Research Square

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Fluke in the grass: forage species influence Fasciola hepatica and Calicophoron daubneyi metacercarial encystment

Rhys Aled Jones
raj22@aber.ac.uk
Aberystwyth University
Serian R. Evans
Aberystwyth University
Susan Girdwood
Aberystwyth University
Cennydd Owen Jones
Aberystwyth University

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Abstract

Background

The trematode parasites *Fasciola hepatica* and *Calicophoron daubneyi* infect grazing livestock following ingestion of metacercariae encysted on herbage. However, the influence of forage species on metacercariae encystment is poorly understood. The aim of this study was to determine whether the encystment of *F. hepatica* and *C. daubneyi* differed among common forage species.

Methods

Infected *Galba truncatula* snails were stimulated to shed cercariae in the presence of forage material. Single-forage assays were conducted for *F. hepatica* and *C. daubneyi* using eight and three forage species, respectively, and a mixed-forage assay compared Italian ryegrass, Timothy and Cocksfoot for *F. hepatica*. Metacercarial recovery from plant and non-plant locations was recorded with encystment rates analysed using beta-binomial models.

Results

Encystment rates differed among forage species with *F. hepatica* encystment rates lower on Timothy (OR = 0.438, $p = 0.036$) and Yorkshire Fog (OR = 0.106, $p < 0.001$) compared to Italian ryegrass. In the mixed-forage assay, *F. hepatica* encystment was also lower on Cocksfoot (OR = 0.519, $p = 0.040$) and Timothy (OR = 0.453, $p = 0.015$) than on Italian ryegrass. Leaf surface analysis indicated lower-surface bias for *F. hepatica* metacercariae on Ryegrass and Yorkshire Fog ($p < 0.05$). In contrast, *C. daubneyi* encystment was higher on Plantain (OR = 38.305, $p < 0.001$) and Yorkshire Fog (OR = 26.862, $p = 0.001$) than on Italian ryegrass.

Conclusions

Forage species influenced the encystment of *F. hepatica* and *C. daubneyi* metacercariae, with contrasting patterns between fluke species. These findings suggest that plant-parasite interactions during the free-living metacercarial stage may influence the spatial distribution of infective stages on pasture. Semi-natural and field-based studies are needed to determine whether these differences affect transmission risk under grazing conditions.

Background

Trematode parasites present a significant challenge to sustainable livestock production globally. The liver fluke *Fasciola hepatica* contributes substantially to the estimated €1.8 billion annual cost of helminth infections to the European livestock industry [1], while the rumen fluke *Calicophoron daubneyi* is an emerging threat across Europe [2]. Like other liver and rumen flukes of veterinary importance, such as *Fasciola gigantica* and *Paramphistomum* spp., these parasites have complex lifecycles involving an intermediate freshwater snail host. Within this host, they undergo asexual reproduction before being released as free-swimming cercariae, which encyst on herbage as free-living metacercariae and facilitate transmission to grazing mammalian hosts upon ingestion.

Although substantial research has focused on intermediate snail host ecology [3], the influence of temperature on trematode infection, development and shedding within the intermediate host, and metacercarial survival [4], there remain remarkably few studies examining interactions between trematode environmental stages and plant species. Given that the infective metacercarial stage adheres to vegetation to increase the likelihood of ingestion by grazing livestock, this represents an important research gap.

The adherence of trematode cercariae to vegetation involves both behavioural and physicochemical processes, with encystment occurring only after suitable settlement cues are encountered [5]. Consequently, plant surface properties and microclimatic conditions are likely to influence metacercarial attachment and persistence [6]. As these characteristics vary among forage species, sward composition may represent an important but largely unexplored determinant of metacercarial distribution and the risk of parasite transmission to grazing livestock.

Control of trematode infections remains heavily reliant on anthelmintic treatment, but this approach is increasingly challenged by triclabendazole resistance, limited treatment options, and wider concerns regarding the sustainability of repeated flukicide use [7, 8]. Liver fluke management is further complicated by diagnostic uncertainty, variable farm-level and seasonal risk and the practical difficulty of implementing non-chemical control measures, often leading farmers to rely on routine or precautionary treatment [9]. In contrast, manipulation of grazing systems and pasture composition is already recognised as a promising component of integrated, non-chemical control for gastrointestinal nematodes [10], with forage and sward characteristics influencing the development, migration, survival and availability of infective larvae on pasture [11–13]. However, whether similar pasture-based approaches could influence the attachment, persistence and availability of infective trematode stages remains largely unexplored.

This study aimed to determine whether forage species influence the encystment of *F. hepatica* and *C. daubneyi* cercariae. Specifically, we compared encystment rates on Italian ryegrass, used here as a ryegrass control representative of commonly used temperate pasture grasses, with those on alternative grass, legume and herb species. We hypothesised that encystment rates would vary between forage species, indicating that pasture composition may influence the availability of infective metacercariae to grazing livestock.

