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DOI 10.2376/1439-0299-2026-10
Clinic for Ruminants with Ambulatory and Herd Health Services, Ludwig-Maximilians-Universität München, Germany
Peer-reviewed | Eingegangen: 03.10.2025 | Angenommen: 27.02.2026 | Veröffentlicht: 31.03.2026

Clinical course of *Calicophoron daubneyi* infection in experimentally infected sheep

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Summary This study investigated the clinical course of *Calicophoron daubneyi* infection in experimentally infected sheep. Three helminth-free White Bavarian Alpine ewe lambs aged 7 to 10 months were infected with 150 *C. daubneyi* metacercariae each, while two lambs served as uninfected controls. All animals were housed without any access to pasture and monitored daily for clinical signs. Weekly blood samples were analysed for biochemical and haematological parameters. Faecal samples were examined using the Flukefinder® technique. Egg excretion began in two infected lambs at 86 days post infection (p. i.), but only persisted in one animal, lasting longer than 30 months. In the other infected lamb, the patency ended after the first shedding. One inoculated lamb showed no egg excretion and was excluded from the analysis. No clinical signs were observed in any of the animals during the study. The average daily weight gain from day 1 to day 108 p. i. was 84.5 g in infected lambs and 108.5 g in control lambs ($P = 0.421$). Blood parameters remained largely within reference ranges, with no significant differences between infected and non-infected lambs. These results indicate that infection with a low number of *C. daubneyi* metacercariae does not lead to clinical paramphistomidosis nor abnormalities in haematology or clinical chemistry.

Keywords rumen fluke, paramphistomidosis, haematology, clinical chemistry, patency

Klinischer Verlauf einer experimentellen Infektion mit *Calicophoron daubneyi* bei Schafen

Zusammenfassung In dieser Studie wurde der klinische Verlauf einer *Calicophoron daubneyi*-Infektion bei experimentell infizierten Schafen untersucht. Drei helminthenfreie Weiße Bayerische Bergschaflämmer wurden im Alter von sieben bis zehn Monaten jeweils mit 150 *C. daubneyi*-Metazerkarien infiziert, während zwei Lämmer als nicht infizierte Kontrollen dienten. Alle Tiere wurden in Ställen ohne Weidegang untergebracht und täglich klinisch untersucht. Wöchentliche Blutproben wurden auf biochemische und hämatologische Parameter untersucht. Die Kotproben wurden mit der Flukefinder®-Technik untersucht. Die Eiausscheidung begann bei zwei infizierten Lämmern 86 Tage nach der Infektion, hielt aber nur bei einem Lamm an und dauerte bei diesem Tier über 30 Monate. Die Patenz endete bei dem anderen Lamm nach der ersten Eiausscheidung. Ein inokuliertes Lamm zeigte keine Eiausscheidung und wurde von der Auswertung ausgeschlossen. Während der Studie wurden keine klinischen Veränderungen beobachtet. Vom 1. bis 108. Tag nach der Infektion betrug die tägliche Gewichtszunahme im Durchschnitt 84,5 g bei den infizierten und 108,5 g bei den Kontrolllämmern ($P = 0{,}421$). Die Blutparameter blieben weitgehend innerhalb der Referenzbereiche und wiesen keine signifikanten Unterschiede zwischen infizierten und nicht infizierten Lämmern auf. Diese Ergebnisse deuten darauf hin, dass eine experimentelle Infektion mit einer niedrigen Dosis *C. daubneyi*-Metazerkarien nicht zu klinischer Paramphistomidose und hämatologischen oder klinisch-chemischen Abweichungen führt.

