukes cause considerable tissue lesions due to the mechanical damage caused by their spiky tegument (Stuen and Ersdal 2022). The immature flukes feed on parenchymal cells and blood (Cwiklinski and Dalton 2022; Yadav 2015), leading not only to traumatic damage to the organ, but also to coagulation necrosis, which might possibly be related to toxic excretions of the young flukes (Rojo-Vázquez et al. 2012). All *F. hepatica* stages secret enzymes, particularly proteases, to degrade the host's blood and hepatic tissue (Williams 2020). The migration of the immature flukes forms an extensive track system with tunnels of 2-3 mm diameter containing flukes of less than 1 mm length (Rojo-Vázquez et al. 2012). At the beginning, the migratory tracks are filled with necrotic debris and blood cells encircled by necrotic hepatocytes with microthrombi in intact sinusoids (Rushton and Murray 1977). In massive infections, severe haemorrhages as well as liver ruptures can occur and free blood may accumulate in the abdomen during the acute phase (Stuen and Ersdal 2022) and even deaths are possible in the stage of acute hepatitis (Rojo-Vázquez et al. 2012). The presence of collagen around the migratory tracks is first observed at week six p.i. and the post-necrotic collagen grows in quantity until the migratory pathways are transformed into wide heterogenous scars (Rushton and Murray 1977). At this time, the ventral hepatic lobes start to become smaller and firmer and display adhesions between the hepatic surfaces and the adjacent serosa (Rushton and Murray 1977). Histiocytes and giant cells eliminate the debris in the migratory tracks and cicatrisation occurs by the formation of granulation tissue. The hepatic scars can amalgamate in heavy infections resulting in a moderate irregular fibrosis of the liver (Rojo-Vázquez et al. 2012). The left hepatic lobe is commonly more affected than the right hepatic lobe (Ashoor and Wakid 2023; Stuen and Ersdal 2022; Sangster et al. 2021; Pérez-Caballero et al. 2018; Rojo-Vázquez et al. 2012), most likely due to its anatomic proximity to the duodenum (Sangster et al. 2021). During their migration, the immature flukes also impair the hepatic blood vessels provoking phlebitis, thrombosis, and stenosis (Rushton and Murray 1977).

When the extensive migration of the young flukes is finalised, thin and elongated shallow scars are apparent on the liver surface as macroscopic lesions in the chronic stage (Stuen and Ersdal 2022). When the flukes reached maturity and have settled in the bile ducts and the gall bladder, they induce cholangiohepatitis. Adult flukes cause traumatic damage by mechanical irritation through the attachment of their suckers, and the spines on their tegument to the host tissue. They may engender obstruction of the bile ducts leading to biliary retention and facilitate bacterial infections (Rojo-Vázquez et al. 2012). A recent study from Ashoor and Wakid (2023) investigated the pathological findings in sheep livers infected with both mature and immature *Fasciola* spp. stages and described hepatomegaly with

rounded edges, thickened liver capsules as well discolouration and an irregular visceral surface with regions of necrosis and fibrosis as well as haemorrhagic foci and whitish rings around the migrated flukes. The bile ducts in a chronically infected liver were whitish and enlarged and show thickened walls. A study from Raadsma et al. (2007) found a significant increase in liver weight in sheep experimentally infected with 250 *F. hepatica* metacercariae compared to noninfected sheep at necropsy at ten weeks p.i. Interestingly, no significant increase in liver weight was seen in sheep challenged with up to 400 *F. gigantica* metacercariae compared to noninfected sheep. The authors also observed macroscopic liver damage with nodules confined to approximately 30% of the liver surface.

Histologically, hyperplasia of the bile duct epithelium and damaged cellular endothelium, inflammatory fibrosis with infiltration of eosinophils, lymphocytes, and plasma cells in portal regions are visible as well as granulomas with necrotic centers and narrowly compressed sinusoids due to swollen hepatocytes (Ashoor and Wakid 2023; Sangster et al. 2021; Pérez-Caballero et al. 2018; Elsheikh et al. 1992).

The liver plays a pivotal role in various physiological functions of the body, e.g. the lipid-, protein- and carbohydrate metabolism, urea synthesis, homeostasis, detoxification and ketogenesis (Sangster et al. 2021). The liver damage induced by *F. hepatica* significantly affects the hepatic metabolism and its vital functions for the host resulting in systemic changes and disorders. Besides its central role in the nutritional metabolism of the host, the liver is also a key part in terms of drug biotransformation. The hepatic damage may therefore negatively impact the physiological hepatic drug/xenobiotic metabolisation pathways. Elsheikh et al. (1992) investigated the effects of natural and artificial *F. gigantica* infections in sheep on the activities of hepatic drug-metabolising enzymes. The authors observed a significant decrease in the activities of these enzymes in infected sheep compared to uninfected control animals. Lacking activities of substantial enzymes can impair the hepatic capacity for the biotransformation of chemotherapeutic drugs and toxic compounds. Hence, the flukicidal efficacy and pharmacokinetics of anthelmintics used for the treatment of fasciolosis may also be compromised (Elsheikh et al. 1992).

2.1.6.2 Haematological and biochemical changes

Fasciola hepatica induces various biochemical and haematological changes in the definitive host. The adult flukes inside the bile system feed on blood, bile, lymph, and hepatic tissue resulting in anemia and hypoproteinaemia (Stuen and Ersdal 2022; Sangster et al. 2021). The reason for the development of anaemia is not only the direct blood loss due to the blood sucking activities of the trematodes, but also alterations in the coagulation system of the host, which presumably facilitate the feeding process (Joachim et al. 2003). Haemorrhagic

anaemia is a major contributor to morbidity and mortality of an infected host and is usually normochromic and normocytic if the blood loss is subtle enough for the host to adapt (Sangster et al. 2021). At the beginning of infection, erythropoiesis is increased and a mild reticulocytosis may be observed, but at later stages the unavailability of iron and protein limits the production of new red blood cells (Sangster et al. 2021).

The mechanical damage to the liver due to *Fasciola* spp. results in destruction of hepatocellular integrity engendering increasing activities of liver enzymes due to the release from damaged cells into the blood (Hodžić et al. 2013). Increased serum levels of the transaminases aspartate aminotransferase (AST) and alanine aminotransferase (ALT) as well as of the glutamate dehydrogenase (GLDH) are sensitive indicators of hepatocellular injury (Raadsma et al. 2007; Hodžić et al. 2013). Moreover, the activity of gamma-glutamyl transferase (GGT) may be altered during fasciolosis. As GGT is an indicator for bile epithelium damage in sheep (Sargison 2008), increased levels suggest the presence of adult flukes in the bile system during the chronic stage of infection (Matanović et al. 2007; Raadsma et al. 2007). Aside from the liver enzymes, increased levels of bilirubin are also an important biochemical parameter altered in *F. hepatica* infected sheep (Hodžić et al. 2013). Infected sheep may suffer from prehepatic icterus due to excessive breakdown of erythrocytes, as well as from intrahepatic icterus due to intrahepatic cholestasis or inadequate bilirubin conjugation due to hepatic injury and resulting metabolic disorders. A posthepatic icterus occurs as a result from extrahepatic cholestasis due to biliary obstruction.

The study from Matanović et al. (2007) investigated the haematological and biochemical differences between naturally *F. hepatica* infected and noninfected sheep flocks raised on organic farms in Croatia. The authors found severe normocytic hypochromic anaemia in the infected animals as well as leucocytosis with neutrophilia and eosinophilia. Regarding the biochemical results, the *F. hepatica* infected sheep presented increased serum GGT levels, hypoalbuminemia and hyperglobulinaemia and a lower concentration of creatinine. The values of blood urea nitrogen (BUN) and AST were also significantly lower compared to the noninfected sheep (Matanović et al. 2007). Since AST is an enzyme with a short half-life, the observed decreased activity in serum indicates recovery after hepatic injury and a normalised liver function. Since GGT levels were elevated, the results suggest that the animals were already in the biliary stage of fasciolosis. Decreased levels of BUN and albumin result most possibly from the impairment of hepatic metabolic cycles such as the urea cycle and the protein synthesis. This is in accordance with the low creatinine concentrations, which might be related to muscle loss due to protein deficiency.

The pathogenic impact of *F. hepatica* infections in sheep was also studied by Hodžić et al (2013). The authors collected blood samples before slaughter of 29 naturally infected sheep and 34 uninfected control sheep and measured biochemical parameters in the blood (GGT, AST, total bilirubin (TBIL), and direct (conjugated plus albumin-bound) bilirubin (DBIL)) to investigate the hepatic functional capacity and examined the livers with a focus on *F. hepatica*-induced changes on the structures of the blood vessels and the biliary tract. Regarding the biochemical parameters, significantly higher levels of GGT, TBIL, and DBIL were observed in the infected sheep compared to the noninfected sheep. These observations suggest the sheep were already in the chronic stage of fasciolosis with damaged bile duct epithelium and increased bilirubin levels due to haemolytic toxins of the flukes and bile duct obstructions resulting in cholestasis. As in the study from Matanović et al. (2007), no significantly higher activities of AST were found in the infected sheep, suggesting lack of hepatocellular damage, which possibly indicates that the livers of the sheep in this study already regenerated after the migratory phase of the juvenile flukes and no acute hepatocellular necrosis was present. The livers of *F. hepatica*-infected sheep exhibited dilatated and wrinkled bile ducts and in some livers also newly formed bile ducts and malformed blood vessels were present.

The study by Raadsma et al. (2007) compared the pathogenicity in terms of immunological and plasma biochemical changes during the early stage of experimental infections of sheep between *F. hepatica* (infection dose of 250 metacercariae per animal) and *F. gigantica* (four groups receiving different doses between 50 and 400 metacercariae per animal). The authors measured the plasma GGT and GLDH levels at day 0 and then at four, six, eight and ten weeks p.i. and compared the values with noninfected sheep. By week six p.i., the study group dosed with 50 *F. gigantica* metacercariae did not demonstrate higher GLDH levels compared to the noninfected sheep, whereas the study groups infected with 125 - 400 *F. gigantica* metacercariae had significantly increased levels of GLDH compared to the noninfected sheep, and the group infected with 250 *F. hepatica* metacercariae showed even higher GLDH levels than all *F. gigantica* infected study groups indicating severe damage of the liver parenchyma. At week ten p.i., the GLDH levels of the *F. hepatica*-infected sheep decreased to the levels of the groups infected with 125 - 400 *F. gigantica* metacercariae, which were still significantly higher compared to the noninfected sheep. Increased values of GGT (as an indicator of epithelial damage in the bile system) were only observed in the group infected with *F. hepatica* metacercariae. Moreover, haematological changes were investigated in this study. All study groups infected with either *F. hepatica* or *F. gigantica* metacercariae showed a significantly increased white blood cell count (WBC) compared to the noninfected sheep at week three p.i.. Haemoglobin levels raised continuously over the

first eight weeks p.i. in all study groups with no observable differences between the non-infected sheep and the groups infected with *F. gigantica*. In the *F. hepatica*-infected group, a significant decline of the haemoglobin levels was seen by week nine p.i.. Overall, the authors concluded that *F. hepatica* appears to be even more pathogenic than *F. gigantica* during the early infection stage due to a faster growth rate causing more damage to the host (Raadsma et al. 2007).

2.1.6.3 Clinical symptoms

In agreement with the pathological changes, the clinical symptoms of fasciolosis vary and depend on a variety of factors: the infective dose and the strain of the parasite as well as the host species, the host's age and its body condition, its general health status and the stage of the infection (Sangster et al. 2021). Generally, fasciolosis may present in an acute, subacute or chronic form and may also be subclinical depending on the quantity of ingested metacercariae over a certain time period (Fiss et al. 2013). Acute presentations of fasciolosis evolve after the ingestion of very high quantities of metacercariae over a short period engendering fatal invasions of the liver and leading to massive hepatic destruction and severe haemorrhages induced by a high number of simultaneously migrating immature flukes (Sangster et al. 2021; Rojo-Vázquez et al. 2012). Acutely infected animals might not show any clinical signs prior to a sudden death, or they display apathy, anorexia, abdominal pain, dyspnoea, fever, pale or icteric mucous membranes, muscle tremors, and drooling (Alvarez Rojas et al. 2014; Fiss et al. 2013; Rojo-Vázquez et al. 2012; Sargison and Scott 2011), but none of these clinical symptoms are pathognomonic for *F. hepatica* infections (Alvarez Rojas et al. 2014). Sudden deaths due to massive infections in susceptible host species (particularly small ruminants) may decimate flocks rapidly (Sangster et al. 2021). Acute forms are usually seen during autumn and early winter (Rojo-Vázquez et al. 2012), shortly after the period of the highest metacercarial burden on the grazing areas. Subacute clinical presentations develop after six to ten weeks after the consumption of smaller doses of metacercariae over a period of several weeks. This form results from migrating juvenile flukes in the hepatic parenchyma and the concurrent presence of mature flukes, which have already settled in the bile ducts leading to cholangitis (Rojo-Vázquez et al. 2012). Chronic forms of *F. hepatica* infections are the most common form of fasciolosis and occur after four to five months after ingestion of moderate quantities of metacercariae (Rojo-Vázquez et al. 2012), e.g. after grazing on only slightly contaminated pastures. In chronic cases, the animals have recovered from the acute hepatic damage and the long-lasting physical stress caused by the emaciating adult flukes is paramount. Chronically infected sheep present a progressive loss of condition, pale mucous membranes, ascites, and submandibular oedema

("bottle jaw", shown in Figure 4) and may die in an emaciated stage (Rojo-Vázquez et al. 2012; Sargison and Scott 2011).

Figure 4: Submandibular oedema in a ram

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Among all ruminants, sheep are generally considered to be the most susceptible and clinically affected species (Pérez-Caballero et al. 2018). The fatal acute clinical course of fasciolosis occurs most frequently in ovines with a major impairment of animal welfare, productivity, and profitability. In cattle, the acute clinical form and sudden deaths are rather uncommon, presumably due to the relatively tough bovine liver tissue (Skuce and Zadoks 2013), which is less vulnerable and prone for severe hepatic damage and haemorrhages as seen in sheep. Furthermore, the bovine bile ducts react with considerable calcification as a response to the presence of flukes limiting the survival time of adult *F. hepatica* stages. The fibrous reaction in the bovine hepatic parenchyma after a primary *F. hepatica* infection engenders a protective effect against further fluke establishments in cattle (Sargison 2008). This is most likely the reason why most flukes are naturally eliminated within nine to twelve months in cattle (Mas-Coma and Bargues 1997; Boray 1969) in contrast to the considerably longer natural lifespan of up to eleven years in sheep (Rojo-Vázquez et al. 2012).

An older study by Hawkins and Morris (1978) investigated the impact of experimental *F. hepatica* infections on productivity of young sheep infected with different doses of *F. hepatica* metacercariae (infection doses ranging from 50-5000 metacercariae) and compared the bodyweight changes, patch wool growth, fleece weights and feed utilisation with a noninfected control group over 24 weeks. The authors observed a statistically significant reduction of wool growth and body weight gain in the infected group compared to the noninfected group and the negative changes correlated with the mean fluke burden. In the groups infected with 1100 metacercariae or more, the authors did not only observe a reduced weight increase, but even a considerable liveweight loss compared to the liveweights at the beginning of the study despite being fed *ad libitum*. All sheep which received 1100 or more metacercariae and two out of three sheep challenged with 500 metacercariae died within six months, before the end of the trial. No deaths occurred in the groups that were infected with up to 230 metacercariae, but the authors described the sheep which received 230 metacercariae as visibly weaker and paler than the sheep which

received 0, 50 or 110 metacercariae. Fasciolosis was confirmed as the reason for the decease by postmortem examinations. Feed intake was not decreased in the groups infected with 50, 110 or 230 metacercariae compared to the control group and the feed utilisation of dry or organic matter was not significantly negatively influenced in the study animals. Nonetheless, the bodyweight gain and the wool growth were significantly reduced, so the authors deduced that the feed conversion efficiency must be substantially depressed due to fasciolosis. The feed intake was significantly reduced in all groups dosed with 500 or more metacercariae and therefore the reduced body weight could not exclusively be attributed to poor feed conversion for groups with more than 500 metacercariae used for infection. However, it is highly likely that *F. hepatica* did also impair the feed conversion efficiency in these groups. These findings are in agreement with the results of the previously mentioned study by Raadsma et al. (2007), who also observed a significant decrease in bodyweight of the sheep artificially infected with 225 or 400 *F. gigantica* or 250 *F. hepatica* metacercariae in relation to the uninfected control group at week two p.i. in spite of an *ad libitum* diet and no significant difference in feed intake between all groups. This means that an impaired feed conversion efficiency must have also occurred in these study animals. Overall, the study from Hawkins and Morris (1978) evinces the severe, infection dose-dependent clinical impact of *F. hepatica* on productivity and physiological status of infected sheep.

A previous study from Pantelouris (1965) also showed that *F. hepatica* takes up and utilises the sulphur-containing amino acid methionine. Methionine is an essential amino acid and one of the main sources of organic sulfur for body processes (Bankov et al. 1996). For sheep, methionine is particularly essential for protein synthesis and wool growth (Hawkins and Morris 1978). Therefore, infected sheep may present with a poor wool and fleece quality compared to healthy sheep.

Another important aspect of livestock productivity is the reproductive performance, which is diminished in *F. hepatica* infected animals. Most likely due to the release of toxic metabolites and inflammatory mediators during the parenchymal injuries, which impede the embryonic implantation process and the maintenance of early pregnancy stages, *F. hepatica* infected sheep demonstrate poor fertility with high barren rates, decreased twinning rates, and prolonged lambing periods (Sargison 2008). For ewes, liver fluke infections are a predisposing factor for mastitis in the post-partum period (Mavrogianni et al. 2014). This is presumably related to the liver damage (as the liver is essential for the acute phase immune responses) and the development of pregnancy toxaemia, a common disease in sheep induced by impaired nutrient availability with a simultaneously elevated energy requirement during gestation (Mavrogianni et al. 2014). Reduced feed intake and impeded feed conversion efficiency due to fasciolosis exacerbate the metabolic state resulting in increased

production of ketone bodies due to abnormal hepatic metabolism during the state of energy deficiency (Mavrogianni et al. 2014). Mavrogianni et al. (2014) hypothesise that the hyperketonaemia contributes to a decreased local cellular immune defence in the teat, provoking a higher risk of infections with pathogens.

Moreover, due to its immunosuppressive and immunomodulating effects on the host, which is necessary for its own longevity, *Fasciola* spp. facilitate infections with other pathogens. Fasciolosis evokes a polarised T helper (Th) type-2 response in the host associated with the production of interleukin (IL)-4 and IL-10 (Martínez-Pérez et al. 2014). Most likely due to secreted molecules, the parasites suppress the host protective Th1 response towards the predominating Th2 response (Cwiklinski et al. 2016; O'Neill et al. 2000; Brady et al. 1999), resulting in an impaired antibacterial immunity. A recent study from Bangladesh (Sultana et al. 2022) concluded that chronic fasciolosis may be associated with tuberculosis in small ruminants due to the downregulated Th1 immune response. In 1981, Aitken et al. (1981) ascertained that the persistence of *Salmonella dublin* in cattle infected with *F. hepatica* as well as the duration of faecal bacterial excretion was longer than in noninfected animals. Brady et al. (1999) showed that the Th1 immune response in mice infected with *Bordetella pertussis*, which normally results in a selectively induced Th1 cell response, was almost fully abrogated in mice concurrently infected with *F. hepatica* and therefore, coinfection with *F. hepatica* also resulted in a protracted bacterial clearance from the lungs.

2.1.7 Diagnosis of *Fasciola* spp. infections

Due to the strong impact and major consequences of fasciolosis on animal welfare as well as its negative effects on productivity and thus economics, reliable methods for diagnosis of infection are essential. Numerous laboratory approaches are available for the diagnosis of fasciolosis with various advantages and disadvantages regarding sensitivity, specificity and practicability under field conditions.

2.1.7.1 Coproscopic diagnosis

The most used routine diagnostic method is the coproscopic detection of *F. hepatica* eggs in faecal material after a sedimentation process. For a standard protocol of this method, usually 10 g of faeces are suspended in cold tap water in a beaker, sieved through a coarse meshed sieve (e.g. a tea strainer) and left for 30 minutes. Due to their high relative density, *F. hepatica* eggs rapidly sink to the bottom of the beaker, whereas other faecal components, mostly lighter plant components, float to the surface or sink only at a lower speed. After 30 minutes, the sieve is removed, and the supernatant is decanted. The remaining sediment at the bottom of the beaker is replenished with fresh tap water followed by another

sedimentation period of 3-15 minutes (depending on the height of the beaker) and subsequently the supernatant is decanted once again. This process is repeated until the supernatant is clean, and the *F. hepatica* eggs are separated from most of the other faecal components. The sediment is then transferred to a petri dish and microscopically examined using 25× or 40× magnification. Due to their unique appearance and their relatively large size, the eggs can be easily discriminated from other parasite eggs. Only rumen fluke eggs might look rather similar under the microscope, as these trematode eggs are of similar shape, but they can be distinguished by their colour (Mazeri et al. 2016), since *F. hepatica* eggs are yellowish brown, whereas rumen fluke eggs are colourless (Taylor et al. 2016, p. 384) (Figure 5). The detection of fluke eggs is facilitated by adding a staining dye to the sediment, e.g. methylene blue (Rojo-Vázquez et al. 2012), since the remaining plant components of the sediment will be coloured in blue making the parasite eggs clearly visible among the debris.

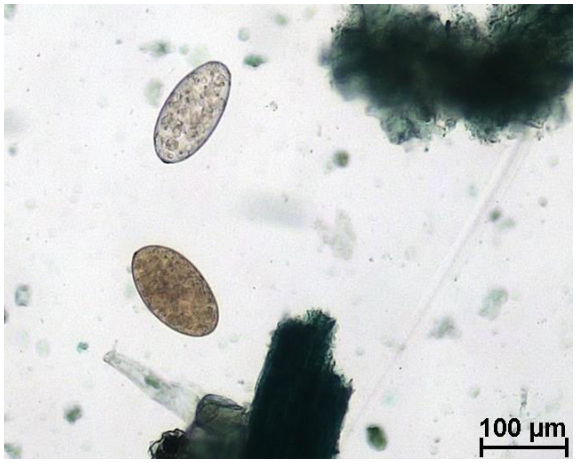

Figure 5: Fasciola hepatica *egg (brownish) and Paramphistominae egg (colourless) The picture was obtained with counterstaining using methylene blue.*

The sedimentation technique with numerous slight adaptations in practice by each laboratory (Charlier et al. 2013) is the standard method for diagnosing *F. hepatica* infections due to its simplicity requiring only inexpensive basic laboratory equipment and low expenditure of hands-on time. However, the greatest disadvantage was the rather low sensitivity particularly for diagnosing low-intensity infections with only a small number of flukes (Hanna et al. 2015).

A modified sedimentation technique was included in the FLUKEFINDER® (FLUKEFINDER® Diagnostic System, Soda Springs, Idaho, USA) protocol, a specifically designed device for differential sieving of the faecal sample before sedimentation. The commercial FLUKEFINDER® device consists of two stacked sieves of different mesh sizes optimised to

specifically separate faecal components of an approximate size of *F. hepatica* eggs from other faecal components. After the sieving process, the debris on the lower sieve is backwashed into a beaker for the following sedimentation process. The resulting sediment is considerably cleaner and easier to evaluate under the microscope, since no coarse plant components obscure the view. Several recent studies used the FLUKEFINDER® method to detect *F. hepatica* eggs in ovine and bovine faecal samples (Bosco et al. 2023; Kurnianto et al. 2022; Kelley et al. 2021; Reigate et al. 2021; Elliot et al. 2015) as well as in human stool samples (Zárate-Rendón et al. 2019).

A different approach for enriching *F. hepatica* eggs from faecal samples for microscopic detection is a flotation technique, e.g. the FLOTAC or MiniFLOTAC technique, which are also used for the microscopic detection of nematode eggs. The FLOTAC apparatus is a device of cylindrical shape consisting of a base, a translation disc, and a reading disc (Cringoli et al. 2010). The homogenised faecal sample (5-10 g) is diluted in tap water with a ratio of 1:10, filtered through a wire mesh and 11 ml of the sieved suspension are transferred into a conic tube followed by a three-minute centrifugation period. Subsequently, the supernatant is discarded. The pellet is resuspended in a specific flotation solution with a defined relative density up to the 11 ml level inside the tube and then filled into the flotation chambers of the FLOTAC apparatus (5 ml in each chamber). After a five-minute centrifugation period, the top parts of the flotation chambers are translated by 90° and examined under a microscope for parasite eggs (Cringoli et al. 2010). A variation of the FLOTAC method is the Mini-FLOTAC technique. The Mini-FLOTAC device is also a round apparatus, but in contrast to the FLOTAC device, it is only made of two physical components (a base and a reading disc) (Cringoli et al. 2017). Five grams of faeces are homogenised with 45 ml of a specific flotation solution with a defined specific gravity and subsequently sieved through a coarse sieve (if the device is not used together with the Fill-FLOTAC kit). One ml of the filtered suspension is then transferred into the flotation chambers. After a flotation period of ten minutes, the reading disc is turned clockwise (90°) and the reading grids are microscopically examined (Cringoli et al. 2017). In contrast to the FLOTAC technique, the Mini-FLOTAC method does not require a centrifuge and is also less time consuming due to fewer work steps. However, the most important disparity is the multiplication factor, as the Mini-FLOTAC method only allows for multiplication factors of 5 and 10 while FLOTAC can be used applying multiplication factors of 1 and 2 (Cringoli et al. 2017). Since *F. hepatica* eggs have a higher relative density than most nematode egg types, flotation solutions with a high relative density must be used for their detection when using a flotation technique. Zinc-sulphate-based flotation solutions with a relative density of 1.35 can be used to float *F. hepatica* eggs (Rinaldi et al. 2015; Duthaler et al. 2010).

The very recent study by Bosco et al. (2023) comparing Mini-FLOTAC, FLUKEFINDER® and sedimentation technique for detection and quantification of *F. hepatica* and *C. daubneyi* eggs in naturally infected and artificially egg-spiked bovine faecal samples observed the highest number of recovered eggs using the Mini-FLOTAC method in highly spiked samples at 50 eggs per gram faeces (EPG) and 100 EPG for both trematodes followed by FLUKEFINDER® and sedimentation, whereas in samples containing only 10 EPG, the FLUKEFINDER® surpassed the other two methods (Bosco et al. 2023).

A further coproscopic approach to detect *F. hepatica* eggs in faecal samples is the Kato-Katz method, a very simple technique for the direct diagnosis of parasite eggs by straining faecal material through a stainless-steel cloth and subsequently smearing onto a microscope slide (Katz et al. 1972).

Coproscopic detection methods cannot only reveal qualitative, but also quantitative results if the amount of faeces is weighed before parasitological examination. The faecal egg count (FEC) measured as EPG is obtained by dividing the counted total egg quantity by the weight of faecal material. This is particularly important for an approximate estimation of infection intensity and for the performance of faecal egg count reduction tests (FECRT) in anthelmintic efficacy trials by comparing the FEC before and after treatment.

Generally, coproscopic techniques are relatively simple to perform and provide the opportunity of a fast and non-invasive examination of the animals for the presence of parasite eggs. Depending on the method, coproscopy may also be conducted directly on farm if a microscope and basic laboratory equipment is available. Especially in rural areas without access to highly equipped laboratories, the simplicity of coproscopic methods – in particular the sedimentation method – is one of the greatest benefits in the field. Moreover, the specificity of coproscopy for the diagnosis of fasciolosis is almost perfect (Mazeri et al. 2016) due to the unique appearance of *Fasciola* spp. eggs. Only rumen fluke eggs resemble *Fasciola* spp. eggs in size and shape but are easily distinguishable by their colour. The relatively low equipment costs of coproscopic examinations present another advantage.

However, coproscopic techniques also have their disadvantages. Regardless of the technique, all coproscopic methods can only detect patent infections and show a negative result in the early stage of the infection (Mezo et al. 2022; Mazeri et al. 2016; Duthaler et al. 2010; Almazán et al. 2001). Since juvenile flukes do not produce eggs, diagnosis of fasciolosis in the particularly harmful acute migration phase cannot be made by coproscopy leading to false-negative results. Only from the beginning of the patency (eight to twelve weeks p.i.) eggs can be detected by coproscopical examination, but at this time the liver parenchyma has already been harmed (Mezo et al. 2022). In contrast to the risk of false-

negative results, the detection of eggs in the faeces may also be false-positive for several weeks after flukicidal treatment since *F. hepatica* eggs remain in the host's gall bladder for a certain time even after successful elimination of the flukes (Fairweather 2011b; Chowaniec and Darski 1970). The study from Chowaniec and Darski (1970) showed that *F. hepatica* eggs may be excreted up to about 35 days after successful flukicidal treatment.

Due to the intermittent shedding from the gall bladder, the excretion of eggs with the faeces is irregular (Kelley et al. 2021; Gordon et al. 2012; Sargison and Scott 2011; Flanagan et al. 2011b; Düwel und Reisenleiter 1990), so that FEC may fluctuate considerably. That may result in relatively low sensitivity of coproscopic methods, particularly in low-intensity infections with only small numbers of flukes (Duthaler et al. 2010). Especially for the most widely used sedimentation technique a rather low sensitivity has been described by various authors (Alstedt et al. 2022; Ploeger et al. 2017; Becker et al. 2016; Hanna et al. 2015; Charlier et al. 2013; Rapsch et al. 2006). According to Rapsch et al. (2006), the sensitivity can be notably increased by repeated testing and the analysis of larger sample volumes, however this is more labour intensive as a natural consequence.

2.1.7.2 Detection of coproantigens in faecal material

A more novel approach for *in vivo* diagnosis of fasciolosis is the detection of coproantigens in faecal material. *Fasciola hepatica* releases metabolic products which are detectable in the faeces with immunological tests (Mezo et al. 2004; Almazán et al. 2001). The most widely used method is a coproantigen enzyme-linked immunosorbent assay (cELISA) based on a monoclonal antibody (mAb) (MM3, IgG1/κ) that captures specific *F. hepatica* excretory-secretory antigens in the faeces. This cELISA was first established by Mezo et al. (2004) and is highly specific showing no cross-reactivities with other helminths frequently parasitising ruminants including gastrointestinal nematodes (GIN), cestodes, and other trematodes such as *Dicrocoelium dendriticum* or rumen flukes (Mazeri et al. 2016; Brockwell et al. 2013; Kajugu et al. 2012; Mezo et al. 2004). This mAB-based cELISA has also been shown to be ultrasensitive, capable of detecting sub-nanogram amounts of specific antigens in faecal material (detection limit of 0.3 ng of the coproantigen per ml of ovine faecal supernatant) and able to detect infections with only one single fluke (Mezo et al. 2004). Nowadays, a commercially available version of this cELISA is the BIO K 201 - Monoscreen AgELISA kit (BIO-X Diagnostics S.A., Rochefort, Belgium), which has already been used in various studies for the detection of *F. hepatica* in ruminants (Kelley et al. 2021; George et al. 2017; Arifin et al. 2016; Mazeri et al. 2016; Novobilský et al. 2016; Elliot et al. 2015; Hanna et al. 2015; Novobilský and Höglund 2015; Brockwell et al. 2014; Palmer et al. 2014; Brockwell et al. 2013; Gordon et al. 2012; Novobilský et al. 2012; Flanagan et al. 2011a; Flanagan et al.

2011b; Charlier et al. 2008). The test principle of the commercial BIO K 201 cELISA is pictured in Figure 6.

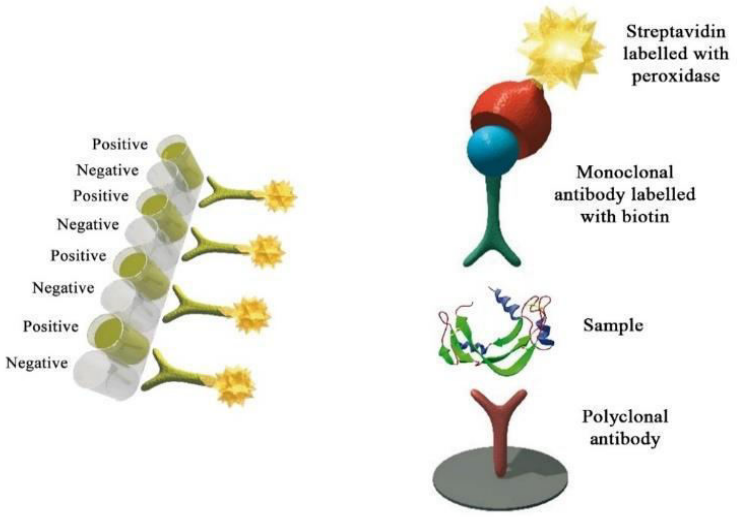

Figure 6: Test principle of the commercial indirect sandwich BIO K 201 cELISA (BIO-X Diagnostics S.A., Rochefort, Belgium). This picture originates from the official website of the company (https://www.biox.com/en/bio-k-201-monoscreen-agelisa-fasciola-hepatica-indirect-sandwich-double-wells-p-257/). The commercial BIO K 201 Sandwich-ELISA kit consists of a 96-well microplate, on which every second row is coated with a specific polyclonal antibody against F. hepatica*, whereas the wells in every other row is sensitised with an antibody, which is not specific for* F. hepatica *and serves as a control to prevent false-positive results. If* F. hepatica *coproantigens are present in the sample, they bind to the specific antibody. After washing the plate, the specific MM3 monoclonal antibody coupled to biotin is incubated on the microplate and binds to a specific coproantigen. Then, a peroxidase-coupled avidine, which specifically binds to biotin is incubated on the plate as a second conjugate. Finally, the chromogen tetramethylbenzidine is added. If coproantigens were present in the faecal sample, the two conjugates remain bound to the plate during the process and the enzyme catalyses the reaction of the colourless chromogen to a blue compound, proportionally to the coproantigen amount in the sample. This reaction is stopped with an acidic stopping solution terminating the reaction by the pH shift and changing the colour from blue to yellow. The resulting optical density (OD) can then be determined at 450 nm using a photometer and the signals measured in the negative control wells are subtracted from the signals of the corresponding test wells.*

The coproantigen detected by the mAb MM3 ELISA is specific to gastrodermal cells of the flukes and is most probably related to the cathepsin family of cysteine proteases, a group of digestive enzymes, which are found in adult as well as in juvenile stages of *F. hepatica* (Flanagan et al. 2011a). This fact constitutes one of the major advantages of the cELISA compared to coproscopy, since the cELISA can already be applied in the prepatent period for an earlier diagnosis of fasciolosis. Coproantigens are detectable at five to six weeks p.i. (Calvani et al. 2018; Brockwell et al. 2013; Flanagan et al. 2011a; Valero et al. 2009; Mezo et al. 2007). Different studies comparing the time of the first positive result in the cELISA with the first coproscopic detection of eggs drew the conclusion that coproantigens are considerably sooner detectable than eggs (Brockwell et al. 2013; Flanagan et al. 2011a; Flanagan et al. 2011b; Valero et al. 2009; Mezo et al. 2004). In contrast, Gordon et al. (2012), did not observe positive cELISA results prior to positive FEC results in their study,

but this finding could be due to a monthly instead of a weekly sampling interval of the sheep in this trial.

