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Establishment of the complete life cycle of *Calicophoron daubneyi* under experimental conditions

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ABSTRACT

The complex life cycle of the rumen fluke *Calicophoron daubneyi* is similar to that of the liver fluke *Fasciola hepatica*. Interestingly, *C. daubneyi* and *F. hepatica* share the same intermediate host, *Galba truncatula*. However, in contrast to its relative, experimental production of metacercariae is a major challenge for *C. daubneyi*, hampering a detailed analysis of its life cycle, especially in the definitive host. *G. truncatula* snails collected from natural habitats were bred in glass Petri dishes and fed dried organic lettuce leaves. *C. daubneyi* eggs were obtained from feces of naturally infected cattle and incubated until miracidia were hatching. Subsequently, these miracidia were allowed to infect snails, which were kept under specific laboratory conditions to monitor the shedding of metacercariae. In total, 177 *G. truncatula* snails were exposed to *C. daubneyi* miracidia during eleven snail infection trials. Sixty-eight of these snails survived for longer than 30 days post-infection (p.i.). From day 35 p.i., seven snails from five trials started shedding an average number of 106 metacercariae (range: 38–186) per snail. Three ewe lambs (aged 7–10 months) were inoculated orally with 150 metacercariae each. A different batch of metacercariae (obtained from three different snail trials) was used for each lamb. Another two lambs served as controls. All animals were regularly examined clinically, hematologically and coproscopically, using sedimentation techniques for the detection of trematode eggs. Low numbers of *C. daubneyi* eggs were detected in fecal samples of two of the three inoculated lambs on day 86 post-inoculation (yielding $\leq$ 2 epg), but only one lamb continued to shed eggs (up to 6 epg) until the end of the experiment (day 104 post-inoculation). None of the animals showed any abnormal clinical findings or blood parameters throughout the course of the study. Production of *C. daubneyi* metacercariae under laboratory conditions is reported, followed by experimental infection of the definitive host, thus completing the full life cycle of this parasite under experimental conditions. However, neither the survival rate of the snails nor the amount of metacercariae produced were comparable to previously published experiments using *F. hepatica*, necessitating further optimization of the laboratory protocols. Nevertheless, the results can serve as a starting point for more in-depth studies of this increasingly important trematode.

1. Introduction

Paramphistomidosis is caused by various rumen fluke species, such as, for instance, *Paramphistomum leydeni*, *P. cervi* or *Calicophoron daubneyi*. In recent years, *C. daubneyi* emerged as the predominant rumen fluke species in Europe (Martinez-Ibeas et al., 2016; Forstmaier et al., 2021; Huson et al., 2017; Cervena et al., 2022; Bosco et al., 2021), and the mud snail *Galba truncatula* is the most important intermediate host (Jones et al., 2015).

As for most trematodes, the life cycle of *C. daubneyi* includes an

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asexual phase, which involves free-living parasite stages and stages within the intermediate host, with multiplication occurring inside the intermediate host. During the sexual phase, reproduction takes place inside the definitive host. Interestingly, *C. daubneyi* and *Fasciola hepatica* share the same intermediate host, *G. truncatula*. Although larval stages of *F. hepatica* or *C. daubneyi* have also been identified in other snail species such as *Lymnaea glabra* or *Planorbis leucostoma*, the prevalence and cercarial productivity were highest in *G. truncatula* (Abrous et al., 2000; Vignoles et al., 2002).

While extensive research has been carried out to establish protocols for the infection of *G. truncatula* with *F. hepatica* under laboratory conditions (Boray, 1969; Taylor and Mozely, 1948; Gold, 1980; Abrous et al., 1999; Dreyfuss et al., 2015; Moazeni et al., 2018), similar knowledge involving *C. daubneyi* is still very limited. To date, no reliable protocol for the experimental production of metacercariae has been established, hampering a detailed and controlled analysis of its life cycle, and experimental infection of its definitive hosts.

There is some, albeit limited, published information regarding the clinical course of paramphistomidosis from previous studies involving experimental infections of small ruminants. The species *P. microbothrium*, *P. cervi, P. ichikawai* and *P. daubneyi* (generic name for *C. daubneyi* used in older publications) were used in these studies (Horak, 1967; Kraneburg and Boch, 1978; Rolfe et al., 1994; Singh et al., 1984; Vignoles et al., 2008): After ingestion of metacercariae, there is an initial intestinal phase, followed by a ruminal phase. The severity of any clinical signs depends on the number of metacercariae ingested. The latter excyst in the abomasum, presumably under the influence of pepsin, and then migrate as newly excysted juvenile (NEJ) flukes to the proximal duodenum (Huson et al., 2018). The immature flukes, pinkish in color and 2–3 mm in size, use their acetabulum for attachment and are thought to feed on the intestinal mucosa (Deorani and Jain, 1969). In sheep naturally infected with *C. daubneyi* by grazing, no clinical signs were observed except for soft feces on day 9 and from day 38–52 after the start of exposure to rumen fluke infested pasture, although hemorrhagic mucosal lesions occurred and several thousand immature paramphistomes were visible at necropsy (Devos et al., 2013). For *P. ichikawai*, it is suggested that the presence of 20.000–25.000 NEJ flukes results in clinical disease (Rolfe et al., 1994). Unlike adult cattle, adult sheep are also affected by the intestinal phase (Mason et al., 2012), because they seemingly do not develop sufficient immunity (Horak, 1967). Attachment of NEJ flukes to the small intestine can lead to thickening of the intestinal wall, necrosis and hemorrhage resulting in watery diarrhea with a fetid smell (Roach and Lopes, 1966). High infectious doses of the Australian species *P. ichikawai* lead to colonization of most of the jejunum and prolonged the time NEJ flukes remained in the intestine (Rolfe et al., 1994).

Laboratory analyses showed a marked drop in total plasma protein concentration, hypalbuminemia and decreased plasma calcium when sheep were infected with high numbers of *P. microbothrium* metacercariae (Horak and Clark, 1963). In contrast, hematological values and plasma proteins remained within the reference ranges in sheep naturally infected with *C. daubneyi*, but packed cell volume (PCV) and red blood cells (RBC) decreased until day nine, and plasma proteins decreased until day 38 after exposure to fluke infested pasture. All parameters subsequently returned to normal values (Devos et al., 2013).