Methods

Parasite material and cercarial collection

The study was conducted at Ridgeway Research Limited (Gloucestershire, UK) a commercial company who maintain the full *F. hepatica* and *C. daubneyi* lifecycle using *G. truncatula* snails and sheep as hosts. A total of 39 shedding *F. hepatica* infected and 20 shedding *C. daubneyi* infected *G. truncatula* snails were supplied for the experiment. As the attainment of *F. hepatica* and *C. daubneyi* metacercariae are undertaken as a commercial activity, we are unable to disclose the exact methodology of *G. truncatula* rearing, infection or maintenance.

Forage species selection and preparation

Eight forages were selected for use in this study. Ryegrasses (*Lolium* spp.) are widely regarded as the prominent productive forage grasses in temperate grassland systems [14]. Accordingly, Italian ryegrass (*Lolium multiflorum*, cv. 'Fabio') was selected as the control species in this study, providing a representative ryegrass comparator against which alternative grass, legume and herb species could be assessed. Cocksfoot (*Dactylis glomerata*, cv. 'Sparta') and Timothy (*Phleum pratense*, cv. 'Comer') were included as additional temperate forage grasses as they are both widely used as companion or alternative grass species in temperate pastures [14]. Yorkshire Fog (*Holcus lanatus*) was included because, although common in temperate pastures, it is often considered undesirable in productive swards due to its relatively low digestibility and palatability [15]. Its distinctive pubescent leaf surface also provided a contrasting morphology with which to test whether leaf trichome density and surface structure influence trematode encystment [16]. Chicory (*Cichorium intybus*, cv. 'Puna II'), Ribwort Plantain (*Plantago lanceolata*, cv. 'Endurance ribgrass') and Sainfoin (*Onobrychis viciifolia*) were selected as they contain bioactive secondary metabolites, which have known antiparasitic effects [10]. Crimson Clover (*Trifolium incarnatum*) was included to provide an additional legume with a pubescent leaf surface [17].

Experimental encystment assay

To evaluate the encystment of *F. hepatica* and *C. daubneyi* on forage material, two assay formats were used. Single-species encystment assays were conducted for both *F. hepatica* and *C. daubneyi*, while a mixed-forage competition assay was conducted for *F. hepatica* only.

Preliminary experiments were first conducted to establish an assay apparatus that maximised the opportunity for cercariae to encyst on plant material rather than on non-biological laboratory surfaces. Under normal commercial conditions, snails would be shed in Visking tubing, however initial trials indicated that *F. hepatica* metacercariae preferentially encysted on this Visking tubing rather than on vegetation. Similar patterns were seen when snails were placed in plastic tubes with metacercariae preferentially encysting on tube walls as opposed to plant material. As such, to minimise non-target encystment, a suitable lining material was sought that would minimise attachment to these surfaces while still allowing assays to be conducted in a controlled apparatus. Although no directly comparable studies were identified for *F. hepatica* or *C. daubneyi*, LeSage and Fried [18] reported that metacercariae of the paramphistome trematode *Zygocotyle lunata* adhered at substantially lower rates to cheesecloth than to plastic, glass or Styrofoam. Preliminary trials were therefore conducted using cheese cloth and muslin cloth. *F. hepatica* metacercariae rarely adhered to these materials and were less likely to pass through muslin, likely due to its smaller pore size. Based on these preliminary observations, the *F. hepatica* single-species assays were conducted in muslin-lined 10 ml plastic tubes. A square of muslin cloth, approximately 10 × 10 cm, was inserted into each plastic tube and pushed down to form a well that covered the inner tube wall. For grass treatments, two leaf blades of the same width (0.5 cm) were cut at the node and placed vertically inside the muslin-lined tube, spanning the muslin well, with the two blades oriented in opposite directions. On one blade, the upper adaxial surface faced inwards, while on the other blade, the lower abaxial surface faced inwards. For broad-leaved herb species, leaves were cut to the same width (0.5 cm) as the grass blades. For the legume treatments, one Sainfoin stem bearing six leaves and two Crimson Clover stems, each bearing three leaves, were inserted into each tube. Each herb or legume sample was oriented in opposing directions as described for the grass treatments. However, for Crimson Clover and Sainfoin, changes in leaf position were observed during incubation meaning that consistent upper- and lower-surface orientation was not seen. In total, replicate numbers were: Italian ryegrass, n = 5; Chicory, n = 4; Cocksfoot, n = 4; Crimson Clover, n = 4; Plantain, n = 5; Sainfoin, n = 3; Timothy, n = 4; and Yorkshire Fog, n = 5. Each tube was then filled with approximately 8 ml distilled water, and a single infected snail, which was shedding cercariae, was placed between the leaf blades and submerged. The tube lid was placed loosely on top to prevent snail escape but was not screwed shut. Tubes were placed randomly in a rack, submerged in a water bath with two ice packs and left overnight to allow cercarial shedding and encystment, with the contents of the assay examined the following morning.