Studies investigating the relationship between the coproantigen concentration and the parasite burden found a positive correlation between the OD signal measured in the cELISA and the number of flukes (George et al. 2017; Brockwell et al. 2013; Charlier et al. 2008; Mezo et al. 2004), so that a rough quantitative estimation of the infection intensity can be provided by the cELISA.

Compared to coproscopic methods, the cELISA is considered to be a more sensitive test to diagnose *F. hepatica* infections (Fairweather et al. 2012; Flanagan et al. 2011a), particularly in low-intensity infections with low EPG values (Mezo et al. 2022; Brockwell et al. 2013). In addition, the cELISA requires less faecal material than coproscopic methods (only 0.5 g for ovines) and is more convenient and time-efficient when analysing large sample quantities allowing numerous individual samples to be run simultaneously (George et al. 2017; Gordon et al. 2012). The collection of faecal samples for the examination is not invasive, a commonality that the cELISA shares with coproscopic diagnostic approaches.

Another advantageous aspect of the cELISA method is that only actively metabolising flukes are detected (Fairweather et al. 2012; Martínez-Pérez et al. 2012) and that the coproantigen release is maintained during the total duration of infection (Valero et al. 2009). Therefore, the cELISA is a suitable approach to verify an active infection status as well as the absence of flukes after flukicidal treatments in anthelmintic efficacy trials (George et al. 2017; Flanagan et al. 2011a; Flanagan et al. 2011b). After successful elimination of the flukes, coproantigen levels decrease to a negative result within 14 days post-treatment (p.t.) (Brockwell et al. 2014; Flanagan et al. 2011a). Hence, for a CRT to evaluate treatment efficacy, an interval of 14 days between treatment and re-sampling is recommended (Brockwell et al. 2014; Fairweather 2011b; Flanagan et al. 2011a; Flanagan et al. 2011b).

Several studies implementing the BIO K 201 - Monoscreen AgELISA (BIO-X Diagnostics S.A., Rochefort, Belgium) revised the threshold value indicated by the manufacturer to discriminate between positive and negative results and used a lowered cut-off value in their experiments to increase sensitivity (Calvani et al. 2017; Novobilský and Höglund 2015; Palmer et al. 2014; Brockwell et al. 2013).

However, the cELISA method is not without limitations. As mentioned above, coproantigens are detectable at five to six weeks p.i., enabling an earlier diagnosis of fasciolosis compared to the coproscopic detection of eggs. Nevertheless, even at five to six weeks p.i., juvenile flukes have already damaged the hepatic parenchyma prior to the coproimmunological diagnosis. Therefore, the cELISA will show a negative result during the first stage of infection

before coproantigens are released. Like egg shedding, the excretion of coproantigens can be inconsistent with fluctuations over time (Brockwell et al. 2013; Valero et al. 2009). Furthermore, reports about persisting coproantigen detection in individual animals even after two weeks p.t. exist (Hanna et al. 2015; Flanagan et al. 2011a). A positive cELISA result despite successful flukicidal treatment presumably originates from the continued coproantigen release by dead and degenerating flukes, which had not been cleared from the bile system at the point of re-sampling and still release detectable amounts of antigens (Hanna et al. 2015; Flanagan et al. 2011a). Regarding the financial aspect, immunodiagnosis using the cELISA kit is more expensive than coproscopic techniques with regard to the working appliances needed. Not only the cELISA kit itself, but also the additionally required laboratory equipment (e.g. centrifuge, microplate shaker, and microplate reader) are more cost-intensive than the basic devices necessary for coproscopy. When analysing only smaller quantities of faecal samples, coproscopy can be performed faster, since the conduction of the cELISA takes several hours due to long incubation periods during the procedure. Nevertheless, for greater sample quantities cELISA might be faster overall since many samples can be processed in parallel. In this case, the equipment costs for the cELISA also relativise the personnel costs necessary for individually analysing large amounts of samples via coproscopy.

2.1.7.3 Detection of specific antibodies against *F. hepatica*

Another immunological approach to diagnose *F. hepatica* infections is the detection of specific anti-*F. hepatica* IgG antibodies in the sera of infected animals. The most common immunological-based method for the serodiagnosis of fasciolosis in ruminants is the immunoassay using excretory-secretory antigens of *F. hepatica* to capture the specific antibodies in ELISA tests (Nur Hafizah et al. 2023). Excretory-secretory antigens are implicated in the migration of the flukes through the host tissue (Drescher et al. 2023) and therefore, antibodies against these antigens can be detected in the early stage of infection.

Fasciola hepatica induces a humoral immune response and specific antibodies are detectable from two weeks p.i. and may persist at high levels after 20 weeks p.i. (Alvarez Rojas et al. 2014). Hence, antibody ELISA tests provide the benefit of an earlier diagnosis compared to coproscopic examinations and cELISA tests. Several serological ELISA kits are commercially available (Beesley et al. 2018). For cattle, a multiplex immunological assay to simultaneously detect antibodies against *Cooperia oncophora, Dictyocaulus viviparus,* and *F. hepatica* has been established for a rapid examination for different parasites (Karanikola et al. 2015).

However, a serious drawback of antibody ELISA examinations in the diagnosis of fasciolosis is not only the higher invasiveness of venipunctures compared to the collection of faeces but predominantly the inability to discriminate between current and past infections since antibodies remain detectable for a long period even after successful elimination of the flukes (Beesley et al. 2018; Mezo et al. 2007). Therefore, the presence of antibodies is not a reliable indicator for an active *F. hepatica* infection inside the host but rather an indicator of previous exposure (Gordon et al. 2012; Munguía-Xóchihua et al. 2006). Particularly in terms of anthelmintic efficacy testing serodiagnosis is an inexpedient method to control the success of treatment since antibody levels do not decrease promptly after treatment. Castro et al. (2000) reported a consistent drop of antibody titres in *F. hepatica* infected cattle starting one month after flukicidal treatment with TCBZ and negative status within four to six months after treatment. In sheep, Sánchez-Andrade et al. (2001) made similar observations with antibodies only starting to decrease from four weeks after successful treatment of sheep with either TCBZ or netobimin. Remarkably, none of the animals reached a negative status throughout the study (16 weeks p.t.). Mezo et al. (2007) also investigated the decline of antibodies in *F. hepatica* infected lambs after successful TCBZ treatment and observed a drop of only 25% in OD values over a period of four weeks p.t.

Accordingly, the serodiagnosis of fasciolosis is more suitable for screening livestock flocks for the herd exposure to *F. hepatica* on the pastures rather than for indicating active infections. The same applies for the detection of antibodies in milk. Specific antibodies against *F. hepatica* can be detected in milk samples from dairy cows since antibodies are secreted in milk and antibody ELISA tests have been adapted to test either individual or bulk milk samples for the presence of antibodies against *F. hepatica* (Beesley et al. 2018). By using existing milk samples collected for monitoring milk quality and productivity instead of blood samples, costs can be reduced, animals experience less stress due to less handling and flocks or individual animals presenting loss of milk productivity associated with fasciolosis can be identified (Mezo et al. 2011). Nevertheless, since the presence of antibodies in the milk is dependent on the presence of antibodies in the serum of the animal, a positive result does not give reliable evidence for a current infection. Therefore, the detection of antibodies is generally better suited for the diagnosis and risk-assessment of fasciolosis on farm-level.

2.1.7.4 Molecular methods

Nowadays, molecular biological methods have been developed as new diagnostic techniques for the genetic identification of *Fasciola* spp. using nuclear or mitochondrial DNA sequences of the parasite (Alvarez Rojas et al. 2014). Various PCR approaches to detect

Fasciola spp. DNA in faecal samples have been published. For instance, Martínez-Pérez et al. (2012) described two PCR assays, a standard PCR and a nested-PCR, for amplifying a 423 base pair (bp) fragment of mitochondrial *F. hepatica* DNA from faecal material and compared these molecular techniques with the results of a coproscopic sedimentation method and the commercially available cELISA (Bio-X Diagnostics, Belgium) in both naturally and experimentally infected sheep. The authors reported a significantly earlier detection of *F. hepatica* DNA (first positive results two- and three-weeks p.i. by nested-PCR and standard PCR, respectively) than eggs (first positive results nine weeks p.i.) and coproantigens (first positive results four weeks p.i.) in the experimentally infected sheep. In naturally infected sheep, the nested-PCR outperformed the standard PCR since not every sheep tested positive by standard PCR whereas the infection was confirmed in all sheep by the nested-PCR. Moreover, the specificity of the PCR techniques was proven due to the absence of cross-reactions with GIN (Martínez-Pérez et al. 2012).

A further molecular diagnostic method for the amplification of fluke DNA is the loop-mediated isothermal amplification (LAMP). This method was originally developed by Notomi et al. (2000) and is an amplification assay based on auto-cycling strand displacement DNA synthesis performed by a DNA polymerase characterised by a high strand displacement activity. This procedure can run under constant temperatures in contrast to PCR (Notomi et al. 2000). The LAMP method has been tested and compared with a standard PCR assay by Martínez-Valladares and Rojo-Vázquez (2016) regarding the diagnosis of natural and experimental *F. hepatica* infections in sheep. The advantages of the LAMP method over PCR were shown to be the lower requirements regarding time and equipment. Both methods were able to detect the experimental *F. hepatica* infections from the first week p.i. onwards, which is even prior to the first detections by PCR in the study from Martínez-Pérez et al. (2012). Martínez-Valladares and Rojo-Vázquez (2016) hypothesised that the DNA detected in this very early stage of infection possibly originates from cellular material resulting from tegumental turnover of the flukes or from metacercariae that did not progress.

Regarding the assessment of anthelmintic efficacy, the same drawbacks as seen using coproscopy might be present. A residue of small numbers of fluke eggs, which can be present even after successful chemotherapy due to delayed release from the gall bladder can lead to false-positive results after treatment since molecular techniques such as PCR and LAMP could amplify the DNA from these residual eggs (Martínez-Valladares and Rojo-Vázquez 2016). It can therefore be concluded that molecular techniques for the diagnosis of the current infection status are also not without limitations due to the risk of false-positive results. A further drawback of molecular methods are the high demands regarding equipment and the examiner’s expertise.

2.1.7.5 Post-mortem examinations

Apart from the above discussed laboratory diagnostic methods, fasciolosis can also be diagnosed post-mortem during the visual meat inspection after slaughter. Mature and immature fluke stages as well as the characteristic macroscopic alterations in the hepatic tissue (as described under 2.1.6.1) can be visually detected. The examination of the liver in European abattoirs for indicators of parasite infections is mandatory according to the Commission Implementing Regulation (EU) 2019/627 (European Commission 2019). Regulation (EU) 2019/627 stipulates visual inspection and palpation of the liver and the hepatic and pancreatic lymph nodes (*Lnn. portales*) as well as the incision of the gastric surface of the liver to examine the bile ducts for the post-mortem examination of domestic sheep and goats. In bovines, visual inspection of the liver and the hepatic and pancreatic lymph nodes (*Lnn. portales*) is mandatory as well. Palpation and incision of the liver must follow in bovines, when there are indications of a possible risk to human health. Visual characteristics indicating the presence of parasites entail the condemnation of the liver.

The diagnostic sensitivity of visual liver inspections of cattle slaughtered in an abattoir in Switzerland was estimated to be 63.2% by Rapsch et al. (2006), which was lower than the sensitivities of coproscopic sedimentation, a commercial serum ELISA and the post-mortem examination of bile fluid for the presence of *F. hepatica* eggs in the same study.

It is in the nature of the matter that post-mortem diagnosis by visual inspection of the liver is not possible without sacrificing the animal. However, findings at the abattoir during meat inspection are often used to assess the prevalence of a disease (Caroll et al. 2017). Moreover, dose-and-slaughter trials can be applied for the evaluation of the anthelmintic efficacy of a flukicide by assessing the definite fluke burden after treatment (Coles and Stafford 2001).

2.1.8 Prophylactic control measures

2.1.8.1 Prophylactic pasture management

Prophylactical actions against fasciolosis target to minimise infection pressure by reducing the metacercarial contamination on the pasture. Strategic measures include the drainage of wet pastures to lower the rate of egg hatching as well as the survival of free-living *F. hepatica* stages and snail populations by making the habitat unsuitable for the completion of the life cycle. Grazing management strategies like pasture rotation or fencing off moist sections to prevent the animals from grazing in particular high-risk areas are further practical solutions to reduce the contact between metacercariae and livestock and the spread throughout pastures (Castro-Hermida et al. 2021; Deplazes et al. 2020, p. 604; Rojo-Vázquez et al. 2012;

Fairweather 2011b; Sargison and Scott 2011). Moreover, an easily cleanable trough for watering the animals on the pasture instead of free access to natural water sources like ditches and ponds lowers the risk of ingesting freely swimming metacercariae. The development of secondary habitats (damp areas around the trough) must be avoided (Deplazes et al. 2020, p. 604).

Another approach to impede the establishment of snail populations is the application of molluscicides to inhibit the transmission of *F. hepatica*. However, synthetic molluscicidal chemicals are expensive and must be viewed critically due to the risk of toxicity to non-target organisms and detrimental long-term environmental effects (Yadav 2015) and are therefore no longer a way to control fasciolosis (Knubben-Schweizer et al. 2021). Hence, the use of plants with molluscicidal potential has received attention in recent years for controlling the intermediate host snail populations (Yadav 2015).
Furthermore, the infection status of livestock flocks should be regularly monitored, and newly arrived animals should be quarantined and examined for current *F. hepatica* infections before being introduced into a flock (Castro-Hermida et al. 2021). In this way, the risk of spreading (potentially drug-resistant) *F. hepatica* populations between farms and countries can be considerably minimised.

2.1.8.2 Immunoprophylaxis

An effective vaccine against fasciolosis would constitute a breakthrough in combatting fasciolosis and its economic consequences. Effective immunoprophylaxis might also decrease the spread of anthelmintic resistance due to reduced treatment requirements. Therefore, the identification of molecules with antigenic capacity has been a major research topic in the last decades, but no vaccine formulation against fasciolosis has been sufficiently effective for a commercial production until now (Zafra et al. 2021; Beesley et al. 2018). Spithill et al. (2021) recently reviewed the current state regarding vaccine trials in ruminants conducted from 2013 to 2020 and the observed vaccine efficacies. A major part of the vaccine trials reported in the last decade investigated previously known immunomodulatory proteins, e.g. cathepsin proteases, leucine aminopeptidase or glutathione S-transferase. Both single and combination vaccines with various adjuvants and different administration routes (subcutaneous, intramuscular, intranasal, and oral) have been tested in cattle, sheep and goats against *F. hepatica* and *F. gigantica* with observed efficacies ranging from 0-56.2% (Spithill et al. 2021).

Another recent review by Cwiklinski and Dalton (2022) stated various reasons for the difficulties in developing an effective vaccine against fasciolosis: Firstly, *Fasciola* spp. are large, multicellular and complex organisms transcribing over 18,000 genes during their

development. That makes the development of a protecting vaccine far more challenging than for example developing a vaccine against viruses with only a small number of proteins being expressed. Furthermore, the active locomotion of *Fasciola* spp. helps in evading the host's immune cells. Another important aspect is the fact that a protective immunity is also not displayed during natural infections. Lastly, the alteration of the host's immune response towards a strong Th2-driven response may aggravate vaccine-induced Th1 responses, so that challenging infections might neutralize the effects of the vaccine (Cwiklinski and Dalton 2022). Summarised, a commercial vaccine cannot be expected in the near future.

2.1.8.3 Natural resistance

Natural resistance against fasciolosis can be found in Indonesian thin tail (ITT) sheep, but only against *F. gigantica* and not against *F. hepatica* (Pleasance et al. 2011; Piedrafita et al. 2004; Roberts et al. 1997a; Roberts et al. 1997b). This breed attains uncommonly low *F. gigantica* fluke burdens (0.3-5% of the infective metacercarial dose) due to innate and acquired resistance mechanism (Pleasance et al. 2011). Roberts et al. (1997b) stated that the natural resistance is determined by a major gene with incomplete dominance and a prevalence of 90% in the ITT sheep population. Due to their unique ability to oppose *F. gigantica* infections, ITT sheep might present an ideal model to study potential mode of actions involved in parasite resistance (Pleasance et al. 2011), so that the aspect of natural resistance against *Fasciola* spp. should be considered in future research regarding livestock breeding.

2.1.9 Therapy

The control of fasciolosis relies heavily on the administration of anthelmintic drugs. A wide range of various drugs with flukicidal activity are used for the treatment of fasciolosis. Chemically, flukicides belong to different major drug classes: (Pro-)Benzimidazoles (e.g. ABZ, netobimin, TCBZ), salicylanilides (e.g. closantel (CLOS), rafoxanide (RAFOX), oxyclozanide (OXYCLO)), halogenated phenols (e.g. nitroxynil (NITROX)), and sulfonamides (e.g. clorsulon (CLOR)) are used in veterinary medicine (Alvarez et al. 2021; Fairweather and Boray 1999). *Fasciola* spp. incorporate anthelmintic drugs either by the oral ingestion during blood sucking or by transtegumental diffusion from the surrounding medium through the high absorption surface of the flukes (Mottier et al. 2004). The developmental stage and the location of the flukes in the definitive host are of major significance regarding the exposure to the different flukicidal drugs, since the flukes are situated in very diverse physiological environments if they are in the migrating phase through the liver parenchyma compared to the establishment in the bile ducts (Alvarez et al. 2021).

2.1.9.1 Benzimidazoles

Benzimidazoles are the most important anthelmintics used to treat fasciolosis and consist of a benzene ring and an imidazole ring as a basic structure. Most benzimidazoles demonstrate a broad efficacy spectrum against nematodes, but only a few representatives of the (pro-) benzimidazole group (TCBZ, ABZ, netobimin) act against trematodes in reasonable dosages (Löscher and Richter 2016, pp. 474-486; Fairweather and Boray 1999). Other benzimidazoles only show flukicidal activity when administered in substantially increased dose rates (e.g. thiabendazole and mebendazole only affect *Fasciola* spp. when given five times the effective dose against GIN) and are therefore impractical for the chemotherapy of fasciolosis (Löscher et al. 2014, p. 390).

Most benzimidazoles act by binding to the beta tubulin of the parasite leading to a disruption of the formation of microtubules. Microtubules are hollow tubular cell organelles consisting of typically 13 protofilaments and are major elements of the cytoskeleton (Lacey 1988). The basic structural components are heterodimers comprised of alpha and beta tubulin proteins. Microtubules are in dynamic equilibrium with constant assembly and disassembly of tubulin units at the opposite end of the emerging tubule (Lacey 1988). At cellular level, microtubules fulfil a wide variety of functions including the formation of the mitotic spindle during cell division, maintenance of cell shape, cell motility, cellular secretion, nutrient absorption, and intracellular transport (Lacey 1988). Benzimidazoles bind to the same binding site on the beta tubulin as colchicine (an alkaloid originating from meadow saffron with a well-recognised microtubule activity inhibitor activity) (Lacey 1988). When the assembly of microtubules is blocked by the binding of benzimidazoles, microtubule-based processes fundamental for cellular homeostasis such as the mitotic spindle formation, the formation of the cytoskeleton as well as the absorption and transportation of nutrients are impaired. Not only the reduced absorption of glucose, but also the inhibition of mitochondrial enzymes leads to a depletion of energy reserves. The stagnation of the energy metabolism of the helminths results in starvation and a delayed lethal effect on the helminths two to three days after administration (Fairweather et al. 2020; Löscher and Richter 2016, p. 476; Taylor et al. 2016, p. 314; Löscher et al. 2014, p. 362). Most benzimidazoles have also ovicidal effects due to the inhibition of mitotic spindle formation and disruption of metabolism during embryogenesis (Löscher et al. 2014, p. 362).

Generally, benzimidazoles are lipophilic compounds with a good enteral resorbability. The active metabolites, which are excreted via the biliary route, can reach high drug concentrations in the bile ducts effecting the fluke stages established within. Due to their slow elimination process, the use of benzimidazoles in livestock often entails long withdrawal periods (Löscher and Richter 2016, p. 476). Another characteristic of benzimidazoles is their

wide therapeutic safety margin with a low toxicity even at over ten times the recommended dose (Löscher and Richter 2016, p. 476; Taylor et al. 2016, p. 314; Löscher et al. 2014, p. 363, 390). However, due to their anti-mitotic activity, benzimidazoles demonstrate teratogenic or embryotoxic effects and should therefore only be used with particular caution in gestating animals (Löscher and Richter 2016, p. 476; Löscher et al. 2014, p. 363).

2.1.9.1.1 Albendazole

Albendazole is a methylcarbamate benzimidazole and a widely used anthelmintic drug effective against all major nematodes. Between 1.3-1.5 times the nematocidal dose of ABZ is effective against adult stages of *F. hepatica*. The recommended flukicidal oral administration dose amounts to 7.5 mg/kg bodyweight (bw) in sheep and 10 mg/kg bw in cattle (Alvarez et al. 2021; Löscher and Richter 2016, p. 476; Löscher et al. 2014, p. 390). The active metabolite is ABZ sulfoxide (ABZ.SO) (Alvarez et al. 2021).

The minimum age of the flukes for an efficacy of ≥90% is twelve weeks (Williams 2020; Fairweather and Boray 1999). The efficacy against flukes after ten to eleven weeks p.i. is 50-70% (Alvarez et al. 2021). Only up to 25% of immature flukes under the age of six weeks are killed by ABZ (Löscher and Richter 2016, p. 484; Löscher et al. 2014, p. 390). Hence, ABZ is inapplicable for the treatment of acute fasciolosis since the particularly pathogenic juvenile flukes are not adequately affected during their migration through the liver parenchyma.

Following oral administration, ABZ is extensively absorbed and metabolised to its sulfoxide metabolite in the liver. Due to its biliary excretion, the concentration in the bile ducts is sufficiently high to kill the mature flukes established in the bile duct system (Löscher and Richter 2016, p. 476).

Albendazole is licensed for dairy sheep and cattle in Germany (Löscher et al. 2014, p. 390), but due to its embryotoxic effect it is contraindicated during the embryogenetic stage of gestation (Löscher et al. 2014, p. 363).

The pro-benzimidazole netobimin is only converted in the host organism to the active benzimidazole metabolite of ABZ by biotransformation activities of the rumen and intestinal microbes (Löscher and Richter 2016, p. 475-476; Taylor et al. 2016, p. 313), so the efficacy spectrum is equivalent to the spectrum of ABZ. Netobimin is currently non-licensed in Germany (Löscher and Richter 2016, p. 476).

2.1.9.1.2 Triclabendazole

Triclabendazole, a halogenated benzimidazole, was introduced in 1983 for the treatment of fasciolosis in livestock (Hanna et al. 2015; Boray et al. 1983). In contrast to all other

benzimidazoles, TCBZ has chloride atoms, and a thiomethyl group and lacks a carbamate component in its structural chemical formula (Fairweather and Boray 1999; Bennett and Köhler 1987).

Among the benzimidazoles, TCBZ holds a special position by reason of its selective efficacy against trematodes, but not against nematodes (Löscher and Richter 2016, p. 485; Löscher et al. 2014, p. 390; Fairweather 2005; Fairweather and Boray 1999). Moreover, it is of particular importance since it is the only flukicidal drug targeting not only the late immature and adult fluke stages, but also the highly pathogenic early immature stages of *Fasciola* spp. (Alvarez et al. 2021; Fairweather et al. 2020; Cwiklinski et al. 2016; Löscher and Richter 2016, p.484-485; Brockwell et al. 2014; Skuce and Zadoks 2013; Fairweather et al. 2012; Fairweather 2011b). Hence, TCBZ is the drug of choice to treat acute fasciolosis. The recommended oral administration dose amounts to 10 mg/kg bw in sheep and 12 mg/kg bw in cattle (Alvarez et al. 2021; Löscher and Richter 2016, p. 485; Löscher et al. 2014, p. 390). Using 10 mg/kg bw in sheep, Boray et al. (1983) observed efficacies of 93-98% against one-week-old *F. hepatica* stages, 99-100% efficacy against two- to four-week-old fluke stages and 100% efficacy against six-week-old flukes. Using 15 mg/kg bw in sheep, the authors observed 98% efficacy against flukes one day after infection.

A high proportion of the administered amount of TCBZ is enterally absorbed (more than 70%) (Löscher and Richter 2016, p. 485; Löscher et al. 2014, p. 390), completely eliminated from the portal blood by the liver and oxidized to TCBZ sulphoxide (TCBZ.SO) and TCBZ sulphone (TCBZ.SO2) (Moreno et al. 2014; Fairweather 2005; Hennessy et al.1987). Triclabendazole sulfoxide and TCBZ.SO2 are the main metabolites in plasma, whereas the parent drug TCBZ is not present in blood (Moreno et al. 2014; Hennessy et al. 1987). TCBZ.SO is considered the main pharmacologically active metabolite (Fairweather 2011b; Virkel et al. 2006; Alvarez et al. 2005; Fairweather 2005; Robinson et al. 2004; Wolstenholme et al. 2004). The maximum plasma concentrations of TCBZ.SO and TCBZ.SO2 occur protracted (18 h and 36 h after intraruminal administration of TCBZ, respectively), most likely due to the strong binding affinity to albumin and its release from the liver (Fairweather 2005; Hennessy et al. 1987). Triclabendazole, TCBZ.SO and TCBZ.SO2 are also hydroxylated in the liver forming the hydroxy metabolites OH-TCBZ, OH-TCBZ.SO and OH-TCBZ.SO2 (Virkel et al. 2006; Fairweather 2005; Hennessy et al. 1987). The hydroxylated metabolites are not present in the plasma but are secreted in the bile fluid, mainly conjugated as glucuronide or sulphoxide, where they represent the main metabolites besides TCBZ.SO and TCBZ.SO2. The parent drug TCBZ is only present in very minor amounts in bile (Mottier et al. 2004; Hennessy et al. 1987). More than 95% of the absorbed amount of TCBZ is biliary excreted and eliminated with the faeces (Löscher et al. 2014, p.

390). Hennessy et al. (1987) measured high concentrations (> 50-60 µg/ml) of TCBZ metabolites in bile providing significant chemical exposure to the flukes.

Due to the strong binding to albumin, TCBZ demonstrates a long elimination half-life resulting in long withdrawal periods of up to 56 days for meat (Löscher and Richter 2016, p. 485; Löscher et al. 2014, p. 390).

Triclabendazole presents a wide therapeutic safety margin and even 200 mg/kg bw are well tolerated by sheep and cattle (Löscher and Richter 2016, p. 485; Löscher et al. 2014, p. 390; Fairweather and Boray 1999). In contrast to ABZ, there is no evidence of teratogenic or embryotoxic effects during gestation (Löscher and Richter 2016, p. 485), but TCBZ is not licensed for dairy sheep and cattle (Löscher and Richter 2016, p. 485).

2.1.9.2 Salicylanilides

Salicylanilides are a large group of weakly acidic, highly lipophilic compounds consisting of a salicylic acidic ring and an anilide ring as a basic chemical structure (Swan 1999). Important representatives of this group used as chemotherapeutics against fasciolosis in ruminants are CLOS, OXYCLO, and RAFOX. Closantel and RAFOX demonstrate a broad antiparasitic spectrum affecting not only trematodes, but also haematophagous nematodes such as *Haemonchus contortus* and arthropods such as *Oestrus ovis*. In contrast, the antiparasitic spectrum of OXYCLO is very narrow and principally limited to trematodes with only minor activity against *H. contortus* (Swan 1999).

The mode of action of salicylanilides is based on the uncoupling of oxidative phosphorylisation, leading to a shortage of ATP in the mitochondria and consequent death of the parasite due to energy deficiency (Deplazes et al. 2020, p. 583; Löscher and Richter 2016, p. 485; Taylor et al. 2016, p. 316; Löscher et al. 2014, p. 387; Swan 1999; Martin 1997). Due to the long half-life of salicylanilides, the exposure time to the drugs is long enough to decrease the availability of energetic compounds in the parasites (Taylor et al. 2016, p. 316). All three flukicidal salicylanilides (CLOS, RAFOX, OXYCLO) show a high plasma:tissue ratio due to their extensive binding to plasma proteins (>97-99%) (Swan 1999; Michiels et al. 1987; Mohammed-Ali and Bogan 1987). This explains the selective toxicity against haematophagous parasites, since the highly protein-bound drugs can concentrate within the parasites without engendering the incorporation of the drugs into the host tissues (Taylor et al. 2016, p. 316; Martin 1997). Salicylanilides are generally poorly metabolised and excreted mostly in the unchanged form (Swan 1999).

2.1.9.2.1 Closantel

Closantel is widely used to control fasciolosis and *H. contortus* infections and shows also activity against several arthropods. It is highly effective against adult *Fasciola* spp. stages (91-99% against flukes over nine weeks) and moderately effective against immature stages (50-90% against flukes over six weeks) (Fairweather and Boray 1999). Alvarez et al. (2021) indicated 23-73% efficacy against fluke stages from three to four weeks p.i, 91% efficacy against flukes at the age of five weeks, 91-95% efficacy against flukes from six to nine weeks and 97-100% efficacy from week ten onwards.

It is slowly absorbed with an oral bioavailability of 50% and poorly metabolised (Michiels et al. 1987). Maximum plasma concentrations occur after two to three days and approximately 80% of CLOS are biliary excreted (Löscher and Richter 2016, p. 485; Löscher et al. 2014, p. 389). The mean plasma half-life of CLOS in sheep is approximately 14.5 days. This long persistence of the drug in the host body is potentially associated with the efficacy against the immature flukes, since the drug may remain in the plasma until those fluke stages mature (Mohammed-Ali and Bogan 1987).

The recommended administration dose amounts to 10 mg/kg bw orally in sheep and cattle or 20 mg/kg bw for cattle when dermally applied. Closantel presents a moderately wide therapeutic safety margin: Cattle do not show clinical symptoms after being administered six times the recommended dose whereas sheep only tolerate four times the recommended dose. Higher rates of overdosing may result in anorexia, uncoordinated body movements, weakness, and impaired vision culminating in blindness. Closantel is not licensed for dairy sheep and cattle. The withdrawal period for meat is between 24 and 65 days (Löscher and Richter 2016, p. 485; Löscher et al. 2014, p. 389).

2.1.9.2.2 Rafoxanide

A further potent flukicide from the group of salicylanilides is RAFOX with an efficacy spectrum comparable to CLOS (Löscher et al. 2014, p. 389), demonstrating a high efficacy not only against trematodes, but also against *H. contortus* and *O. ovis* (Swan 1999). Fairly similar to CLOS, RAFOX affects flukes before they reach maturity in sheep (45-98% efficacy against flukes from four to five weeks p.i., 85-99% efficacy against flukes from six to nine weeks p.i. and 99-100% efficacy from week ten p.i onwards) (Alvarez et al. 2021). The average plasma half-life of RAFOX in sheep is 16.6 days, which might be a possible reason for the efficacy against immature flukes similar to CLOS (Mohammed-Ali and Bogan 1987).

The recommended administration dose amounts to 7.5 mg/kg bw orally in sheep and cattle. The maximum tolerated dose in sheep is 45 mg/kg bw (Fairweather and Boray 1999).

Rafoxanide is currently not approved and not available in Germany (Löscher et al. 2014, p. 389).

2.1.9.2.3 Oxyclozanide

In contrast to the broad antiparasitic spectrum of CLOS and RAFOX, OXYCLO demonstrates a very narrow anthelmintic spectrum with only minor efficacy against adult *H. contortus* stages and no efficacy against arthropods (Swan 1999).

Unlike CLOS and RAFOX, which eliminate premature fluke stages to some extent, OXYCLO only affects flukes, which have entered the bile system (Löscher et al. 2014, p. 389). Oxyclozanide kills 50-70% of flukes at the age of ten to eleven weeks p.i. and 80-99% of flukes from week twelve p.i. onwards (Alvarez et al. 2021; Fairweather and Boray 1999). With a mean plasma half-life of 6.4 days, OXYCLO is eliminated faster from the host body (Mohammed-Ali and Bogan 1987).

The recommended administration dose amounts to 15 mg/kg bw orally in sheep and 13-16 mg/kg bw (Fairweather and Boray 1999) or 10 mg/kg bw (Deplazes et al. 2020, p. 606) in cattle. The dosage of 15 mg/kg bw has also been proven to be efficient against rumen flukes (Paramphistomidae) in cattle (Arias et al. 2013) and sheep (García-Dios et al. 2020). The maximum tolerated dose in sheep is 60 mg/kg bw (Fairweather and Boray 1999). Oxyclozanide was not available on the German market for a long period, but only recently it was re-licensed in Germany (Distocur®, Dopharma). In sheep, withdrawal periods amount to 14 days and seven days for meat and milk, respectively (Deplazes et al. 2020, p. 614). In cattle, withdrawal periods amount to 13 days and five days for meat and milk, respectively (Deplazes et al. 2020, p. 606).