Schlüsselwörter Pansenegel, Paramphistomidose, Hämatologie, klinische Chemie, Patenz

Introduction

In recent years, an increase in the prevalence of the rumen fluke *Calicophoron daubneyi* has been observed in Europe. This trematode is a member of the Paramphistomidae family and infects sheep and other grazing ruminants (Huson et al. 2017). *Calicophoron daubneyi* has a heteroxenic life cycle, with the aquatic snail *Galba truncatula* serving as intermediate host. After ingestion of metacercariae, the infection progresses in two phases: in the first, intestinal phase, newly excysted juvenile (NEJ) flukes adhere to the duodenal mucosa and undergo a maturation phase which lasts several weeks. They then migrate back into the rumen to attach themselves to the ruminal and reticular mucosa as adult flukes. Mortality in sheep depends on the severity of infection, but it is assumed that the second, ruminal phase is clinically inapparent even with a high rumen fluke burden (Busin et al. 2023). However, sheep are relatively susceptible to clinical disease during the intestinal phase throughout their lives. To the authors' knowledge, cases of intestinal paramphistomidosis in sheep have so far only been published from the British Isles (Mason et al. 2012, Toolan et al. 2015, SAC Consulting Veterinary Services 2018, AFBI 2023) but not from mainland Europe. Heavy rainfall, which favoured a strong accumulation of metacercariae on pasture in the intermediate host habitats, was suspected as the cause of an increased mortality due to paramphistomidosis in Ireland (Toolan et al. 2015).

So far, the clinical course of paramphistomidosis in sheep has predominantly been studied in species other than *C. daubneyi* within the Paramphistomidae family. For *Paramphistomum microbothrium,* 170,000 metacercariae led to progressive anorexia from the sixth day post infection (p. i.). From day 14 p. i., liquid, foetid diarrhoea occurred. At the same time, blood samples showed a drop in total protein and albumin, and subsequently also in calcium levels. Due to dehydration, the plasma volume decreased and haemoglobin, haematocrit, red cell count and the total volume of circulating erythrocytes increased. Submandibular oedema was rare and anaemia never occurred. All untreated animals died following this high infection dose. In contrast, no clinical signs were observed following infection with less than 20,000 *P. microbothrium* metacercariae. The clinical course was however fatal for infection doses of 40,000 metacercariae or more (Horak 1967). In a lamb infected with 40,000 *Paramphistomum ichikawai* metacercariae, severe destruction of the intestinal mucosa was seen at necropsy on day 42 p. i., whereas inappetence, weight loss and lethargy only occurred from day 65 p. i. onwards (Rolfe et al. 1994). Infections with *Paramphistomum cervi*, which was assumed to be the dominant rumen fluke species in Germany in the last century, led to temporary diarrhoea and apathy following an infection dose of 30,000 metacercariae. Haematocrit, haemoglobin, erythrocyte and leukocyte counts were unchanged compared to control animals, but weight gain was lower in the infected group (Boch et al. 1983). Pathological-anatomical evidence of haemorrhagic enteritis could already be seen following a dose of 1,500 *P. cervi* metacercariae (Kraneburg and Boch 1978). Specific information on the clinical course, haematological and necropsy findings of *C. daubneyi* infections has been investigated in one French study. In 14 lambs naturally infected with 'several thousand' *C. daubneyi* flukes, three histopathological phases were observed during the course of the infection: an early non-specific eosinophilic reaction, followed by an extension of parasite-induced lesions showing a significant inflammatory reaction, and finally a healing phase associated with parasite migration to the rumen (Devos et al. 2013).

Since information on the subclinical course, haematological parameters, and weight gain associated with *C. daubneyi* infection in sheep is currently very limited, the aim of this study was to analyse data from a small number of lambs infected with a low dose of *C. daubneyi* metacercariae in order to obtain initial indications for assessing the significance of subclinical levels of infection in terms of their effects on blood values and weight gain. In addition, a contribution is made to the longevity of *C. daubneyi* in sheep.

Material and methods

All experimental procedures involving lambs were approved by the government of Upper Bavaria under the reference number ROB55.2–2532.Vet_02–20–175. Three indoor-reared helminth-free White Bavarian Alpine ewe lambs aged 7 – 10 months originating from the university's own sheep flock were each inoculated with 150 *C. daubneyi* metacercariae. The metacercariae were produced under laboratory conditions (Wenzel et al. 2025), and the low dose was intentionally chosen to avoid the induction of clinical signs (Paraud et al. 2009), allowing the assessment of whether subclinical effects, such as haematological changes, could nevertheless be detected.