For *C. daubneyi*, preliminary observations indicated limited adherence to Visking tubing, whereas shedding in plastic tubes was low and inconsistent. Therefore, *C. daubneyi* assays were conducted in Visking tubing rather than muslin-lined plastic tubes, following the same shedding protocol used for the *F. hepatica* assay. The exception was that *G. truncatula* snails were removed from the Visking tubing after 4 h, as preliminary observations indicated that *C. daubneyi* metacercariae commonly encysted on the snail shell when snails remained in the assay for longer periods. Due to variable *C. daubneyi* shedding observed at the research site, and limited snail availability, assays for this species were focused on three forage species, Italian ryegrass (n = 7), Yorkshire Fog (n = 7) and Plantain (n = 6). These species were selected to compare the ryegrass control with a pubescent grass surface and a bioactive forage herb which has a complex leaf surface. Visking tubing was cut into approximately 5 cm lengths, and forage material was positioned horizontally to cover the bottom of the Visking tube surface. This placement was chosen as *C. daubneyi* cercariae are reported to exhibit positive geotactic and/or negative phototactic responses [5], causing them to move downwards following emergence, a trait observed in the preliminary observations. Positioning the forage along the bottom of the tube was therefore intended to maximise contact between newly shed cercariae and plant

material and better represent how *C. daubneyi* may come into contact with vegetation in the field. For Italian ryegrass and Yorkshire Fog, six grass blades of 0.5 cm in width were used per assay, while two Plantain leaves (1.5 cm width) were used to provide the same surface area of plant material. A clip was attached to one end of the Visking tube to secure the forage material, distilled water was added until the tube overflowed, and a single infected snail was inserted. The second end of the tube was then clipped closed.

In both instances, the contents of each tube were examined using a Brunel stereomicroscope. The muslin and/or plant material and snail were removed from the respective tubes and placed into a petri dish. The snail was examined for encysted metacercariae, as were both sides of each forage blade, followed by both sides of the muslin lining. The remaining water was poured into a petri dish and examined for metacercariae, as was the inner surface of the tube and lid or Visking tube. The number of metacercariae recovered from each location was recorded. Metacercariae recovered from forage material were classified as plant-associated, while those recovered from the snail, water, muslin, tube wall or lid were classified as non-plant-associated.

To assess forage choice under mixed-species conditions, a competition assay was conducted in which *F. hepatica* cercariae were shed from snails in the simultaneous presence of Italian ryegrass, Timothy and Cocksfoot within the same muslin-lined plastic tube assay. In total five replicate tubes were used, each containing one blade of each grass species of a standardised 0.5 cm width. These species were selected to compare the ryegrass control with two widely used alternative temperate forage grasses. Within each tube, all three leaf blades were presented with the same surface orientation. In two replicate tubes the upper (adaxial) surface faced inwards, whereas in the remaining three tubes the lower (abaxial) surface faced inwards. Cercarial shedding, incubation and metacercarial recovery were conducted as described for the single-species *F. hepatica* assay. However, in the mixed-species assay, metacercariae encysted on each forage species were counted separately, along with those recovered from non-plant locations within the assay environment.

Scanning electron microscopy of leaf surfaces

Leaf-surface morphology was examined using scanning electron microscopy (SEM) to provide a qualitative characterisation of structural differences among forage species and between upper and lower leaf surfaces. Representative leaves from each forage species were fixed in 2.5% glutaraldehyde prepared in 0.1 M sodium cacodylate buffer (pH 7.2). Following fixation, samples were rinsed in 0.1 M sodium cacodylate buffer (pH 7.2) and dehydrated through a graded aqueous ethanol series (30%, 50%, 70%, 95% and 100%), followed by treatment with hexamethyldisilazane (HMDS). Leaf material was then mounted on aluminium stubs using self-adhesive carbon tape, with either the upper or lower leaf surface exposed, and coated with a 4 nm layer of Pt/Pd using a high-resolution sputter coater.

Upper and lower surfaces of each forage species were examined separately using a JEOL JSM-IT200 scanning electron microscope under high-vacuum conditions. Images were acquired at ×100 magnification using an accelerating voltage of 10 kV. Representative micrographs were selected to illustrate qualitative differences in leaf-surface structure, including longitudinal ridging and striation and the presence and arrangement of trichomes. SEM images were used for qualitative morphological comparison only and were not subjected to quantitative statistical analysis.

Statistical analysis

All statistical analyses were conducted in R. For the single-forage assays, the total number of recovered metacercariae per replicate was first compared among forage treatments using negative binomial regression. This approach was selected as recovered metacercarial counts were discrete and highly variable between replicate snails. Plant species was included as a fixed effect, with Italian ryegrass set as the reference treatment. Separate models were fitted for *F. hepatica* and *C. daubneyi*. Incidence rate ratios (IRR), 95% confidence intervals and *p* values were calculated to describe differences in total metacercarial recovery relative to Italian ryegrass.

Plant-associated encystment was analysed as a proportional count response using beta-binomial models fitted with the glmmTMB package separately for *F. hepatica* and *C. daubneyi*. For each replicate, the response was specified as a two-column matrix using the cbind function, with metacercariae recovered from plant material treated as successes and all remaining recovered metacercariae treated as failures. Plant species was included as a fixed effect, with Italian ryegrass again set as the reference treatment. Log-transformed total metacercarial recovery was included as an additional covariate to assess whether proportional encystment was influenced by the total number of metacercariae recovered per replicate. Replicates in which no metacercariae were recovered were excluded from proportional encystment analyses as no encystment opportunity occurred. For the *F. hepatica* encystment analysis, Cocksfoot was also excluded as only one metacercaria was recovered across this treatment, providing insufficient information for robust estimation of encystment probability. Odds ratios (OR), 95% confidence intervals and *p* values were calculated to compare the odds of encystment on each forage species relative to Italian ryegrass.