The NEJ flukes undergo a maturation phase in the small intestine. After 1.5 to two months, this is followed by retrograde migration to the rumen (Devos et al., 2013), where they attach to the ruminal wall and start egg production. Another study showed that once the migration of juvenile flukes into the rumen had been completed, the intestinal mucosa no longer showed any lesions (Busin et al., 2023). The colonization of the rumen is considered to be clinically insignificant. This is in accordance with pathological studies, which did not report any changes to ruminal papillar density and length (Busin et al., 2023). The only macroscopic lesions observed during the ruminal phase were irregular papillae and a slightly edematous and inflamed ruminal mucosa (Devos et al., 2013), but nutrient digestibility in chronically infected sheep remained unaffected (Rutherford et al., 2022).

Although experimental infections of small ruminants with *C. daubneyi* have been attempted, many basic questions remain unanswered, such as the parasite's prepatent period, which is assumed to be 10–12 weeks (Fenemore et al., 2021). In naturally infected lambs, no eggs were detected up to 80 days after exposure to rumen fluke infected pasture (Devos et al., 2013). A goat kid infected with 1.500 *C. daubneyi* metacercariae excreted eggs in week 12 and 13 post-inoculation (Sey, 1977). In another study, goats infected with 250 *C. daubneyi* metacercariae started shedding eggs on day 131 (i.e., at about 18 weeks post-inoculation) (Paraud et al., 2009). Variations in the prepatent period were also seen in studies on *P. microbothrium* (Horak, 1967).

In order to facilitate future studies regarding the biology, epidemiology, pathogenicity, diagnostic methods and immunology of *C. daubneyi* infections (Atcheson et al., 2020), the primary aim of this study was the establishment of the entire life cycle of this parasite under standardized experimental conditions. In doing so, we aimed to determine the prepatency of *C. daubneyi* in sheep, the infectivity of metacercariae of different ages, and to confirm if low parasite burdens do indeed result in no clinical signs in sheep.

2. Material and methods

2.1. Origin of the intermediate host snails, miracidia and adult rumen flukes

The second generation of *G. truncatula* bred under controlled laboratory conditions with a shell height of at least 4 mm was used for eleven infection trials of the intermediate host. The population was originally collected from three natural habitats in Bavaria (Southern Germany), which were identified based on indicator plants and the snails' preferred environmental characteristics (Mehl, 1932; Rondelaud et al., 2011). From these sites, a total of 242 *G. truncatula* snails with a shell size of 2–5 mm had been initially collected. The identification of the snail species was carried out based on shell morphology (Mehl, 1932) directly on site.

To obtain miracidia, eggs were extracted from bovine fecal samples using the sedimentation method. These samples originated from herds known to be heavily infected with *C. daubneyi* (Forstmaier et al., 2021; Alstedt et al., 2022). Sediments containing only rumen fluke eggs, which were differentiated from other trematode eggs by their morphology (colorless, operculated, large eggs) (Alstedt et al., 2022; Küchler, 2021), were stored in an upright position in 10 mL test tubes filled with tap water at 4 °C for a maximum of one year. The percentage of miracidia hatching in the stored samples was checked monthly.

Adult rumen flukes were obtained during the slaughter of cattle from the same farms which provided fecal samples for harvesting *C. daubneyi* eggs.

2.2. Rumen fluke species identification

Species identification of *C. daubneyi* was carried out on adult flukes obtained from the cattle farms, on rumen fluke eggs isolated from fecal samples of the infected lambs, and on infected snails by polymerase chain reaction (PCR) as previously published (Küchler, 2021). In all cases, the DNA was extracted using the DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) as specified by the manufacturer. The entire snail tissue was removed from the shells and crushed using Lysis Tube P with ceramic beads (IST Innuscreen, Berlin, Germany), 180 µL ATL buffer and the homogenization system (SpeedMill PLUS, Analytic Jena, Jena, Germany) for four minutes. Twenty µL proteinase K was then added to the supernatant and incubated for 15 hours at 56 °C. The same protocol was used for the DNA extraction from adult rumen flukes and rumen fluke eggs. *C. daubneyi* was identified using primers to amplify a 167 bp strand from the cytochrome c oxidase subunit 1 (cox1) gene

(GenBank JQ815200) (*C. daubneyi* COX-1, fwd 5'- GTT TGT GTG GTT TGC CAC GG –3'and rev. 5'- CTA CCC CAA GCA GCC ACT AC -'3) (Jones et al., 2015). PCR was conducted using a reaction volume of 50 µL containing 10 µL GoTaq® Reaction buffer (Promega, Madison, WI, USA), 0.25 µL GoTaq® G2 DNA polymerase (Promega, Madison, WI, USA), 1 µL dNTP Mix (Promega, Madison, WI, USA), 0.625 µL per primer, 32.5 µL distilled water and 5 µL DNA template. The PCR was performed in a thermal cycler (Biometra TOptical Gradient 96, Analytik Jena, Jena, Germany) under the following conditions: initial denaturation at 95 °C for two minutes, followed by 40 cycles at 95 °C for one minute, 59 °C for one minute and 73 °C for one minute, and a final elongation step at 73 °C for five minutes. The PCR product was visualized using an automated electrophoresis system (D1000 ScreenTape, Agilent Technologies, Santa Clara, CA, USA) according to the manufacturer's instructions (Fig. 1). To increase the reliability of species identification, DNA from adult rumen flukes was Sanger sequenced (Eurofins Genomics, Ebersberg, Germany) and the identification as *C. daubneyi* was confirmed.