Two lambs from the same flock served as controls. The lambs were randomly mixed and housed in two free range pens on straw bedding without any access to pasture. Clinical examiners and laboratory staff were blinded to the infection status of the animals. In one inoculated lamb, no rumen fluke eggs were detected in the faeces at any time during the trial. It was therefore assumed that it had not become patently infected. This lamb was thus excluded from the following analyses. Data analysed for this study therefore originated from two infected and two control lambs. All animals were clinically examined daily (general condition, cardiac and pulmonary auscultation, rectal temperature), and faecal consistency and feed intake were noted by observation. Once a week, blood samples were taken and analysed for calcium, phosphorus, total protein, and albumin (Cobas c311, Roche, Basel, Suisse) according

to Mavenyengwa et al. (2010), as well as for erythrocytes, leucocytes, haematocrit, and haemoglobin (VetScan HM5, Abaxis, Union City, CA, USA) according to Boch et al. (1983). The lambs were weighed four times during the study: at the time of infection, and on days 36, 78, and 108 p. i. From day 65 p. i. onwards, faecal samples were taken and examined using the Flukefinder® technique (Soda Springs, ID, USA) to detect trematode eggs according to the manufacturer's instructions. The trematode faecal egg count (FEC) was assessed in eggs per gram (EPG) faeces.

Statistical analyses

Although the sample size was low for statistical analyses, haematological parameters and weight gain of the lambs were analysed using R version 4.3.1 (The R Foundation for Statistical Computing, Vienna, Austria) in order to explore potential effects and generate hypotheses for future studies with greater statistical power. To differentiate between different phases of the infection, the blood values were assigned to the following four phases according to necropsy findings by Devos et al. (2013): pre-infection (sample taken 5 days prior to infection), intestinal phase (samples taken on days 6, 14, 20, 27, 35 p. i.), migration phase (samples taken on days 42, 48, 55, 62, 69, 76, 83 p. i.), and patency (samples taken on days 90, 97, 104 p. i.). The data were expressed as estimated marginal means of blood values from every phase. Daily weight gain was calculated for each animal with the formula:

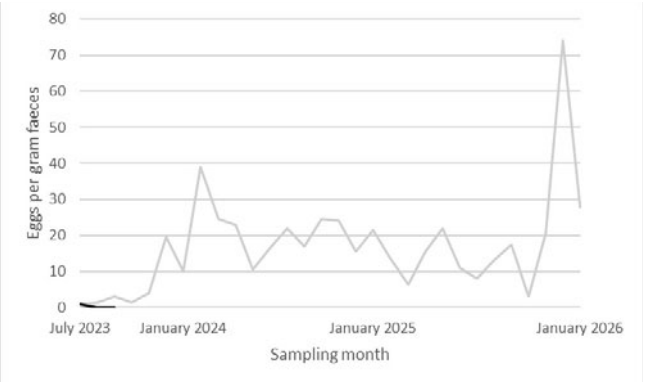
$$\text{Weight gain (g)} = \frac{\text{live weight at day 108 - live weight at day 1}}{108}$$

and expressed as estimated marginal means of infected and non-infected lambs. The significance of any differences between the infected and non-infected animals were assessed via linear mixed effects models (daily weight gain) or via robust linear mixed effects models (haematological parameters) to account for the repeated measures on the same animals. P values lower than 0.05 were considered significant, and all P values were corrected for multiple comparisons by the Tukey method.