The *F. hepatica* mixed-forage competition assay was analysed using a beta-binomial model fitted with the glmmTMB package. For each assay tube and forage species, the response compared the number of metacercariae recovered from the focal forage species with the number recovered from all remaining locations within the same tube, including the other forage species and non-plant locations. Forage species was included as a fixed effect, with Italian ryegrass set as the reference treatment, and replicate tube identity was included as a fixed blocking factor to account for variation among assay tubes. A secondary analysis assessed whether the relative effect of forage species differed according to leaf orientation by including forage species-by-orientation interaction terms while retaining tube identity as a blocking factor. Model coefficients were interpreted as differences in the odds of metacercarial recovery on Cocksfoot or Timothy relative to Italian ryegrass.

An analysis was also conducted to assess whether *F. hepatica* metacercariae were distributed differently between the upper and lower leaf surfaces in the single-forage assay. For each replicate, counts recovered from the two leaf blades were summed separately for the upper and lower surfaces, and these were specified as a two-column proportional count response, with upper-surface counts treated as successes and lower-surface counts as failures. Replicates in which no metacercariae were recovered from either leaf were excluded, including all Cocksfoot replicates, while Plantain was excluded because only two metacercariae were recovered from leaf surfaces, providing insufficient information for model estimation. Sainfoin and Crimson Clover were also excluded because movement of the leaves during incubation meant that upper- and lower-surface orientation was not consistently maintained, preventing reliable assignment of recovered metacercariae to leaf surface. The remaining data were analysed using a beta-binomial model fitted with the glmmTMB package to account for overdispersion in proportional count data, with forage species included as a fixed effect. Estimated marginal means were obtained for each forage species on the logit scale using the emmeans package and tested against zero, corresponding to an equal probability (0.5) of metacercarial recovery from the upper and lower leaf surfaces.

Model diagnostics were assessed for all fitted models using simulated scaled residuals generated with the DHARMa package. Models were checked for convergence, residual uniformity, overdispersion, zero inflation and outliers. All figures were produced using ggplot2. For these, model-predicted means and proportions, with 95% confidence intervals, were generated using the emmeans package. For models including log-transformed total metacercarial recovery as a covariate, predicted proportions were estimated at the mean value of the covariate.

Results

F. hepatica and C. daubneyi recovery

Total metacercariae recovery varied among forage treatments for both trematode species, full results are presented and visualised in Table 1, Fig. 1 and Fig. 2. For *F. hepatica*, the highest total numbers of metacercariae were recovered in the presence of Yorkshire Fog, Timothy and Ryegrass, while very low numbers were recovered in the presence of Cocksfoot and Plantain. Negative binomial regression indicated that, relative to the Ryegrass control, total metacercariae recovery was significantly lower in the presence of Cocksfoot (IRR = 0.007, 95% CI: 0.000–0.130, $p < 0.001$) and Plantain (IRR = 0.030, 95% CI: 0.003–0.255, $p = 0.001$). No significant differences in total metacercariae recovery were detected between Ryegrass and Chicory, Crimson Clover, Sainfoin, Timothy or Yorkshire Fog.

For *C. daubneyi*, total metacercariae recovery was highest in the presence of Ryegrass and Plantain, whereas substantially fewer metacercariae were recovered in the presence of Yorkshire Fog. Negative binomial regression showed that total metacercariae recovery was significantly lower in presence of Yorkshire Fog than in presence of Ryegrass (IRR = 0.022, 95% CI: 0.003–0.157, $p < 0.001$). Total metacercariae recovery in the presence of Plantain did not differ significantly from Ryegrass (IRR = 0.643, 95% CI: 0.090–4.572, $p = 0.659$).

Table 1
Total metacercariae recovery results for F. hepatica and C. daubneyi. Predicted means were estimated using negative binomial regression with Ryegrass as the reference/control treatment. IRR, incidence rate ratio; CI, confidence interval.

Plant	Mean number of metacercariae (95% CI)	IRR vs Ryegrass (95% CI)	p value
F. hepatica			
Ryegrass (n = 5)	33.60 (8.37 to 134.94)	1.000	Reference
Chicory (n = 4)	11.75 (2.44 to 56.56)	0.350 (0.043 to 2.851)	0.326
Cocksfoot (n = 4)	0.25 (0.02 to 3.03)	0.007 (0.000 to 0.130)	< 0.001
Crimson Clover (n = 4)	15.50 (3.24 to 74.14)	0.461 (0.057 to 3.743)	0.469
Plantain (n = 5)	1.00 (0.19 to 5.14)	0.030 (0.003 to 0.255)	0.001
Sainfoin (n = 3)	29.67 (4.92 to 178.81)	0.883 (0.091 to 8.559)	0.914
Timothy (n = 4)	99.25 (21.10 to 466.83)	2.954 (0.369 to 23.667)	0.308
Yorkshire Fog (n = 5)	92.40 (23.13 to 369.15)	2.750 (0.386 to 19.573)	0.312
C. daubneyi			
Ryegrass (n = 7)	65.86 (17.39 to 249.46)	1.000	Reference
Plantain (n = 6)	42.33 (10.03 to 178.75)	0.643 (0.090 to 4.572)	0.659
Yorkshire Fog (n = 7)	1.43 (0.33 to 6.19)	0.022 (0.003 to 0.157)	< 0.001