2.3. Infection of the intermediate host

G. truncatula was kept and bred in glass Petri dishes (all Petri dishes from Roth, Karlsruhe, Germany) with a diameter of 14 cm filled with 60 mL of filtered tap water (Brita® water filters, Taunusstein, Germany), four stems of greater water moss (*Fontinalis antipyretica*), and sweet grasses (*Hierochloe odorata*) (Fig. 2a). The water was either changed every two days using an accu-jet pipetting aid and a 25 mL serological pipette, or the Petri dishes were connected to a water flow system with a flow rate of 80 mL per hour (Fig. 2b). A maximum of 15 snails were kept per Petri dish and a maximum of four Petri dishes were stacked on top of each other. The glass Petri dishes were autoclaved after each use. The lighting (NICREW ClassicLED G2) provided a constant 3000–4000 lux for 12 hours. The room temperature was mostly around 20 °C, although this rose to 24 °C in the summer months. The snails were fed dried organic lettuce leaves every two days. The lettuce leaves were divided into pieces approximately 0.5 cm in size, and the snails were given three to four pieces per Petri dish. Unconsumed food from the previous feeding was removed. Additionally, the snails fed on the algae that formed on the bottom of the Petri dish. This setup led to successful reproduction of the snails (Fig. 2c and d).

In the next step, 500 µL of the stored sediment containing rumen fluke eggs were placed in a Petri dish and diluted with 1000 µL distilled water. The eggs (Fig. 3a) were then picked up with a 200 µL micropipette (Brand, Wertheim, Germany) at 40x magnification under an inverted microscope (all microscopical work was performed using Leica DM IL, Wetzlar, Germany), and transferred into one well of a 24-well plate (all plates from Greiner Bio-One, Kremsmünster, Austria) with 1000 µL distilled water per well. Between 20 and 50 eggs were used per well. The 24-well plate was then incubated in the dark at 27 °C (Forma Series II Water Jacketed CO_2 Incubator, Thermo Electron LED, Langenselbold, Germany) (modified according to Magalhaes et al., (2004). After 12–15 days, the plate was removed from the incubator and exposed to a light stimulus of 3000–4000 lux for about 10 minutes at room temperature. After 10 minutes, hatching of the miracidia was checked microscopically at 40x magnification (Fig. 3b). Immediately after hatching, either two or four miracidia were pipetted into a well of a 24-well plate under microscopic control at 40x magnification. The wells were filled with either 1 mL filtered tap water or 1 mL distilled water. Snails were added individually to each of the prepared wells and exposed to the miracidia for 4 hours. Following this, the vitality of the snail and the miracidia was checked again microscopically at 40x magnification. Penetration of the miracidia into the snail could also be observed (Fig. 3c). After 4 hours, the snails were transferred to a glass Petri dish (modified according to Vignoles et al., (2002), where they were kept for 30 days under the laboratory conditions described.

On day 30 post-infection (p.i.), the snails were individually transferred to 3.5 or 9 cm glass Petri dishes. The 9 cm Petri dishes contained 40 mL of filtered tap water and 2 stems of *F. antipyretica*, while the 3.5 cm Petri dishes each contained 3 mL filtered tap water and one stem of *F. antipyretica*. The snails continued to be fed dried lettuce, and the water was changed every two days. From day 30 p.i. onwards, all Petri dishes hosting presumably infected snails were examined microscopically every two days at 40x magnification for cercariae or metacercariae. If cercariae (Fig. 3d) or metacercariae (Fig. 3e) were visible microscopically, the snail was transferred to a new Petri dish. The previous Petri dish was stored at 4 °C for two days. Under an inverted microscope at 40x magnification, the metacercariae were transferred (individually or in pairs) to a 5 mL Eppendorf tube (Eppendorf, Hamburg, Germany) containing 2.5 mL of filtered tap water using a micropipette. A maximum of 100 metacercariae were placed into one vessel and stored

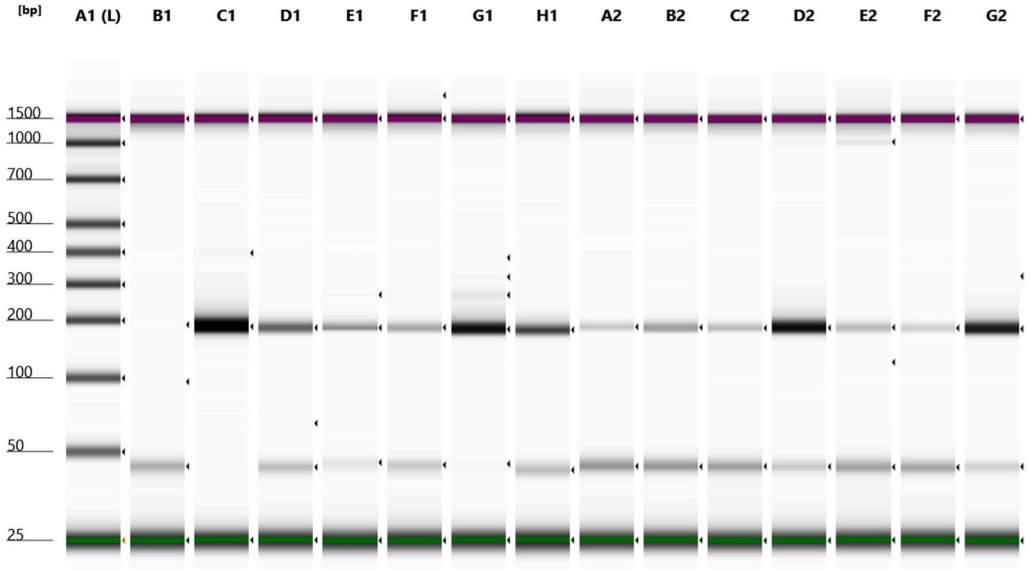

Fig. 1. Virtual gel electrophoresis following PCR amplification of a 167 base pair (bp) strand from *Calicophoron daubneyi* DNA (GenBank JQ815200): A1 DNA ladder (L), B1 negative control, C1 adult rumen fluke, D1, E1, F1, G1, H1, A2, B2, C2, D2, E2 and F2 infected *G. truncatula* snails and G2 rumen fluke eggs from infected sheep.