Results

Prepatency, patency, clinical signs and daily weight gain

Egg excretion was first detected on day 86 p. i. in two lambs. Only one animal, however, continued to excrete and has consistently been shedding eggs until the time of writing (i.e. for 30 months) (▶ Fig. 1). No clinical signs were observed in any lamb during this study. The mean rectal temperature during prepatency was 38.8°C (SD 0.2°C) for infected and 39.0°C (SD 0.2°C) for non-infected lambs. Heart and lung auscultation remained normal and feed intake was rated as good throughout the observation period. The faeces were always normal in the form of isolated or agglomerated droppings. From the day of infection until day 108 p. i., the control lambs gained 108.5 grams (lower confidence level [LCL] 36.0 – upper confidence level [UCL] 181.0) and the infected lambs gained 84.5 grams (LCL 12.1 – UCL 157.0) body weight per day ($P = 0.421$).



Graphic: Christoph Wenzel

Figure 1: Egg excretion in eggs per gram faeces of two lambs, each experimentally infected with 150 *Calicophoron daubneyi* metacercariae. Grey line: Lamb with continuous egg excretion, black line: Lamb with single egg excretion in July 2023. Faecal sampling was completed in this animal in October 2023.

Clinical chemistry and haematology

Mean blood values from the different phases of the infection remained within the reference ranges (Bostedt et al. 2019), except for elevated albumin values in both groups in all phases. Estimated marginal means of the biochemical and haematological parameters of the different phases showed no significant differences between infected and non-infected animals (▶ Table 1).

Discussion

Several studies have investigated the effects of infection with different species of rumen flukes on blood parameters in ruminants (Horak and Clark 1963, Horak 1966, Singh et al. 1984, Misra et al. 1996, Mavenyengwa et al. 2010, Devos et al. 2013, Chauhan et al. 2015). However, all selected infection doses were within a range at which clinical signs would be expected. In the case of infection with *C. daubneyi*, the infection dose to induce clinical signs in experimentally infected sheep is currently unknown. We therefore deliberately chose a dose low enough that it was not expected to cause any clinical signs when infecting the lambs (Paraud et al. 2009). This expectation was confirmed. Regarding the question whether changes in selected biochemical and haematological parameters can already be observed in subclinical infections, our detailed analysis suggests that there are no

Tab. 1: Estimated marginal means (with lower and upper confidence limits in parentheses) of selected blood chemistry and haematology values of two lambs, each inoculated with 150 *Calicophoron daubneyi* metacercariae, from phases before (pre-phase) and after (intestinal, migration, patency phase) inoculation, compared to non-infected lambs (Reference ranges according to Bostedt et al. 2019).

		Infection Phase			
	Infected/ non-infected	Pre	Intestinal	Migration	Patency
Calcium	inf.	2.5 (2.3–2.7)	2.6 (2.5–2.8)	2.6 (2.4–2.7)	2.6 (2.4–2.7)
(2.0–3.0 mmol/L)	n. inf.	2.6 (2.4–2.8)	2.6 (2.4–2.8)	2.6 (2.4–2.7)	2.6 (2.4–2.8)
Phosphorus	inf.	2.4 (1.7–3.2)	2.1 (1.5–2.7)	2.2 (1.7–2.8)	2.5 (1.9–3.1)
(0.7–3.1 mmol/L)	n. inf.	2.5 (1.8–3.3)	2.1 (1.5–2.7)	2.3 (1.8–2.9)	2.3 (1.7–3.0)
Total Protein	inf.	63.4 (55.7–71.0)	62.3 (53.0–71.5)	62.5 (53.0–72.0)	64.8 (56.0–73.6)
(51–73 g/L)	n. inf.	65.0 (57.4–72.7)	61.2 (51.9–70.5)	62.4 (52.9–71.9)	67.8 (59.1–76.6)
Albumin	inf.	39.9 (37.6–42.1)	40.6 (38.8–42.3)	40.3 (38.6–41.9)	40.5 (38.7–42.3)
(35–38 g/L)	n. inf.	41.0 (38.7–43.2)	38.8 (37.1–40.6)	39.7 (38.0–41.4)	41.3 (39.5–43.1)
Erythrocytes	inf.	12.3 (10.5–14.1)	11.2 (9.51–12.9)	10.9 (9.17–12.5)	12.0 (10.6–13.7)
(8.7–12.9 x103/µL)	n. inf.	14.4 (12.4–16.3)	11.1 (9.4–12.8)	10.4 (8.7–12.1)	12.1 (10.4–13.8)
Leucocytes	inf.	10.8 (7.4–14.2)	7.8 (5.3–10.4)	7.3 (4.8–9.8)	8.0 (5.3–10.7)
(2.7–13.0 x103/µL)	n. inf.	10.1 (5.9–14.4)	6.8 (4.2–9.3)	6.2 (3.8–8.7)	7.4 (4.7–10.1)
Haematocrit	inf.	33.5 (26.3–40.6)	32.4 (23.8–41.0)	31.7 (22.8–40.5)	32.5 (24.4–40.7)
(25.0–41.0 %)	n. inf.	38.4 (31.4–45.3)	32.1 (23.5–40.7)	29.9 (21.0–38.7)	33.4 (25.2–41.6)
Haemoglobin	inf.	12.0 (10.1–13.9)	10.6 (8.8–12.3)	10.2 (8.4–11.9)	10.4 (8.6–12.2)
(8.5–13.3 g/dL)	n. inf.	14.3 (12.3–16.4)	10.6 (8.8–12.4)	9.9 (8.1–11.7)	10.8 (9.0–12.6)