2.1.9.3 Halogenated phenols

Substituted/halogenated phenols can be regarded as close analogues to the group of salicylanilides. Like salicylanilides, the mode of action is the uncoupling of oxidative phosphorylation in the mitochondria resulting in a lack of energetic phosphate compounds, such as ATP, and NADH and a consequent death of the fluke (Taylor et al. 2016, p. 315). Since no representative of this group is currently licensed and available in Germany, only one compound of this group is briefly mentioned.

2.1.9.3.1 Nitroxynil

A representative of this group is NITROX with an efficacy of 50-90% against flukes aged six to nine weeks and 91-99% efficacy against flukes from week nine p.i. onwards (Fairweather and Boray 1999). The recommended subcutaneous administration dose of NITROX amounts to 10 mg/kg bw in sheep and cattle (Alvarez et al. 2021; Fairweather and Boray 1999). Due

to the microflora-mediated nitro-reduction in the rumen, NITROX must be given by parenteral route (Alvarez et al. 2021).

2.1.9.4 Sulphonamides

Clorsulon is the only compound from the group of sulphonamides, which is used to treat fasciolosis and is therefore the only drug briefly mentioned here.

2.1.9.4.1 Clorsulon

Clorsulon is a benzene sulphonamide compound (Taylor et al. 2016, p. 317) and is more effective in cattle than in sheep (Fairweather and Boray 1999). Clorsulon exerts its flukicidal activity by inhibiting glycolytic enzymes of the parasites leading to their death due to energy depletion (Deplazes et al. 2020, p. 583; Taylor et al. 2016, p. 317; Löscher et al. 2014, p. 388). The recommended dose amounts to 2 mg/kg bw when administered subcutaneously in cattle or 7 mg/kg bw when given orally to sheep (Alvarez et al. 2021). Clorsulon is only effective against fluke stages over eight weeks p.i. (Taylor et al. 2016, p. 317; Löscher et al. 2014, p. 388).

2.2 Present knowledge of triclabendazole resistance

Increasing reports about anthelmintic resistance of *F. hepatica* pose a threat to livestock production and animal welfare worldwide (Beesley et al. 2023; Beesley et al. 2018; Kelley et al. 2016; Sargison and Scott 2011). Particularly resistance to TCBZ is of major concern since it is currently the only available flukicide for the treatment of acute fasciolosis. Moreover, it is the drug of choice for the treatment of human *Fasciola* spp. infections (Cwiklinski et al. 2016; Fairweather 2009). Until now, reports of TCBZ resistance only relate to *F. hepatica*, but not *F. gigantica* populations (Fairweather 2020; Cwiklinski et al. 2016).

The definition of anthelmintic resistance according to Prichard et al. (1980) reads as follows: “Resistance is present when there is a greater frequency of individuals within a population able to tolerate doses of a compound than in a normal population of the same species and is heritable.” Consequently, the compound is either no longer effective in resistant populations or increased doses are required for the drug to be effective (Wolstenholme et al. 2004; Prichard et al. 1980). General factors influencing the selection pressure are for example the parasite’s biology (e.g. short generation times and high fecundity levels enhance the probability of resistance development and distribution) and parasite genetics (e.g. whether resistance alleles are inherited dominantly or recessively and how many genes are involved) (Wolstenholme et al. 2004). One of the most important management factors contributing to the development of drug resistance is the frequent use of a flukicide, particularly at sub-therapeutic doses (Tabari et al. 2022; Castro-Hermida et al. 2021). With regard to TCBZ, the emergence of resistance has been promoted by an over-reliance on the drug itself due to its unique efficacy range against immature flukes (Fairweather 2020; Kelley et al. 2016; Hanna 2015; Hanna et al. 2015; Moreno et al. 2014; Fairweather 2005; Robinson et al. 2002). According to Fairweather (2020), the initial selection of TCBZ-resistant fluke isolates has most probably occurred in sheep due to the huge amount of TCBZ frequently used to control fasciolosis in this species in the past and the first detection of TCBZ resistance in sheep (Overend and Bowen 1995). Co-grazing, the movement of livestock as well as the presence of wildlife reservoir hosts might have aggravated the spread of resistant *F. hepatica* populations (Fairweather 2020). Additionally, resistant miracidia or cercariae may be disseminated between farms in the natural passage of water streams or following flooding (Sargison and Scott 2011).

Particularly for sheep production, the spread of resistance is considered a serious problem since farmers will always be reliant on effective flukicides for the therapy of fasciolosis in sheep (Sargison and Scott 2011). Therefore, prophylactic control measures to reduce the infection pressure on the pastures (as mentioned under 2.1.8.1) as well as lowering the

selection pressure for resistance by ensuring the correct drug administration dose and avoiding unnecessary flukicidal treatments are of major importance.

2.2.1 Occurrence of triclabendazole resistance

Resistance to TCBZ in livestock has been reported in many countries worldwide, e.g. in Argentina (Larroza et al. 2023; Olaechea et al. 2011), Brazil (Oliveira et al. 2008), Bolivia (Mamani and Condori 2009), Chile (Romero et al. 2019), Peru (Ortiz et al. 2013; Chávez et al. 2012), Australia (Elliot et al. 2015; Brockwell et al. 2014; Overend and Bowen 1995) as well as in European countries such as the United Kingdom including Wales (Daniel et al. 2012; Thomas et al. 2000), Scotland (Kamaludeen et al. 2019; Daniel et al. 2012; Gordon et al. 2012; Mitchell et al. 1998) and England (Kamaludeen et al. 2019), Ireland (Hanna et al. 2015; Mooney et al. 2009; Coles et al. 2000), the Netherlands (Borgsteede et al. 2005; Gaasenbeck et al. 2001; Moll et al. 2000), and Spain (Martínez-Valladares et al. 2010; Alvarez-Sánchez et al. 2006).

Not only in animals, but also in humans lacking efficacy of TCBZ has been observed, e.g. in the Netherlands (Winkelhagen et al. 2012), Chile (Gil et al. 2014), and Peru (Cabada et al. 2016), emphasising the implications of extensive anthelmintic treatments on human health (Winkelhagen et al. 2012) since it is the only flukicide licensed for treatment of human fasciolosis (Beesley et al. 2023).

2.2.2 Mode of flukicidal action of triclabendazole

The exact fasciolicidal drug action of TCBZ is still uncertain and remains to be completely elucidated. It is assumed that TCBZ also targets the microtubules in the flukes by binding to the beta tubulin, impairing microtubule-based processes in the parasites as demonstrated for other benzimidazoles (Davis et al. 2020; Fairweather 2020; Fairweather 2011b; Brennan et al. 2007; Mestorino et al. 2007; Wolstenholme et al. 2004; Robinson et al. 2002; Bennett and Köhler 1987). Treatment with TCBZ results in changes accounting for microtubule inhibition (stoppage of the movement of secretory vesicles, inhibition of mitotic and meiotic division, and loss of tubulin immunostaining), which are absent in TCBZ-r *F. hepatica* isolates (Fairweather 2020; Wolstenholme et al. 2004). However, it is supposed that the TCBZ binding site in the beta tubulin molecule must be a different one compared to the binding site for other benzimidazoles or colchicine due to the narrow range of activity of TCBZ (Fairweather 2020).

2.2.2.1 Drug entry into *F. hepatica*

Since flukicidal agents must reach their target molecules within the parasite for exerting fasciolicidal efficacy, knowledge about drug entry and accumulation inside the target parasite

is essential for optimising chemotherapeutic strategies for the control of fasciolosis (Alvarez et al. 2021). Given the haematophagous nature of *F. hepatica* and the strong binding of TCBZ metabolites to plasma proteins, one might assume that oral ingestion is the principal route of drug entry into the fluke (Fairweather 2009; Toner et al. 2009). However, two independent *in vitro* studies by Mottier et al. (2006) and Toner et al. (2009) asserted that transtegumental diffusion rather than oral ingestion is the predominant entry route of TCBZ into the fluke. The authors of the respective studies blocked the oral entry route of isolated flukes by ligation and incubated ligated and non-ligated flukes *in vitro* in TCBZ.SO, which is assumed to be the active form of the drug (Fairweather 2011b; Virkel et al. 2006; Alvarez et al. 2005; Fairweather 2005; Robinson et al. 2004; Wolstenholme et al. 2004). The pharmacological study by Mottier et al. (2006) compared the concentration of TCBZ.SO in orally ligated and non-ligated flukes after *in vitro* incubation and measured equivalent concentrations of TCBZ-SO in both groups. The morphological study by Toner et al. (2009) contrasted the TCBZ-induced alterations to the teguments of orally ligated and non-ligated flukes after *in vitro* incubations in TCBZ.SO and observed no apparent differences in the degree of drug-induced disruption to the teguments. Additionally, Toner et al. (2009) did not detect TCBZ-induced changes to the gut morphology in both ligated and non-ligated flukes. The results of both studies indicate that closing of the oral route does not impair the ability of the drug to enter the fluke for exerting its flukicidal activity. To confirm these observations, both studies included additional experiments by adding bovine serum albumin (BSA) to the incubation medium, since TCBZ shows a strong binding affinity to plasma proteins. In the presence of BSA, TCBZ.SO concentrations measured inside both ligated and non-ligated flukes were significantly lower (Mottier et al. 2006) and the damage to the tegument was noticeably smaller (Toner et al. 2009), meaning that drug uptake is inhibited when TCBZ is bound to plasma proteins.

Even though the *ex vivo* studies suggest that absorption through the external surface is the main route of drug entry, the study by Moreno et al. (2014) showed the opposite under *in vivo* conditions. After infecting sheep with a TCBZ-susceptible (TCBZ-s) *F. hepatica* isolate and treating the sheep with 10 mg/kg bw at week 16 p.i., animals were sacrificed at 3, 24, 48 and 60 h p.t. and samples of blood, bile, liver tissue, and adult *F. hepatica* specimens were collected for measurement of the concentrations of TCBZ and its metabolites. TCBZ.SO and TCBZ.SO2 were the only metabolites detectable in plasma (corroborating the previous result by Hennessy et al. (1987)) and the main molecules measured in the liver flukes. Although high concentrations of TCBZ.SO, OH-TCBZ, TCBZ.SO2 and TCBZ were detected in bile providing potential for considerable chemical contact to the adult flukes, only very low concentrations of OH-TCBZ and TCBZ were measured in the *F. hepatica* specimens despite

a similar ability of TCBZ and its sulpho-metabolites to penetrate through the external surface (Mottier et al. 2004). It is assumed, that the presence of amphiphilic bile components inside the host leads to micellar solubilisation of TCBZ and TBCZ metabolites. This might decrease the proportion of free drug concentrations impeding the transtegumental diffusion capacity under *in vivo* conditions (Moreno et al. 2014). Moreover, due to a high correlation between the TCBZ.SO and TCBZ.SO2 concentrations in plasma and in *F. hepatica* specimens on the concentration versus time curve, the authors concluded that oral ingestion of the compounds must be the main mechanism of drug entry under *in vivo* conditions (Moreno et al. 2014).

2.2.2.2 Flukicidal effects on *F. hepatica*

Bennett and Köhler (1987) investigated the *in vitro* action of TCBZ on immature and adult *F. hepatica* stages to elucidate the molecular mechanisms by which the drug impairs biochemical processes and vital functions inside the fluke. The authors examined the effect of TCBZ and TCBZ.SO on motility, resting membrane potential, bioenergetics, microtubular protein, and secretory processes of three-week-old and mature *F. hepatica* stages. In these *in vitro* experiments, 24 h incubation periods at high concentrations of 25-50 µM TCBZ were needed to totally immobilise mature flukes, whereas lower concentrations had only little impact on motor activity. Immature flukes appeared to be more sensitive since the exposure to 10 µM TCBZ for 24 h already sufficed for total immobilisation (Bennett and Köhler 1987). Complementing the *in vitro* results by Mottier et al. (2006) and Toner et al. (2009), the effects on the motility of immature and mature flukes were reduced when BSA was added to the incubation medium. The authors also detected a drug-induced suppression of the release of proteolytic enzymes before the total drug-induced immobilisation occurred (Bennett and Köhler 1987). TCBZ and TCBZ.SO also produced a significant effect on the resting membrane potential of immature flukes by compromising the maintenance of a normal gradient of inorganic ions between the syncytial compartment and its environment, most likely due to direct interactions with the fluke's tegument structures (Bennett and Köhler 1987). In order to investigate if TCBZ exerts its flukicidal effect by inhibiting ion pumps necessary to maintain the ion gradients across the external surface, the authors specifically assessed the effect of TCBZ and TCBZ.SO on various ATPases isolated from membrane fractions of *F. hepatica*. However, no alterations in the activities of these pumps directly or indirectly involved in maintaining the fluke's resting membrane potential were observed under the influence of the drugs (Bennett and Köhler 1987). A further unexpected finding in this study was a TCBZ-induced stimulation of the carbohydrate catabolism in the flukes and ATP levels remained basically unaffected compared to control levels, even though a decrease in motor activity was concurrently ascertained. This indicates that the lethal effect of TCBZ cannot be conceived as a suppression of the energy generating pathway (Bennett and

Köhler 1987). The authors also investigated the influence of TCBZ on the binding of colchicine (a well-recognised microtubule activity inhibitor binding to the same site of the beta tubulin as benzimidazoles (Lacey 1988)) to microtubular protein isolated from *F. hepatica* and observed a potent TCBZ-induced displacement of the binding of colchicine to *F. hepatica* tubulin (Bennett and Köhler 1987).

Besides from its assumed role as a microtubule inhibitor, TCBZ.SO has been shown to exert a potent inhibition of protein synthesis within *F. hepatica* exacerbating the effects due to the anti-tubulin activity (Stitt et al. 1995).

Histologically, cells from the testis tubules of TCBZ-treated flukes exhibit signs of apoptosis (multiple nuclei, pyknotic or karyorrhectic nuclei, eosinophilic cytoplasm, and rounded profiles) and the total amount of cells in the testis tubules is reduced compared to non-treated flukes (Hanna 2015). This most likely results from a TCBZ-induced inhibition of mitosis since benzimidazoles inhibit microtubule-mediated processes such as the spindle formation during mitosis (Hanna 2015). In the female reproductive tract, TCBZ induces interruption of ovigenesis and no normal eggs, but free vitelline cells, free ova, and irregular masses of coagulated shell protein in the uterus are detectable after exposition to TCBZ (Hanna 2015).

The tegument of TCBZ-treated flukes is also severely affected after drug exposure with considerable signs of disruption. The surface of TCBZ-treated flukes is swollen with visible blebbing on the surface (a common stress reaction to anthelmintic treatment) and submerged spines lying flat against the tegument (Toner et al. 2009; Robinson et al. 2002).

Further research to elucidate the metabolism of TCBZ is essential to fully understand its exact mode of action. That is particularly important with regard to the development of resistance (Virkel et al. 2006).

2.2.3 Assumed resistance mechanisms

Generally, mechanisms of acquired anthelmintic resistance are either categorised into pharmacokinetic-mediated or pharmacodynamic-mediated mechanisms (Alvarez et al. 2005). Current assumptions about potential mechanisms of TCBZ resistance have recently been reviewed by Fairweather (2020). As mentioned above, the putative drug target of TCBZ is the beta tubulin (Fairweather 2020; Fairweather 2011b; Brennan et al. 2007; Mestorino et al. 2007; Robinson et al. 2002; Bennett and Köhler 1987) and inhibition of the microtubule assembly by the binding of benzimidazoles leads to the loss of cellular homeostasis with a lethal outcome for the parasite (Lacey 1988). One conjecture regarding TCBZ resistance is an altered tubulin binding of the drug in resistant *F. hepatica* isolates (Fairweather 2020).

Changes in the target molecule pertain to pharmacodynamic-mediated resistance mechanisms and increasing the dosage of the drug may have no effect on a resistant parasite isolate (Alvarez et al. 2005). In nematodes, mutations in the amino acid sequences of the beta tubulin molecule have been identified as a reason for benzimidazole resistance (Venkatesan et al. 2023; Dilks et al. 2020; Keegan et al. 2017; Wolstenholme et al. 2004; Kwa et al. 1994). However, the study by Robinson et al. (2002) suggested that no comparable mechanism is involved in TCBZ resistance in *F. hepatica* since no differences in the primary amino acid sequences of the beta tubulin from TCBZ-s and TCBZ-r isolates have been demonstrated (Robinson et al. 2002). The very recent results from Beesley et al. (2023) substantiate that the beta tubulin can be excluded as a candidate directly involved in TCBZ resistance. Moreover, lacking cross-resistance between TCBZ and ABZ has been ascertained: Coles and Stafford (2001) observed high efficacy of ABZ against adult TCBZ-r *F. hepatica*, whereas Sanabria et al. (2013) reported high efficacy of TCBZ against an isolate resistant to ABZ. Hence, if it is presumed that both ABZ and TCBZ exert flukicidal activity by binding to the beta tubulin, other ways of resistance must be taken into consideration (Alvarez et al. 2005). Thus, current presumptions regarding possible resistance mechanisms shifted more towards altered drug uptake or altered drug metabolism in TCBZ-r flukes (Fairweather 2020), which are pharmacokinetic-mediated mechanisms resulting in a decreased drug accumulation inside the parasite (Alvarez et al. 2005).

Altered drug uptake in TCBZ-r flukes is assumed since studies showed that TCBZ-r *F. hepatica* isolates absorb significantly less TCBZ and TCBZ.SO than TCBZ-s flukes, so that resistant flukes are exposed to lower concentrations of the flukicide (Mottier et al. 2006; Alvarez et al. 2005). This might involve P-glycoprotein (Pgp)-linked drug efflux pumps, which transport drug molecules through the membrane out of the cells (Fairweather 2020; Mottier et al. 2006; Alvarez et al. 2005), since the Pgp inhibitor verapamil enhanced the flukicidal effect of TCBZ in a TCBZ-r *F. hepatica* isolate (Meaney et al. 2013).

Recently, the study by Davis et al. (2020) ascertained that *F. hepatica* specimens exposed to TCBZ and its metabolites release five times higher concentrations of extracellular vesicles (EVs) than non-treated flukes. Extracellular vesicles are heterogeneous structures surrounded by a lipid bilayer incorporating components from the cytoplasm (Davis et al. 2020; Mathivanan et al. 2010). These EVs have been shown to contain TCBZ compounds after drug exposure (Davis et al. 2020). Hence, the authors assumed that EVs are implicated in the natural reduction of TCBZ metabolites from the microenvironment of the fluke. This is probably an indirect process due to the TCBZ-induced tegument disorganisation, but it cannot be fully excluded that TCBZ and its metabolites are actively eliminated by EVs as a direct detoxification approach, contributing to the survival of *F. hepatica* after being exposed

to the drug. Consequently, this observation raises the question whether the sequestration by EVs is also involved in TCBZ resistance as a pharmacokinetic-mediated mechanism. This possibility should be part of future research (Davis et al. 2020).

Another possibility to explain TCBZ resistance is an altered drug metabolism by the fluke. *Fasciola hepatica* is able to metabolise TCBZ to TCBZ.SO and the pharmacologically less active sulphone (TCBZ.SO2) and the rate of metabolism is higher in TCBZ-r than in TCBZ-s flukes (Alvarez et al. 2005; Robinson et al. 2004). An enhanced oxidative metabolism of TCBZ in TCBZ-r *F. hepatica* isolates might result in a lower exposition to pharmacologically potent metabolites (Alvarez et al. 2005).

Pharmacokinetic-mediated resistance mechanisms might enable possibilities to reverse drug resistance because drug uptake, drug efflux, and drug metabolism could be saturated by a surplus of the flukicide (Alvarez et al. 2005). Nevertheless, overdosing as a solution to the problem is impractical due to limitations in terms of drug toxicity, drug solubility, as well as financial and environmental impacts. Moreover, increasing the administered drug doses might also intensify the selection pressure for resistant populations (Alvarez et al. 2005).

Very recently, the study from Beesley et al. (2023) identified a single locus comprised of 30 genes in the genome of *F. hepatica* conferring TCBZ resistance. Pooled genotyping of *F. hepatica* eggs before and after TCBZ exposure of naturally infected sheep showed that this locus was also under selection after TCBZ treatment in the field. Within this locus, several genes implicated in membrane transport, transmembrane signalling and signal transduction, DNA and RNA binding and transcriptional regulation as well as drug storage and sequestration have been detected as possible candidates for conferring TCBZ resistance (Beesley et al. 2023). The authors also showed that TCBZ resistance is inherited dominantly, what emphasises the potential for a fast spread once resistance has developed among a *F. hepatica* population (Beesley et al. 2023).

In conclusion, there are different hypotheses and possibilities regarding TCBZ resistance mechanisms in *F. hepatica*, but the actual mechanism has not been clearly identified yet. It could also be possible that resistance in *F. hepatica* is multifactorial and polygenic in nature (Fairweather 2020; Meaney et al. 2013). The study by Beesley et al. (2023) is a fundamental basis for further investigations elucidating the genetic background and the involved mechanisms of TCBZ resistance.

Chapter 3: Coproscopical diagnosis of patent *Fasciola hepatica* infections in sheep - A comparison between standard sedimentation, FLUKEFINDER® and a combination of both

3.1 Publication

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Coproscopical diagnosis of patent *Fasciola hepatica* infections in sheep – A comparison between standard sedimentation, FLUKEFINDER® and a combination of both

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ABSTRACT

The liver fluke *Fasciola hepatica* is a highly pathogenic and zoonotic trematode with a cosmopolitan distribution. In livestock, infections may lead to significant economic losses if not diagnosed promptly and treated effectively. Particularly for small ruminants, the standard method for the detection of fluke infection is based on coproscopical methods such as the sedimentation method, which detects *F. hepatica* eggs in faecal samples. In this respect a recent innovative coproscopical approach to diagnose patent infections is the FLUKEFINDER® method, which relies on differential sieving before sedimentation. These two methods and a combination of both methods that allows larger amounts of faeces to be processed with the FLUKEFINDER® apparatus were compared, to assess which method is most appropriate to determine the prevalence and intensity of *F. hepatica* egg shedding. The methods were compared for their ability to recover eggs from ovine faecal samples containing different numbers of fluke eggs per gram (EPG) of faeces and diluting the samples further by mixing with faeces from uninfected sheep. To compare the specificity of the test procedures, positive and negative samples with a low EPG were analysed in parallel by an investigator blinded to the nature of the samples. Significant differences concerning the EPG outcome were found: The FLUKEFINDER® method demonstrated the highest EPG values ($p < 0.001$) in the undiluted samples as well as in all mixing levels, followed by the modified FLUKEFINDER® method. The standard sedimentation showed the lowest EPG values and the highest variability between technical replicates. The precision of the FLUKEFINDER® method and the modified FLUKEFINDER® method were significantly higher than the precision of the standard sedimentation as determined by comparison of variability between technical replicates. The highest raw egg counts were detected using the modified FLUKEFINDER® method. The FLUKEFINDER® method and the combined method showed a sensitivity of 100 % even at the lowest egg concentrations, whereas the sensitivity of the standard sedimentation was 98.1 % for the same set of samples (i.e. one false negative sample). In a separate investigation aiming to estimate the specificity no differences were found between the three methods: all protocols showed 100 % specificity and were able to correctly distinguish between truly positive and truly negative samples without any evidence of cross-contamination between positive and negative samples processed in parallel.

1. Introduction

The common liver fluke *Fasciola hepatica* is a trematode with a worldwide distribution. It is of great importance for sheep farmers and infections may result in acute to chronic disease with for example wasting and significant production losses but also acute mortality (Forbes, 2017; Hayward et al. 2021; Kahl et al., 2021; Williams, 2020; Stuen and Ersdal, 2022). Fasciolosis has a major economic impact leading to considerable financial losses in both small and large ruminants (Charlier et al. 2020). The diagnosis of patent infections is important but often challenging since egg shedding occurs intermittently (Düwel und Reisenleiter, 1990; Sargison and Scott, 2011) and the

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number of eggs in the faeces is generally low (e.g. considerably lower than the number of eggs shed by major gastrointestinal nematodes) and not necessarily correlated with worm burden or the degree of clinical disease.

Reports about emerging resistance of the parasite to different flukicidal agents have increased during recent years (Moll et al. 2000; Alvarez-Sánchez et al. 2006; Mooney et al. 2009; Gordon et al. 2012; Ortiz et al. 2013; Kelley et al. 2016; Novobilský et al. 2016; Kamaludeen et al. 2019). Resistance monitoring requires accurate coproscopical methods with a high sensitivity to identify patent infections reliably and good precision to determine the trematode egg shedding intensity in eggs per gram (EPG) faeces. Precise data and high numbers of eggs observed directly under the microscope in contrast to extrapolated EPGs were shown to be highly relevant to diagnose anthelmintic resistance in parasitic gastrointestinal nematodes using faecal egg count reduction tests (FECRT) (Levecke et al. 2011; Levecke et al. 2012; Torgerson et al. 2012; Torgerson et al. 2014; Levecke et al. 2015; Levecke et al. 2018; Nielsen, 2021).

The most frequently used method for examining faecal samples for the presence of *F. hepatica* eggs is the conventional sedimentation method ("standard sedimentation" in the following), which is a simple and inexpensive method requiring only basic laboratory equipment (Boray, 1969). Up to 10 g of faeces are usually examined with this method. A recently established technique for *F. hepatica* diagnosis is the "FLUKEFINDER®" method (FLUKEFINDER® Diagnostic System, Soda Springs, Idaho, USA), which is based on differential sieving followed by a sedimentation procedure. Two g of faeces can be processed per sample (Zárate-Rendón et al. 2019; Reigate et al. 2021) with a detection limit of one egg per gram of faeces according to the manufacturer. The low egg detection limit is promising for an accurate diagnosis even at low egg concentrations in faecal samples. The first use of the FLUKEFINDER® was promising with every single *F. hepatica* egg visible in the sediment, even without the use of methylene blue, which is often applied in sedimentation protocols to enhance the visibility by counter-staining plant particles in a blue colour. When performing the standard sedimentation, a large amount of coarse faecal components in the sediment frequently impairs the visibility of the eggs and the probability of missing individual eggs during the microscopical examination is high. However, the FLUKEFINDER® method only allows a sample size of 2 g of faeces, whereas up to 10 g of faeces per procedure can be examined using the standard sedimentation. Hence, a combination of both methods was implemented by performing the first steps equal to the standard sedimentation using 10 g of faeces and sieving the resulting sediment through the FLUKEFINDER® device ("Modified FLUKEFINDER®").

The aim of the present study was to compare the faecal egg counts obtained from repetitive analysis of the same ovine faecal sample using these three different coproscopical methods. Precision was investigated by analysing multiple technical replicates for each biological replicate. Biological replicates contained eggs with various *F. hepatica* EPG values to be able to investigate technical variability between these methods and its dependency on egg counts. Furthermore, a comparison of the sensitivity and specificity of the methods was conducted to investigate whether the methods are able to distinguish between truly positive and truly negative samples or if cross-contamination was likely to occur during parallel handling of both sample types in the laboratory.

2. Materials and methods

2.1. Collection of faeces

Faecal samples were collected from patently *F. hepatica*-infected sheep. The first five experimental runs were performed using the faeces of a two-year-old naturally *F. hepatica*-infected dairy sheep (~ 65 kg bodyweight) with moderate egg shedding, collected at different days post infection. The last repetition was conducted using the faeces of a one-year-old experimentally *F. hepatica*-infected crossbred sheep (~ 55 kg bodyweight) with a comparatively high egg shedding intensity. All animal experiments were in agreement with both the European Directive 2010/63/EU and the German Animal Welfare Act (Tierschutzgesetz) and were approved by the Landesamt für Gesundheit und Soziales of the federal state Berlin under the reference number H0337/17.

Since a large amount of defined faecal material was needed for the comparison (286 g of *F. hepatica*-positive faeces per experimental round), faecal samples from infected sheep were collected over several hours using self-made cotton bags placed underneath the sheep's tail (Fig. 1). Since egg shedding may be uneven over time, all samples were thoroughly kneaded for several minutes to obtain homogenous distribution before further analysis. Between collection and analysis, samples were stored at 4 °C in the dark. For the mixing procedure to obtain samples with lower *F. hepatica* egg counts than the original sample, *F. hepatica*-negative faeces from non-infected animals were collected in the same way.

In order to evaluate how the three different methods performed with low faecal egg counts, four dilutions of each original sample were made for each repetition by mixing the *F. hepatica* positive samples with faeces from non-infected sheep using defined ratios.

2.2. Mixing procedure

For the examination of the undiluted *F. hepatica*-positive faeces, 10 g

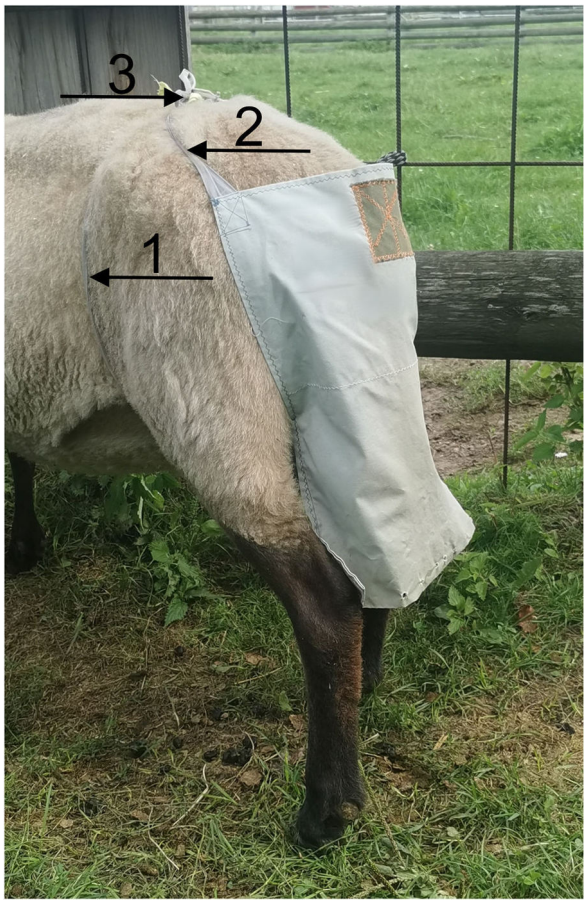

Fig. 1. Cotton bag placed underneath a sheep's tail for collecting faecal samples. Strips below (1) and above (2) the hind legs from both sides were brought together at the hind back and knotted together (3).

of faeces (standard sedimentation and modified FLUKEFINDER®) or 2 g of faeces (standard FLUKEFINDER®) were weighed into a 250 ml beaker using a digital scale (accuracy: 0.1 g) and a wooden spatula.

In order to compare the EPG in samples containing a lower number of eggs than the original samples using each of the three methods, the *F. hepatica*-positive faeces were mixed and homogenised with *F. hepatica*-negative faeces in defined ratios. For this purpose, a defined amount of *F. hepatica*-positive faeces was individually weighed into a 250 ml-beaker and mixed with a defined amount of *F. hepatica*-negative faeces to obtain proportions of faeces with eggs of 80 %, 50 %, 20 % and 10 % (Table 1). Each sample ratio (100 %, 80 %, 50 %, 20 %, 10 % of *F. hepatica*-containing faeces) was examined five times (technical replicates) with each of the three methods. Such a mixing series was set up on six different days with sample material differing in starting EPG, leading to six biological replicates for each of the five ratios (n = 30 in total for biological and technical replicates).

2.3. Analyses for sensitivity and specificity estimation

The required amount of faeces for each method was homogenised and weighed as described above to obtain 20 technical replicates of *F. hepatica*-positive samples (mean EPG obtained by five FLUKEFINDER® analyses of 14.6 EPG) and 20 technical replicates of *F. hepatica*-negative samples for each method under investigation. Individual replicates were labelled in a blinded manner by a third party before coproscopic analyses. Samples were only investigated under a microscope until the first *F. hepatica* egg was found and then scored as positive. If no egg was found, the complete sediment was thoroughly inspected.

2.4. Coproscopic methods

2.4.1. Standard sedimentation

After weighing the defined amounts of faeces (in total: 10 g) into a labelled 250 ml beaker, the faecal material was thoroughly suspended in approximately 50 ml cold tap water using a wooden spatula. The suspension was transferred into a second 250 ml beaker and subsequently sieved through a tea strainer (mesh size: >300 μm) back into the first 250 ml beaker until the beaker was completely filled. The inner surface of the transfer-beaker was thoroughly rinsed to avoid losing eggs. After 30 min, the tea strainer was removed from the beaker and the supernatant was decanted. One drop of detergent was added to the sediment and the beaker was replenished with tap water until the 250 ml mark was reached. After a three-minute sedimentation period, the supernatant was decanted again and the beaker was refilled with tap water. This procedure was repeated twice (in total: 3 × 3 min of sedimentation).

The sediment usually consisted of two fractions: the lower part containing the faecal components with the highest specific weight (including *F. hepatica* eggs) and a lighter layer on top mostly containing coarse plant components. Since no further sieving followed the decantation, each decantation had to be conducted extensively to remove coarse components of the sediment at this point. Only the bottom part of the sediment remained in the beaker during the decantation process and the upper layer of the sediment was poured off, so that fewer plant components might obscure the microscopical view. Subsequently, the sediment was transferred into a petri dish (6 cm diameter) marked with coloured lines on the bottom. Following the coloured lines, the complete petri dish was microscopically (25x magnification) examined in a meandering pattern and all eggs were counted using a manual counter. The petri dish was reused for multiple samples but thoroughly rinsed with a strong water jet between every sample to avoid cross-contamination.

2.4.2. Standard FLUKEFINDER® method

The commercial FLUKEFINDER® device consists of two sections forming a column. Each section contains a sieve: The upper sieve has a larger mesh size, so that *F. hepatica*-eggs can pass through this sieve. The second sieve has a finer mesh size, which holds the eggs back. Howell (2011) indicated the mesh sizes with approximately 125 μm and 30 μm. However, the exact mesh sizes are proprietary and not declared by the manufacturer. The two units of the column fit together with the larger meshed sized sieve on top and the part with the finer meshed sieve on the bottom.