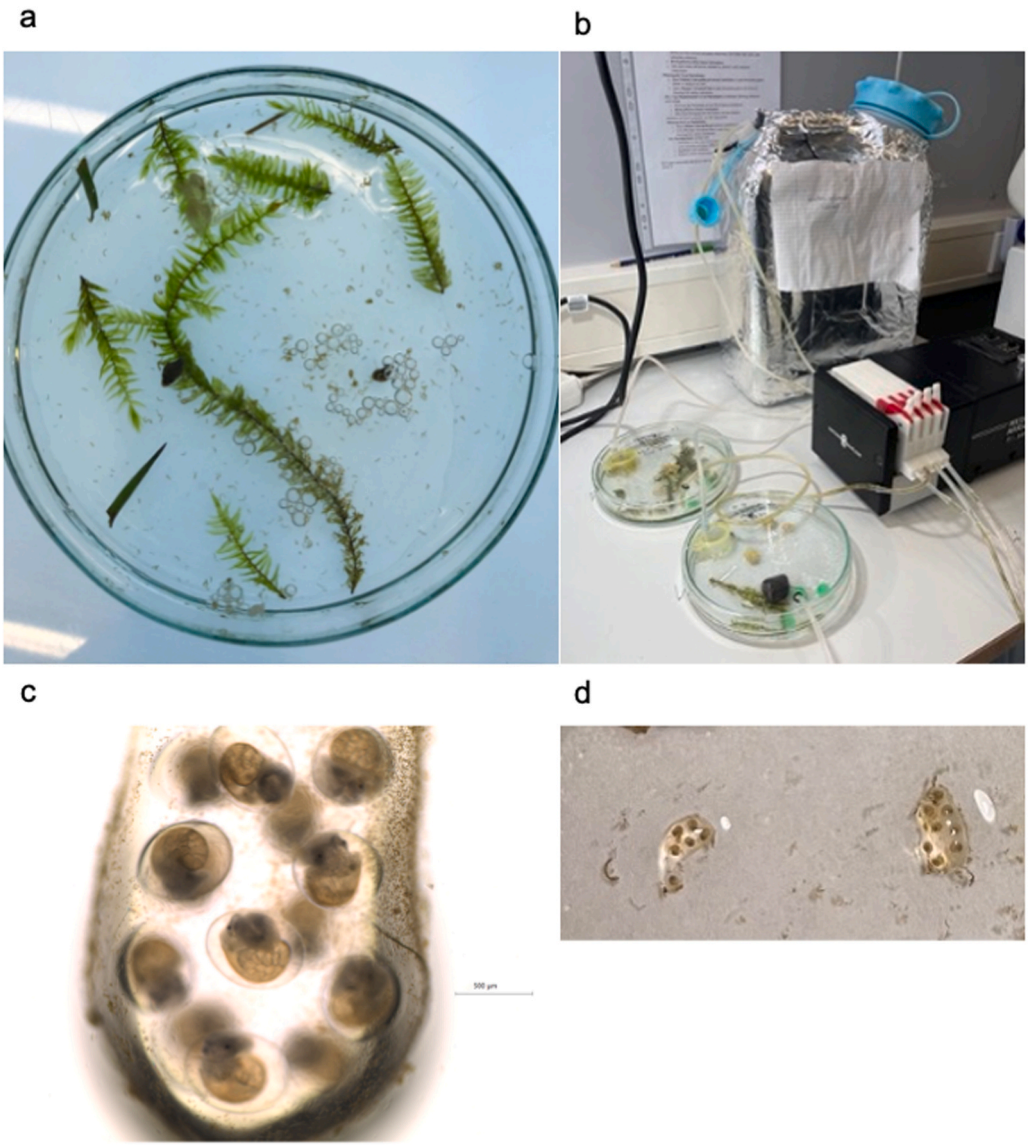

Fig. 2. Establishment of a *Galba truncatula* cultivation system. a) Glass Petri dishes (diameter 14 cm) with filtered tap water, greater water moss (*Fontinalis antipyretica*) and sweet grasses (*Hierochloe odorata*) in order to keep *Galba truncatula* under standardized laboratory conditions. b) Arrangement for automatically circulating filtered tap water in the 14 cm glass Petri dishes with a flow rate of 80 mL per hour (rear: silver covered container with tap water, front: water pump). c) Microscopic image of *Galba truncatula* egg masses (magnification: 40x, BZ-X810, KEYENCE, Neu-Isenburg, Germany) and d) *Galba truncatula* egg masses in a Petri dish, showing the successful reproduction of the snails.

in an upright position at 4 °C until used for inoculation of the lambs.

2.4. Inoculation of the ovine host

Five healthy White Bavarian Alpine ewe lambs aged 7–10 months were used for the study. The animals originated from the teaching and research flock of the Clinic for Ruminants with Ambulatory and Herd Health Services, Ludwig-Maximilians-Universität in Munich and were selected on the basis of age and sex. Random selection was not possible due to a low number of available animals. The lambs were weaned from their dams when four months old and subsequently raised in a young-stock group. They had been housed on deep straw bedding without any access to pasture or fresh forage during their entire lifetime. At the beginning of the study, the lambs were weighed and checked for parasitic infections using the sedimentation and McMaster techniques to ensure that the animals were free from gastro-intestinal nematode or trematode infections. Following randomized assignment to the inoculation or control groups, three lambs (Ear tag No. 376, 377, 379) were used for inoculation with *C. daubneyi* metacercariae (group i), while two animals (Ear tag No. 378, 380) served as controls (group c) (Table 1). They were randomly mixed and housed in two free range stalls on straw bedding without any access to pasture and were fed hay, minerals and commercial concentrates. After three weeks of habituation, three lambs were inoculated with 150 *C. daubneyi* metacercariae of different ages: 261 days (No. 376), 109 days (No. 379) and 14 days (No. 377), respectively (Table 1). After inoculation, all animals were clinically examined daily (heart rate, respiratory rate, temperature, fecal consistency, feed intake). Once a week, blood samples were taken from the jugular vein, and feces were sampled directly from the rectum. From day 65 post-inoculation until the beginning of patency, fecal samples were taken every other day except Saturdays and Sundays, and the sampling pattern returned to a weekly routine after the onset of egg excretion. Coproscopical examination was carried out either using 10 g feces for the sedimentation technique (days 65 – 84) or 2 g feces for the Flukefinder® technique (from day 86 onwards) to determine the quantity of *C. daubneyi* eggs per gram feces (epg). Additionally, pooled fecal samples