differences between infected and non-infected sheep. Total protein, albumin, calcium, and phosphorus are important parameters in rumen fluke infections, as the pathogenic mechanism of attachment to the intestinal mucosa by NEJ flukes leads to strangulation, necrosis and haemorrhage (Rolfe et al. 1994), resulting in protein loss and loss of protein-bound minerals (Horak and Clark 1963, Mavenyengwa et al. 2010). The significance of a blood count is no less important than biochemical variables in parasitic infections. The mean values for erythrocytes, leucocytes, haemoglobin, and haematocrit varied within the normal reference ranges in all phases in the examined sheep, a finding which is in accordance with the results of Boch et al. (1983), while other studies did show a tendency towards anaemia in sheep and cattle (Mavenyengwa et al. 2010, Dorny et al. 2011, Devos et al. 2013). The results suggest that infection with more than 150 metacercariae is required to cause haematological alterations. In future studies, it would be helpful to link changes in haematological parameters (e.g., the haematocrit value) to subclinical infections in order to take appropriate preventive and control measures (Dorny et al. 2011). For practical use, a complete blood count without differential appears to be sufficient. However, since eosinophils in particular play an important role in the response to the attachment of NEJ flukes in the intestine (Rolfe et al. 1994), a differential should be performed in subsequent trials.

Weight data has shown no differences between infected and control animals over the whole observation period. Although sheep were weighed according to the infection phases of Devos et al. (2013), number of weight data was too small for statistical evaluation. However, weight gain should be taken into account in the future in order to assess the significance of subclinical infection levels in terms of their impact on productivity. Patency was short-lived in one infected animal, but has been persistent to the time of writing (30 months) in the other, indicating the potential longevity of the parasite and emphasizing the important role of sheep in the maintenance of the infection cycle.

Acknowledgments

The authors thank the staff of the small ruminant department, the laboratory team and the veterinary care team at the Clinic for Ruminants with Ambulatory and Herd Health Services of LMU Munich, for their help in conducting this study.

Ethical approval

The authors hereby declare that they have followed the accepted guidelines of good scientific and good veterinary practice while preparing the present paper. All relevant international, national and institutional ethical guidelines for the handling of the animals used in the study were fol-

lowed. Details of the animal welfare applications and their approval are given in the Materials and Methods section of the published text.

Conflict of interest

The authors hereby declare that they have no proprietary, professional or other personal interests in any product, service and/or company that could have influenced the contents or opinions expressed in this publication.

Funding

No external funding was received for this study.

Authors‘ contribution

Conceptualization and study design: CW, GKS; Data acquisition: CW, VE, KV, SH; Data analysis and interpretation: CW, VE, KV, GKS, FW, YZ; First draft of manuscript: CW; Critical revision and final approval of manuscript: VE, KV, GKS, FW, SH, YZ.

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