The protocol used here followed the directions given by the manufacturer with slight modifications. The defined amount of faecal material (in total: 2 g) was weighed into a labelled 250 ml beaker (instead of using the 100 ml beaker provided) and suspended in 30 ml cold tap water. The suspension was poured into the top section of the FLUKEFINDER® column. After the suspension had completely passed the upper sieve, the upper section of the FLUKEFINDER® apparatus was half refilled with cold tap water. After repeating this step three times, the two sections of the column were separated. The debris on the upper sieve were removed and the debris on the lower sieve were backwashed with a strong water jet into a labelled 250 ml beaker. After a sedimentation period of three minutes, the supernatant was decanted, and the fine sediment was transferred into a 50 ml-centrifugation tube with conical bottom (instead of using the provided 15 ml tube of equal height). The inner surface of the beaker was thoroughly rinsed using a wash bottle to wash out remaining *F. hepatica*-eggs into the centrifugation tube. The centrifugation tube was filled with cold tap water to the 50 ml mark (water level: approximately 10 cm). After a three-minute sedimentation period, the supernatant was carefully poured off and the centrifugation tube was refilled again up to the 50 ml-mark, and the sediment was well resuspended in 50 ml tap water. After another three-minutes sedimentation period, the supernatant was decanted, and the sediment was transferred into a petri dish. Differing from the instruction manual of the FLUKEFINDER®, a petri dish with a diameter of 6 cm and coloured lines on the bottom was used instead of the small petri dish supplied. This petri dish was also used for the two other methods. The microscopical egg counting was performed using a 25-fold magnification.

The FLUKEFINDER® column and the petri dish were thoroughly rinsed with a strong water jet between every sample to avoid cross-contamination.

2.4.3. Modified FLUKEFINDER® method

The first steps were identical to the protocol for the standard

Table 1
Procedure to generate artificial samples with different levels of low *F. hepatica* faecal egg counts.

F. hepatica-positive fraction	Standard sedimentation (faeces per replicate)		Standard FLUKEFINDER® (faeces per replicate)		Modified FLUKEFINDER® (faeces per replicate)	
	F. hepatica-positive (g)	*F. hepatica*-negative (g)	*F. hepatica*-positive (g)	*F. hepatica*-negative (g)	*F. hepatica*-positive (g)	*F. hepatica*-negative (g)
100 %	10	0	2	0	10	0
80 %	8	2	1.6	0.4	8	2
50 %	5	5	1	1	5	5
20 %	2	8	0.4	1.6	2	8
10 %	1	9	0.2	1.8	1	9

sedimentation (see 2.3.1) using 10 g of faeces. Altogether, two sedimentations were performed. Due to the fact that further sieving followed the decanting and the sediment did not have to be fine enough for microscopical evaluation at this point, each decantation was performed very carefully and the decantation was immediately stopped when the thin apex of the top sediment fraction reached the edge of the beaker, meaning that the lighter superficial layer of the sediment was retained. The sediment was then poured into the top section of the FLUKEFINDER® and the process as described under 2.3.2 was conducted.

2.5. Statistical analyses

The statistical analyses were performed using GraphPad Prism 5.03 (Graph - Pad Software, San Diego, CA, USA) and Microsoft Excel 2019 (Microsoft Corporation, Redmond, WA, USA). First, the raw egg counts were entered into an Excel spreadsheet and the means and the standard deviations for all technical replicates for each method and each biological replicate were calculated. Linear regressions and Pearson correlations were calculated in GraphPad. In order to determine, if a slope was significantly different from 1, a curve based on the sedimentation data was added on the x and y axis, which results in a perfect line with a slope of 1. Then, slopes of data for FLUKEFINDER® and modified FLUKEFINDER® were compared with this line. A Friedman test followed by the Dunn's multiple comparison post hoc test were performed to evaluate whether the paired EPG values obtained with the three methods were significantly different from each other.

To determine if the mixing of eggs from *F. hepatica*-positive faeces with non-infected animals resulted in deviations from linearity at low egg counts, data were normalised to the mean EPG in the undiluted sample for a given method. Pearson's linear regression analyses were performed based on these normalised EPG values and the slopes of the regression lines and Y intercepts for each coproscopic method were compared with the tests implemented in GraphPad. Wald–Wolfowitz runs tests were performed to detect potential deviations from linearity.

The coefficient of variation (CV) was determined for each of the three methods to identify the relative dispersion around the mean EPG value in each level of dilution with *F. hepatica*-negative faeces. Correlation between the normalised mean EPG and the CV values for the different methods were calculated as Spearman's ρ. Paired CV values for each sample obtained with the different coproscopic methods were pairwise subtracted (sedimentation - FLUKEFINDER, sedimentation - modified FLUKEFINDER®, FLUKEFINDER® - modified FLUKEFINDER®). Then GraphPad was used to conduct a Wilcoxon signed rank test to determine if the median value was significantly different from zero.

The specificity and sensitivity as well as the positive and negative predictive values with their 95 % confidence intervals (95 % CIs) were calculated using the 20 true positive and 20 true negative replicates for each method using the BDtest function from the R package bdpv 1.3 and analyses were conducted in R version 4.1.1.

3. Results

3.1. Comparison of raw egg counts and EPG values

The raw egg counts and the calculated EPGs were compared for the three different methods (Fig. 2 and Supplementary Tables S1 and S2). Mean raw egg counts and EPGs were calculated from the five technical replicates for each mixing replicate (n = 30) and plotted in Fig. 2. Using the data obtained from the well established sedimentation method as reference, scatter plots for raw egg counts (Fig. 2A) as well as calculated EPG values (Fig. 2B) were plotted. Linear regressions and Pearson correlations were calculated for all combinations of methods (Table 2). All Pearson r coefficients were > 0.978. For raw egg counts, the slope for FLUKEFINDER® was almost twice as high as for modified FLUKEFINDER®. Both slopes were significantly higher than 1 ($p < 0.0001$) and significantly different from each other ($p < 0.0001$). These data indicate

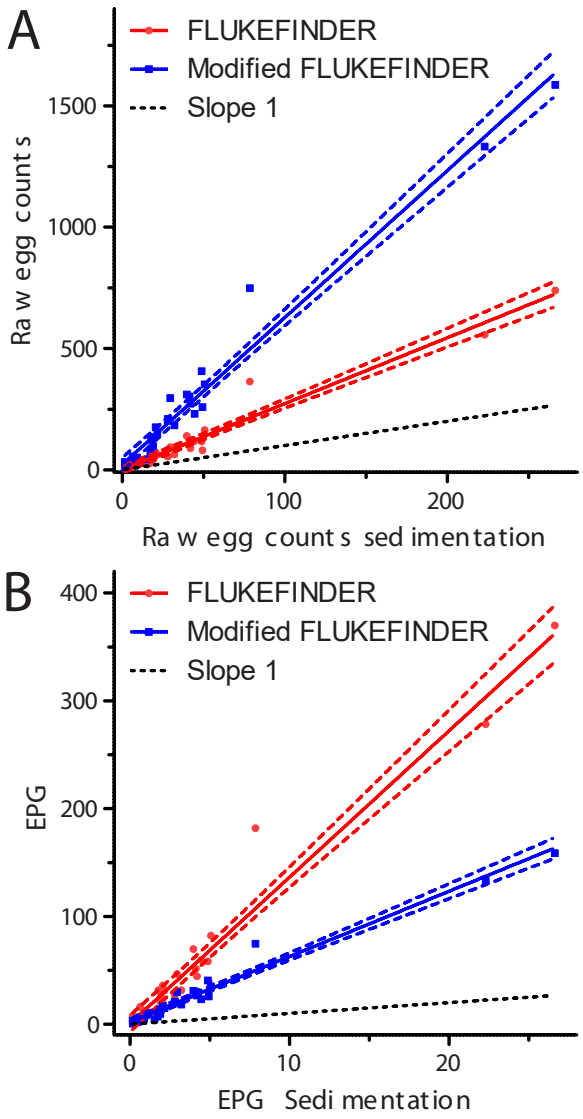

Fig. 2. Pearson correlation and linear regression of raw egg counts (A) and calculated EPG values (B) for each biological replicate from six mixing series with five mixing steps (n = 30) using standard sedimentation (10 g faeces), FLUKEFINDER® (2 g faeces) and modified FLUKEFINDER® (10 g faeces). Lines and dashed lines indicate regression lines and 95 % confidence bands, respectively. The dotted black lines indicate a slope of 1.

that raw egg counts for the modified FLUKEFINDER® and the FLUKEFINDER® methods were approximately 6- and 14-fold higher than for the sedimentation method. For EPG values, slopes for FLUKEFINDER® and modified FLUKEFINDER® were 2.7 and 6.1, respectively, corresponding to 2.7 and 6.1-fold higher EPG values obtained for these methods compared to sedimentation. Slopes were again significantly different between all methods and also significantly different from 1 ($p < 0.0001$).

In addition, the Friedman test followed by Dunn's multiple comparison post-hoc test detected highly significant differences between all three methods for the raw egg counts as well as the calculated EPG values ($p < 0.0001$). While raw egg counts were highest for the modified FLUKEFINDER® followed by FLUKEFINDER® and standard sedimentation, calculated EPG values were highest for FLUKEFINDER® followed

Table 2
Correlation and linear regression results between sedimentation, FLUKEFINDER® and modified FLUKEFINDER® for raw egg counts and EPG values.

Raw egg counts	FLUKEFINDER®				Modified FLUKEFINDER®			
	Slope (95 % CI[a])	Intercept (95 % CI[a])	r	P value[b]	Slope (95 % CI)	Intercept (95 % CI)	r	P value[b]
Sedimentation	13.58 (12.45 – 14.70)	0.54 (−7.33 to 8.41)	0.978	< 0.0001	6.06 (5.65 – 6.46)	2.13 (−0.71 to 4.97)	0.985	< 0.0001
FLUKEFINDER®					0.44 (0.42 – 0.46)	2.28 (0.06 – 4.51)	0.991	< 0.0001
EPG values								
Sedimentation	2.72 (2.49 – 2.94)	1.08 (14.66 – 16.81)	0.978	< 0.0001	6.06 (5.65 – 6.46)	2.13 (−0.71 to 4.97)	0.985	< 0.0001
FLUKEFINDER®					2.19 (2.08 – 2.31)	22.83 (0.55 – 45.11)	0.991	< 0.0001

[a] CI, confidence interval.
[b] Significance of correlation (slope ‡ 0).

by modified FLUKEFINDER® and sedimentation.

3.2. Dependency of egg enrichment efficacy on egg concentration

For each dilution series, EPG data were normalised to the mean EPG of the technical replicates of the undiluted sample for the same method. These normalised EPG values were plotted against the fraction of *F. hepatica*-positive faeces in the samples (Fig. 3). Pearson regression analyses were performed for each coproscopic method and revealed a significant linear effect of dilution on the EPG ($p < 0.0001$). Regression lines for all three methods were almost identical although normalised EPGs were slightly higher for FLUKEFINDER® for low fractions of *F. hepatica*-positive faeces in the samples. However, the differences between the three slopes were not significant ($p = 0.3054$) and the y intercepts of the regression lines ($p = 0.2351$) were not significantly different. The Wald–Wolfowitz runs test did not detect any significant deviations from linearity ($p = 0.5$). Therefore, egg recovery of all three methods did not vary depending on the EPG.

3.3. Comparison of reproducibility between methods

Analyses of variances regarding the egg recovery rate between the biological replicates were performed by calculating the CV values from the technical replicates. The CV values were plotted against the mean EPG values followed by calculation of Spearman correlations (Fig. 4 A).

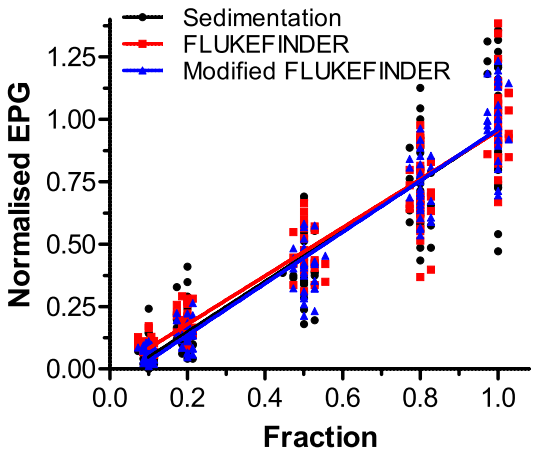

Fig. 3. Comparison of linearity of normalised EPG data between methods. EPG values for each replicate were normalised to the mean EPG of the same dilution series obtained with the same method. Pearson regression analyses revealed a linear relationship between the fraction of *F. hepatica*-positive faeces in the sample and the normalised EPG. Slope and y intercepts were not significantly different between data sets. The calculated linear equations (and correlation coefficients) were $y = -0.054 + 1.011x$ (0.839), $y = -0.015 + 0.968x$ (0.895) and $y = -0.071 + 1.031x$ (0.930) for sedimentation, FLUKEFINDER® and modified FLUKEFINDER®, respectively.

As expected, Spearman ρ coefficients revealed a significant negative correlation between the mean EPG and the CV in all three methods with $\rho = -0.5609$ for the standard sedimentation ($p = 0.001$), $\rho = -0.5519$ for the FLUKEFINDER® method ($p = 0.002$) and $\rho = 0.6854$ for the modified FLUKEFINDER® method ($p < 0.0001$).

A direct comparison of CVs between methods was used to compare precision of methods and is provided in Fig. 4B. The difference of CVs for each biological replicate was calculated and plotted for the pairwise comparison of the three methods. Using a Wilcoxon rank-sum test it was shown that the median difference was significantly higher than zero for the comparison of the sedimentation with either FLUKEFINDER® or modified FLUKEFINDER® methods. In contrast, the median difference in CV values was not significantly different from zero for the comparison of the two FLUKEFINDER® based methods. This shows that the technical variation is significantly higher when sedimentation is used compared to any of the FLUKEFINDER® methods.

3.4. Comparison of sensitivity and specificity at low egg concentrations

3.4.1. Initial estimation of sensitivities from highly diluted positive samples

To compare the sensitivity of the three methods in samples with low egg concentration, the FLUKEFINDER® method was taken as a reference and all data from biological replicates with a FLUKEFINDER® mean EPG < 10 in the respective dilution levels (Supplementary Table S2) were pooled. In total, seven biological replicates showed a mean EPG < 10 EPG in the FLUKEFINDER® method, so that 35 biological replicates per method were qualitatively assessed whether the result was positive (≥ 1 EPG) or negative (0 EPG). All technical replicates with a low egg concentration examined using the FLUKEFINDER® and the modified FLUKEFINDER® methods showed a positive result (each 105/105 = 100 %). In contrast, for the standard sedimentation two technical replicates showed a negative result and the other 103 replicates were positive (103/105 = 98.1 %).

3.4.2. Systematic evaluation of sensitivity and specificity with defined positive and negative sample

For each method, twenty true positive samples (mean EPG of 14.6 as determined by five FLUKEFINDER® analyses) and twenty true negative samples were analysed. These data were used to calculate sensitivity, specificity, predictive positive and predictive negative values with 95 % CIs. Results are summarised in Table 3 and Supplementary Table S3.

4. Discussion

The standard sedimentation is a simple and inexpensive method to detect *F. hepatica* eggs in faecal samples. However, the method is generally considered to have limitations regarding examination of faecal samples with low egg counts (Conceição et al. 2002; Becker et al. 2016; Ploeger et al. 2017; Alstedt et al. 2022). Egg shedding by *F. hepatica* as well as by other liver flukes is known to be intermittent and thus, EPGs might show considerable variation over time (Düwel and Reisenleiter, 1990; Sargison and Scott, 2011). Hence, sensitive methods to detect

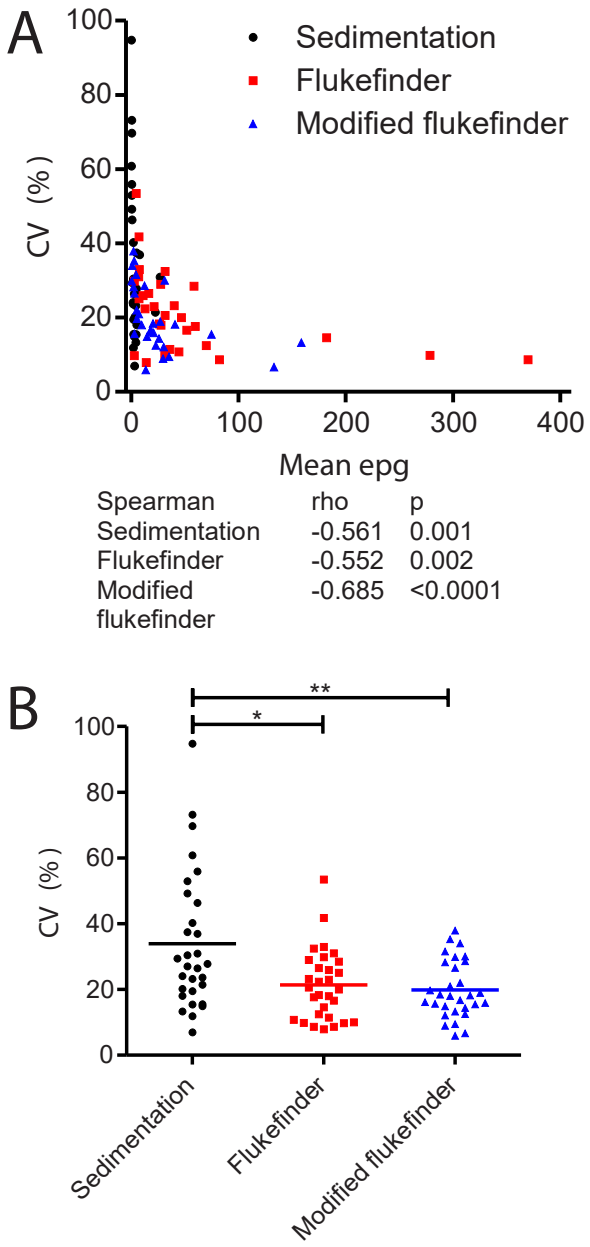

Fig. 4. Dependency of the coefficient of variation (CV) on the mean egg concentration (A) and the coproscopic method used (B). (A), The CV was calculated from the five technical replicates analysed for each biological replicate and plotted against the mean EPG of the biological replicate. Spearman correlation analyses revealed that variation decreased with increasing egg counts. (B), The paired CV values were compared for each biological replicate using the different methods as pairs. For this purpose, pairwise comparisons were made by subtracting from the CV for sedimentation the CVs for FLUKEFINDER® or the CV for modified FLUKEFINDER® for the same biological replicate. For the third comparison, the CV for modified FLUKEFINDER® was subtracted from the CV for FLUKEFINDER®. The values for differences were plotted (B) and a Wilcoxon signed rank test was conducted to determine if the median of the differences was significantly different from 0. ***, $p < 0.001$.

infected animals and precise methods to evaluate the efficacy of flukicidal drugs are needed. In human and veterinary medicine, several other methods have been used to detect *F. hepatica* eggs such as Kato-Katz, Mini-FLOTAC and more recently the FLUKEFINDER®. Comparison of FLUKEFINDER® with Kato-Katz and Mini-FLOTAC for human samples spiked with *F. hepatica* eggs revealed that FLUKEFINDER® outcompeted the other two methods in terms of low variation between technical replicates as determined by CV and sensitivity, particularly at low EPGs (Zárate-Rendón et al. 2019). Surprisingly, however, EPGs obtained with FLUKEFINDER® were much lower than expected and also lower than those obtained with the other two methods, resulting in poor accuracy (Zárate-Rendón et al. 2019). In our preliminary investigations, the Mini-FLOTAC method using zinc sulfate (specific gravity: 1.3) as a flotation medium for the detection of *F. hepatica* eggs in faecal samples performed worse than the standard sedimentation (unpublished data), so we did not pursue this method.

Among the methods compared in the present study, FLUKEFINDER® data showed the highest EPG counts. However, since the true EPG was unknown for the samples used here, it was not possible to estimate and compare accuracy between the methods.

Using the initial data set without negative samples, a slightly lower sensitivity of standard sedimentation compared to the other two methods was observed. However, due to the absence of negative samples, this data set was not suitable to calculate sensitivity and specificity values. Therefore, another sample set, which was blinded for the examiner by a third party, was analysed to compare the sensitivity and specificity of all three methods. Using these truly positive and truly negative samples, no differences were detected regarding the performance of the three methods in terms of false positive and false negative outcomes. All truly positive samples showed a positive result for all methods and all truly negative samples showed a negative result for all methods. Since all test results were in complete agreement with the true status of the sample, sensitivity and specificity were 100 % for the standard sedimentation, FLUKEFINDER® and modified FLUKEFINDER® in this second data set resulting from the systematic evaluation of sensitivity and specificity. The PPV and NPV were 0.94 for all methods.

Noteworthy, a thorough cleaning process of every repeatedly used equipment after each sample is an absolute prerequisite to preclude cross-contamination when examining many samples in a row. Our experiments showed that a meticulous rinsing of the equipment using a strong jet of tap water is sufficient to clean the FLUKEFINDER® column, beakers and the petri dish between the samples in order to avoid an adulteration of the examination results.

Generally, the EPG values detected with the standard sedimentation turned out to be significantly lower than the EPG values detected with the other two methods in the same biological replicate. This may lead to an underestimation of the severity of infection or even false negative results as seen here for two technical replicates at the highest dilution with faeces from non-infected animals, so that a flukicidal treatment may potentially not appear necessary to the farmer or the veterinarian. Moreover, for evaluating the flukicidal effect of an anthelmintic through a FECRT, coproscopic methods that result in high numbers in raw egg count data have a strong advantage since this decreases the size of the 95 % confidence interval of the faecal egg count reduction estimate (Torgerson et al. 2005; Levecke et al. 2015).

Finally, the size of the CV was significantly higher in the standard sedimentation compared to the other two methods, resulting in a higher relative variability of the EPG outcome.

The lower egg counts detected with the standard sedimentation could possibly be caused by eggs remaining in the faecal debris in the tea strainer or by loss of eggs in the decantation process, needed to remove the coarse components of the sediment.

The FLUKEFINDER® method showed the highest EPG values independently of the extent by which the positive samples were diluted with negative faeces, most likely because the mesh sizes of the two sieves are

Table 3
Sensitivity, specificity, predictive positive (PPV) and predictive negative values (NPV) for standard sedimentation, FLUKEFINDER® and modified FLUKEFINDER® methods on samples with low EPG.

	Standard sedimentation		FLUKEFINDER®		Modified FLUKEFINDER®	
	True pos.	True neg.	True pos.	True neg.	True pos.	True neg.
n/N	20/20	0/20	20/20	0/20	20/20	0/20
	Estimate	95 % CI	Estimate	95 % CI	Estimate	95 % CI
Sensitivity	1	0.83–1	1	0.83–1	1	0.83–1
Specificity	1	0.83–1	1	0.83–1	1	0.83–1
PPV	0.94	0.75–0.97	0.94	0.75–0.97	0.94	0.75–0.97
NPV	0.94	0.75–0.97	0.94	0.75–0.97	0.94	0.75–0.97

EPG, eggs per gram faeces; pos., positive; neg., negative; n, number of positive samples; N, number of investigated samples; 95 % CI, 95 % confidence interval.
For positive samples: Mean EPG: 14.6; median EPG: 14.0; range: 12–18.5.

optimally adapted to the size of *F. hepatica* eggs, so that only components with an approximate size above that of *F. hepatica* eggs are filtered out and then *F. hepatica* eggs retained in the two sieving steps, respectively. In contrast to the standard sedimentation, the faecal debris on the sieve, in which the eggs may get caught, is only rinsed three times, with no decanting, so the chance of losing eggs in the sieving process is kept very low. Since the backwashed sediment from the second sieve is very clean once the coarse components have been removed on the first sieve of the FLUKEFINDER®, the subsequent decanting steps in the centrifugation tube have a lower risk of losing eggs when pouring off the supernatant. The sensitivity of this method was 100 % in our experiments, even at low egg concentrations. To implement the FLUKEFINDER® method, only the one-time purchase of the FLUKEFINDER® device and centrifugation tubes are necessary aside from the basic equipment which is also required for the standard sedimentation. As no specialised laboratory equipment is needed, it is a simple and low-cost method applicable for the use in basically equipped laboratories.

The present study also evaluated the combination of both methods (modified FLUKEFINDER®) for the first time and found that it leads to the recording of higher EPG values compared to the EPG values detected with the standard sedimentation. That is presumably because only two decantation steps must be performed after the coarse sieving through the tea-strainer. Moreover, also the upper layer of the sediment containing mostly lighter plant components is retained in the beaker during these two decantation steps. As the sediment is further filtered through the FLUKEFINDER® column, coarse plant components do not have to be removed through decantation at this point and thereby the chance of losing eggs, which might have not completely settled down to the bottom of the beaker, decreases. In contrast to the standard sedimentation in the initial data set, the sensitivity of the combined method was 100 %, even in the samples with the lowest egg concentration.

The calculated EPG values were significantly lower than the EPG values detected with the FLUKEFINDER® method. As already stated above, it is likely that eggs adhere to the faecal debris in the tea strainer as the debris is only rinsed once, so that a proportion of the eggs does not get into the sediment. Both FLUKEFINDER® and modified FLUKEFINDER® methods showed a significantly lower variability in recovering eggs from faecal samples compared to the standard sedimentation and thus, have a higher precision.

Looking at the actual raw egg counts without downscaling the results to EPG level, the highest raw egg counts were found using the modified FLUKEFINDER®. This reveals that the use of 10 g of faeces in the modified FLUKEFINDER® method has a positive effect on the results but that losses during the sedimentation processes are still higher than during the FLUKEFINDER® method alone.

A high raw egg count is crucial for the performance of the FECRT to statistically determine the egg count reduction (Torgerson et al. 2005; Levecke et al. 2015). Considering this perspective, the examination of composite samples instead of individual samples is superior as raw eggs counts increase depending on the number of patently infected animals included in the composite sample. For performing FECRT to evaluate the flukicidal activity of triclabendazole against *F. hepatica*, a protocol using 5 g of faeces from 10 individual sheep (50 g in total) has been validated (Daniel et al. 2012). For the evaluation of anthelmintic efficacy on herd level, this approach was shown to narrow the 95 % confidence intervals and it is time-saving compared to the examination of individual samples. However, information on variability of egg shedding and drug efficacy is lost when composite samples are used. Use of so large amounts of faeces is not possible with the FLUKEFINDER® approach. However, pooling of data from multiple FLUKEFINFER® investigations might help to overcome this limitation. The modified FLUKEFINDER® method was able to handle larger amounts of faeces than the standard FLUKEFINDER® by adding two sedimentation steps at the beginning. This led to increased raw egg counts but apparently eggs were lost in the sedimentation step and EPG values were significantly lower. Moreover, the amount of 10 g faeces might sometimes be difficult to collect from small ruminants under field conditions.

5. Conclusion

Although the sensitivity of the three methods used in the study was comparable, both FLUKEFINDER® and modified FLUKEFINDER® are superior to sedimentation in terms of egg count data. The FLUKEFINDER® method had its strength in resulting in the highest EPG value, since loss of eggs during enrichment was minimised. The modified FLUKEFINDER® method resulted in higher raw egg counts, which is beneficial for the FECRT. FLUKEFINDER® based approaches should replace sedimentation in all studies for which egg counts are relevant.

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CRediT authorship contribution statement

Alexandra Kahl: Conceptualization, Methodology, Formal analysis, Investigation, Writing – original draft. **Jürgen Krücken:** Conceptualization, Methodology, Formal analysis, Writing – original draft, Validation, Visualisation; Project administration. **Georg von Samson-Himmelstjerna:** Conceptualization, Methodology, Formal analysis, Writing – original draft, Project administration. **Christina Helm:** Conceptualization, Investigation, Writing – original draft. **Stephan Steuber:** Conceptualization, Funding acquisition, Writing – review & editing. **Wiebke Weiher:** Funding acquisition, Writing – review & editing. **Werner Terhalle:** Funding acquisition, Writing – review & editing. **Jane Hodgkinson**: Resources, Writing – review & editing. **Diana Williams:** Resources, Writing – review & editing.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Georg von Samson-Himmelstjerna reports financial support was provided by Federal Office of Consumer Protection and Food Safety.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.vetpar.2023.109956.

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3.2 Supporting information

Table S1. Raw egg counts of all biological and technical replicates for sensitivity estimation.

Fraction	Sedimentation						SD	Mean	FLUKEFINDER®						SD	Mean	Modified FLUKEFINDER®						SD	Mean
1	36	43	67	40	61		13.7222447	49.4	111	85	68	69	68		18.6735107	80.2	252	309	273	207	251		37.1187284	258.4
0.8	29	34	24	24	29		4.18330013	28	53	56	72	45	52		10.0149888	55.6	249	182	156	194	229		37.1416209	202
0.5	17	19	14	18	19		2.07364414	17.4	27	28	27	25	31		2.19089023	27.6	60	91	55	67	80		14.7749788	70.6
0.2	6	2	2	3	4		1.67332005	3.4	12	11	21	11	13		4.21900462	13.6	23	15	12	30	30		8.336666	22
0.1	0	2	2	3	2		1.09544512	1.8	7	6	3	7	5		1.67332005	5.6	7	6	3	7	5		1.67332005	5.6
1	59	64	23	36	62		18.294808	48.8	78	108	145	96	157		33.2671009	116.8	466	283	460	407	419		73.8410455	407
0.8	31	27	32	28	30		2.07364414	29.6	95	79	70	114	108		18.6735107	93.2	293	317	274	268	329		26.4896206	296.2
0.5	19	20	17	27	25		4.21900462	21.6	70	65	53	64	61		6.26897121	62.6	171	119	164	152	131		21.9613297	147.4
0.2	16	17	20	14	10		3.71483512	15.4	26	21	30	16	18		5.76194412	22.2	27	51	45	62	34		13.8094171	43.8
0.1	2	5	1	3	3		1.4832397	2.8	12	15	20	11	13		3.56370594	14.2	28	17	32	32	38		7.79743548	29.4
1	46	60	38	59	51		9.20326029	50.8	175	149	152	164	182		14.2583309	164.4	350	353	337	315	405		33.1963853	352
0.8	44	45	53	47	34		6.87749955	44.6	104	102	121	114	154		21.023796	119	242	197	207	269	237		28.8582744	230.4
0.5	19	26	18	19	21		3.20936131	20.6	69	69	86	69	65		8.23407554	71.6	148	143	200	184	202		28.227646	175.4
0.2	9	10	5	5	3		2.96647939	6.4	34	37	27	21	43		8.59069264	32.4	48	69	47	47	65		10.8719823	55.2
0.1	4	2	1	1	0		1.51657509	1.6	9	21	18	11	16		4.94974747	15	37	33	24	30	34		4.92950302	31.6
1	50	33	51	43	30		9.6072889	41.4	78	112	114	93	119		17.1959298	103.2	311	301	354	252	294		36.5691126	302.4
0.8	29	18	37	24	33		7.46324326	28.2	38	53	73	69	41		15.880806	54.8	244	210	237	195	162		33.21596	209.6
0.5	14	14	19	22	28		5.89915248	19.4	60	46	45	45	65		9.5760117	52.2	147	130	134	137	126		7.98122798	134.8
0.2	9	12	8	5	10		2.58843582	8.8	30	29	17	22	29		5.6833089	25.4	52	39	41	65	59		11.2338773	51.2
0.1	6	4	2	2	10		3.34664011	4.8	6	11	7	17	5		4.91934955	9.2	26	25	22	11	24		6.10737259	21.6
1	34	46	43	39	48		5.61248608	42	101	75	92	91	85		9.60208311	88.8	194	322	314	273	257		51.4149784	272
0.8	30	33	24	42	32		6.49615271	32.2	71	56	49	57	81		12.9305839	62.8	232	192	154	165	173		30.6055551	183.2
0.5	20	10	13	23	29		7.64852927	19	42	36	38	59	36		9.70566845	42.2	115	88	77	115	88		17.3867766	96.6
0.2	5	11	4	3	5		3.13049517	5.6	24	14	13	12	8		5.93295879	14.2	35	40	23	22	17		9.65919251	27.4
0.1	1	3	1	3	6		2.04939015	2.8	5	6	5	6	6		0.54772256	5.6	16	6	10	10	13		3.74165739	11
1	351	324	229	284	144		82.4093441	266.4	838	767	697	716	680		63.9476348	739.6	1456	1518	1545	1458	1957		210.491568	1586.8
0.8	198	236	300	203	178		47.8225888	223	486	613	584	511	590		55.02454	556.8	1300	1457	1355	1213	1335		88.5268321	1332
0.5	116	97	48	52	80		29.0826409	78.6	417	422	314	314	352		53.1902247	363.8	754	615	767	681	923		115.325626	748
0.2	55	26	35	39	44		10.7563934	39.8	158	120	122	145	152		17.4298594	139.4	242	188	354	352	418		93.3659467	310.8
0.1	13	20	23	19	14		4.20713679	17.8	49	79	83	35	68		20.376457	62.8	176	105	115	84	141		35.4781623	124.2

Table S2. Eggs per gram levels of all biological and technical replicates for sensitivity estimation.