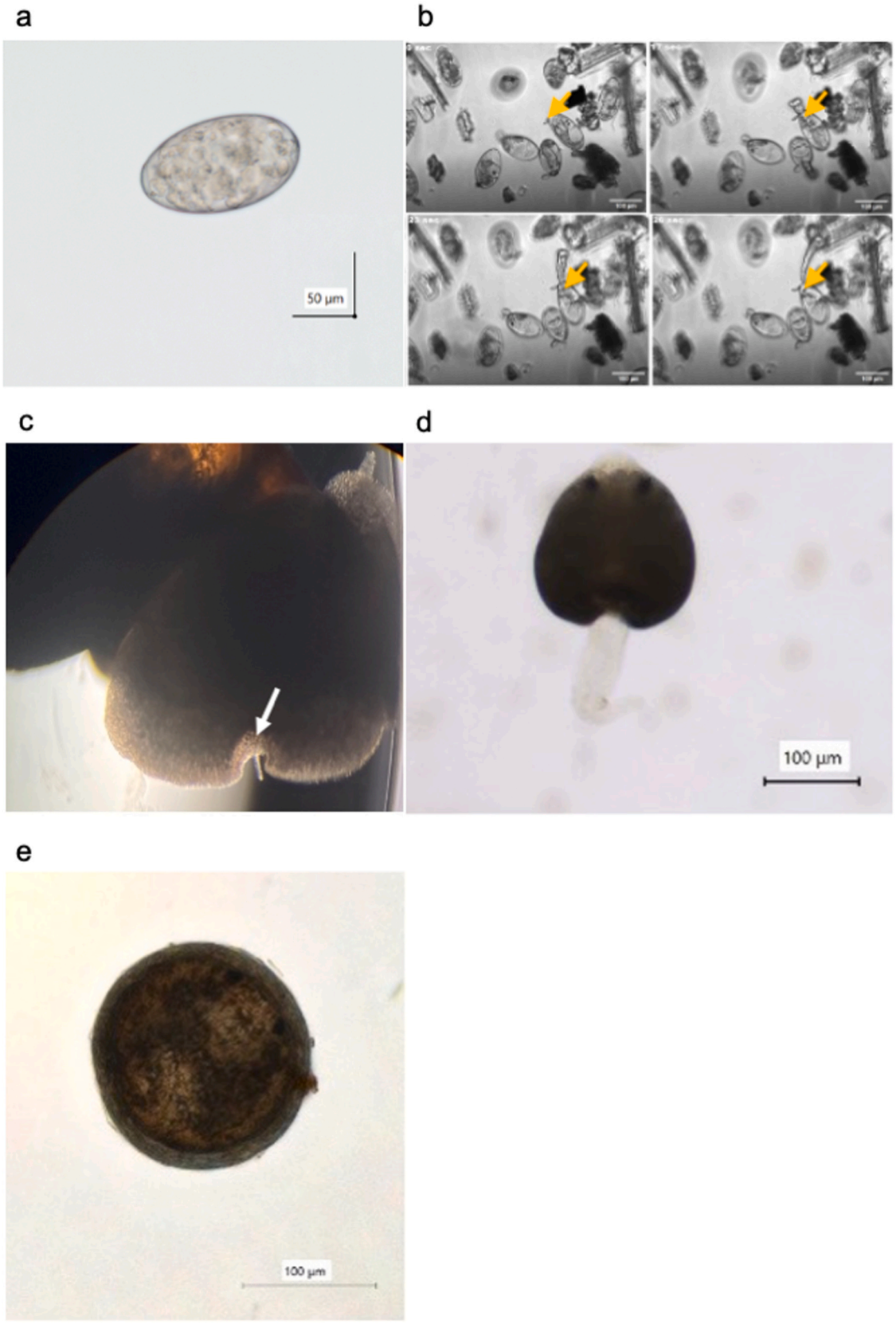

Fig. 3. Various stages of the *Calicophoron daubneyi* life cycle under laboratory conditions a) Egg extracted from feces of a naturally infected cow. b) Hatching process of the developed miracidia (yellow arrows) following incubation. c) Photography through eyepiece of the microscope at 40x magnification shows penetration of a *Calicophoron daubneyi* miracidium into the foot of *Galba truncatula* (white arrow). d) Microscopic image (100x) of a *Calicophoron daubneyi* cercaria shed by experimentally infected *Galba truncatula* in a Petri dish. e) Microscopic image (200x) of encysted *Calicophoron daubneyi* metacercaria in a Petri dish.

Table 1
Age and weight of the study animals (White Bavarian Alpine ewe lambs), and age of *Calicophoron daubneyi* metacercariae at inoculation.

Ear tag	Lamb age at inoculation (days)	Lamb weight at inoculation (kg)	Age of metacercariae at inoculation (days)
376	310	70.5	261
377	314	64.5	14
378	274	64	Not inoculated
379	238	60.5	109
380	235	54.5	Not inoculated

were examined by a modified McMaster technique with a diagnostic sensitivity of 50 epg (using a saturated sodium chloride solution with specific gravity 1.2) to rule out any potential concurrent infection with gastrointestinal nematodes. EDTA blood samples were collected for hematological examination including leukocyte and erythrocyte counts, hemoglobin and hematocrit (VetScan HM5, Abaxis, Union City, CA, USA). Biochemical parameters analyzed from serum samples included calcium, phosphorus, total protein, albumin (Cobas c311, Roche, Basel, Suisse) and haptoglobin (Monoscreen Quant ELISA Bovine Haptoglobin, Bio-X, Rochefort, Belgium). The lambs were weighed on the day of inoculation (day 0), as well as on days 36 and 77, after reaching patency and at the end of the study period. The inoculation or control status of the lambs was unknown to the clinical investigators and laboratory staff at all times.