F. hepatica and C. daubneyi metacercarial encystment

The proportion of metacercariae encysting on plant material differed among forage species, full results are presented and visualised in Table 2, Fig. 3 and Fig. 4. For *F. hepatica*, the highest predicted encystment proportions were observed on Chicory, Sainfoin, Crimson Clover and Ryegrass, whereas

lower predicted proportions were observed on Timothy and Yorkshire Fog. Relative to Ryegrass, the odds of encystment on plant material were significantly lower on Timothy (OR = 0.438, 95% CI: 0.202–0.948, p = 0.036) and Yorkshire Fog (OR = 0.106, 95% CI: 0.047–0.241, p < 0.001). Encystment on Chicory, Crimson Clover, Plantain and Sainfoin did not differ significantly from Ryegrass (Table 2; Fig. 3). Log-transformed total metacercarial recovery was not significantly associated with the proportion of *F. hepatica* metacercariae recovered from plant material (estimate = -0.144, p = 0.607), indicating that the lower proportional recovery on Timothy and Yorkshire Fog was not explained by higher total recovery in those treatments.

For *C. daubneyi*, predicted encystment on plant material was lowest on Ryegrass and highest on Plantain. The odds of metacercarial encystment were significantly higher on Plantain than on Ryegrass (OR = 38.305, 95% CI: 16.229–90.414, p < 0.001). Yorkshire Fog also had higher predicted encystment than Ryegrass (OR = 26.862, 95% CI: 3.664–196.929, p = 0.001), although total metacercariae recovery in this treatment was low. Log-transformed total metacercarial recovery was positively associated with proportional *C. daubneyi* encystment on plant material (estimate = 0.771, p = 0.027). However, Plantain and Yorkshire Fog remained significantly associated with higher plant-associated recovery than Ryegrass after adjustment for total recovery, indicating that these forage effects were not explained solely by differences in the number of metacercariae recovered per replicate.

Table 2
Modelled metacercarial encystment by forage species. Modelled encystment results for *F. hepatica* and *C. daubneyi*. Predicted proportions were estimated using beta-binomial models. OR, odds ratio; CI, confidence interval.

Plant	Total recovered	Total encysted on plant	Predicted proportion (95% CI)	OR vs Ryegrass (95% CI)	p value
F. hepatica					
Ryegrass (n = 4)	168	110	0.671 (0.531 to 0.785)	1.000	Reference
Chicory (n = 3)	47	34	0.712 (0.523 to 0.848)	1.215 (0.439 to 3.360)	0.708
Crimson Clover (n = 2)	62	43	0.672 (0.474 to 0.824)	1.006 (0.387 to 2.615)	0.991
Plantain (n = 3)	5	2	0.342 (0.066 to 0.793)	0.255 (0.029 to 2.253)	0.219
Sainfoin (n = 2)	89	57	0.685 (0.498 to 0.826)	1.067 (0.423 to 2.690)	0.891
Timothy (n = 3)	397	157	0.471 (0.301 to 0.649)	0.438 (0.202 to 0.948)	0.036
Yorkshire Fog (n = 4)	462	72	0.177 (0.090 to 0.318)	0.106 (0.047 to 0.241)	< 0.001
C. daubneyi					
Ryegrass (n = 5)	461	95	0.115 (0.057 to 0.220)	1.000	Reference
Plantain (n = 4)	254	230	0.833 (0.710 to 0.910)	38.305 (16.229 to 90.414)	< 0.001
Yorkshire Fog (n = 3)	10	6	0.778 (0.430 to 0.942)	26.862 (3.664 to 196.929)	0.001

Across assays, metacercariae not recovered from plant material were distributed among several non-plant locations, including water, snail surfaces, muslin and assay surfaces (Fig. 5). In the *F. hepatica* single-forage assay, non-plant recovery occurred mainly from assay surfaces, particularly in the Timothy, Cocksfoot and Yorkshire Fog treatments, whereas recovery from muslin and water was generally lower. In the *C. daubneyi* Visking tube assay, the distribution of non-plant metacercariae differed between forage treatments, with a greater proportion recovered from water in the Ryegrass treatment and from snail surfaces in the Yorkshire Fog treatment.