Fraction	Standard Sedimentation					SD	Mean	CV%	FLUKEFINDER®					SD	Mean	CV%	Modified FLUKEFINDER®					SD	Mean	CV%
1	3.6	4.3	6.7	4	6.1	1.37222447	4.94	27.7778233	55.5	42.5	34	34.5	34	9.33675533	40.1	23.2836791	25.2	30.9	27.3	20.7	25.1	3.71187284	25.84	14.364833
0.8	2.9	3.4	2.4	2.4	2.9	0.41833001	2.8	14.9403576	26.5	28	36	22.5	26	5.00749438	27.8	18.0125697	24.9	18.2	15.6	19.4	22.9	3.71416209	20.2	18.386941
0.5	1.7	1.9	1.4	1.8	1.9	0.20736441	1.74	11.917495	13.5	14	13.5	12.5	15.5	1.09544512	13.8	7.93800808	6	9.1	5.5	6.7	8	1.47749788	7.06	20.9277321
0.2	0.6	0.2	0.2	0.3	0.4	0.16733201	0.34	49.2152957	6	5.5	10.5	5.5	6.5	2.10950231	6.8	31.0220928	2.3	1.5	1.2	3	3	0.8336666	2.2	37.8939364
0.1	0	0.2	0.2	0.3	0.2	0.10954451	0.18	60.8580619	3.5	3	1.5	3.5	2.5	0.83666003	2.8	29.8807152	0.7	0.6	0.3	0.7	0.5	0.16733201	0.56	29.8807152
1	5.9	6.4	2.3	3.6	6.2	1.8294808	4.88	37.4893607	39	54	72.5	48	78.5	16.6335504	58.4	28.4821069	46.6	28.3	46	40.7	41.9	7.38410455	40.7	18.142763
0.8	3.1	2.7	3.2	2.8	3	0.20736441	2.96	7.00555451	47.5	39.5	35	57	54	9.33675533	46.6	20.0359556	29.3	31.7	27.4	26.8	32.9	2.64896206	29.62	8.94315348
0.5	1.9	2	1.7	2.7	2.5	0.42190046	2.16	19.5324288	35	32.5	26.5	32	30.5	3.1344856	31.3	10.014331	17.1	11.9	16.4	15.2	13.1	2.19613297	14.74	14.8991382
0.2	1.6	1.7	2	1.4	1	0.37148351	1.54	24.122306	13	10.5	15	8	9	2.88097206	11.1	25.9547032	2.7	5.1	4.5	6.2	3.4	1.38094171	4.38	31.5283495
0.1	0.2	0.5	0.1	0.3	0.3	0.14832397	0.28	52.9728463	6	7.5	10	5.5	6.5	1.78185297	7.1	25.0965207	2.8	1.7	3.2	3.2	3.8	0.77974355	2.94	26.5218894
1	4.6	6	3.8	5.9	5.1	0.92032603	5.08	18.1166541	87.5	74.5	76	82	91	7.12916545	82.2	8.67295067	35	35.3	33.7	31.5	40.5	3.31963853	35.2	9.43079129
0.8	4.4	4.5	5.3	4.7	3.4	0.68774995	4.46	15.4204026	52	51	60.5	57	77	10.511898	59.5	17.6670555	24.2	19.7	20.7	26.9	23.7	2.88582744	23.04	12.5252927
0.5	1.9	2.6	1.8	1.9	2.1	0.32093613	2.06	15.5794238	34.5	34.5	43	34.5	32.5	4.11703777	35.8	11.5001055	14.8	14.3	20	18.4	20.2	2.8227646	17.54	16.0932988
0.2	0.9	1	0.5	0.5	0.3	0.29664794	0.64	46.3512405	17	18.5	13.5	10.5	21.5	4.29534632	16.2	26.5144835	4.8	6.9	4.7	4.7	6.5	1.08719823	5.52	19.6956202
0.1	0.4	0.2	0.1	0.1	0	0.15165751	0.16	94.7859431	4.5	10.5	9	5.5	8	2.47487373	7.5	32.9983165	3.7	3.3	2.4	3	3.4	0.4929503	3.16	15.5996931
1	5	3.3	5.1	4.3	3	0.96072889	4.14	23.2060118	39	56	57	46.5	59.5	8.59796488	51.6	16.6627226	31.1	30.1	35.4	25.2	29.4	3.65691126	30.24	12.0929605
0.8	2.9	1.8	3.7	2.4	3.3	0.74632433	2.82	26.4654016	19	26.5	36.5	34.5	20.5	7.94040301	27.4	28.979573	24.4	21	23.7	19.5	16.2	3.321596	20.96	15.8473092
0.5	1.4	1.4	1.9	2.2	2.8	0.58991525	1.94	30.4080025	30	23	22.5	22.5	32.5	4.78800585	26.1	18.34485	14.7	13	13.4	13.7	12.6	0.7981228	13.48	5.92079227
0.2	0.9	1.2	0.8	0.5	1	0.25884358	0.88	29.4140434	15	14.5	8.5	11	14.5	2.84165445	12.7	22.3752319	5.2	3.9	4.1	6.5	5.9	1.12338773	5.12	21.9411667
0.1	0.6	0.4	0.2	0.2	1	0.33466401	0.48	69.7216689	3	5.5	3.5	8.5	2.5	2.45967478	4.6	53.4711908	2.6	2.5	2.2	1.1	2.4	0.61073726	2.16	28.2748731
1	3.4	4.6	4.3	3.9	4.8	0.56124861	4.2	13.3630621	50.5	37.5	46	45.5	42.5	4.80104155	44.4	10.8131567	19.4	32.2	31.4	27.3	25.7	5.14149784	27.2	18.9025656
0.8	3	3.3	2.4	4.2	3.2	0.64961527	3.22	20.1743873	35.5	28	24.5	28.5	40.5	6.46529195	31.4	20.5901018	23.2	19.2	15.4	16.5	17.3	3.06055551	18.32	16.706089
0.5	2	1	1.3	2.3	2.9	0.76485293	1.9	40.2554172	21	18	19	29.5	18	4.85283422	21.1	22.9992143	11.5	8.8	7.7	11.5	8.8	1.73867766	9.66	17.9987335
0.2	0.5	1.1	0.4	0.3	0.5	0.31304952	0.56	55.9016994	12	7	6.5	6	4	2.96647939	7.1	41.7813999	3.5	4	2.3	2.2	1.7	0.96591925	2.74	35.2525274
0.1	0.1	0.3	0.1	0.3	0.6	0.20493902	0.28	73.1925055	2.5	3	2.5	3	3	0.27386128	2.8	9.78075996	1.6	0.6	1	1	1.3	0.37416574	1.1	34.0150672
1	35.1	32.4	22.9	28.4	14.4	8.24093441	26.64	30.9344385	419	383.5	348.5	358	340	31.9738174	369.8	8.64624592	145.6	151.8	154.5	145.8	195.7	21.0491568	158.68	13.2651605
0.8	19.8	23.6	30	20.3	17.8	4.78225888	22.3	21.4451071	243	306.5	292	255.5	295	27.51227	278.4	9.88228089	130	145.7	135.5	121.3	133.5	8.85268321	133.2	6.64615857
0.5	11.6	9.7	4.8	5.2	8	2.90826409	7.86	37.0008154	208.5	211	157	157	176	26.5951123	181.9	14.6207325	75.4	61.5	76.7	68.1	92.3	11.5325626	74.8	15.4178644
0.2	5.5	2.6	3.5	3.9	4.4	1.07563934	3.98	27.0261142	79	60	61	72.5	76	8.71492972	69.7	12.503486	24.2	18.8	35.4	35.2	41.8	9.33659467	31.08	30.0405234
0.1	1.3	2	2.3	1.9	1.4	0.42071368	1.78	23.6356	24.5	39.5	41.5	17.5	34	10.1882285	31.4	32.4465876	17.6	10.5	11.5	8.4	14.1	3.54781623	12.42	28.5653481

Table S3. Results of systematic evaluation of sensitivity and specificity with defined positive and negative samples.

Standard Sedimentation			**FLUKEFINDER®**			**Modified FLUKEFINDER®**		
Sample Number	True quality	Detected quality	Sample Number	True quality	Detected quality	Sample Number	True quality	Detected quality
1	neg	neg	1	pos	pos	1	pos	pos
2	neg	neg	2	neg	neg	2	neg	neg
3	pos	pos	3	neg	neg	3	pos	pos
4	pos	pos	4	pos	pos	4	pos	pos
5	neg	neg	5	pos	pos	5	neg	neg
6	neg	neg	6	pos	pos	6	neg	neg
7	neg	neg	7	neg	neg	7	neg	neg
8	pos	pos	8	pos	pos	8	pos	pos
9	neg	neg	9	neg	neg	9	neg	neg
10	pos	pos	10	neg	neg	10	pos	pos
11	neg	neg	11	pos	pos	11	neg	neg
12	neg	neg	12	pos	pos	12	neg	neg
13	pos	pos	13	neg	neg	13	pos	pos
14	neg	neg	14	pos	pos	14	neg	neg
15	pos	pos	15	neg	neg	15	neg	neg
16	pos	pos	16	pos	pos	16	pos	pos
17	neg	neg	17	neg	neg	17	neg	neg
18	pos	pos	18	pos	pos	18	pos	pos
19	pos	pos	19	pos	pos	19	neg	neg
20	pos	pos	20	neg	neg	20	pos	pos
21	pos	pos	21	neg	neg	21	pos	pos
22	neg	neg	22	pos	pos	22	pos	pos
23	pos	pos	23	neg	neg	23	neg	neg
24	pos	pos	24	pos	pos	24	neg	neg
25	neg	neg	25	pos	pos	25	pos	pos
26	neg	neg	26	neg	neg	26	pos	pos
27	pos	pos	27	pos	pos	27	neg	neg
28	pos	pos	28	neg	neg	28	neg	neg
29	pos	pos	29	neg	neg	29	pos	pos
30	neg	neg	30	pos	pos	30	pos	pos
31	neg	neg	31	neg	neg	31	pos	pos
32	pos	pos	32	neg	neg	32	neg	neg
33	neg	neg	33	pos	pos	33	pos	pos
34	pos	pos	34	pos	pos	34	neg	neg
35	neg	neg	35	neg	neg	35	neg	neg
36	neg	neg	36	neg	neg	36	pos	pos
37	neg	neg	37	pos	pos	37	neg	neg
38	neg	neg	38	pos	pos	38	pos	pos
39	pos	pos	39	neg	neg	39	neg	neg
40	pos	pos	40	neg	neg	40	pos	pos

3.3 Authors contributions

Author contributions to the published article according to the CRediT scheme:

Alexandra Kahl	First author	Conceptualisation Methodology Validation Formal analysis Investigation Data curation Writing – original draft Writing – review & editing Visualisation
Georg von Samson-Himmelstjerna	Co-author	Conceptualisation Writing – review & editing Supervision Project administration Funding acquisition
Christina Helm	Co-author	Conceptualisation Resources Writing – review & editing
Jane Hodgkinson	Co-author	Conceptualisation Resources Writing – review & editing
Diana Williams	Co-author	Conceptualisation Writing – review & editing
Wiebke Weiher	Co-author	Conceptualisation Writing – review & editing
Werner Terhalle	Co-author	Conceptualisation Writing – review & editing
Stephan Steuber	Co-author	Conceptualisation Supervision Funding acquisition Writing – review & editing
Jürgen Krücken	Co-author	Conceptualisation Methodology Validation Formal analysis Data curation Writing – original draft Writing – review & editing Visualisation Supervision Project administration Funding acquisition

Chapter 4: Efficacy of flukicides against *Fasciola hepatica* and first report of triclabendazole resistance on German sheep farms

4.1 Publication and Corrigendum

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Efficacy of flukicides against *Fasciola hepatica* and first report of triclabendazole resistance on German sheep farms

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ABSTRACT

Fasciola hepatica infections lead to severe health problems and production losses in sheep farming, if not treated effectively. Triclabendazole has been used extensively over decades due to its unique efficacy range against all definitive hostfluke stages but published data about the susceptibility of *F. hepatica* to anthelmintics in Germany are lacking. This study aimed to identify current *F. hepatica* infections in German sheep flocks by coproscopic examinations and to evaluate the efficacy of anthelmintics with a focus on triclabendazole in a field study conducted from 2020 to 2022. Initial screening included 71 sheep farms, many of them with known history of fasciolosis. In this highly biased sample set, the frequency of *F. hepatica* infection at individual sheep and farm level were 12.8% and 35.2%, respectively. Additionally, eggs of Paramphistominae were found at frequencies of 4.8% and 15.5% at individual sheep and farm level, respectively. Due to low egg shedding intensity, faecal egg count reduction (FECR) tests could only be conducted on a few farms. The efficacy of triclabendazole was tested on 11 farms and albendazole on one farm, including 3–53 sheep/farm. Individual faecal samples were collected before and two weeks after treatment to evaluate the FECR using the sedimentation or FLUKEFINDER® or a modified FLUKEFINDER® method. On all farms a coproantigen reduction test was conducted in parallel. Lacking efficacy of triclabendazole even at double dosage was shown on one farm associated with a high number of animal losses due to acute fasciolosis. On this farm, the *Fasciola* miracidium development test was additionally performed, revealing a high *in vitro* ovicidal activity of albendazole while closantel was effective *in vivo*. On all other farms, sufficient efficacy of triclabendazole was observed. In conclusion, triclabendazole resistance appears not to be widespread on German sheep farms but, when present, can have serious effects on animal health.

1. Introduction

The common liver fluke *Fasciola hepatica* is a trematode parasitising the liver of mammals. The parasite is distributed worldwide (Rojo-Vázquez et al., 2012; Howell and Williams, 2020; Fairweather et al., 2020) and affects in particular ruminants, but also a large range of different other hosts (Beesley et al., 2018; Mas-Coma et al., 2019), including humans (Saba et al., 2004). Lymnaeid snails serve as intermediate hosts for the diheteroxenic life cycle of the parasite (Beesley et al., 2018). *Galba truncatula* is the most important snail species being involved in the transmission of *F. hepatica* in Europe (Charlier et al., 2014). Since the intermediate host is dependent on moist environmental conditions, *F. hepatica* typically occurs in humid regions. Liver fluke infections are becoming increasingly common in ruminants (Skuce and Zadoks, 2013; Beesley et al., 2018; John et al., 2019) and may lead to considerable economic losses in sheep and cattle (Schweizer et al., 2005; Rojo-Vázquez et al., 2012; Gordon et al., 2012; Charlier et al., 2014; Beesley et al., 2018) and is a severe, potentially life-threatening health problem particularly in sheep (Skuce and Zadoks, 2013; Pérez-Caballero et al., 2018). Traditionally, there are three courses of the disease to be

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distinguished: acute, subacute and chronic clinical manifestations (Rojo-Vázquez et al., 2012), based on the quantity of metacercariae ingested by the host animal (Fiss et al., 2013) and its genetic susceptibility. In sheep, clinical signs extend from chronic wasting associated with emaciation, oedema and anaemia (Sargison and Scott, 2011) to sudden deaths in acute cases (Sargison and Scott, 2011; Rojo-Vázquez et al., 2012; Fiss et al., 2013; Skuce and Zadoks, 2013; Forbes, 2017).

For the treatment of *F. hepatica* infections there are several anthelmintics from different drug classes available. The most commonly used anthelmintic is triclabendazole (TCBZ) (Walker et al., 2004; Halferty et al., 2009) since it is the only compound effective against mature and juvenile mammalian stages of the parasite (Fairweather, 2011a; Fairweather et al., 2012; Skuce and Zadoks, 2013). Other established flukicides, including albendazole (ABZ), closantel (CLOS), nitroxynil, clorsulon, oxyclozanide and rafoxanide only demonstrate high efficacy against the mature parasites (Fairweather, 2011a), making TCBZ the drug of choice to control acute *F. hepatica* infections (Brockwell et al., 2014). The unique efficacy of TCBZ against all fluke stages in the host resulted in an over-reliance on this substance and the development of resistance (Fairweather et al., 2020). The first incidence of TCBZ resistance was reported in 1995 in Australia (Overend and Bowen, 1995). During recent years, there have been numerous reports about lack of TCBZ efficacy against *F. hepatica* in ruminants worldwide and Flanagan et al. (2011b) described resistance to TCBZ as a spreading but under-recognised problem. In Europe, lack of efficacy of TCBZ in ruminants has been reported in Wales (Thomas et al., 2000; Daniel et al., 2012; Kamaludeen et al., 2019), England (Kamaludeen et al., 2019); Scotland (Mitchell et al., 1998; Daniel et al., 2012; Gordon et al., 2012), Ireland (Mooney et al., 2009; Hanna et al., 2015), the Netherlands (Moll et al., 2000; Gaasenbeek et al., 2001) and Spain (Alvarez-Sánchez et al., 2006). Further reports originate from Australia (Brockwell et al., 2014; Elliott et al., 2015), Argentina (Olaechea et al., 2011; Larroza et al., 2023) and Peru (Ortiz et al., 2013).

Different diagnostic methods can be applied for the evaluation of anthelmintic efficacy; however, no standard protocol has been established for *F. hepatica* (Fairweather, 2011a; Fairweather et al., 2012; Brockwell et al., 2014; Novobilský and Höglund, 2015; Solana et al., 2016). The most frequently practiced method to determine drug efficacy is the faecal egg count reduction test (FECRT), with anthelmintic treatment being defined effective if a 95% faecal egg count (FEC) reduction is ascertained on day 14 (or 21) post-treatment (Daniel et al., 2012; Fairweather, 2011a; Fairweather et al., 2012; Solana et al., 2016). However, the exclusive implementation of the FECRT for drug efficacy analysis against *F. hepatica* has practical limitations: *Fasciola hepatica* shows a long prepatent period ranging from 8 to 12 weeks (Calvani et al., 2018; Mezo et al., 2022), so that eggs can only be coproscopically detected at a later stage of the infection, making the FECRT not applicable for animals in the prepatent stage (Almazán et al., 2001; Duthaler et al., 2010; Fairweather, 2011b; Skuce and Zadoks, 2013; Mazeri et al., 2016; Mezo et al., 2022). Moreover, *F. hepatica* egg shedding is intermittent (Gordon et al., 2012; Kelley et al., 2021), and FEC can potentially be false-negative and highly variable in a single animal over time. In addition, TCBZ is the only currently available drug effective against juvenile flukes, thus, the efficacy of adulticidal drugs is more difficult to determine, as juvenile or immature flukes are not effectively treated and may mature within the two weeks until the post-treatment sample is collected (Coles et al., 2006; Novobilský et al., 2016). Furthermore, coproscopic methods may show false-positive results following effective treatment and elimination of the parasites, as fluke eggs are stored in the gallbladder and can be shed in the faeces for weeks even if the flukes have been successfully killed by the anthelmintic (Chowaniec and Darski, 1970; Fairweather, 2011a).

Hence, it is best practice to combine the FECRT with another diagnostic method to evaluate anthelmintic efficacy reliably (Fairweather, 2011b; Hanna et al., 2015) such as the coproantigen reduction test (CRT), which has been proven to show adequate results for the diagnosis of anthelmintic efficacy in *F. hepatica* in the field (Flanagan et al., 2011a, 2011b; Gordon et al., 2012; Brockwell et al., 2013; Hanna et al., 2015). *Fasciola hepatica* releases antigens that are excreted with the faeces of its host (Almazán et al., 2001) and a coproantigen-ELISA (cELISA) is able to detect infections with a single fluke in sheep (Mezo et al., 2004). In contrast to FECs, a positive cELISA proves the presence of metabolically active flukes (Martínez-Pérez et al., 2012). Moreover, various studies reported that fluke antigens can be detected earlier than eggs (Almazán et al., 2001; Flanagan et al., 2011a; Flanagan et al., 2011b; Valero et al., 2009). In contrast, Gordon et al. (2012) did not observe that coproantigens can be detected before parasite eggs are shed during their field study, but overall, the authors still evaluated the cELISA as suitable for efficacy tests and more convenient than FECs.

Another method to evaluate anthelmintic efficacy is the *Fasciola* miracidium development (MDT) or egg hatch test (FEHT), a low-cost *in vitro* method, which is able to distinguish between ABZ-susceptible and ABZ-resistant *F. hepatica* isolates by incubating the isolated *F. hepatica* eggs in ABZ solutions of different concentrations and evaluating the development and hatching rates of the treated eggs compared to untreated control eggs (Alvarez et al., 2009; Canevari et al., 2014; Robles-Pérez et al., 2014; Novobilský et al., 2016; Ceballos et al., 2019). According to Alvarez et al. (2009), this test does not work for TCBZ. Reasons for this might be the highly lipophilic nature of TCBZ or the high binding affinity for different proteins, both impeding the penetration of the drug through the eggshell, or a non-microtubule related mode of action of TCBZ, different from the mode of action of other benzimidazoles (Alvarez et al., 2009).

Until now, no published data are available regarding the susceptibility of *F. hepatica* to flukicides in Germany. Therefore, the primary aim of this study was to investigate the efficacy of TCBZ in German sheep flocks. Unexpectedly, the number of sheep flocks with sufficient prevalence and egg shedding intensity to conduct a FECRT was very low during the evaluation period (most likely due to the extremely dry weather conditions in 2020 in Germany). Therefore, many sheep flocks were screened and data on frequency and intensity of egg shedding are also reported.

2. Materials and methods

2.1. Investigation on occurrence of F. hepatica on German sheep farms

To identify sheep flocks suitable to conduct a FECRT, data about the current occurrence of *F. hepatica* on German sheep farms were collected. In total, 1673 individual faecal samples from 71 German sheep farms located in different regions of the country were coproscopically examined for the presence of *F. hepatica* eggs between December 2020 and August 2022. At the beginning of the study, the authors focussed on the examinations of farms located in Lower Saxony due to the collaboration with the University of Veterinary Medicine in Hannover and a high number of *F. hepatica* findings in the past in this federal state. Since the frequency of *F. hepatica* findings turned out to be lower than expected and a sufficient number of infected flocks was not detected in Lower Saxony during the winter season 2020/2021, the geographic range for coproscopic examinations was expanded from the beginning of 2021 on and eligible farms in other regions of the country were examined. Farms were specifically selected due to previous *F. hepatica* infections (as recorded by the Clinic for Swine and Small Ruminants in Hannover or by local veterinarians). Moreover, farms with a high probability of *F. hepatica* occurrence due to wet pasture conditions were contacted by systematically calling sheep farms in the coast regions in Lower Saxony and Schleswig-Holstein. After identifying three highly infected sheep farms in the region of Paderborn (North Rhine Westphalia), a local veterinarian collected faecal samples from several more farms in this region and sent them to Berlin for coproscopic examinations. In addition, the objectives of the study were published in three national veterinary and sheep farmer journals from 2021 to 2022 addressing the

target groups of sheep farmers and veterinarians and asking to contact the authors if sheep farms with anamnestic *F. hepatica* infections or a high probability of *F. hepatica* occurrence are known.

Individual faecal samples were either submitted by farmers by post or the farms were visited by the authors to collect individual samples personally. The number of examined individual samples ranged from 3 to 79 samples per farm. Farmers were asked to specifically select individual animals with a poor body condition, oedema, or poor wool quality for sampling since poor body conditions can result from chronic fasciolosis (Kahl et al., 2021).

The coproscopic examination was offered free of charge for the farmers. Inclusion criteria were access to a pasture on which the occurrence of *F. hepatica* is likely due to moist conditions and/or previous *F. hepatica* infections on the farm and a farm location in Germany.

Each faecal sample was individually analysed using either a standard sedimentation method, the FLUKEFINDER® method or a combination of both methods ("Modified FLUKEFINDER®") (Kahl et al., 2023). Trematode eggs were counted separately for *F. hepatica* and Paramphistominae (rumen flukes) which were distinguished according to their different colours (Mazeri et al., 2016).

2.2. Investigation of flukicide resistance

The field trial to evaluate anthelmintic efficacy in German sheep flocks was conducted from February 2021 to June 2022. In total, 12 sheep farms from different federal states of Germany were included in the trial. Three of the farms were located in Lower Saxony, three in North Rhine-Westphalia, two in Brandenburg, two in Schleswig-Holstein, one in Mecklenburg-Western Pomerania and one in Baden-Wuerttemberg. The number of treated animals with available samples before and 14 days after treatment varied from 9 to 71 sheep, including sheep that turned out to have a FEC of zero in the first faecal examination. The number of sheep coproscopically *F. hepatica*-positive before treatment varied from 3 to 35 per farm. In 11 out of 12 flocks, the efficacy of TCBZ was evaluated. However, since TCBZ is non-licensed for dairy livestock, ABZ was tested on the only included dairy sheep farm instead. All farms were visited at least twice. Sheep were individually treated with the anthelmintic according to the individual bodyweight (bw) and rectally sampled on day 0. On day 14 post treatment, the farms were revisited for the collection of the post treatment samples to evaluate the treatment success. On one farm, the collection of the post-treatment samples was performed on day 15 post treatment. A two-week-interval between the collection of the pre- and post-treatment samples was described in the literature for the FECRT (Mooney et al., 2009; Fairweather 2011a; Fairweather et al., 2012; Solana et al., 2016) and the CRT (Fairweather, 2011a; Flanagan et al., 2011b; Brockwell et al., 2014) and Flanagan et al. (2011a) assessed day 14 post-treatment as a robust re-sampling time when combining FECRT and CRT.

The collection faecal samples and oral treatment were approved by each visited federal state of Germany, confirmed in written form, that rectal sampling and oral treatment were not considered to be an animal experiment within the framework of the German law (Tierschutzgesetz) and the EU directive 2010/63/EU.

2.2.1. Clinical examination, weighing, treatment, sampling

On day 0, sheep were individually weighed on a mobile animal balance (resolution: 0.1 kg) and orally treated with a licensed TCBZ preparation (ENDOFLUKE® 100 mg/ml, Livisto aniMedica GmbH, Senden-Boesensell, Germany or Cydectin® TriclaMox 1 mg/ml + 50 mg/ml, Zoetis Deutschland GmbH, Berlin, Germany) in the dosage of 10 mg TCBZ/kg body weight (bw) or ABZ preparation (Valbazen® 1,9%, Elanco Deutschland GmbH, Bad Homburg, Germany) in the dosage of 7.5 mg ABZ/kg bw. A disposable syringe (size: 5 ml, 10 ml, or 20 ml) was used to ensure dosing exactly adjusted to the individual bodyweight. Finally, a faecal sample was taken rectally from each individual animal. On day 14 (day 15 on one farm) post treatment, each individual sheep was re-sampled.

A second set of post-treatment samples was collected on one farm on day 21 and on another farm on day 28, since *F. hepatica* eggs were still present in the first post-treatment samples (14/15 days post treatment).

On one farm, on which TCBZ did not lead to a FECR nor to a decrease of coproantigen levels at day 14 post treatment, a second oral treatment of the study population with the double dose of TCBZ (20 mg/kg bw) was conducted on day 21 post first treatment and further post treatment samples were collected on day 35 (14 days after the second treatment).

Faecal samples were stored in a transportable electric cool box and transferred to the laboratory in Berlin within one to two days.

2.2.2. Faecal egg count reduction test (FECRT)

The FECRT was performed on a farm level using paired data pre- and post-treatment for individual animals. After arrival in the laboratory, samples were stored at 4 °C until coproscopic examinations within at least seven days after sample collection.

In contrast to the coproscopic examinations conducted for screening sheep flocks for the presence of *F. hepatica* eggs, the FECRT was performed using only two different sedimentation techniques ("Modified FLUKEFINDER®" and FLUKEFINDER®). The standard sedimentation method was not applied for the FECRT. The pre- and post-treatment examinations of the first three farms included were conducted using a method combining a standard sedimentation technique with the FLUKEFINDER® method (Soda Springs, Idaho, USA) using 10 g of faeces per sample as described recently ("Modified Flukefinder") (Kahl et al., 2023). However, since the collection of 10 g of faeces turned out to be a practical constraint, the standard protocol of the FLUKEFINDER® using only 2 g of faeces was applied on farms 4–12 as described in Kahl et al. (2023).

2.2.3. Coproantigen reduction test (CRT)

The CRT was also performed at the individual animal level. After arrival in the laboratory, samples were either stored at 4 °C or frozen at −20 °C until examination (maximum storage time at 4 °C or −20 °C: 3 months) as recommended by Flanagan et al. (2011a).

For the CRT, a commercially available cELISA kit (BIO K 201/2 – Monoscreen AgELISA Fasciola hepatica, Bio-X Diagnostics S.A., 5580 Rochefort, Belgium, batches FASA20L03, FASA21M11, FASA21L23) was used according to the manufacturer's instructions. Absorbances were read at 450 nm using an Epoch microplate spectrophotometer (Bio Tek Instruments, Winooski, Vermont, USA). After subtracting the OD of the negative control from sample and positive control Ods, a relative OD was calculated as: (Delta OD Sample * 100)/Delta OD positive control = relative OD (%)

According to the manufacturer's instructions, the sample's status (positive or negative for *F. hepatica*) was determined using a lot-specific threshold value, which was 8.0% relative OD. However, during the course of the study, several coproscopically positive pre-treatment samples with a calculated relative OD value of less than 8.0% were found. Hence, it was decided to decrease the threshold value for this study to 2.0% of the positive control OD value for all samples to avoid false negative assertions referring to the cELISA. At 2% relative optical density, all FEC positive samples were also positive in cELISA.

2.2.4. Fasciola miracidium development test (FMDT)

The FMDT was performed with the eggs collected on one farm, on which TCBZ failure was observed. This was done to evaluate, whether ABZ was still effective against this field population.

The protocol from Alvarez et al. (2020) was implemented with slight modifications. For the assay, *F. hepatica* eggs were isolated from faecal samples: The sediments from positive individual samples were pooled after coproscopic examination as part of the FECRT. The pooled samples were further cleaned by another round of purification on the FLUKEFINDER® followed by numerous 3-min sedimentation cycles with tap water in a 250 ml beaker until the sediment was macroscopically as

clean as possible. The sediments were transferred into a 50 ml centrifugation tube. An egg suspension of 200 eggs/ml was prepared using 10 mM sodium phosphate buffer (pH 7) (NaPi) as a solvent instead of tap water as indicated in the protocol from Alvarez et al. (2020). The suspension was subdivided and transferred into twenty 7 ml-cell culture tubes (Nunc™ Thermo Fisher Scientific™, Waltham, Massachusetts, USA) with 1 ml egg suspension per tube. A 5 mM ABZ stock solution was prepared (13.26 mg ABZ dissolved in 10 ml methanol acidified with 20 µl HCl (37%)). Three different ABZ working solutions (500 µM; 50 µM and 5 µM) were generated originating from the 5 mM stock solution. The ABZ efficacy was tested in three different final concentrations (5 µM, 0.5 µM and 0.05 µM) by adding 10 µl of the different working solutions to the respective tubes. Each concentration was assessed using five replicates. Untreated eggs (5 replicates) exposed only to 1% methanol were included as negative control. The tubes were incubated in the dark at 25 °C for 12 h and subsequently washed three times with 10 mM NaPi to remove the drug. Afterwards, the washed eggs were incubated in 1 ml NaPi in the dark at 25 °C for 15 days. On day 16, eggs were exposed to daylight for 4 h before adding 10 µl 10% buffered formalin (pH 7.8) to each egg suspension to stop the development. The eggs were carefully inspected using a microscope (400 × magnification) to identify completely undeveloped eggs and discriminate them from partially developed eggs and egg shells after hatching of miracidia. Fully embryonated eggs and empty eggshells after hatching of miracidia were counted together as "developed eggs". The percentage of developed eggs was calculated for each replicate in each ABZ concentration and the negative controls. The egg development rate in the negative controls in each assay was required to be at least 70% to consider the assay as valid. The ABZ ovicidal activity was calculated by means of the following formula:

Ovicidal activity = [(% eggs developed in control - % eggs developed after drug incubation)/% eggs developed in control] x 100.

2.3. Statistical analyses

The statistical analyses were performed using R version 4.2.0 in R Studio version 2022.07.1.

The results of the faecal examinations regarding the occurrence of *F. hepatica* and Paramphistominae on German sheep farms were grouped according to the time points of the sampling: December 2020 (start of the examinations)-March 2021; April 2021–September 2021; October 2021–March 2022; April 2022–August 2022 (end of the examinations). The visualisation of the occurrence of the parasites on a map was performed using Microsoft Excel 2019 with Microsoft 3D Maps (Microsoft Corporation, Redmond, WA, USA).

The 95% confidence intervals (CI) for the occurrence frequencies of *F. hepatica* and Paramphistominae were calculated using the binom.wilson function in the R package epitools (version 0.5–10.1). Tests for statistical significance of the differences between *F. hepatica* and Paramphistominae occurrences were performed using the tab2by2.test in the R package epitools. Odds ratios were calculated using the mid-p exact method implemented in the or.midp command from the same package.

The reduction in egg excretion after anthelmintic treatment in the efficacy trial was calculated with 95% credibility intervals (the Bayesian equivalent to confidence intervals) using the R package eggCounts version 2.3–2 (Wang et al. 2017, 2018), which implements Bayesian hierarchical models to assess the efficacy of treatment (using the model common efficacy on herd level without zero-inflation).

The calculation of the net optical densities in the cELISA and the final percentual result using the formula given by the manufacturer was performed using Michiels et al., 1987 (Microsoft Corporation, Redmond, WA, USA).

Cohen's kappa coefficients were calculated using the CohenKappa function from the DescTool package (version 0.99.48) to measure the level of agreement between the results detected by the FLUKEFINDER®method and the cELISA (farms no. 3–12) depending on the threshold value used for assessing the cELISA result. Cohen's kappa values were calculated independently for cELISA results with thresholds set to 2.0% and 8.0%, respectively. The results of both calculation approaches were compared using the table by Landis and Koch (1977).

For visualisation of the results in graphs, Graph Pad Prism 5.03 (GraphPad Software, San Diego, CA, USA) was used.

3. Results

In total, 71 farms were included in the initial screening. Supplemental Table S1 provides information about the locations, the time of examination, the FEC method applied on each respective farm and the FEC results of all farms included in the study.

3.1. Occurrence of Fasciola hepatica and Paramphistominae on German sheep farms

The results of the investigation of the current occurrence of *F. hepatica* on German sheep farms are summarised in Table 1 and visualised in Fig. 1. Out of the 1673 individual sheep investigated during the study, 214 sheep (12.8%, 95% CI 11.3–14.5%) on 25 out of 71 farms (35.2%, 95% CI 25.1–46.8%) were shedding *F. hepatica* eggs in their faeces. The frequency at which eggs of Paramphistominae were found was lower: only 81 individual animals (4.8%, 95% CI 3.9–6.0%) on 11 out of 71 farms (15.5%, 95% CI 8.9–25.7%) were coproscopically positive for Paramphistominae, resulting in a significantly lower occurrence of Paramphistominae on farm level as well as on individual animal level ($p = 0.007$ and $p < 0.001$, respectively) (Table 1). In addition, there were considerable differences between seasons. *Fasciola hepatica* was found significantly more often than rumen flukes in winter 2021/22 (OR 1.8) and winter 20/21 (OR 4.4), while in summer 2021 Paramphistominae were found significantly more often than *F. hepatica* (OR 0.5). Co-infections with both groups of trematodes were observed on 8 of 71 farms (11.3%, 95% CI 5.8–20.7%) and in 21 of 1673 individual sheep (1.3%, 95% CI 0.8–1.9%). The odds to find Paramphistominae on farms that were positive for *F. hepatica* was 3.6-fold higher than on *F. hepatica* negative farms but this difference was not significant ($p = 0.070$, mid-p exact test).