2.5. Data analysis

The biochemical and hematological data were managed using Microsoft Excel 2019 (Microsoft Corporation, Redmond, WA, USA). Statistical analyses were performed using R 4.3.1 (The R Foundation for Statistical Computing, Vienna, Austria). Due to the presence of repeated measures, generalized linear mixed effects models with the individual animal as a random effect were chosen for analysis. The following model assumptions were always checked: (1) the normality of residuals was checked by the Shapiro-Wilk normality test, (2) the homogeneity of variances between groups was checked using the Bartlett test, and (3) the heteroscedasticity (constancy of error variance) was checked with the Breusch-Pagan test. In case the assumptions were satisfied, generalized linear mixed effects models were used (R package - lmer). In case the assumptions were violated, robust linear mixed effects models were applied (R package - robustlmm). Additionally, both linear and robust linear models were compared amongst each other using six main performance quality indicators: Akaike's Information Criterion (AIC), Bayesian Information Criterion (BIC), Conditional coefficient of determination R2, Marginal coefficient of determination R2, the intraclass-correlation coefficient (ICC) and Root Mean Square Error (RMSE). The model showing the best combination of predictive (AIC and BIC) and fitting (R2, ICC, RMSE) power was preferred. All contrasts (differences) between particular groups (infected vs. non-infected) were assessed after model-fitting by the estimated marginal means (R package - emmeans) with Tukey p-value correction for multiple comparisons. Results with a P-value < 0.05 were considered statistically significant.

3. Results

3.1. Survival rates and cercarial shedding of the intermediate host

Of the 177 snails exposed to *C. daubneyi* miracidia, 68 (38.4 %) survived the first 30 days p.i. Of these 68 surviving snails, seven (10.3 %) shed a total number of 38–186 (mean: 106) cercariae, starting from day 35 p.i. on the earliest occasion and lasting to day 75 on the latest (Fig. 4). Eventually, all the snails died. In all eleven infection trials, snails which did not produce cercariae died between 5 and 58 days p.i. Those which produced cercariae died within one week of the last shedding (Table 2). PCR analysis for *C. daubneyi* DNA was carried out on a selection of dead snails which did not produce any cercariae (n = 80). Of these, 41 snails were positive. No PCR analysis was performed on the remaining 97 snails which were exposed to miracidia but died without shedding. When the seven snails that shed cercariae are added to the PCR positive snails, 48 snails out of 87 snails with known infection status (55.2 %) were identified as infected.

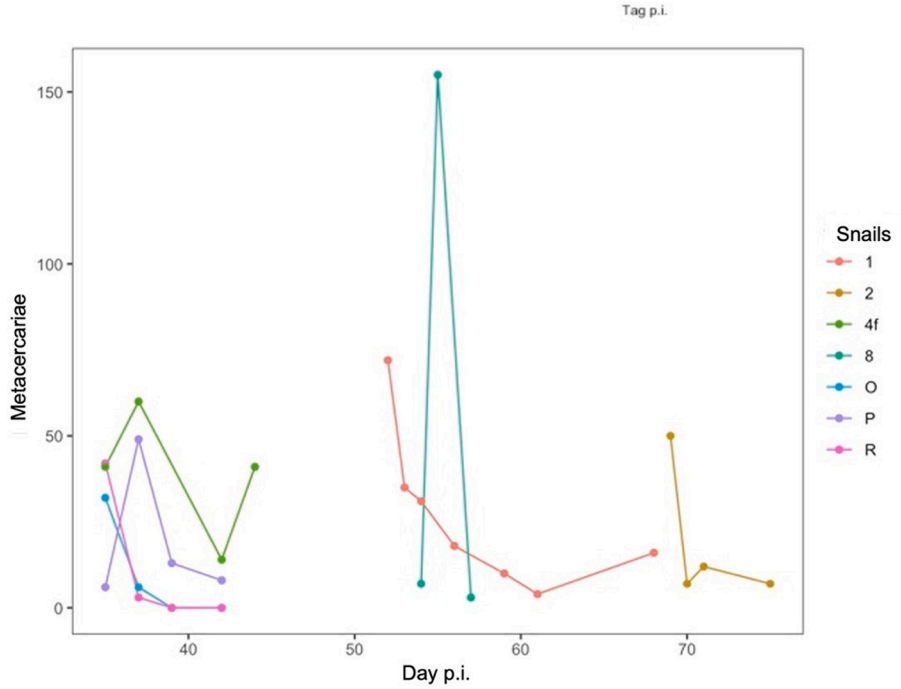

Fig. 4. Number and duration (days post infection) of shedding of *Calicophoron daubneyi* cercariae by experimentally infected *Galba truncatula* (n = 7) under standardised laboratory conditions (Legend: Snails O, P, R were infected in infection trial 1, snail 4 f in infection trial 4, snail 2 in infection trial 5, snail 1 in infection trial 6 and snail 8 in infection trial 10).

Table 2
Survival rate of the snails and success of cercarial shedding in 11 experimental infection trials of *Galba truncatula* with *Calicophoron daubneyi* miracidia.

Trial No.	Total No. of snails	No. of miracidia per snail	No. of snails per 14 cm-Petri dish (day 0–29 p.i.)	No. of snails surviving past day 30 p.i. (survival rate)	No. of snails per 3.5 cm-Petri dish (> day 30 p.i.)	No. of snails shedding cercariae	No. of meta-cercariae per snail
1[a]	38	2	2 × 10 (3 ×6[b])	9 (23.7 %)	1 (4[b])	3	76/ 38/ 45
2	40	2	10	6 (15.0 %)	1	0	
3	16	2	8	5 (31.3 %)	1	0	
4[a]	15	2	15	14 (93.3 %)	1	1	156
5	12	2	12[c]	8 (66.7 %)	1	1	76
6[a]	8	2	8[c]	3 (37.5 %)	1	1	186
7	4	4	4[c]	3 (75.0 %)	1	0	
8	4	2	4[c]	2 (50.0 %)	1	0	
9	16	2	10[c], 6[c]	6 (37.5 %)	1	0	
10	12	2	12[c]	4 (44.4 %)	1	1	165
11	12	2	12[c]	8 (80.0 %)	3 (2[b], 3[b])	0	
Total	177			68 (38.4 %)		7 (45.5 %)	

Abbreviations: p.i. post-infection, No. number
[a] Trials used for inoculation of lambs (see Table 3)
[b] Number of snails kept in 9 cm-Petri dish
[c] Petri dish with additional water flow system

3.2. Coproscopy

C. daubneyi eggs were first detected in the feces of lambs No. 376 and 377 on day 86 post-inoculation (Table 3). Lamb No. 376 was only positive on this one occasion, with a rumen fluke egg count of 2 epg. No more rumen fluke eggs were detected in this animal throughout the course of the study. Lamb No. 377, however, continued excretion until the end of the study on day 104 post-inoculation, with rumen fluke egg counts ranging from 1 to 6 epg. The fecal samples from the third inoculated lamb were always negative for rumen fluke eggs throughout the whole study. During the experiment, coccidial oocysts were detected irregularly at low levels in all animals from both groups. All fecal samples were always negative for gastrointestinal nematode eggs.