Mixed forage competition assay

Across the five replicate tubes, 224 metacercariae were recovered from Ryegrass, 177 from Cocksfoot and 174 from Timothy. A further 368 metacercariae were recovered from non-plant locations within the assay environment, with 123 encysted on the muslin, 234 on the tube, 10 were floating in water and 1 encysted on a snail's shell (Fig. 5). Relative to Ryegrass, the odds of metacercarial encystment were significantly lower on Cocksfoot (OR = 0.519, 95% CI: 0.278–0.971, p = 0.040) and Timothy (OR = 0.453, 95% CI: 0.240–0.858, p = 0.015). (Table 3; Fig. 6). There was no evidence that the relative effect of forage species differed according to whether the upper or lower leaf surface faced inwards in a replicate tube (likelihood-ratio test: $\chi^2 = 4.07$, df = 2, p = 0.131).

Table 3
Distribution of F. hepatica metacercariae in the mixed-forage competition assay. Predicted proportions were estimated using a beta-binomial model with Ryegrass as the reference/control treatment and replicate tube identity included as a blocking factor. OR, odds ratio; CI, confidence interval.

Plant	Total metacercariae recovered	Predicted proportion (95% CI)	OR vs Ryegrass (95% CI)	p value
Ryegrass	224	0.271 (0.199 to 0.358)	1.000	Reference
Cocksfoot	177	0.162 (0.106 to 0.239)	0.519 (0.278–0.971)	0.040
Timothy	174	0.144 (0.093 to 0.217)	0.453 (0.240–0.858)	0.015

Upper- and lower-leaf surface distribution of F. hepatica metacercariae

Significant lower-surface bias for *F. hepatica* metacercariae encystment was detected for Ryegrass (OR = 0.431, 95% CI: 0.215–0.906, $p = 0.026$), and on Yorkshire Fog (OR = 0.214, 95% CI: 0.082–0.561, $p = 0.002$). No significant upper- or lower-surface bias was detected for Chicory or Timothy.

All fitted models converged successfully. DHARMa residual diagnostics did not indicate substantial misspecification of any of the negative binomial or beta-binomial models reported in this paper ($p > 0.05$).

Leaf structure analysis

Scanning electron microscopy showed clear differences in leaf-surface structure among forage species and, for some species, between the upper and lower surfaces (Fig. 8). Italian ryegrass showed clear ridging on the upper surface, whereas the lower surface appeared smoother and more regular, with closely aligned, continuous longitudinal lines running in parallel and relatively few projecting structures. Timothy and Cocksfoot also showed ridges and grooves on both leaf surfaces, but these were less uniformly arranged and did not show the same consistent parallel longitudinal pattern seen on the lower surface of ryegrass. Yorkshire Fog had prominent trichomes on both the upper and lower leaf surfaces, with Crimson Clover similarly pubescent on both surfaces. However, the upper surface of Crimson Clover appeared to have fewer prominent trichomes than the lower surface, and trichomes overall appeared less prominent than those observed on Yorkshire Fog. Sainfoin showed relatively few prominent trichomes on the upper surface but numerous long trichomes on the lower surface, while Chicory showed a more irregular surface pattern on both sides and Plantain had a comparatively open upper surface but a more densely textured lower surface.

Discussion

This study provides novel evidence that forage species influences the encystment of infective metacercariae of trematodes *F. hepatica* and *C. daubneyi* on plant material. For *F. hepatica*, encystment was strongly reduced on Yorkshire Fog and, to a lesser extent, Timothy relative to Italian ryegrass. In the mixed-forage competition assay, *F. hepatica* metacercarial encystment was also significantly lower on Timothy and Cocksfoot than on Italian ryegrass. In contrast, *C. daubneyi* showed higher encystment on Plantain and Yorkshire Fog than on Italian ryegrass. Leaf-surface analysis further indicated lower-surface bias for *F. hepatica* metacercariae on Ryegrass and Yorkshire Fog, suggesting that encystment may vary not only among forage species but also within individual plants. Together, these results indicate that forage species can affect where on pasture metacercariae encyst, and that these effects differ between *F. hepatica* and *C. daubneyi*. This identifies sward composition as a previously underexplored factor that may influence the distribution of infective trematode stages on pasture and, consequently, transmission risk to grazing livestock.

The clearest effect observed for *F. hepatica* was the markedly lower encystment on Yorkshire Fog. Yorkshire Fog has a distinctive pubescent leaf surface, and it is plausible that this dense trichome layer reduced the ability of cercariae to establish sufficiently close contact with the leaf surface during settlement. Encystment in free-living metacercariae is thought to involve a sequence of initial contact, temporary attachment, surface exploration and permanent fixation, with cercariae detaching and searching elsewhere if settlement cues are inadequate [5]. Once suitable cues are encountered, the parasite orients its ventral surface towards the substrate, releases adhesive material and forms the cyst wall [5, 6]. A hairy or highly textured surface may interfere with this process by preventing close ventral contact, reducing effective adhesion, or generating an unsuitable tactile cue.