A seasonal variation in the occurrence of *F. hepatica* was observed: The highest number of patently *F. hepatica* infected sheep was found in the winter seasons with 20.2% and 16.9% of the examined samples containing at least one *F. hepatica* egg in winter 2020/2021 (December 2020–March 2021) and 2021/2022 (October 2021–March 2022), respectively. In contrast, only 3.9% and 6.3% of the examined samples were tested positive for *F. hepatica* during summers 2021 (April 2021–September 2021) and 2022 (April 2022–August 2022), respectively. The difference between the occurrences of *F. hepatica* in the winter seasons and the summer seasons was significant in the mid-p exact test ($p < 0.001$).

The highest number of animals testing positive for Paramphistominae, in relation to the total number of investigated animals, was found in winter 2020/2021 (12.1%), whereas the percentage of positive samples in the following winter 2021/2022 was much lower (1.0%) and even lower compared to both summers 2021 and 2022 (7.8% and 5.0%, respectively). For rumen flukes, no statistically significant difference was detected between winter and summer ($p = 0.823$).

The geographical occurrence of *F. hepatica*-positive and *F. hepatica*-negative farms and the occurrence of Paramphistominae-positive and Paramphistominae-negative farms is shown on maps in Fig. 1.

Table 1
Comparisons between frequency of infection with *Fasciola hepatica* and Paramphistominae and between seasons in coproscopic data before treatment.

		F. hepatica		Paramphistominae			
	N[a]	n[b]	Frequency % (95% CI)	n[b]	Frequency % (95% CI)	P value[c]	OR[c] (95% CI)
Comparisons between trematode groups							
Farm level							
Winter 20/21[d]	12	5	44.67 (19.3–68.0)	3	25.0 (8.9–53.2)	0.430	2.0 (0.35–13.9)
Summer 21	10	6	60.0 (31.3–83.2)	3	30.0 (10.8–60.3)	0.220	3.2 (0.5–24.8)
Winter 21/22	39	12	30.8 (18.6–46.4)	2	5.1 (1.4–16.9)	0.003	7.6 (1.8–56.6)
Summer 22	10	2	20.0 (5.7–51.0)	3	20 (5.7–51.0)	0.652	0.6 (0.1–5.2)
Total winter	51	17	33.3 (22.0–47.0)	5	33.3 (22.0–47.0)	0.004	4.5 (1.6–15.0)
Total summer	20	8	40.0 (21.9–61.3)	6	30.0 (14.5–51.9)	0.531	1.5 (0.4–6.0)
Total	71	25	35.2 (25.1–46.8)	11	15.5 (8.9–25.7)	0.007	2.9 (1.3–6.8)
Individual level							
Winter 20/21	248	50	20.2 (15.6–25.6)	30	12.1 (8.6–16.7)	0.015	1.8 (1.1–3.0)
Summer 21	411	16	3.9 (2.4–6.2)	32	7.8 (5.6–10.8)	0.018	0.5 (0.3–0.9)
Winter 21/22	792	134	16.9 (14.5–19.7)	8	1.0 (0.5–2.0)	<0.001	4.4 (3.0–6.4)
Summer 22	222	14	6.3 (3.8–10.3)	11	5.0 (2.8–8.7)	0.783	1.3 (0.6–3.0)
Total winter	1040	184	17.7 (15.5–20.1)	38	3.7 (2.7–5.0)	<0.001	5.6 (4.0–8.2)
Total summer	633	30	4.7 (3.3–6.7)	43	6.8 (5.1–9.0)	0.119	0.7 (0.4–1.1)
Total	1673	214	12.8 (11.3–14.5)	81	4.8 (3.9–6.0)	<0.001	2.9 (2.2–3.8)
Comparisons between seasons on individual level						P value[c]	OR[c] (95% CI)
F. hepatica							
Total winter	1040	184	17.7 (15.5–20.1)			<0.001	4.3 (2.9–6.5)
Total summer	633	30	4.7 (3.3–6.7)				
Paramphistominae							
Total winter	1040			38	3.7 (2.7–5.0)	0.004	0.5 (0.3–0.8)
Total summer	633			43	6.8 (5.1–9.0)		

[a] Total investigated.
[b] Total positive.
[c] Odds ratios and p values calculated using the mid-p exact method and *F. hepatica* as reference level.
[d] Definition of seasons: Winter 20/21: December 2020–March 2021; Summer 21: April 2021–September 2021; Winter 21/22: October 2021–March 2022; Summer 22: April 2022–August 2022.

3.2. Inter rater agreement between FLUKEFINDER® and coproantigen ELISA

Cohen's kappa coefficients were calculated twice to measure the level of agreement between the coproscopic FLUKEFINDER® method and the copro-immunological method using the results from farms no. 3–12. In total, 612 individual faecal samples (pre- and post-treatment samples) were analysed with both methods in parallel to assess whether an individual animal was positive or negative for a patent *F. hepatica* infection.

When considering only samples with an OD value of ≥8.0% of the positive control OD value (i.e. the lot-specific threshold value) as positive for *F. hepatica*, the calculated kappa coefficient was 0.797 (95% CI: 0.749–0.846). According to the nomenclature of Landis and Koch (1977), this can be described as a "substantial" strength of agreement. Compared to that, when all samples with an OD value of ≥2.0% of the positive control OD value (i.e. the threshold value applied within this study) were considered as positive for *F. hepatica*, the calculated kappa coefficient was 0.805 (95% CI: 0.757–0.852), which is slightly higher than the kappa value in the first calculation, but also a "substantial" agreement according to the nomenclature of Landis and Koch (1977). When looking at the 95% CI values, the increase in Cohen's kappa was not significant.

3.3. Efficacy study to evaluate the flukicidal activity of TCBZ and ABZ

The results of the field trial are summarised in Table 2 and Fig. 2. Sufficient efficacy of TCBZ as evidenced by negative cELISA (cELISA result below the chosen threshold value of 2.0% relative OD) of all originally positive animals two weeks after treatment was shown on nine out of 11 farms, on which TCBZ efficacy was tested. Sufficient efficacy of TCBZ based on the FECRT results (>95% FECR two weeks after treatment) was also shown on nine out of 11 farms, on which TCBZ efficacy was tested.

High efficacy of ABZ was proven on one dairy sheep farm (farm no. 1) with negative cELISA results and FECR >95% three weeks after treatment. On this farm, a negative coproscopic as well as a negative cELISA result was observed in 12 out of 13 post-treatment samples from day 14. However, one of the post treatment samples still contained a few parasite eggs in the coproscopic examination (0.8 EPG on day 14 post treatment compared to 8.5 EPG before treatment) and therefore, an egg count reduction of 95% was not obtained on the farm level on day 14. Moreover, the cELISA result (4.7% of positive control) was slightly above the chosen threshold value of 2.0% for the egg-positive sample. Therefore, a second set of post-treatment samples was collected on day 21 from all four individual animals that had originally been positive for *F. hepatica* on day 0. No parasite eggs were found in the samples from day 21, leading to an egg count reduction of 100%. Furthermore, the cELISA result was negative for all individual samples on day 21 post treatment. The *F. hepatica* population on this farm was therefore considered to be ABZ susceptible.

On one out of the 11 TCBZ-treated farms (farm no. 2), the egg count reduction already exceeded 95% on day 15 post treatment (FECR: 98.4%). Nevertheless, a second set of post-treatment samples was collected on day 28 as some parasite eggs were still present in the samples on day 15. On day 28, the FECR was >99.9%. Moreover, one individual animal also showed a cELISA result slightly above the chosen threshold of 2% on day 15 (4.58% of positive control), but a negative coproscopic result at the same time. A second set of post-treatment samples from all animals was collected on this farm on day 28 and the coproscopic result of this individual animal (and all other animals) was negative again. The cELISA was not repeated with the second post-

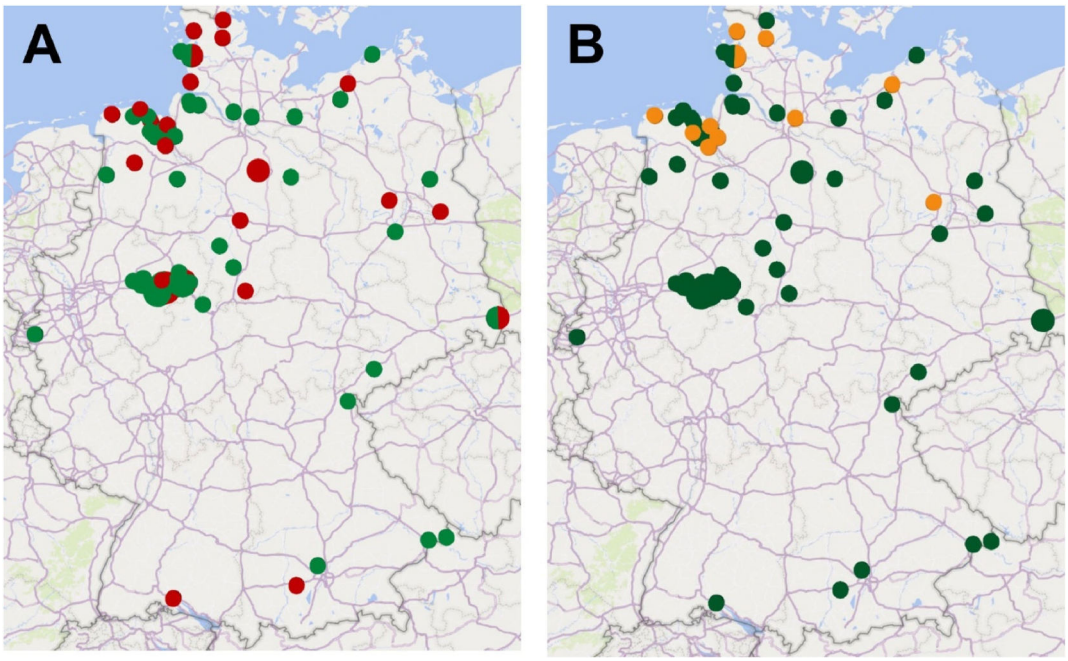

Fig. 1. Occurrence of *F. hepatica* (A) and Paramphistominae (B) on German sheep farms from 2020 to 2022 using coproscopic methods. Individual farm locations were defined by the combination of the village name and the ZIP code. If two farms were located in the same village, they are represented by a single, larger circle. Locations with detection of *F. hepatica* are marked in red on map A, locations with detection of Paramphistominae are marked in orange on map B and locations where trematodes were not detected, are marked in green. Red-green coloured dots on map A show the occurrence of *F. hepatica*-positive and *F. hepatica*-negative farms in the same location. Orange-green coloured dots on map B show the occurrence of Paramphistominae-positive and Paramphistominae-negative farms in the same location.

Table 2
Faecal egg count reduction (FECR) (calculated using the EggCounts package in R Studio, assuming a common drug efficacy for all animals) and coproantigen reduction test (CRT) results for *F. hepatica* on all 12 farms included in the efficacy trial.

Farm	Federal state	Method	Drug	Product	N total	N paired FEC	n	FECR %	2.5% CL	97.5% CL	N paired cELISA	cELISA positive before/after[d]
1a[a]	Lower Saxony	Mod. FLUKEFINDER®	ABZ	Valbazen 1.9%	13	13	4	94.92	90.51	97.73	13	3/1
1 b[b]	Lower Saxony	Mod. FLUKEFINDER®	ABZ	Valbazen 1.9%	13	4	4	99.87	98.15	100	4	3/0
2a[a]	Mecklenburg-Western Pomerania	Mod. FLUKEFINDER®	TCBZ	Cydectin TriclaMox	46	43	35	98.4	98.07	98.69	44	35/1
2 b[b]	Mecklenburg-Western Pomerania	Mod. FLUKEFINDER®	TCBZ	Cydectin TriclaMox	46	41	33	99.94	99.85	99.99	n.d	n.d.
3	Schleswig-Holstein	Mod. FLUKEFINDER®	TCBZ	Cydectin TriclaMox	79	71	3	99.81	97.12	100	4	3/0
4	North Rhine Westphalia	FLUKEFINDER®	TCBZ	Endofluke	55	46	8	83.42	80.73	86.11	49	9/0
5	Brandenburg	FLUKEFINDER®	TCBZ	Endofluke	32	32	12	99.41	98.54	99.82	32	12/0
6a[a]	Lower Saxony	FLUKEFINDER®	TCBZ	Endofluke	53	36	24	0.01	0	0.11	37	30/33
6 b[c]	Lower Saxony	FLUKEFINDER®	TCBZ	Cydectin TriclaMox	53	27	22	0	0	0.06	29	27/27
7	North Rhine-Westphalia	FLUKEFINDER®	TCBZ	Endofluke	48	43	28	99.55	99.39	99.69	45	33/0
8	Baden-Wuerttemberg	FLUKEFINDER®	TCBZ	Endofluke	30	30	30	99.71	99.64	99.77	30	30/0
9	Schleswig-Holstein	FLUKEFINDER®	TCBZ	Cydectin TriclaMox	21	13	7	99.92	98.82	100	13	5/0
10	North Rhine-Westphalia	FLUKEFINDER®	TCBZ	Cydectin TriclaMox	17	17	8	99.98	99.93	100	17	9/0
11	Brandenburg	FLUKEFINDER®	TCBZ	Endofluke	9	9	5	99.82	97.34	100	9	4/0
12	Lower Saxony	FLUKEFINDER®	TCBZ	Endofluke	27	27	13	99.96	99.35	100	27	12/0

N total, total number of coproscopically examined individual animals on the farm before flukicidal treatment; N paired, total number of paired samples pre and post treatment; n, number of coproscopically F. hepatica-positive animals before treatment; CL, credibility limit; ABZ, albendazole; TCBZ, triclabendazole.

[a] FECR/coproantigen reduction from day 0 to day 14 (1a and 6a)/to day 15 (2a).

[b] FECR/coproantigen reduction from day 0 to day 21 (1 b)/to day 28 (2 b).

[c] FECR/coproantigen reduction from day 21 to day 35 after treatment with the double dose of TCBZ on day 21.

[d] Number of samples positive in the coproantigen ELISA before/after treatment using the 2% of the positive control threshold.

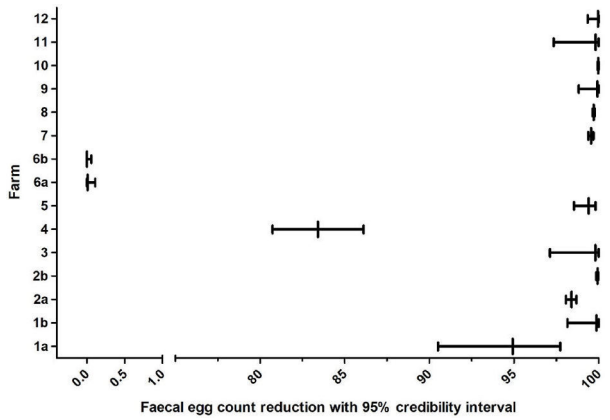

Fig. 2. Calculated faecal egg count reduction (FECR) with 95% credibility intervals for *F. hepatica* on all 12 farms. The EggCounts package was used in R to calculate FECR and 95% credibility limits assuming a common efficacy for all animals. Albendazole was used as flukicide on farm 1 while triclabendazole was tested on farms 2–12. Farm 1a: FECR day 0 to day 14 post treatment, farm 1 b: FECR day 0 to day 21 post treatment, farm 2a: FECR day 0 to day 15 post treatment, farm 2 b: FECR day 0 to day 28 post treatment, farm 6a: FECR day 0 to day 14 post treatment, farm 6 b: FECR day 21 to day 35 post treatment.

treatment samples, since the authors had not decided to lower the threshold for the cELISA from 8.0% to 2.0% of positive control at this point of the study.

On another one out of the 11 TCBZ-treated farms (farm no. 4), a negative cELISA result on day 14 post treatment was seen in all individual post-treatment samples, but one individual sheep of this flock with the highest pre-treatment egg count (154 EPG) still showed a high number of *F. hepatica* eggs in the sample 14 days post treatment (81 EPG), leading to an egg count reduction of only 83.42 % (95% CL 80.73%–86.11%) on herd level. Unfortunately, it was not possible to obtain further samples from this farm since the farmer did not provide further samples from this animal.

On another farm (farm no. 6), a high number of sudden deaths was observed daily among recently clinically healthy animals. Some of the deceased animals were pathologically examined and massive infections with juvenile *F. hepatica* stages were observed. On this farm, 53 out of 1300 ewes and lambs of the flock were enrolled in the efficacy trial as a study population and the animals were treated with TCBZ at the recommended dose (10 mg/kg bw) on day 0. The egg counts and coproantigen levels of those 48 animals that were still alive on day 14 post treatment were even higher compared to the pre-treatment results. Hence, a second treatment attempt with twice the recommended dose (20 mg/kg bw) was conducted on day 21 (42 surviving animals) and post-treatment samples were collected on day 35 (35 survivors). However, the egg counts and coproantigen levels increased further (Table 2). Not only among the study animals, but in the whole flock many sheep perished within a few weeks (roughly 300 animals out of originally 1300 ewes and lambs according to the farmer). On day 46, the farmer treated the surviving sheep with closantel (CLOS) in various doses (8.8–20.3 mg/kg bw, same amount of drug (750 mg) independently of the body weight). No rectal samples from day 46 were available, but samples from the 11 surviving sheep of the study population were taken on day 70 (i.e. 24 days post treatment with CLOS). These showed a negative cELISA result and only one of the samples contained *F. hepatica* eggs (16 EPG on day 70 compared to 1223 EPG on day 35). For the surviving 11 sheep, this corresponds to a FECR of 100% (95% CL 99.89–100%) from day 35 to day 70. The FEC development and survival of the animals in the course of the study is visualised in Fig. 3.

To test the efficacy of ABZ against this phenotypically TCBZ-resistant *F. hepatica*-population, a FMDT was performed using eggs isolated from the pooled sediments after the coprological examination on day 35. The results are shown in Fig. 4. An adequate level of development of the non-treated eggs (negative control) was detected in all replicates (mean: 90.2%) and a high ovicidal activity (85.0%) of ABZ was calculated for the discriminating dose (eggs exposed to an ABZ concentration of 0.5 μM) (Alvarez et al., 2020). Therefore, this isolate can be considered to be ABZ-susceptible according to the FMDT results.

4. Discussion

The present study showed seasonal variations regarding the occurrence of *F. hepatica* from 2020 to 2022 on German sheep farms. A significantly higher percentage of coproscopically *F. hepatica*-positive individual animals was found in the winter seasons. *Fasciola hepatica* is a diheteroxenic parasite and a major proportion of its life cycle occurs in the environment including intermediate host stages. Furthermore, the development in the intermediate host is heavily dependent on climate conditions like warm temperatures and moisture. Hence, a seasonal pattern is typical for the epidemiology of *F. hepatica* (Luzón-Peña et al., 1995). The embryonation of *F. hepatica* eggs only occurs when the environmental temperatures exceed 5 °C, and the rate of development increases with higher temperatures (Andrews et al., 2021). In temperate climates of the northern hemisphere, snails are usually infected in spring and summer when the weather conditions are favourable for the miracidia to hatch. After 6–8 weeks, infected snails begin to shed multiple cercariae, which subsequently encyst on plants as metacercariae (Hodgkinson et al., 2018). The infection of the definitive host typically takes place during the end of the grazing season and positive coproscopic results are seen after the prepatent period from the end of autumn onwards and during the winter period.

The number of individual animals infected with Paramphistominae was lower than the number of individual animals positive for *F. hepatica* in the respective season. Interestingly, no significant differences were found between summer and winter seasons regarding the occurrence of Paramphistominae, although the life cycle of the most frequently observed rumen fluke in sheep in Germany, *Calicophoron daubneyi*, is similar to *F. hepatica* with the same intermediate host (Alstedt et al., 2022; Forstmaier et al., 2021). An explanation for that might be, that treatments against rumen flukes are possibly not as frequently conducted in the annual anthelmintic management on many farms compared to treatments against *F. hepatica.* In the routine parasitological monitoring of the sheep and goat health service of the Clinic for Swine and Small Ruminants in Hannover eggs of rumen flukes were not found before 2016 on the farms at the shore of the North Sea (Roden, 2022). In contrast to fasciolosis, infections with rumen flukes in the adult stage are mostly subclinical (Kahl et al., 2021), so that a specific therapy against Paramphistominae was unknown or might not appear necessary for the veterinarians and the farmers and animals remain infected throughout the year. The specific anthelmintic that targets rumen flukes, oxyclozanide, was first licensed in 2019 and most veterinarians only used TCBZ for routine treatment of all flukes. Moreover, we found the occurrence of Paramphistominae limited to the northern part of Germany in our investigations. Most farms, which were coproscopically positive for Paramphistominae in our examinations, are located in the federal States Lower Saxony and Schleswig-Holstein close to the North Sea (Fig. 1). In contrast to that, *F. hepatica*-positive farms were identified throughout the country as shown in the map (Fig. 1). That agrees with a recent study from Alstedt et al. (2022). The authors investigated the geographical distribution of rumen and liver flukes in small ruminants in Germany and found a lower prevalence of patent paramphistomidosis in the southern part of Germany and a higher prevalence in Lower Saxony in the north. In contrast, the prevalence of fasciolosis was higher in the southern federal state Bavaria than in the northern state Lower Saxony. Another recent publication from Forstmaier et al. (2021) investigated the distribution and prevalence of liver and rumen flukes in cattle in Germany. This study also demonstrated a higher rumen fluke prevalence

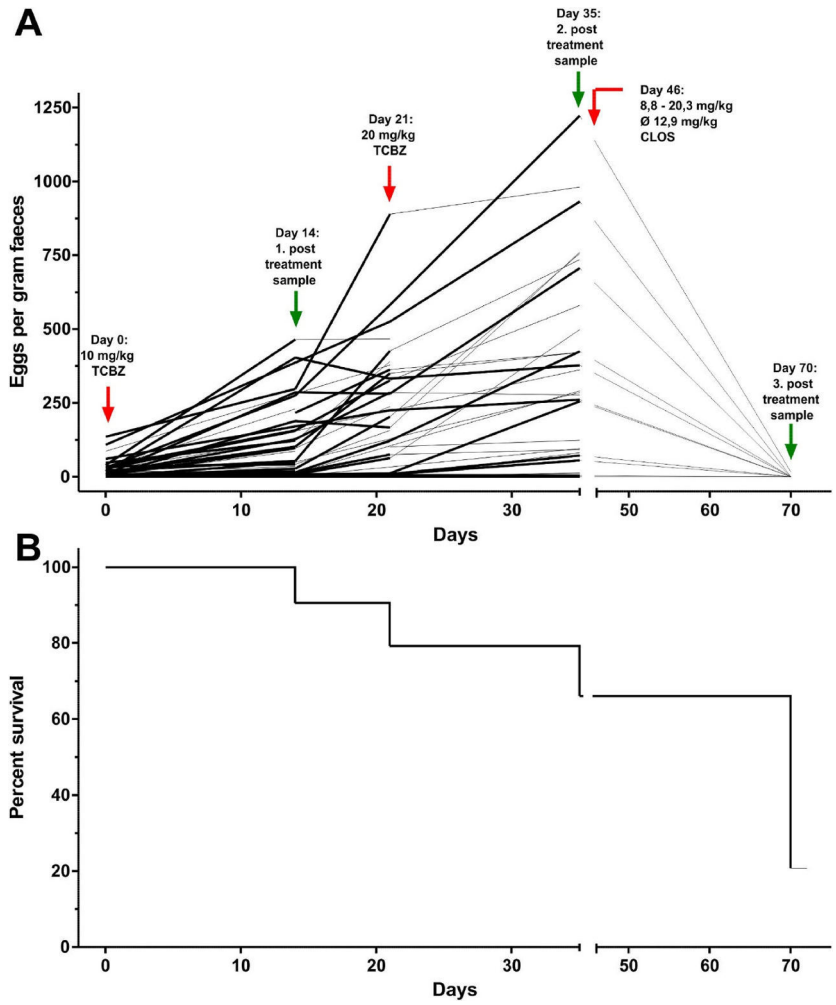

Fig. 3. Faecal egg count development of individual sheep (A) and survival (B) shown as Kaplan-Meier plot of the study group on farm no. 6 during the course of the study. The CLOS treatment on day 46 was conducted by the farmer. No faecal samples were available for examination from that particular day.

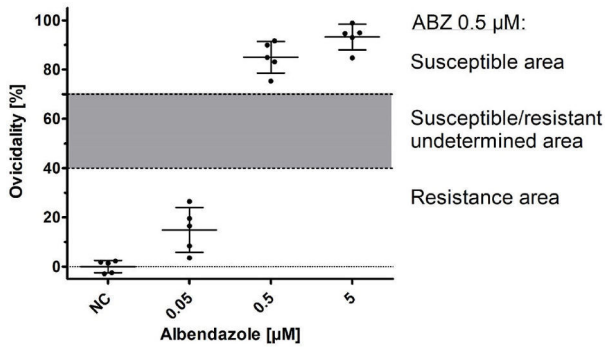

Fig. 4. Ovicidal activity (%) of ABZ on *F. hepatica* eggs isolated from faecal samples on farm no. 6: 5 technical replicates per albendazole concentration (0.05; 0.5 and 5 µM) and negative control (NC).

in the north compared to the south while they found the opposite for the occurrence of *F. hepatica*. There are different hypotheses to explain the different distribution of the two trematode groups. In the past, *Paramphistomum cervi* was generally considered to be the predominant rumen fluke species occurring in Europe (Wiedermann et al., 2021). However, recent molecular studies identified *C. daubneyi* as the main cause for rumen fluke infections in Germany (Forstmaier et al., 2021; Wiedermann et al., 2021; Alstedt et al., 2022). The same was evidenced in the UK by Gordon et al. (2013), who identified all rumen flukes isolated from sheep and cattle in Scotland as *C. daubneyi* by DNA sequencing. In contrast to *P. cervi*, which uses molluscs of the family Planorbidae as intermediate hosts, *C. daubneyi* includes the same intermediate host snail as *F. hepatica* in its life cycle (Kahl et al., 2021). Hence, assuming that *C. daubneyi* is the predominant rumen fluke species in Germany, competitions between liver and rumen flukes might be the reason for the disparate occurrence of these two trematode species, since they compete for *G. truncatula* as an intermediate host (Forstmaier et al., 2021). According to Forstmaier et al. (2021), another reason – especially related to cattle farming – might be the more local livestock breeding system with lesser international animal trading in the southern region leading to a less frequent introduction of rumen flukes into this area. This might also apply for sheep farming. If this is the primary reason, spread of rumen flukes in southern Germany can be expected in the future following the trend to more extensive animal trading.

Generally, the percentage of *F. hepatica*-positive farms was

noticeably higher than the percentage of individual animals which were coproscopically positive for *F. hepatica*. On many farms, only individual animals from the flock showed a positive coproscopic result with often very low egg counts. This is noteworthy, since the flocks were specifically selected due to moist pasture conditions or anamnestic *F. hepatica* infections in the past and individual animals in a poor body condition were selected for sampling. The findings of our extensive coproscopic examinations of 1699 individual faecal samples indicate that *F. hepatica* seems to be widely distributed at herd level in German sheep flocks, but in view of the rather low prevalences within the herds, does currently not seem to have a major impact on sheep farming on most farms. One reason for this unexpected finding might be the very dry summers in Germany in the recent years 2018–2020. This might have limited the local habitats where *G. truncatula* snails could live and reproduce and thus, reduced the pressure of *F. hepatica* infection.

Regarding the threshold value for discriminating between a positive and a negative cELISA result, we found a slightly higher level of agreement between FLUKEFINDER ® coproscopy and cELISA, when the cut-off was reduced from 8.0% to 2.0% of the positive control. During the course of the study, we found several individual pre-treatment samples containing at least one *F. hepatica* egg in the coproscopic examination and a concurrent cELISA result clearly below 8.0% of the positive control. The value of 2.0% was the lowest value detected in the cELISA examinations of pre-treatment samples containing at least one *F. hepatica* egg. Therefore, we decided to lower the threshold value according to our personal experience to avoid false-negative cELISA interpretations in low-intensity infections. The Cohen's Kappa increased from 0.797 to 0.805, which both counts as "substantial agreement" according to Landis and Koch (1977), but this difference was not significant since 95% CIs for Cohen's Kappa values were widely overlapping. Palmer et al. (2014) evaluated the sensitivity and specificity of the same commercially available BIO K 201-cELISA kit with a concurrent coproscopic examination of the same samples (using an officially approved sedimentation method using 4 g of faeces for small ruminants and 10 g of faeces for large ruminants and horses and sieving the faecal sample through three sieves of different mesh sizes before sedimentation). The authors also concluded, that the threshold values for the cELISA can be set much lower than the recommendation of the manufacturer without losing specificity, which is in agreement with results in the present study.

Due to the detected low prevalence and egg shedding intensity, only 12 out of 71 examined sheep farms were eligible for the anthelmintic efficacy study. A sufficient efficacy of the respective anthelmintic was observed on most farms (for TCBZ on 10/11 farms and for ABZ on 1/1 farms).

Farm no. 1 was a dairy sheep farm and therefore the use of TCBZ was not allowed. The adulticide ABZ was used as an alternative for this *F. hepatica* infected sheep flock. In fact, the implemented methods to determine anthelmintic efficacy are not optimal to evaluate the efficacy of an adulticide in the field, if the sheep are not kept indoors under fluke-free conditions for at least eight weeks after the last day when infection could have occurred. Adulticides only affect mature flukes, whereas remaining juvenile flukes, if present, may mature within the period of 14 days and influence the outcome of the FECRT and CRT, resulting in misleading results. This problem has already been addressed by Novobilský et al. (2016). In this study, one out of the four initially infected animals was still positive for *F. hepatica* using coproscopy and cELISA on day 14 after ABZ treatment but all animals were negative on day 21. This indicates that delayed clearance of eggs and antigen from the gall bladder caused the positive result on day 14 but was apparently not the result of ABZ-unaffected juvenile flukes that matured into adults after treatment. Fairweather (2011a) defined successful flukicidal treatment as the absence of coproantigens in faecal samples on day 14 post treatment. However, Flanagan et al. (2011b) also observed one individual animal in their study, which had tested negative at earlier sampling-points before, showing a positive cELISA result on day 14 post treatment. They explained positive cELISA values on day 14 post treatment as a result of continued coproantigen release from disintegrating flukes. Similarly, in the present study there was a single sheep positive in the cELISA on day 21 post TCBZ treatment that was negative on day 28, which can be explained by the same phenomenon. Continued coproantigen release might have also occurred in one individual animal on farm no. 2, which still showed a slightly elevated cELISA result on day 15 and a negative coproscopic result on days 15 and 28.

On one of the other potentially TCBZ-treated farms (farm no. 4), the sheep with the highest pre-treatment egg count (154 EPG) and one of the highest cELISA values of the flock (25.04% relative OD value) on day 0 still demonstrated a large number of *F. hepatica* eggs in the post-treatment sample on day 14 post treatment (81 EPG) with a negative cELISA result (0.32% relative OD value) at the same time, leading to a FECR of 83.42% at herd level. The farmer was asked to submit another faecal sample of that respective animal from day 21, but due to unknown reasons, he did not comply with the request. Hence, no further sample of that individual sheep was analysed to determine the egg shedding three weeks after treatment. Since the cELISA result of this sample was clearly negative, it is highly probable, that this individual sheep was still shedding parasite eggs that had been stored in the gallbladder until day 14 post treatment, despite successful flukicidal treatment. Using flukicidal treatment of experimentally infected rabbits, Chowaniec and Darski (1970) concluded that *F. hepatica* eggs were shed in the faeces for up to 35 days after treatment. This means that the chance for false-positive FEC results on day 14 post treatment is high and remaining eggs stored in the gallbladder despite efficient flukicidal treatment may influence the outcome of a FECRT. Exclusively based on the FECRT result, treatment failure would have been suspected for this individual animal and the fluke population on this farm considered to be TCBZ resistant. However, taking the cELISA result into account and also the fact that poor treatment efficacy was only observed for a single animal in the flock, it is highly likely that all flukes were eliminated and only the liver fluke eggs were still present in a high number in the bile system. Therefore, the TCBZ treatment on this farm was considered to be effective. This observation highlights the importance of combining two different diagnostic approaches in field studies on the efficacy of flukicides.

In contrast to farm no. 4, TCBZ resistance was clearly demonstrated on farm no. 6. In the entire examined study group from this flock TCBZ treatment clearly failed, even at twice the recommended dose (20 mg/kg bw). To the knowledge of the author's, this is the first documented case showing that even a double dose of TCBZ is ineffective against a *F. hepatica* population in sheep. Despite TCBZ treatment, a high mortality and an increase in faecal egg counts as well as coproantigen levels were observed on day 14. Since the pathological examination of several perished animals revealed massive infections with immature flukes, the use of a different fasciolicide without activity on juvenile flukes was not indicated. Therefore, a second TBCZ treatment with twice the recommended dose (20 mg/kg bw) was administered on day 21. However, faecal egg counts and coproantigen levels continued to rise even further until day 35 and also the clinical signs of acute fasciolosis associated with a high number of daily animal losses persisted, leading to the reasonable suspicion of TCBZ resistance on this farm.