3.3. Clinical signs

No clinical signs were observed in any lamb during this study. General condition and food intake were unaffected; fecal consistency was always firm. Body temperature measurements were mostly within the normal range for juvenile sheep (38.3–39.9 °C) (Bostedt et al., 2019). Values between 38.0 °C and 38.3 °C were measured in only three occasions, while values above 39.9 °C were never recorded. All five lambs gained between 10 % and 20 % of their initial body weight until the end of the experiment.

3.4. Clinical chemistry

The mean values for calcium and phosphorus for both groups remained within the reference ranges of 2.0–3.0 mmol/L for calcium and 0.7–3.1 mmol/L for phosphorus (Bostedt et al., 2019), without a significant difference between both groups ($P > 0.05$; Table 4). Similarly, the mean total plasma protein levels remained within the reference range (51–73 g/L) (Bostedt et al., 2019) throughout the experiment for all animals, and no significant differences were noted between both groups ($P > 0.05$; Table 4). The mean albumin levels were mostly within the reference range (35–38 g/L) (Bostedt et al., 2019). Occasionally, values above 38 g/L were measured, with a maximum of 41.75 g/L (day 76, control group), but no significant differences could be noted between the groups ($P > 0.05$; Table 4). Details of the clinical chemistry parameters from the weekly blood collections are presented in Supplementary Table 1. For haptoglobin, blood samples from days 35, 55, 62 post-inoculation were missing for analyses due to accidental disposal. On the remaining days, the values were below the detection limit (5.6 ng/mL) in the inoculated and non-inoculated lambs.

Table 4
Estimated marginal means (Emmean), lower (LCL) and upper confidence limits (UCL) of blood chemistry and hematology values from *Calicophoron daubneyi* inoculated and non-inoculated lambs throughout the trial period.

Parameter	Group	Emmean	LCL-UCL
(reference range[a])			
Calcium	i	2.53	2.4–2.67
(2.0–3.0 mmol/L)	c	2.58	2.41–2.74
Phosphorus	i	2.37	1.93–2.82
(0.7–3.1 mmol/L)	c	2.25	1.71–2.8
Total protein	i	62.9	59.9–66
(51–73 g/L)	c	63.1	59.3–66.8
Albumin	i	39.8	38.1–41.4
(35–38 g/L)	c	39.8	37.8–41.8
Leucocytes	i	7.89	6.24–9.54
(2.7–13.0 x10^3/µL)	c	6.64	4.62–8.66
Erythrocytes	i	10.9	9.59–12.3
(8.7–12.9 x10^3/µL)	c	10.9	9.24–12.5
Hematocrit	i	31.6	27.8–35.3
(25–41 %)	c	31.4	26.8–35.9
Hemoglobin	i	10.3	9.07–11.5
(8.5–13.3 g/dL)	c	10.3	8.82–11.8

Abbreviations: i inoculated, c control
[a] Reference values according to Bostedt et al., (2019)

3.5. Hematology

Mean leukocyte and erythrocyte counts and mean hemoglobin and

Table 3
Data from three different successful infection trials of *Galba truncatula* with *Calicophoron daubneyi* miracidia used for inoculation of the definitive host (ewe lambs).

Trial No. (see Table 2)	Month of shedding	Start of cercarial shedding (day p.i.)	End of cercarial shedding (day p.i.)	Total No. of metacercariae produced by snails	Age of metacercariae at inoculation of lambs	Ear tag of lambs	Start of egg excretion (day post- inoculation)
1	Aug. 2022	35	42	159[a]	261	376	86
4	Dec. 2022	35	44	156	109	379	n.a.
6	April 2023	52	68	186	14	377	86

Abbreviations: p.i. post-infection, No. number, n.a. not applicable
[a] Total harvest from three *G. truncatula* snails

hematocrit values remained mostly within the reference ranges (Bostedt et al., 2019) of 2.7–13 x 10^3/μL for leukocytes, 8.7–12.9 x 10^3/μL for erythrocytes, 8.5–13.3 g/dL for hemoglobin and 25–41 % for hematocrit, and did not differ significantly between inoculated and non-inoculated lambs ($P > 0.05$; Table 4). Only at the first blood collection five days prior to inoculation, the mean erythrocyte count and hemoglobin value were slightly increased in the control animals. Details of the hematology results from the weekly blood collections are presented in Supplementary Table 2.

4. Discussion

Establishment of the complete life cycle of *C. daubneyi* under experimental conditions has been achieved, although our results also highlight challenges regarding reliable and repeatable production of metacercariae in the laboratory environment. Currently, the experimental infection of the intermediate host still represents a bottleneck for the production of metacercariae (Atcheson et al., 2020). Firstly, infected snails had a high mortality of 61.6 % within the first 30 days following exposure to *C. daubneyi* miracidia. A low survival rate was also described by Abrous et al. (1999). This can be explained by a possibly suboptimal adaption of *C. daubneyi* to *G. truncatula* compared to its relative, *F. hepatica* (Abrous et al., 1997). Secondly, even though cercarial shedding was achieved in nearly half of the snail trials, it was unpredictable and seemed to occur by chance, and in individual snails only. Therefore, with current protocols, the production of *C. daubneyi* cercariae by *G. truncatula* is less efficient compared to *F. hepatica*, as described previously. For example, Sey (1977) reported that infected snails did not shed any cercariae even though there was an accumulation of rediae inside the snails. In our experiments, only seven of the 68 snails that survived past day 30 p.i. went on to shed cercariae.