The apparent preference of *F. hepatica* for smoother surfaces is also supported by previous work. Pecheur [19] reported that, of nine plant species investigated, greater cercarial attachment occurred on *Juncus* sp., the upper surface of *Ranunculus repens* and the lower surface of *Glyceria fluitans*, all of which were described as offering smooth surfaces that allowed better fixation than the other plants tested. Dreyfuss et al. [20] reported higher recovery of *F. hepatica* metacercariae from cellophane sheets than from *Juncus* stems under natural conditions, while cellophane floats or rafts have been used to monitor *F. hepatica* metacercarial contamination in pasture-associated environments [21, 22]. In the present study, preliminary trials similarly indicated that *F. hepatica* readily encysted on plastic or Visking tube surfaces when these were available. Together, these observations suggest that artificial smooth surfaces can provide strong settlement cues for *F. hepatica*. To date, such surfaces have primarily been used for detection or surveillance rather than as active control tools. However, artificial trapping has been developed for schistosome cercariae using fatty-acid or oil-based stimulants incorporated into artificial matrices [23, 24]. Whether a comparable approach could be developed for *F. hepatica* metacercariae remains untested, but the tendency of *F. hepatica* to encyst on smooth artificial surfaces suggests that attractive matrices could be further explored as monitoring tools, and potentially as sacrificial or removable trap substrates.

The lower *F. hepatica* recovery on Timothy and Cocksfoot relative to Italian ryegrass suggests that surface structure may influence encystment even among grass species that lack the dense pubescence of Yorkshire Fog. Although Italian ryegrass is not entirely smooth, Cope and Gray [16] describe it as having flat leaf blades, while Timothy is described as being flat and glabrous but also scabrid on the upper surface and margins, with Cocksfoot described as being a coarser, densely tufted grass with blades ranging from smooth to coarsely scabrid. These descriptions were broadly consistent with the qualitative SEM observations in the present study, which showed a particularly regular, parallel longitudinal pattern on the lower surface of Italian ryegrass, whereas the surfaces of Timothy and Cocksfoot displayed less uniformly arranged ridges and grooves. These differences in blade texture and coarseness, together with the pubescence of Yorkshire Fog, may provide less favourable settlement conditions than the flatter ryegrass comparator. However, the high encystment rates observed on Crimson Clover, despite its pubescent leaf surface, indicate that trichome density alone is unlikely to explain the observed patterns. Qualitative SEM observations in this study suggested that trichomes on the upper leaf surface of Crimson Clover were generally less prominent than those observed on Yorkshire Fog. Furthermore, metacercariae on Crimson Clover were observed in clusters in areas of lower trichome density (Fig. 9). Together, these observations suggest that the effect of pubescence may depend on trichome density, length, rigidity and orientation, as well as the wider physical and chemical context of the leaf surface.

Within-plant spatial variation in encystment was also seen when comparing the upper and lower leaf surfaces of plant-associated *F. hepatica* metacercariae, with significant lower-surface bias detected for Ryegrass and Yorkshire Fog. This pattern may again reflect differences in leaf-surface traits. In Ryegrass, the upper leaf surface has been described as scaberulous or distinctly ridged, while the lower surface is smooth and glossy, which may provide a more favourable surface for cercarial attachment [25]. These descriptions were broadly consistent with the SEM observations in the present study, in which the upper surface showed pronounced ridging, whereas the lower surface appeared more regular, with closely aligned, continuous longitudinal structures and relatively few projecting features. This more regular lower surface may therefore provide more favourable conditions for cercarial contact, attachment and subsequent encystment. For Yorkshire Fog, the explanation is likely to be more complex, as this species is pubescent on both leaf surfaces [26]. Prominent trichomes were observed on both surfaces by SEM, indicating that the lower-surface preference in this species cannot be explained by trichome presence alone. More subtle differences in trichome arrangement, underlying epidermal structure or other anatomical characteristics, including differences in stomatal distribution, may instead have influenced attachment [26].

Pecheur [19] similarly reported differences between the upper and lower surfaces of several plant leaves, including greater attachment on the upper surface of *Ranunculus repens* and the lower surface of *Glyceria fluitans* which were attributed to the smoother properties of these surfaces. Hodasi [27] also reported that metacercariae were more frequently found on leaves than stems and were also more common on the green than the brown or dead parts of *Dactylis glomerata* suggesting that factors beyond surface texture may influence within-plant encystment location. In his review, Morley [5] emphasised that encystment by free-living metacercariae is likely to be shaped by multiple settlement cues, including tactile, visual and chemical signals. Colour and light responses also appear to vary between trematode groups, with several paramphistomes reported to show attraction to yellow light or yellow surfaces [28, 29], whereas *F. hepatica* has shown more inconsistent responses to colour [5]. *F. gigantica* cercariae meanwhile respond to both light wavelength and chemical stimuli, including amino acids and carbohydrates [30]. Therefore, differences between forage species in the present study may reflect a combination of surface structure, leaf orientation, leaf condition, colour, chemical cues and physical retention, rather than a single morphological trait. Although SEM provided qualitative evidence of differences in leaf-surface microstructure among forage species, these features were not quantitatively measured, and surface chemistry and colour were not directly assessed.