According to Fairweather (2011a), the term "resistance" should be used with caution, since no standardised protocols and tests are available in *F. hepatica* to prove resistance in the field. Field cases with observations like on farm no. 6 should rather be indicated as "treatment failures" unless a controlled clinical trial confirms the resistance or sensitivity status. Moreover, Fairweather (2011a) lists other reasons for a treatment failure apart from resistance: "(…) e.g., incorrect (under-) dosing, faulty drenching equipment, product failure, reduced metabolism as a result of liver damage, inadequate and incorrect diagnostic tests, even variable quality of drug formulations."

Regarding farm no. 6 in the present study, under-dosing or faulty

drenching equipment can be excluded as being the reason for the unsuccessful treatment since each individual sheep was weighed before treatment and was dosed by AK with exactly 10 mg or 20 mg TCBZ/kg bw, respectively. A disposable syringe was used for the oral administration of the individually calculated dose for each animal. Each animal was monitored after the oral administration to ensure the complete swallowing of the anthelmintic.

Failure of the used product due to quality issues can also be excluded, as the treatments of other flocks from other farms using the same batch of the flukicide were successful. The bottle with the anthelmintic used on farm no. 6 was always stored under the recommended conditions indicated by the manufacturer and was used within the expiry date. The second treatment with the double dose of TCBZ (20 mg/kg bw) was performed using a different TCBZ-containing preparation from a different manufacturer and also the same bottle of this product was successfully utilised on other farms of the study leading to a sufficient efficacy. Regarding the diagnostic tests, a combination of FECRT and CRT was declared as appropriate for evaluating the anthelmintic efficacy against *F. hepatica* (Flanagan et al., 2011a; Hanna et al., 2015; Novobilský et al., 2016). Hence, the only possible alternative explanation to TCBZ resistance in this case is a lack of anthelmintic efficacy due to the induced liver damage and reduced hepatic metabolism of the active substance as a consequence. Juvenile flukes migrate through the liver parenchyma for the first weeks post-infection. During that phase, the migrating flukes cause severe mechanical damage to the liver tissue due to the sharp spines of the tegument as well as the secretion of proteolytic enzymes by the flukes, impairing the organ's vital functions and altering the function of drug-metabolising systems in the liver (Rojo-Vázquez et al., 2012). However, the large number of animals that were included on farm no. 6 in the study and the fact that treatment failed in every single animal, exclude that liver damage is a reasonable explanation for the lack of efficacy of TCBZ.

A final assessment of the TCBZ resistance status would require experimental infection using metacercariae obtained from the population suspected to be resistant. Indeed, eggs collected from these sheep after TCBZ treatment have now been used to infect snails and when metacercaria become available, such experimental infection trials are planned for the future.

The most likely explanation of our observations is an over-use of TCBZ in inadequate dosages in the previous anthelmintic treatments conducted by the farmer. According to the farmer's own statement, TCBZ has been used seven times within the two recent years prior to the examinations described herein. In these previous TCBZ treatments, the sheep were not individually weighed to calculate the exact dosage for each animal. The farmer provided the information that all ewes were roughly dosed for 100 kg bw and all lambs were roughly dosed for 40 kg bw. However, the farmer dosed CLOS at the end of the study using exactly the same amount of drug for a wide range of weights resulting in dosages of CLOS ranging from 8.8 to 20.3 mg/kg bw with a recommended dosage of 10 mg/kg bw. The very frequent use and probably also sometimes underdosing of TCBZ has most probably promoted the emergence of a TCBZ-resistant *F. hepatica* population on the pasture of this farm over the years. According to the farmer, *F. hepatica* has been diagnosed on this farm every year in routine faecal examinations, even in remarkably dry years such as 2020. In contrast to many other farms included in this study, this flock seems to be under infection pressure independent of the climatic conditions. This particular pasture, on which the flock has been grazing, is located behind a dyke and is permanently moist, so that the pasture contamination does not naturally decrease in dry years. Additionally, the summer 2021 was marked by exceptionally heavy rainfalls in this part of the country, resulting in optimal environmental conditions for the intermediate host snails. That might have engendered an overproportional reproduction of the snails and consequently an extraordinary high infection pressure with the arisen TCBZ-resistant *F. hepatica* population on the respective pasture leading to the massive infections we observed in this flock. Another aspect that should be taken into consideration is the geographic proximity of this farm to The Netherlands, where TCBZ resistance is already widespread (Rose Vineer et al., 2020). Since – potentially resistant – *F. hepatica* populations can be unwittingly imported in infected livestock, it cannot be excluded that the first introduction of this resistant *F. hepatica* isolate was due to acquisition of infected animals from The Netherlands. The farmer of farm no. 6 did not purchase sheep from abroad according to his own statement, but it remains unknown, whether neighbouring livestock farms might have purchased infected animals from The Netherlands. This also includes cattle farms since the same *F. hepatica* population can infect different definitive host species and move amongst different hosts according to Beesley et al. (2017).

On that basis, there might also be the possibility that migrating wild ruminants from the neighbouring country carried and shed *F. hepatica*, so that this isolate reached this area.

This field case clearly illustrated the consequences of a lacking TCBZ efficacy: Since TCBZ is the only currently available flukicide affecting the mature as well as the immature fluke stages inside the definitive host, there is no alternative anthelmintic, which could have been used against the early, highly pathogenic stages to prevent further damage caused by the migrating juvenile flukes. Importantly, this field case suggests that whatever the mechanism by which fluke become resistant to TCBZ, it is not possible to overcome this resistance using double doses of the drug. In the absence of available drugs active against the highly pathogenic juvenile stages, flukicides targeting only adult worms obviously have a limited effect on animal welfare in the case of acute infection.

In comparison to all other currently available flukicides, CLOS is the compound which exerts its effects the earliest. A study from George et al. (2017) revealed 90.2% efficacy against *F. hepatica* at a dose of 7.5 mg/kg bw 6 weeks after experimental infection. An older review from Fairweather and Boray (1999) indicated 90% efficacy of CLOS against flukes 6–8 weeks post infection, whereas other available adulticides such as ABZ and oxyclozanide only affect flukes at least 12 weeks post infection. Other flukicides like rafoxanide and nitroxinil show efficacies of $\geq$90% against immature flukes of 6–8 weeks, respectively (Fairweather and Boray, 1999), but they are currently not approved and available in Germany.

Hence, CLOS should be the drug of choice when TCBZ resistance is suspected to treat fasciolosis as early as possible. The effectiveness of CLOS against TCBZ-resistant *F. hepatica* populations has been reported several times before (Coles et al., 2000; Gordon et al., 2012; Hanna et al., 2015).

The *in vitro* FMDT demonstrated a high ovicidal activity of ABZ against the isolated eggs of this *F. hepatica* population, strongly suggesting that ABZ still has a sufficient efficacy *in vivo*. Coles and Stafford (2001) reported a fluke count reduction of 94% after treating lambs infected with a TCBZ resistant *F. hepatica* isolate with ABZ at a dose of 7.5 mg/kg bw, meaning that TCBZ resistance does not inevitably also lead to a cross-resistance against ABZ. Inversely, there are also reports about sufficient efficacy of TCBZ against ABZ-resistant *F. hepatica* populations (Novobilský et al., 2012; Sanabria et al., 2013).

Observations comparable to this case of TCBZ treatment failure in Germany were very recently reported from Argentina (Larroza et al., 2023). The authors identified a TCBZ-resistant *F. hepatica* population highly susceptible to CLOS *in vivo* and to ABZ as determined by a Fasciola Egg Hatch Test *in vitro*, substantiating that TCBZ resistance does not necessarily lead to cross-resistance to other flukicides.

On all other TCBZ-treated farms, treatment with the recommended dose of 10 mg/kg bw led to a sufficient FECR as well as negative results in the cELISA two weeks after treatment. Since the FMDT was only established in the authors's laboratory during the course of the study, capacities for performing the FMDT with all other field isolates were lacking. Therefore, the FMDT was only conducted with the eggs isolated from farm no. 6.

5. Conclusion

The anthelmintic efficacy field trial revealed a sufficient efficacy of the respective flukicide on most of the farms (TCBZ on 10/11 farms and ABZ on the only included dairy sheep farm). Overall, flukicidal resistance does not seem to be a widespread problem on German sheep farms at the moment. However, on one farm there was no apparent activity of TCBZ and this was associated with serious clinical progression, leading to massive losses of animals in the study population and the wider sheep flock on this affected farm. Suspected resistance was corroborated by no faecal egg count reduction at the double recommended dose (20 mg/kg bw) of TCBZ. This is highly relevant regarding animal welfare and economic sheep production and spread of flukicide resistance, especially against TCBZ, should be considered as an emerging and serious problem, as TCBZ is currently the only flukicide effective against all stages of the liver fluke.

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Declaration of interests

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Georg von Samson-Himmelstjerna reports financial support was provided by Federal Office of Consumer Protection and Food Safety Berlin Mitte. Member of the editorial board of Int. J. Parasitol. Drugs Drug Rest. GvSH.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijpddr.2023.11.001.

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Update

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Corrigendum to "Efficacy of flukicides against *Fasciola hepatica* and first report of triclabendazole resistance on German sheep farms" [Int. J. Parasitol. Drugs Drug Resist. 23 (2023) 94–105]

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The authors regret that there were several typing errors in the manuscript, which they would like to correct. The correct sentences should read as follows:

Abstract:

"Individual faecal samples were collected before and two weeks after treatment to evaluate the FECR using the sedimentation or FLUKEFINDER® or a modified FLUKEFINDER® method." Correct: "Individual faecal samples were collected before and two weeks after treatment to evaluate the FECR using the FLUKEFINDER® or a modified FLUKEFINDER® method."

2.3 Statistical analyses

"The calculation of the net optical densities in the cELISA and the final percentual result using the formula given by the manufacturer was performed using Michiels et al., 1987 (Microsoft Corporation, Redmond, WA, USA)." Correct: "The calculation of the net optical densities in the cELISA and the final percentual result using the formula given by the manufacturer was performed using Microsoft Excel 2019 (Microsoft Corporation, Redmond, WA, USA).

3. Results

"In addition, there were considerable differences between seasons. *Fasciola hepatica* was found significantly more often than rumen flukes in winter 2021/22 (OR 1.8) and winter 20/21 (OR 4.4), while in summer 2021 Paramphistominae were found significantly more often than *F. hepatica* (OR 0.5)." Correct: "In addition, there were considerable differences between seasons. *Fasciola hepatica* was found significantly more often than rumen flukes in winter 20/21 (OR 1.8) and winter 21/22 (OR 4.4), while in summer 2021 Paramphistominae were found significantly more often than *F. hepatica* (OR 0.5)."

4. Discussion

"The findings of our extensive coproscopic examinations of 1699 individual faecal samples indicate that *F. hepatica* seems to be widely distributed at herd level in German sheep flocks, but in view of the rather low prevalences within the herds, does currently not seem to have a major impact on sheep farming on most farms." Correct: "The findings of our extensive coproscopic examinations of 1673 individual faecal samples indicate that *F. hepatica* seems to be widely distributed at herd level in German sheep flocks, but in view of the rather low prevalences within the herds, does currently not seem to have a major impact on sheep farming on most farms."

The authors would like to apologise for any inconvenience caused.

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4.2 Supporting information

Appendix A. Supplementary data: Overview about all farms, methods and EPGs.

Farm number in efficacy tr	Federal State	FEC method	Date	Drug used	Total individual samples	viduals positive for *F. hepa*	*F. hepatica* EPG	ndividuals positive for Paramphistomina	Paramphistominae EPG
	Lower Saxony	Standard Sedimentation	Dec 20		43	1	1.7	0	
	Lower Saxony	Standard Sedimentation	Jan 21		13	1	1	0	
	Hamburg	Standard Sedimentation	Feb 21		29	0		5	0.1-0.8
	Lower Saxony	Standard Sedimentation	Feb 21		7	0		0	
	Schleswig-Holstein	Modified FLUKEFINDER®	Feb 21		47	7	0.1-0.7	21	0.2- 52
	Schleswig-Holstein	Standard Sedimentation	Feb 21		9	0		0	
	Lower Saxony	FLUKEFINDER®	Feb 21		8	0		0	
1	Lower Saxony	Modified FLUKEFINDER®	Feb 21	Albendazole	17	4	0.1-8.5	0	
2	Mecklenburg Western Pomerania	Modified FLUKEFINDER®	Feb 21	Triclabendazole	45	37	0.2-110	4	0.2- 3
	Lower Saxony	Standard Sedimentation	Mar 21		11	0		0	
	Mecklenburg Western Pomerania	Standard Sedimentation	Mar 21		6	0		0	
	Mecklenburg Western Pomerania	Standard Sedimentation	Mar 21		13	0		0	
	Lower Saxony	Modified FLUKEFINDER®	Apr 21		30	1	1	0	
	Schleswig-Holstein	Modified FLUKEFINDER®	Apr 21		22	1	3.4	2	0.2 -2.5
3	Schleswig-Holstein	Modified FLUKEFINDER®	Apr 21	Triclabendazole	79	3	2.2-7.0	17	0.1- > 100
	Bavaria	Modified FLUKEFINDER®	May 21		9	1	1	0	
	Mecklenburg Western Pomerania	Modified FLUKEFINDER®	May 21		78	0		0	
	Mecklenburg Western Pomerania	FLUKEFINDER®	Jun 21		54	0		0	
	Hesse	FLUKEFINDER®	Jul 21		9	0		0	
	Lower Saxony	FLUKEFINDER®	Aug 21		50	1	8	13	1.5-150
	North Rhine-Westphalia	Modified FLUKEFINDER®	Aug 21		25	0		0	
4	North Rhine-Westphalia	FLUKEFINDER®	Aug 21	Triclabendazole	55	9	2-154	0	
	Saxony	FLUKEFINDER®	Oct 21		11	0		0	
	Saxony	FLUKEFINDER®	Oct 21		21	1	1	0	
	Schleswig-Holstein	FLUKEFINDER®	Oct 21		15	0		0	
5	Brandenburg	FLUKEFINDER®	Oct 21	Triclabendazole	32	12	1.0-70	6	1.0- 12.0
	Bavaria	FLUKEFINDER®	Nov 21		15	0		0	
	Lower Saxony	FLUKEFINDER®	Nov 21		41	0		0	
	Lower Saxony	FLUKEFINDER®	Nov 21		43	0		0	
	Lower Saxony	FLUKEFINDER®	Nov 21		10	0		0	
	Lower Saxony	FLUKEFINDER®	Nov 21		18	1	1	0	
	Brandenburg	FLUKEFINDER®	Nov 21		22	0		0	
	Lower Saxony	FLUKEFINDER®	Nov 21		22	0		0	
	Brandenburg	FLUKEFINDER®	Nov 21		25	0		0	
	Lower Saxony	FLUKEFINDER®	Nov 21		40	0		0	
6	Lower Saxony	FLUKEFINDER®	Nov 21	Triclabendazole	45*	30*	2-1223	0	
7	North Rhine-Westphalia	FLUKEFINDER®	Nov 21	Triclabendazole	48	31	3-531	0	
	Lower Saxony	FLUKEFINDER®	Dec 21		38	0		0	
	Lower Saxony	FLUKEFINDER®	Jan 22		15	0		0	
	Lower Saxony	FLUKEFINDER®	Jan 22		35	3	2.5-3.5	0	
	Lower Saxony	FLUKEFINDER®	Jan 22		23	1	0.5	2	0.5-11
	North Rhine-Westphalia	FLUKEFINDER®	Jan 22		10	0		0	
	North Rhine-Westphalia	FLUKEFINDER®	Jan 22		10	0		0	
	North Rhine-Westphalia	FLUKEFINDER®	Jan 22		13	0		0	
	North Rhine-Westphalia	FLUKEFINDER®	Jan 22		13	0		0	
	North Rhine-Westphalia	FLUKEFINDER®	Jan 22		14	0		0	
	North Rhine-Westphalia	FLUKEFINDER®	Jan 22		11	1	16	0	
	Lower Saxony	FLUKEFINDER®	Jan 22		14	0		0	
	North Rhine-Westphalia	FLUKEFINDER®	Jan 22		3	0		0	
	North Rhine-Westphalia	FLUKEFINDER®	Jan 22		13	0		0	
	North Rhine-Westphalia	FLUKEFINDER®	Jan 22		5	0		0	
	North Rhine-Westphalia	FLUKEFINDER®	Jan 22		15	0		0	
	North Rhine-Westphalia	FLUKEFINDER®	Jan 22		16	0		0	
	North Rhine-Westphalia	FLUKEFINDER®	Jan 22		29	0		0	
	North Rhine-Westphalia	FLUKEFINDER®	Jan 22		10	0		0	
	North Rhine-Westphalia	FLUKEFINDER®	Jan 22		8	0		0	
8	Baden-Wuerttemberg	FLUKEFINDER®	Jan 22	Triclabendazole	35	34	145-1537	0	
9	Schleswig-Holstein	FLUKEFINDER®	Jan 22	Triclabendazole	21	7	0.5-66	0	
10	North Rhine-Westphalia	FLUKEFINDER®	Feb 22	Triclabendazole	17	8	64-1355	0	
11	Brandenburg	FLUKEFINDER®	Feb 22	Triclabendazole	9	5	0.5-48	0	
	North Rhine-Westphalia	FLUKEFINDER®	Mar 22		7	0		0	
	Lower Saxony	FLUKEFINDER®	Apr 22		19	0		2	0.5-6
	Lower Saxony	FLUKEFINDER®	Apr 22		19	0		0	
	Lower Saxony	FLUKEFINDER®	Apr 22		22	0		0	
	Bavaria	FLUKEFINDER®	Apr 22		9	0		0	
	Lower Saxony	FLUKEFINDER®	Apr 22		19	0		0	
	Lower Saxony	FLUKEFINDER®	Apr 22		32	0		1	40
	Lower Saxony	FLUKEFINDER®	Apr 22		56	1	2.5	0	
	Bavaria	FLUKEFINDER®	Jun 22		13	0		0	
12	Lower Saxony	FLUKEFINDER®	Jun 22	Triclabendazole	27	13	2.0-52.0	8	0.5-49
	Bavaria	FLUKEFINDER®	Aug 22		6	0		0	
				TOTAL:	1673	214		81	

* This data refers to the findings on day 0 before treatment. During the course of the study, 44 out of 53 investigated animals on that farm started *F. hepatica* egg shedding despite of triclabendazole treatment.

4.3 Authors contributions

Author contributions to the published article according to the CRediT scheme:

Alexandra Kahl	First author	Conceptualisation Methodology Validation Formal analysis Investigation Data curation Writing – original draft Writing – review & editing Visualisation
Georg von Samson-Himmelstjerna	Co-author	Conceptualisation Writing – review & editing Supervision Project administration Funding acquisition
Christina Helm	Co-author	Conceptualisation Writing – review & editing
Jane Hodgkinson	Co-author	Conceptualisation Methodology Writing – review & editing
Diana Williams	Co-author	Conceptualisation Writing – review & editing
Wiebke Weiher	Co-author	Conceptualisation Writing – review & editing
Werner Terhalle	Co-author	Conceptualisation Writing – review & editing
Stephan Steuber	Co-author	Conceptualisation Supervision Funding acquisition Writing – review & editing
Martin Ganter	Co-author	Conceptualisation Resources Writing – review & editing
Jürgen Krücken	Co-author	Conceptualisation Methodology Validation Formal analysis Data curation Writing – original draft Writing – review & editing Visualisation Supervision Project administration Funding acquisition

Chapter 5: Discussion

At the beginning of the project, in the summer of 2020, the frequency of *F. hepatica* infections in Germany was unexpectedly low. Originally, it was planned to confine the anthelmintic efficacy trials to sheep farms located in Lower Saxony, a federal state of Germany with a high probability of *F. hepatica* infections due to a high sheep farming intensity, good environmental conditions for the parasite to multiply (proximity to the coast as well as moist weather and soil conditions suitable for the intermediate host snails and free-living *F. hepatica* stages) and a high number of *F. hepatica* infections in the past as recorded by the Clinic for Swine and Small Ruminants in Hannover. On the basis of the results of routine examinations conducted by the Clinic for Swine and Small Ruminants in Hannover, currently infected sheep flocks should have been elected throughout Lower Saxony to carry out efficacy tests on the aimed number of 10-15 sheep farms starting in the summer of 2020.

However, the number of *F. hepatica* infections in Lower Saxony confirmed by the Clinic for Swine and Small Ruminants in Hannover was considerably lower in 2020 compared to previous years. Also, large-scale telephone research by contacting numerous livestock practitioners and sheep farmers in Lower Saxony did not yield contact to farms with currently infected sheep flocks eligible for the study. Therefore, at the beginning of 2021, the project area was first expanded to Mecklenburg-Western Pomerania and Schleswig-Holstein, two neighbouring federal states with direct proximity to the sea and a multitude of huge dyke sheep farms. Since the number of patent *F. hepatica* infections for the efficacy study was still insufficient in those three federal states alone, the project area was then extended to all of Germany in the summer of 2021.

5.1 Systematic comparison of three coproscopical methods

Not only the quantity of patent *F. hepatica* infections at the beginning of the project was low, but also the infection intensities by means of only minimal egg shedding in the few identified infected sheep were low. This fact constituted a major difficulty for the intended field trials to test flukicidal efficacy, since a sufficiently high FEC before treatment is essential for a meaningful result regarding the egg count reduction after drug administration (Levecke et al. 2018). Hence, the necessity of a coproscopical method capable of detecting very low egg shedding intensities had arisen, since no standardised guidelines for the conduction of FECRT in *F. hepatica* exist (Solana et al. 2016; Novobilský and Höglund 2015; Fairweather et al. 2012; Gordon et al. 2012; Fairweather 2011a; Fairweather 2011b; Coles et al. 2006). The standard sedimentation, which was applied for the first screening examinations within the project, is generally considered to have limitations when the egg counts in faecal

samples are low (Alstedt et al. 2022; Ploeger et al. 2017; Becker et al. 2016; Conceição et al. 2002). In view of the situation, the identification of a more suitable method to detect low FEC represented a supplementary aim of the project.

The systematic comparison between the three coproscopical methods (standard sedimentation, FLUKEFINDER®, and a self-developed modified FLUKEFINDER® protocol) was conducted in parallel to the screening examinations and the first flukicidal efficacy trials on the farms. Since the first test runs using the modified FLUKEFINDER® method yielded better results (higher EPG results in the same sample) than the originally envisaged standard sedimentation method (unpublished results), it was initially decided to apply this method for the first FECRT on farms 1-3.

The results of the comparison confirm, that the standard sedimentation method is inferior to the other two methods including differential sieving through the FLUKEFINDER® column and demonstrates the lowest EPG values and the highest relative variability of the EPG outcome, making this method less suitable for a FECRT. The highest raw egg counts were detected with the modified FLUKEFINDER®. High raw egg counts are advantageous for anthelmintic efficacy testing with a FECRT, since it shortens the width of the 95% confidence interval of the FEC reduction estimate (Levecke et al. 2015). Regarding that aspect, the modified FLUKEFINDER® method can be expected to provide the best conditions for a reliable statistical analysis of the anthelmintic efficacy. However, on the basis of own experiences during the first three farm visits within the course of the study, the reliable collection of 10 g faeces from each individual sheep included in the efficacy test turned out to be a practical constraint under field conditions. Sheep tend to defaecate when they are herded together and penned up for sampling as they often become stressed during the process. Consequently, only a smaller amount of faeces was often obtainable. In many instances, individual sheep of the flock had to be postponed and separated for a second sampling attempt, when 10 g of faeces were not obtainable at the first attempt. This complication is time-consuming, when a large number of sheep must be sampled in a short time span. The necessity of a second sampling attempt also increases the stress for the animal when being handled and sampled twice. In contrast, 2 g of faecal material as used for the FLUKEFINDER® method is an amount which was reliably obtainable from most sheep in the field at the first attempt. Moreover, the FLUKEFINDER® protocol contains fewer steps than the modified FLUKEFINDER® protocol, and therefore overall, the faecal examination requires less time, which is of relevance especially when a large quantity of samples must be individually examined.

Thus, depending on the situation how samples are collected for a FECRT, the modified FLUKEFINDER® method might be better suited for laboratory conditions, when e.g. a faeces collection bag can be used. The reliable collection of 10 g of faeces will also not be an issue when a FECRT under field conditions is conducted in cattle. In contrast, from a practical point of view, the original FLUKEFINDER® appears to have advantages for field studies in small ruminants, when many faecal samples might have lower weights than 10 g. Therefore, it was concluded that the original FLUKEFINDER® method is better applicable for the intended field study in sheep and the method for the FECRT was changed from the modified FLUKEFINDER® protocol to the original FLUKEFINDER® protocol for farms 4-12.

In sum, the systematic comparison of the three coproscopical approaches was an initially unplanned, but very valuable subproject to identify the best suitable method for conducting the FECRT in view of the circumstances at the beginning of the project.

5.2 Climate-dependent occurrence of *F. hepatica* on German sheep farms

The large-scale coproscopical screening examinations of suspected farms from December 2020 onwards were initially not envisaged to this extent. However, due to the initial difficulties to acquire currently infected sheep flocks for the efficacy tests, this was a crucial approach to detect sheep whose current infection status was unknown. Thus, sheep farms with either a known history of fasciolosis (as recorded by the Clinic for Swine and Small Ruminants in Hannover or local practitioners) or a high probability of fasciolosis due to their localisations were contacted as described in the Materials and methods section of the second publication. In total, 1673 individual faecal samples from 71 sheep farms were coproscopically examined using either the standard sedimentation method (at the very beginning of the project), the modified FLUKEFINDER® or the original FLUKEFINDER® protocol. Additionally, several composite faecal samples were analysed (sectioned in several sub-samples depending on the size of the composite sample to increase the sensitivity), when farmers did not comply with the request to send individual faecal samples. The composite samples were not included in the statistical analysis in the second publication, since the analysis refers to infections on the individual level.

These screening examinations did not only establish contact to farms, which subsequently could be included in the anthelmintic efficacy trials, but did also provide data regarding the current occurrence of *F. hepatica* on German sheep farms. This is of interest for farmers, clinical veterinarians and parasitologists in order to estimate the current distribution of *F. hepatica*. Due to the fact, that the examined farms were not randomly chosen, but specifically selected on the basis of a high probability of fasciolosis, the sample set is generally highly biased. Thus, the occurrence of *F. hepatica* relating to all German sheep farms including

farms with a lower likelihood of fasciolosis, e.g. due to arid soil conditions, might be markedly lower than the infection frequencies observed within the project. Moreover, since the screening examinations were not statistically designed to calculate a prevalence, the wording “occurrence” was used instead.

The low overall quantity and egg shedding intensity of patent *F. hepatica* infections at the beginning of the project can most probably be traced back to extremely dry and hot weather conditions in the previous years. According to the information on the official website of the German meteorological service (Deutscher Wetterdienst, https://www.dwd.de/DE/leistungen/zeitreihen/zeitreihen.html?nn=480164#buehneTop), the rainfall in 2018, 2019 and 2020 (amounting to 586.3 mm, 735.0 mm and 704.9 mm per year, respectively) was below the average of the last 30 years (791.4 mm per year). Moreover, the number of hot days (daily maximum of ≥ 30 °C air temperature) in 2018, 2019 and 2020 (amounting to 20.4 days, 17.0 days and 11.4 days, respectively) was above the average of the last 30 years (8.9 days per year). As mentioned in the literature review, moist and mild environmental conditions are pivotal for the survival of the intermediate host snails and the free-living *F. hepatica* stages to maintain the life cycle of the parasite. Under dry conditions, *F. hepatica* eggs rapidly desiccate in the environment (Howell and Williams 2020) and at temperatures exceeding 30 °C, embryonation inside the egg is suppressed with no development occurring at 37 °C (Rowcliffe and Ollerenshaw 1960). Encysted metacercariae are only moderately resistant to hot and dry environmental conditions (Yadav 2015; Mas Coma and Bargues 1997). In laboratory experiments, temperatures of 38 °C exerted a lethal effect on metacercarial cysts due to reduced glycogen concentrations inside the cyst. After two days at 38 °C, the viability was already decreased to only 13.2% and after three days at 38 °C, the metacercariae died off (Andreyanov et al. 2021). The occurrence and activity of *G. truncatula*, the most important intermediate host snail species in Europe (Andrews et al. 2021; Mas-Coma et al. 2019; Beesley et al. 2018; Hodgkinson et al. 2018; Charlier et al. 2014; Caron et al. 2007; Bargues and Mas-Coma 2005; Bargues et al. 2003; Bargues et al. 2001), is also influenced by climate conditions. Even though *G. truncatula* seems to be able to withstand dry weather conditions during summer by burrowing in soil before aestivation (Goumghar et al. 2001), drought is a major factor limiting the reproduction capacity of this species. Aestivation decelerates the development of *F. hepatica* stages inside the snail decreasing the number of rediae and cercariae (Rondelaud 1994). Moreover, desiccation of the snail’s spawn may decimate the subsequent snail generations with effects being seen in the following year (Roessler et al. 2022).

The assumption, that unfavourable climate conditions during 2018-2020 were the reason for the unexpectedly low rate of patent *F. hepatica* infections at the beginning of the project is

corroborated by a noticeable increase of infected sheep flocks eligible for the efficacy test after a higher precipitation rate in 2021 with a rainfall of 801.1 mm throughout the year according to the official website of the German meteorological service (Deutscher Wetterdienst) (average of the last 30 years: 791.4 mm per year). Particularly during the summer season in 2021, the rainfall was noticeably higher (305.1 mm) compared to the summer seasons in the previous years (129.4 mm, 174.6 mm and 228.2 mm during summer seasons in 2018, 2019 and 2020, respectively) and the average of the last 30 years (240.5 mm during the summer seasons). Concurrently, the number of hot days (daily maximum of ≥ 30°C air temperature) in 2021 only amounted to 4.5 days, which was clearly below the number of hot days in 2018-2020 and below the average of the last 30 years (8.9 days per year). It is therefore highly likely that moist and mild weather conditions in 2021 favoured an appreciably higher reproduction and transmission of *F. hepatica* in Germany. Generally, the seasonal transmission observed within the study with higher frequencies of fasciolosis in the winter seasons (20.2% and 16.9% on individual level in the winter seasons 2020/2021 and 2021/2022, respectively) compared to the summer seasons (3.9% and 6.3% in the summer seasons 2021 and 2022, respectively) is typical for *F. hepatica* under European conditions (Mas-Coma et al. 2018). Moreover, there was a clear temporal link between the considerably wet weather conditions during the summer season in 2021 and a sudden increase of *F. hepatica* infected sheep flocks with sufficiently high pre-treatment FEC eligible for efficacy testing from October 2021 on. Outbreaks of fasciolosis are usually linked with the peak time of metacercarial pasture contamination (Knubben-Schweizer et al. 2021). Most probably, a high metacercarial contamination during and after the summer season 2021 resulted in much higher infection rates compared to the previous years, presenting in a higher number of patent infections in autumn following the prepatent period. This was highly beneficial for the project after a rather hesitant start during the first year of the project. From October 2021 on, a sufficient number of *F. hepatica* infected sheep flocks eligible for the efficacy trial was available. Hence, the peak period of the efficacy trials concerned the months from October 2021 until February 2022. Eight out of the twelve examined sheep flocks have been included in the efficacy study during that period.

The high dependence of *F. hepatica* on weather conditions displays that climate change may strongly influence the future spread of fasciolosis (Mas-Coma et al. 2009a). A climate-driven increase of fasciolosis, as for example forecasted for most regions in the UK (Fox et al. 2011), might also occur in Germany. The prediction modelling by Caminade et al. (2015) suggested that the risk for fasciolosis in central and western Europe significantly increased during the 2000s and that this trend will continue in the future due to climate change. Caminade et al. (2015) concluded, that the availability of soil moisture is a key determinant

for *F. hepatica* infection levels, which coincides with the observations made within the course of this project. Increasing prevalences of fasciolosis might also exacerbate anthelmintic resistance problems due to a higher frequency of flukicide administration.

5.3 Occurrence of Paramphistominae on German sheep farms

Out of scientific interest, the occurrence of rumen flukes (Paramphistominae) was recorded as additional finding in the coproscopical examinations since rumen fluke eggs can be concurrently detected with the same methods due to a similar relative density and size as *F. hepatica* eggs. Compared to *F. hepatica*, Paramphistominae were detected at a lower frequency and with no significant differences between summer and winter seasons. Moreover, rumen flukes were only found in the northern regions of Germany, whereas *F. hepatica* was present throughout the country. This finding coincides with the recent results from Alstedt et al. (2022), who indicated a higher prevalence of rumen flukes in small ruminants in the north compared to the south of Germany. Besides environmental and climate-associated factors, larger herd sizes and regular treatments against *F. hepatica* have been shown to be positive predictors for rumen fluke infections (Jones et al. 2017). This is consistent with the situation in the north of Germany, where many huge dyke sheep farms are located and regular fasciolicidal treatments are part of the anthelmintic management on these farms. Several associations of owners of dyked land provide an annual financial budget for the sheep farmers responsible for dyke maintenance. This budget can be used for veterinary treatments and medication such as anthelmintics. If this budget is not used up, it lapses at the end of the year (Prof. Dr. Martin Ganter, personal communication). Consequently, this might contribute to an even more frequent use of anthelmintics and particularly fasciolicides in the high-risk areas in the north, facilitating the spread of Paramphistominae due to lower competition for the intermediate host. Moreover, frequent fasciolicidal treatments increase the risk of the development of flukicide resistance.

Paramphistomosis is considered an emerging infectious disease with rising prevalences in ruminants in western Europe (Huson et al. 2017). Since rumen flukes and *F. hepatica* have a similar life cycle (Alstedt et al. 2022) and the most frequently found species in Germany, *C. daubneyi*, share the same intermediate host (Knubben-Schweizer et al. 2021; Jones et al. 2015), a further climate-driven increase of rumen fluke prevalences can also be expected in the future. Many farmers are unaware of the presence of rumen flukes on their farms, but high immature fluke burdens have the potential to cause severe clinical courses (Huson et al. 2017). However, according to own observations, treatments against rumen flukes are presently rather uncommon on German sheep farms. Hence, data about the current occurrence of rumen flukes are valuable in order to suggest the inclusion of treatments

against Paramphistominae in the anthelmintic management of infected flocks and prevent further spreading of these trematodes in this way.