Shedding took place from day 35 p.i. under our experimental conditions. This is earlier than described by other authors, who observed cercarial shedding from day 49, 55, 57, 66 or 70 p.i (Abrous et al., 1999; Dinnik, 1962; Kumar, 1999; Augot et al., 1996). However, this seems to be temperature dependent, since in our trials snails were kept at a relatively stable temperature between 20 and 25 °C, while in other experiments temperature varied between 6 and 20 °C (Dinnik, 1962; Kumar, 1999; Augot et al., 1996). Different factors have been described as triggers for cercarial shedding, such as natural light or temperature (Abrous et al., 1999; Titi et al., 2014). We performed a small trial involving temperature changes (data not shown), but these did not show a significant effect on cercarial shedding. In summary, we established a protocol that allows the production of *C. daubneyi* cercariae using *G. truncatula* as intermediate host. We hypothesize that optimization of culture conditions for *G. truncatula* will subsequently result in better shedding rates, and we currently investigate several parameters to increase the fitness of the intermediate host bred under laboratory conditions (data not shown).

Concerning the definitive host phase of the parasite life cycle, the assumption that the prepatent period of *C. daubneyi* is approximately 10–12 weeks can be supported by our results. We detected eggs in the feces of two out of three inoculated lambs after 12 weeks (on day 86 post-inoculation). The first detection of rumen fluke eggs coincided with the first use of the Flukefinder® technique, since the sedimentation technique was used until day 84. This is most likely true coincidence although it cannot be ruled out that an earlier detection of rumen fluke eggs might have been possible with Flukefinder®. The prepatency seen in our trial is similar to results reported by a French study, where sheep did not shed eggs until day 80 after being moved onto rumen fluke infected pasture even though flukes could be detected in the rumen from day 38 at necropsy (Devos et al., 2013). For goats, previous studies yielded contradictory results. This may be due to differences in the infectious doses used by the different investigators. When infected with 1.500 *C. daubneyi* metacercariae, the prepatent period was 12–13 weeks (Sey, 1977), while it was around 18 weeks when goats were infected with 250 metacercariae (Paraud et al., 2009). The results of both studies are, however, based on a very small number of experimental animals. When the two small ruminant species were infected with the same dose of *P. microbothrium* metacercariae, the prepatency differed between 69 days (goats) and 93 days (sheep) (Horak, 1967).

An age-dependent infectivity was shown for *P. microbothrium* metacercariae. Infectivity decreased slightly from 47 to 63 days of age (percentage take (pt, i.e., the number of adult flukes in the rumen expressed as a percentage of the number of metacercariae dosed): 33.7 %) and rapidly thereafter with increasing metacercarial age (70–77 days: pt 12.9 %, 122 days: pt 0.07 %, 239–240 days: pt 0.6 %, 508–539 days: pt 0.06 %) (Horak, 1967). In goats infected with 250 *C. daubneyi* metacercariae, which were aged up to 56 days, the pt was 12 % (Paraud et al., 2009). It is therefore surprising that 261-day-old metacercariae (lamb No. 376) were infectious in our study, but the inoculation with 109-day-old metacercariae (lamb No. 379) was not successful. The contrary would have been expected. The rapid termination of egg excretion in lamb No. 376, however, also showed that the oldest metacercariae were not highly infectious.

The clinical observations reported in this paper are similar to those described in sheep naturally infected with *C. daubneyi* (Devos et al., 2013), but no differences in blood count and blood chemistry between inoculated and non-inoculated sheep could be observed in our study. This is most likely due to the small number of animals inoculated with a low number of metacercariae. Lambs infected with 5.000 *P. ichikawai* metacerariae did not show any clinical signs (Rolfe et al., 1994), while lambs infected with 170.000 *P. microbothrium* metacercariae showed severe illness and died (Horak, 1971). In order to increase our knowledge regarding the pathogenicity of *C. daubneyi,* inoculation trials with significantly higher doses of metacercariae are therefore necessary. The limited availability of metacercariae also limited the number of lambs used in this pilot study, and, therefore, the representativeness of our results. In addition, the experimental licence did not include euthanasia and necropsy of the lambs, thus limiting proof of infection to egg excretion, and precluding ruminal parasite counts and the assessment of a percentage take. To better understand the definitive host phase of *C. daubneyi*, further studies are therefore necessary to include higher numbers of animals, higher inoculation doses and necropsy of lambs at different stages of infection.

5. Conclusion

To the authors' knowledge, this is the first study since 1977 (Sey, 1977) which describes production of *C. daubneyi* metacercariae and infection of *G. truncatula* in detail and succeeded in establishing the entire life cycle under experimental conditions. Naïve lambs inoculated with low metacercarial doses showed no clinical and hematological signs, and rumen fluke eggs were first detectable in feces after 12 weeks. Both observations confirmed earlier assumptions regarding prepatency and dose dependent severity of paramphistomidosis. However, the production of larger numbers of experimentally produced *C. daubneyi* metacercariae remains a challenge due to the high mortality of infected snails, and unknown triggers for cercarial shedding. Both problems have also been reported by other authors. Nevertheless, the protocols described in this study can serve as a starting point to further improve the conditions for snails kept in the laboratory, especially when infected with *C. daubneyi*.

Ethical approval

All experimental procedures involving lambs were approved by the government of Upper Bavaria under the reference number ROB-55.2–2532.Vet_02–20–175.

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CRediT authorship contribution statement

Christoph Wenzel: Writing – original draft, Visualization, Methodology, Formal analysis, Data curation, Conceptualization. **Verena K. Elbert:** Writing – original draft, Visualization, Methodology, Investigation, Formal analysis. **Sandra Haug:** Writing – review & editing, Methodology, Investigation, Conceptualization. **Katja Voigt:** Writing – review & editing, Resources, Methodology. **Frank Weber:** Writing – review & editing, Resources, Methodology, Conceptualization. **Viktoria Balasopoulou:** Writing – review & editing, Investigation. **Eva Roden:** Writing – review & editing, Investigation. **Yury Zablotski:** Writing – review & editing, Visualization, Formal analysis, Data curation. **Markus Meissner:** Writing – review & editing, Supervision, Project administration, Methodology, Conceptualization. **Gabriela Knubben-Schweizer:** Writing – original draft, Supervision, Resources, Project administration, Methodology, Conceptualization.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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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.2025.110391.

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