5.4 Investigations on the current anthelmintic resistance situation of *F. hepatica* on German sheep farms

The key subject of the project was the assessment of the current occurrence and distribution of anthelmintic resistance amongst *F. hepatica* populations in Germany with a focus on TCBZ. Triclabendazole resistance constitutes a growing concern worldwide, including European countries close to Germany, e.g. Wales (Daniel et al. 2012; Thomas et al. 2000), Scotland (Kamaludeen et al. 2019; Daniel et al. 2012; Gordon et al. 2012; Mitchell et al. 1998), England (Kamaludeen et al. 2019), Ireland (Hanna et al. 2015; Mooney et al. 2009; Coles et al. 2000), the Netherlands (Borgsteede et al. 2005; Gaasenbeck et al. 2001; Moll et al. 2000), and Spain (Martínez-Valladares et al. 2010; Alvarez-Sánchez et al. 2006) and leads to severe treatment problems, since it is the only flukicide targeting the highly pathogenic immature fluke stages. However, at the beginning of the project, data about the TCBZ susceptibility of *F. hepatica* isolates on German farms was lacking. Hence, the conducted efficacy trials provided valuable first data regarding the efficacy of this important anthelmintic drug in Germany.

Since TCBZ is not licensed to be administered to dairy sheep, an exemption was made for the only dairy sheep farm participating in the study (farm no. 1). On this farm, the efficacy of ABZ against *F. hepatica* was tested instead. Out of the 13 sheep of this flock, four animals were shedding *F. hepatica* eggs on day 0, but only three of these animals showed a cELISA result above the threshold of 2% of the positive control. Since this animal below the threshold also showed a very low FEC of only 0.3 EPG before treatment, the amount of coproantigens might have been too low to reach a cELISA result above the threshold. Like the parasite egg shedding, also the rate of release of coproantigens may fluctuate over time (Brockwell et al. 2013; Valero et al. 2009) and coproantigens might also not be evenly distributed within the faeces. That may explain the discrepancy between the FEC and cELISA results. On this farm, a faecal egg count reduction (FECR) of 94.9% was observed on day 14 with one single animal still showing an EPG of 0.8 and a slightly positive result in the cELISA two weeks p.t. Therefore, all four originally positive animals were resampled again on day 21 and a FECR of 99.87% and a negative cELISA result in all samples was observed three weeks p.t. Thus, it can be concluded that ABZ was fully effective on this farm. The fact, that *F. hepatica* eggs are shed despite successful anthelmintic treatment is a well-known problem regarding the validity of a FECRT (Fairweather 2011b). Due to sequestration of *F. hepatica* eggs in the gall bladder of the host leading to ongoing shedding even after elimination of the flukes (Gordon et al. 2012; Fairweather 2011b; Chowaniec and Darski 1970), the FECRT might provide

false-positive results and may rise doubts regarding the flukicidal efficacy. A similar problem has been observed with the CRT: In individual cases coproantigens can still be detectable even after successful treatment as a result of continued coproantigen release from dead and disintegrating flukes (Hanna et al. 2015; Flanagan et al. 2011a). This was most probably the case with this animal, since both tests showed a negative result on day 21 without further treatment.

On all other farms, the efficacy of TCBZ was evaluated. On farm no. 2, a FECR of 98.4% was already reached on day 15 p.t. Nonetheless, since a few individuals were still shedding a modest number of eggs and one single sample was still slightly above the cELISA threshold on that day, all available sheep were resampled on day 21. Since the FECR increased further up to 99.9% and the FEC of that particular sheep with a slightly positive cELISA result on day 15 was completely negative, the cELISA was not rerun and TCBZ was assessed to be fully effective on this farm.

On farm no. 3, the whole flock consisting of 79 sheep was coproscopically examined on day 0 and day 14 with 71 paired faecal samples in total. Only three individuals showed a positive FEC result on day 0 and all other sheep were negative in the pre- and post-treatment coproscopical examination. The pre-treatment cELISA results confirmed, that only the three coproscopically positive sheep were infected with *F. hepatica*. Therefore, the p.t. cELISA was only conducted with the samples of those respective three sheep as well as with the sample of a ram, even though the ram was negative in both coproscopic analyses but was suspected of fasciolosis due to its poor body condition. All samples were negative on day 14 p.t. The FECR amounted to 99.8%. Thus, TCBZ was evaluated to be fully effective on this farm as well.

An interesting observation regarding the validity of the FECRT was made on farm no. 4. Nine sheep out of the 55 sheep from this flock were positive in the coproscopical examination on day 0. All nine sheep were also positive in the cELISA. A further sheep, which was coproscopically negative, was slightly positive in the cELISA on day 0. This particular sheep might have either been in the prepatent period or only infected with a minimal number of flukes, resulting in an egg shedding intensity below the detection limit. Since one of the coproscopically positive sheep was not available for resampling on day 14, this sheep had to be excluded from the analysis. All of the faecal samples turned out to be negative in the cELISA on day 14, including the sheep which only tested positive in the cELISA before treatment. Regarding the coproscopical analysis, seven out of the eight remaining originally positive animals showed a distinct egg shedding reduction. However, the individual animal with the highest FEC on day 0 (154 EPG) still demonstrated an EPG of 81 on day 14. Since

the p.t. cELISA result of this sheep was negative, it is highly likely that the high amount of parasite eggs did not result from an ongoing infection, but rather from the continued release of eggs retained in the gall bladder as discussed for farms no. 1 and 2. This observation emphasises the necessity of two different diagnostic approaches to test flukicidal efficacy rather than relying on the FECRT alone. When only referring to the coproscopic analysis, the resulting FECR of only 83.4% on herd level would suggest an inadequate efficacy of TCBZ on this farm. Nonetheless, considering the complete reduction of coproantigen release as well as the fact that only one individual animal strongly influenced the FECRT outcome, TCBZ can be assessed as fully effective on this farm. The respective farmer was kindly asked to provide another control sample from this individual animal from day 21 p.t., but due to unknown reasons he did not comply with the request and the egg shedding reduction in this animal three weeks p.t. remains unknown.

The most remarkable efficacy trial within the study was the one on farm no. 6. On this farm, a sudden series of acutely perished animals has been observed by the farmer from the end of October 2021 on. Acute fasciolosis with a multitude of immature flukes was confirmed by pathological examination of several deceased sheep. From this flock, originally consisting of roughly 1300 ewes and lambs, 53 individual animals have been randomly selected as a study population. Most sheep presented in a rather poor condition and with pale mucous membranes. Exactly like on the other farms, the study animals were rectally sampled and treated with 10 mg/kg bw TCBZ on day 0. However, despite the TCBZ treatment, a severe increase instead of a decrease regarding the egg counts as well as the cELISA values until day 14 p.t. was seen. Clinically, a further decline in the condition of the animals was observed and the occurrence of deaths continued. Therefore, failure of the flukicidal treatment was suspected. In order to evaluate, if only a partial TCBZ resistance against the recommended dose of 10 mg/kg bw was present on this farm, the surviving animals of the study population were treated with twice the recommended dose (20 mg TCBZ/kg bw) on day 21. Nonetheless, egg counts as well as cELISA values rose even further in the second set of control samples taken 14 days after the second treatment (day 35) with an ongoing reduction of the study population due to a high mortality within the flock (Figure 7). The fact that animals tested positive (either coproscopically or in the cELISA), which were negative on a previous sampling point, further underlined that the flock was in the early phase of the infection with more and more animals reaching the detection limits at each resampling day.

Figure 7: Sheep died from acute fasciolosis on farm no. 6

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According to these findings it was assumed that the *F. hepatica* isolate on that farm was fully resistant against TCBZ. The respective farmer treated all surviving sheep of this flock on his own with CLOS in various dosages (750 mg per sheep independent of the body weight, resulting in approximately 8.8-20.3 mg/kg bw) on day 46. No samples were available from that particular day of treatment and no information could be obtained about how many individuals of the study population were still alive on day 46. Nonetheless, the farm was revisited on day 70. Eleven out of 53 individuals of the study population were still alive on that day and were resampled again. All cELISA results were negative and all but one of the faecal samples were also coproscopically negative. The only animal still shedding eggs (16 EPG) on day 70 was one of the animals with the highest FEC on day 35 (1223 EPG), so a considerable FECR was also proven in this sheep.

Overall, a drastic clinical course with a high mortality due to massive infections with juvenile TCBZ-resistant flukes has been witnessed on this farm. In total, roughly 300 out of the 1300 sheep died within a few weeks according to the information provided by the farmer.

On the basis of the results from this trial, this *F. hepatica* isolate can be considered to be TCBZ-resistant and CLOS-susceptible, however the CLOS-susceptibility cannot be reliably ascertained since the CLOS treatment was conducted by the farmer himself with heavy deviations from the recommended treatment dosage and the lack of faecal samples from day

46, on which the CLOS treatment was conducted. Still, activity of CLOS against TCBZ-resistant flukes has already been confirmed in the past (Gordon et al. 2012; Coles et al. 2006; Moll et al. 2000). To validate the resistance status of this newly identified field isolate, a controlled efficacy test was conducted. The preserved *F. hepatica* eggs from day 35 (14 days after treatment with twice the recommended TCBZ dosage) were sent to the collaborating laboratory in Liverpool, where *G. truncatula* specimens are kept for laboratory infections. After the infection of the snails, the engendered metacercariae were sent back to our laboratory in Berlin for artificial infections of two lambs. Following administration of TCBZ done twice after 18 and 22 weeks p.i, one of the lambs was euthanised and dissected. The other lamb was euthanised and dissected without prior treatment as a negative control. A complete lack of flukicidal activity of TCBZ against the flukes was confirmed (Pfeiffer, Krücken et al., personal communication). Hence, the first report of TCBZ resistance of a *F. hepatica* field isolate in Germany has been confirmed.

In parallel to the field study, the *Fasciola* miracidium development test (FMDT) was established in our laboratory. The FMDT, also known as *Fasciola* egg hatch test (FEHT) is an *in vitro* assay to test the ovicidal activity of ABZ by means of an inhibitory effect on the development of *F. hepatica* eggs after drug incubation (Ceballos et al. 2019; Novobilský et al., 2016; Canevari et al. 2014; Robles-Pérez et al. 2014; Alvarez et al. 2009). According to Alvarez et al. (2009), this test only works with ABZ, but not with TCBZ due to its high lipophilicity resulting in a poor solubility and thus lacking ability to penetrate the egg shell and accumulate inside the egg. The protocol by Alvarez et al. (2009) was used for the first-time establishment of the FMDT in our laboratory. After the establishment of the FMDT (using *F. hepatica* eggs of an isolate known to be ABZ-susceptible) the FMDT was conducted using the *F. hepatica* eggs originating from farm no. 6, which had been preserved in the dark at 4°C. The test results clearly demonstrated a noticeable ovicidal effect of ABZ on the development of the eggs of this TCBZ-resistant field isolate. This suggests lacking cross-resistance between ABZ and TCBZ in this isolate, which also has already been demonstrated before in current literature: Coles and Stafford (2001) observed a 94% reduction of adult fluke burdens in sheep after ABZ treatment in a TCBZ-resistant *F. hepatica* isolate in their experiments. Very recently, a field study from Argentina showed insufficient efficacy of TCBZ in sheep against a *F. hepatica* field isolate, which was susceptible to CLOS and NITROX *in vivo* as indicated by a FECRT and susceptible to ABZ *in vitro* as indicated by FMDT results (Larroza et al. 2023), so this isolate demonstrated a rather similar response to anthelmintics (TCBZ, ABZ and CLOS) like the German isolate found on farm no. 6.

On all other farms, on which TCBZ was tested (farms no. 5 and 7-12), the drug showed excellent efficacy with FECR reaching up to 99% and no positive samples in the cELISA after 14 days p.t.

5.5 Retrospective assessment of the methods applied for anthelmintic efficacy testing

The combination of two complementary diagnostic approaches (FECRT and cELISA) proved to be appropriate for flukicidal efficacy testing in the field. Several authors questioned the sole use of the FECRT for a valid assessment of the resistance status and suggested the combination with a different diagnostic approach, e.g. the CRT or the FMDT (Novobilský et al. 2016; Hanna et al. 2015; Novobilský and Höglund 2015; Fairweather 2011a; Flanagan et al. 2011b). The results from this study support this recommendation and corroborate that the combination of FECRT and cELISA was highly suitable for the field trial since the methods mutually increase their respective reliability. Both methods show limitations: FEC as well as coproantigen levels must exceed respective thresholds to be detectable and therefore, false-negative results may occur, particularly in low-intensity infections. Those limitations have been demonstrated within the study since the number of coproscopically positive animals and animals positive in the cELISA did not always completely coincide. This may be due to very low egg quantities or very low coproantigen content, so that only one of both methods indicated a positive result in some cases. A further explanation regarding animals, which only tested positive in the cELISA, is that those animals were still in the prepatent period. On the other hand, false-positive results may be found due to ongoing egg shedding from the gall bladder (Gordon et al. 2012; Fairweather 2011b; Chowaniec and Darski 1970) or due to continued coproantigen release from disintegrating flukes (Hanna et al. 2015; Flanagan et al. 2011a) despite successful flukicidal treatment. The latter was shown in two sheep within the course of the study (on farms no. 1 and 2, respectively). Remaining *F. hepatica* eggs, which were still being shed (mostly at a very low level) two weeks p.t., were detected in each of the twelve flocks. Anthelmintic efficacy trials are usually conducted by assessing the efficacy in preferably as many individuals of the flock as possible. The more individuals included in the trial, the lower the impact of false-positive p.t. FEC results in single individual animals on the FECR result for calculating a common efficacy on herd level. However, if specifically single individuals of the flock with a high pre-treatment FEC show no sufficient FEC reduction two weeks p.t., this may strongly influence the FECR outcome on herd level. This was clearly illustrated on farm no. 4, where only one single individual with a false-positive high FEC p.t. decreased the FECR on herd level to only 83.4%, so no sufficient drug efficacy would have been assumed when only relying on the FECRT. In this case, the cELISA results were very useful to figure out if this individual sheep was the only animal in the flock demonstrating an

unsuccessful TCBZ treatment. Regarding this individual sheep, underdosing due to deficient swallowing during drug administration or an infection with a different *F. hepatica* isolate, for instance, if this sheep had newly been introduced to the flock whilst being infected with a TCBZ-r external isolate could have been the reason for an unsuccessful TCBZ treatment. However, the negative cELISA clearly demonstrated that the elimination of the flukes in this animal was as successful as in the other infected sheep of the flock.

At the beginning of the project, several coproscopically positive pre-treatment samples with a cELISA result below the lot-specific cut-off value given by the manufacturer (8% of the positive control in all batch numbers used) were found. Since finding parasite eggs in the faeces is the most reliable diagnostic method for the identification of a patent *F. hepatica* infection (provided that the animal has not been recently treated with a flukicide), the cELISA results were false-negative in these cases when referring to the indicated threshold. No coproscopically positive pre-treatment samples with a cELISA result below 2% of the positive control were found. Therefore, it was decided to use a revised cut-off value of 2% to discriminate between positive and negative cELISA results. This resulted in an enhanced agreement between coproscopy and cELISA, even though the increase in Cohen's kappa coefficient was not significant.

In the current literature, other authors also re-evaluated and lowered the positive cut-off value to increase the diagnostic sensitivity of the BIO-X cELISA (Calvani et al. 2017; Novobilský and Höglund 2015; Brockwell et al. 2014; Palmer et al. 2014; Brockwell et al. 2013). Since two changes have been made to the kit from September 2015 on (personal communication with the manufacturer), a direct comparison with the studies using the prior version of the kit is not possible. The batch of the newer kit used by Calvani et al. (2017) also indicated samples with an OD of > 0.08 (= 8%) as positive. However, the authors observed a poor diagnostic sensitivity of 6-63% in the analysis of bovine faecal samples when referring to the recommended cut-off value. The authors re-evaluated the cut-off value by comparing the manufacturer-recommended threshold to four other calculation approaches and found the highest sensitivity using a threshold, which was calculated by averaging the scaled OD of negative samples and adding two standard deviations of the known negative samples using the FEC data as the assumed gold standard. Using this calculated cut-off value, the sensitivity increased to 65-88%. However, the authors did not indicate the exact calculated threshold values.

Referring to the data collected for this project, no standardised statistical approach was used to accurately calculate a revised diagnostic cut off-value to distinguish between positive and negative cELISA results, but that would be a way for future projects. However, Calvani et al.

(2017) state that the implementation of arbitrary statistical methods is complicated, since these methods might not be usable for each new population being tested and an individual positive threshold must be determined for each batch number and examined species.

5.6 Conclusions and outlook

The key subject of the project was the assessment of the current anthelmintic resistance situation of *F. hepatica* in Germany with a focus on TCBZ. Within the study, the first officially documented incidence of TCBZ resistance in Germany was reported. According to the results, it can be concluded, that TCBZ resistance does occur in Germany, but is currently not widespread.

The observations due to massive infections with TCBZ-resistant juvenile flukes witnessed on farm no. 6 drastically visualise the fatal consequences of flukicide resistance, particularly the consequences of resistance to the currently only available drug effective against the highly pathogenic immature flukes. The demonstrated therapeutic gap poses a serious threat to animal welfare and the economy of sheep farming. In case of an outbreak of acute fasciolosis due to a TCBZ-resistant *F. hepatica* population, causal therapy by means of anthelmintic treatment with an alternative flukicide is only possible after several weeks p.t. From all alternative flukicides currently licensed in Germany, CLOS is the drug which can be used the earliest due to its moderate efficacy against (late) immature flukes (Alvarez et al. 2021; Fairweather and Boray 1999). However, in the very acute phase of infection characterised by liver invasion and migration of the young flukes, TCBZ remains the only flukicide for eliminating the flukes to prevent further physical damage. On farm no. 6, many sheep did not survive this period and died, before the flukes were mature enough to be affected by CLOS.

The results of the field trial indicate, that the efficacy of TCBZ is still sufficient in most regions of the country. To maintain this situation, it is absolutely essential to prevent the spread of TCBZ-resistant *F. hepatica* populations across Germany, so that outbreaks as on farm no. 6 do not recur in the future. Prophylactic measures (grazing management, quarantining newly bought animals, regular coproscopic examinations) as well as a reasonable use of flukicides with subsequent assessment of efficacy is essential. Generally, TCBZ should be used with particular caution and only in cases of infections with predominantly immature flukes in order to reduce the selection pressure for resistance due to frequent administration. When chronic infections with adult fluke stages are present, adulticides (e.g. CLOS, ABZ, OXYCLO) should be the preferred treatment option to spare TCBZ as a reserve anthelmintic for cases of acute fasciolosis.

It is important to continue the investigation with follow-up projects to ensure constant monitoring of flukicidal efficacy throughout the country. This study proved that the FLUKEFINDER® method is well suited for conducting FECRT under field conditions in small ruminants and confirmed that the combination of FECRT and CRT with an interval of 14 days (potentially 21 days) between the sampling points supplies reliable results regarding the treatment success. Hence, follow-up projects can build upon these methods and procedures, complemented by the FMDT, which was established in our laboratory within the course of the project to concurrently test the efficacy of ABZ *in vitro*. Moreover, a key point for a follow-up project is the genetic characterisation by genome sequencing of the *F. hepatica* field isolate found on farm no. 6 to investigate which loci were under selection after the TCBZ treatment and whether genetic similarities to the results from Beesley et al. (2023) can be identified or not. Molecular investigations are pivotal to elucidate resistance mechanisms and to comprehend how resistance develops and spreads among a *F. hepatica* population.

Farmers as well as veterinarians must be sensitised to the consequences of TCBZ resistance. For that reason, a detailed report about the incidents on farm no. 6 has been published in several national journals in 2022 (“Schafzucht”, “Badische Bauern Zeitung”, “Deutsches Tierärzteblatt”, “Vet Impulse”) addressing sheep farmers and veterinarians. It must be made clear that anthelmintic efficacy testing is not only essential in a scientific context, but should be a fundamental element of the routine anthelmintic management on every farm. The regular implementation of efficacy tests is important to immediately notice unsuccessful treatments and to decrease the selection for resistance by adapting the anthelmintic management strategies and the use of flukicides according to the results of the efficacy tests.

The collected data regarding the current occurrence of *F. hepatica* on German sheep farms is also highly valuable in this context to determine, in which regions of the country monitoring is of particular importance due to a high incidence of fasciolosis.

Chapter 6: Summary

Investigations on the current occurrence and anthelmintic resistance situation of *Fasciola hepatica* in German sheep flocks

The common liver fluke *Fasciola hepatica* is an important endoparasite of herbivore mammals with rising prevalence due to climate change. Especially in sheep, fasciolosis constitutes a menace for efficient livestock production and animal welfare due to the severe pathogenic impact the parasite may exert in this species when not treated effectively. Hence, increasing reports of anthelmintic resistance of *F. hepatica* populations pose a threat to sheep farming worldwide. Particularly resistance to triclabendazole (TCBZ), the only flukicide effective against the highly pathogenic juvenile flukes, is of major concern and impedes efficient and immediate treatment of fasciolosis in many countries worldwide including European countries close to Germany.

Since data about the susceptibility of *F. hepatica* to flukicides in Germany were lacking, this was the first study investigating anthelmintic efficacy against this parasite on German sheep farms using Faecal Egg Count Reduction Tests (FECRT) and Coproantigen Reduction Tests (CRT). In parallel to the field trials, data about the current frequency of *F. hepatica* infections on sheep farms with a known history of fasciolosis or a high probability due to moist environmental conditions were collected.

Data regarding the current occurrence of *F. hepatica* clearly show a climate-dependent presence of fasciolosis in Germany. The hot and dry summers in 2018, 2019 and 2020 most probably led to a low infection pressure due to unfavourable conditions for the intermediate host snails and free-living *F. hepatica* stages. Following a mild summer associated with a substantially higher precipitation rate in 2021, a noticeable increase of sheep flocks eligible for the efficacy trial was observed in the following autumn and winter. Generally, *F. hepatica* was found significantly more often in the winter seasons than in the summer seasons. Paramphistominae were found at a lower percentage than *F. hepatica* and without significant differences between summer and winter seasons. In contrast to *F. hepatica*, Paramphistominae were only found in the northern part of the country, whereas *F. hepatica* was found also in the central and southern regions.

Sufficient fasciolicidal efficacy of albendazole (ABZ) was proven on 1/1 farm and TCBZ was efficient on 10/11 farms. On one farm, complete failure of TCBZ, i.e. increasing faecal egg counts and coproantigen levels even in response to double the recommended dose, was observed and was associated with massive animal losses due to acute fasciolosis. The efficacy of ABZ against this isolate was demonstrated *in vitro* using the *Fasciola* miracidium

development test (FMDT). This is the first report of confirmed TCBZ resistance in Germany. The results show, that TCBZ resistance is not widespread in Germany so far, but the observations clearly illustrate the drastic consequences of TCBZ resistance, particularly in case of massive infections with juvenile flukes. Due to the lack of alternative drugs to eliminate juvenile flukes, TCBZ resistance should be considered a serious therapy emergency in sheep and resistance might spread in the future. Hence, follow-up projects are essential for a further monitoring of flukicidal efficacy in Germany. This project constitutes an ideal foundation to build upon the herein presented results. Especially regions, where a higher abundance of *F. hepatica* has been detected should be closely monitored in the future since the development of resistance is more likely there. A particular eye needs to be kept on the region in Lower Saxony, where the first incidence of TCBZ has now been confirmed, as the TCBZ-resistant *F. hepatica* population might spread to neighbouring farms.

Follow-up projects can base on the diagnostic methods (FECRT, CRT, FMDT), which have been established and tested within the course of the study. This study proved that diagnosis of resistance should not be based on one single method only and that the combination of FECRT and CRT is well suitable for testing flukicidal efficacy in the field. The systematic comparison between three coproscopical methods showed that the original FLUKEFINDER® method is the most appropriate approach for conducting FECRT under field conditions on sheep farms since only 2 g of faeces are necessary for this method. When FECRTs are conducted on cattle farms or when faecal material can be gathered using a faecal collection bag, the collection of a sufficient amount of faecal material does not constitute a practical constraint. In these cases, the modified FLUKEFINDER® method using 10 g of faeces would provide even more precise results due to higher raw egg counts.

Molecular investigations and identification of resistance-associated genes in the TCBZ-resistant field isolate should be subject of further research in a follow-up project in order to elucidate the genetic background behind the phenotypic resistance. The key priority for the future should be preventing the spread or new development of TCBZ-resistant *F. hepatica* populations in Germany. Increasing risk of fasciolosis due to climate change in Europe (Caminade et al. 2015) might further aggravate the spread of flukicide resistance. The currently still propitious situation regarding TCBZ efficacy must absolutely be maintained, so that fatal therapy emergency situations as witnessed on farm no. 6 will not be repeated. It is the responsibility of clinical veterinarians, parasitologists, and farmers to avert a nationwide critical resistance situation in the future. Treatment failures must be analysed in detail. When chronic infections with mostly adult flukes are suspected, other flukicides than TCBZ should be the first choice of treatment in order to spare TCBZ as a reserve anthelmintic for cases of acute fasciolosis.

Chapter 7: Zusammenfassung

Untersuchungen zum Vorkommen und zur aktuellen Resistenzsituation von *Fasciola hepatica* in deutschen Schafherden

Der große Leberegel *Fasciola hepatica* ist ein bedeutsamer Endoparasit herbivorer Säugetiere mit wachsender Verbreitung aufgrund klimatischer Bedingungen. Insbesondere bei Schafen stellt die Fasciolose aufgrund der schwerwiegenden klinischen Folgen eine Bedrohung für wirtschaftliche Nutztierhaltung sowie das Tierwohl dar, wenn die Infektion nicht wirksam behandelt wird. Aufgrund dessen sind die zunehmenden Berichte über Anthelminthika-Resistenzen bei *F. hepatica*-Populationen eine weltweite Gefahr für die Schafhaltung. Besorgniserregend sind vor allem Resistenzen gegenüber Triclabendazol (TCBZ), dem einzigen flukiziden Wirkstoff, der auch die besonders pathogenen Juvenilstadien bekämpft, da diese bereits in vielen Ländern einschließlich europäischen Nachbarländern Deutschlands effiziente und sofortige Behandlungsmöglichkeiten erschweren.

Da es an Daten über die Suszeptibilität von *F. hepatica* gegenüber Flukiziden in Deutschland fehlte, war dies die erste Studie, welche die Anthelminthika-Wirksamkeit gegen diesen Parasiten auf deutschen Schafbetrieben untersucht hat. Hierfür wurden Eizahl-Reduktionstests (Faecal Egg Count Reduction Tests, FECRT) und Coproantigen-Reduktionstests (Coproantigen Reduction Tests, CRT) durchgeführt. Parallel zu den Feldversuchen wurden Daten zur aktuellen Vorkommenshäufigkeit von *F. hepatica*-Infektionen auf Schafbetrieben mit bekannten Infektionen in der Vergangenheit oder einer hohen Infektionswahrscheinlichkeit aufgrund feuchter Weidebedingungen erhoben.

Die Daten zur aktuellen Verbreitung von *F. hepatica* zeigen eine deutlich wetterabhängige Vorkommenshäufigkeit der Fasciolose in Deutschland. Die heißen und trockenen Sommer in den Jahren 2018, 2019 und 2020 führten mit hoher Wahrscheinlichkeit zu einem sehr niedrigen Infektionsdruck aufgrund ungünstiger Überlebensbedingungen für die Zwischenwirtsschnecken und freilebende *F. hepatica*-Stadien. Nach einem sehr milden und niederschlagsreichen Sommer im Jahr 2021 wurde im darauffolgenden Herbst und Winter ein deutlicher Anstieg der Anzahl infizierter Schafherden beobachtet, welche für die Feldstudie geeignet waren. Insgesamt wurde *F. hepatica* signifikant häufiger in den Winter- als in den Sommersaisons gefunden. Paramphistominae wurden mit geringerer Vorkommenshäufigkeit und ohne signifikante Unterschiede zwischen Winter- und Sommersaisons detektiert. Im Gegensatz zu *F. hepatica* wurden Paramphistominae nur in

den nördlichen Regionen Deutschlands gefunden, wohingegen *F. hepatica* auch in Mittel- und Süddeutschland festgestellt wurde.

Eine adäquate Wirksamkeit von Albendazol (ABZ) wurde auf 1/1 Betrieb nachgewiesen. Triclabendazol zeigte auf 10/11 Betrieben eine gute Wirksamkeit. Auf einem Betrieb wurde ein vollkommenes Therapieversagen von TCBZ durch steigende Eizahlen und Coproantigen-Werte trotz einer zweiten Behandlung mit der doppelten Dosis von TCBZ festgestellt. Auf diesem Betrieb kam es zu einer massiven Anzahl an Todesfällen durch akute Fasciolosen. Die vorhandene Wirksamkeit von ABZ gegen dieses Isolat wurde *in vitro* durch den *Fasciola*-Miracidium-Entwicklungstest (*Fasciola* miracidium development test, FMDT) demonstriert. Dies ist der erste Nachweis von bestätigter TCBZ-Resistenz in Deutschland. Die Ergebnisse der Studie zeigen, dass TCBZ-Resistenz noch nicht weitverbreitet in Deutschland ist, jedoch bilden die Beobachtungen deutlich die drastischen Konsequenzen einer TCBZ-Resistenz ab, insbesondere bei Vorliegen von Masseninfektionen mit juvenilen Leberegeln. Aufgrund des Mangels an Alternativwirkstoffen zur Bekämpfung der Juvenilstadien muss die Resistenz gegenüber TCBZ als ernstzunehmender Therapie-Notfall angesehen werden und Resistenzen könnten sich in der Zukunft noch weiterverbreiten. Daher sind Folgeprojekte essentiell, um die Anthelminthika-Wirksamkeit in Deutschland weiter zu beobachten. Dieses Projekt stellt eine ideale Grundlage hierfür dar, um auf den hier dargestellten Ergebnissen aufzubauen. Insbesondere Regionen mit einer hohen Vorkommenshäufigkeit von *F. hepatica* sollten genau überwacht werden, da die Entstehung von Resistenzen dort wahrscheinlicher ist. Ein besonderes Augenmerk muss auf die Region in Niedersachsen gelegt werden, wo der erste TCBZ-Resistenzfall nun nachgewiesen wurde, da sich diese TCBZ-resistente *F. hepatica*-Population auf benachbarte Betriebe ausbreiten könnte.

Folgeprojekte können auf den diagnostischen Methoden (FECRT, CRT und FMDT), welche im Rahmen dieser Studie etabliert worden sind, aufbauen. Diese Studie bestätigte, dass sich Resistenzdiagnostik nicht nur auf eine einzelne Methodik stützen sollte und dass die Kombination aus FECRT und CRT gut geeignet ist, um die Flukizid-Wirksamkeit im Rahmen von Feldstudien zu untersuchen. Der systematische Vergleich von drei koproskopischen Methoden zeigte, dass das Standardprotokoll der FLUKEFINDER®-Methode für die Durchführung eines FECRT bei Schafen unter Feldbedingungen am geeignetsten ist, da nur 2 g Kot für diese Methode benötigt werden. Wenn ein FECRT bei Rindern durchgeführt wird oder wenn das Kotmaterial mithilfe eines Kotbeutels gesammelt werden kann, stellt die Gewinnung einer ausreichenden Menge an Kot keine praktische Einschränkung dar. In solchen Fällen würde die modifizierte FLUKEFINDER®-Methode, bei der 10 g Kot genutzt wird, aufgrund höherer Eizahlen in den Rohdaten noch präzisiere Ergebnisse liefern.

Molekularbiologische Untersuchungen und die Identifikation von resistenzassoziierten Genen in dem gefundenen TCBZ-resistenten Isolat sollen Teil eines Folgeprojektes werden, um den genetischen Hintergrund hinter der phänotypisch beobachteten Resistenz aufzuklären.

Die Vorbeugung der Ausbreitung oder Neuentstehung von TCBZ-resistenten *F. hepatica*-Populationen in Deutschland sollte oberste Priorität für die Zukunft sein. Das steigende Risiko von Fasciolosen aufgrund von Klimawandel in Europa (Caminade et al. 2015) könnte die weitere Verbreitung von Flukizid-Resistenz verstärken. Die aktuell noch günstige Situation hinsichtlich der Wirksamkeit von TCBZ in Deutschland muss unbedingt aufrechterhalten werden, damit fatale Therapienotstand-Situationen wie auf Betrieb Nr. 6 sich nicht wiederholen. Es liegt in der Verantwortung von Tierärzt*innen, Parasitolog*innen und Landwirt*innen, eine landesweit kritische Resistenzsituation in der Zukunft zu vermeiden. Ein Vorkommen von Therapieversagen muss genau analysiert werden. Sofern chronische Fasciolosen mit hauptsächlich adulten Leberegel-Stadien vermutet werden, sollten andere Flukizide anstatt TCBZ die erste Wahl zur Behandlung sein, um TCBZ als Reserve-Anthelminthikum für Fälle von akuten Fasciolosen zu bewahren.

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Alexandra Kahl, Georg von Samson-Himmelstjerna, Christina Helm, Jane Hodgkinson, Diana Williams, Wiebke Weiher, Werner Terhalle, Martin Ganter, Stephan Steuber, Jürgen Krücken (2023) **First occurrence of triclabendazole resistance in *Fasciola hepatica* on German sheep farms.** Oral presentation by Dr. Jürgen Krücken. 29th International Conference of the World Association for the Advancement of Veterinary Parasitology 20th - 24th August 2023, Chennai, India, abstract book page 120

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Conflicts of interest

In the context of this work, there are no conflicts of interest due to contributions from third parties.

Statement of authorship

I hereby confirm that I have prepared this thesis independently. I certify that I have used only the sources and assistance indicated. No other person’s work has been used without due acknowledgement in this dissertation. This dissertation has not been submitted for the award of any other degree or diploma in any other tertiary institution.

Berlin, 20.12.2024 Alexandra Kahl