In contrast to *F. hepatica, C. daubneyi* showed higher encystment rates on Plantain and Yorkshire Fog than on Italian ryegrass, suggesting that forage effects on metacercarial attachment may differ between fluke species. Both Plantain and Yorkshire Fog present more structurally complex surfaces than ryegrass. As noted, Yorkshire Fog has a pubescent leaf surface, whilst Plantain leaves have prominent longitudinal veins and in the present assay, *C. daubneyi* metacercariae were observed to pool and aggregate along these (Fig. 9), suggesting that physical leaf structure may have influenced where cercariae settled. One possible explanation is that the positive geotactic behaviour reported for Paramphistome cercariae caused newly shed cercariae to sink onto the nearest available substrate, with Plantain veins or Yorkshire Fog trichomes acting as retention structures. By contrast, smoother ryegrass surfaces may have provided fewer physical points at which sinking cercariae could be retained before encystment.

However, these apparent species differences should be interpreted cautiously as the *F. hepatica* and *C. daubneyi* assays differed in both apparatus and plant orientation. The horizontal placement of forage material in the *C. daubneyi* Visking tube assay may have increased the importance of sinking behaviour and physical retention, whereas the vertical arrangement used for *F. hepatica* may have favoured different settlement dynamics. Nevertheless, differences in geotactic behaviour are likely to be ecologically relevant under field conditions, where the position of vegetation within the water column may influence which surfaces cercariae encounter. Positively geotactic *C. daubneyi* cercariae would be expected to sink towards basal vegetation, submerged plant structures or sediment, particularly in deeper or more permanently flooded microhabitats, whereas *F. hepatica* may be more likely to encyst on emergent or near-surface vegetation. This interpretation is supported by previous studies showing species-specific differences in encystment location and aggregation. Dreyfuss et al. [20] found that *F. hepatica* metacercariae were more frequently recovered from cellophane sheets than from *Juncus* stems, whereas *C. daubneyi* showed the opposite pattern and was concentrated lower on submerged substrates. Similarly, Abrous et al. [31] reported that *F. hepatica* was more frequently recovered from dish walls and formed larger aggregates, while *C. daubneyi* was more often found on dish bottoms and in smaller groups.

These species-specific differences in encystment behaviour may help explain why two parasites with broadly similar life cycles can nevertheless show divergent epidemiological patterns. Although *F. hepatica* and *C. daubneyi* share grazing ruminants as definitive hosts and *G. truncatula* as an intermediate host, their field patterns are not identical. Co-infection is common, but negative associations in infection intensity within flocks or herds [32] and contrasting changes in prevalence over time, have been reported [33]. These differences are often discussed in relation to treatment history, changing control practices and potential competition within the shared snail host [32], but species-specific ecology outside the definitive host may also contribute and remains comparatively underexplored [34]. Temporally, divergent field patterns may reflect differing climatic effects on egg hatching, cercarial emergence and metacercarial survival in *F. hepatica* and *C. daubneyi* [35]. Spatially, the present findings suggest that pasture composition and habitat structure may provide an additional source of divergence by determining whether water depth, vegetation architecture and available plant surfaces are more conducive to the geotactic behaviour and encystment requirements of one fluke species than the other.

The pasture-level relevance of these findings is currently difficult to determine because the botanical composition of typical fluke-risk habitats remains poorly characterised. Liver fluke risk is usually considered in relation to environmental suitability for the intermediate snail host, particularly shallow slow-moving water, boggy poached ground and general wet pasture microhabitats [3]. Some studies have used plant indicator species to identify habitats suitable for *G. truncatula*, but to our knowledge there have been no studies evaluating the full botanical composition of *G. truncatula* habitats and how it differs from non-*G. truncatula* habitats, with such differences likely to also influence grazing behaviour which could further influence infection risk. Furthermore, to our knowledge, there has been little systematic investigation of the botanical distribution of infective metacercariae in grazed pasture habitats. The main exceptions are studies of aquatic or semi-aquatic plants associated with human fascioliasis, particularly watercress [36–38].

Future field studies should therefore combine fine-scale botanical surveys of fluke-risk areas with direct recovery or molecular detection of metacercariae from individual plant species. This would help determine whether the forage effects observed under laboratory conditions translate into meaningful differences in the spatial distribution of infective stages in real swards.

These findings may also have potential implications for pasture management and the development of non-chemical approaches to trematode control. Yorkshire Fog appeared to be the clearest candidate for a plant surface associated with reduced *F. hepatica* encystment, but Yorkshire Fog itself is not an obvious candidate for deliberate inclusion in productive grazing swards because it is often regarded as undesirable in improved pasture, partly due to low palatability and lower digestibility [15]. Timothy and Cocksfoot may be more agronomically realistic alternatives as both are established temperate forage grasses, but the reductions in *F. hepatica* recovery observed on these species were more moderate. It therefore remains uncertain whether increasing these species in pasture would produce a biologically meaningful reduction in infection pressure under field conditions. Nevertheless, the results suggest that forage surface traits, rather than forage identity alone, may be important. If specific traits associated with reduced metacercarial attachment can be identified, these could ultimately provide targets for conventional breeding, genomic selection or genome-editing approaches in productive forage grasses [39–41]. However, their genetic basis and any consequences for agronomic performance would first need to be established. Further questions would also need to be answered regarding the fate of metacercariae that do not encyst on vegetation in the field, as unattached or weakly attached cysts may float, sink into sediment, detach under water movement, or remain available for ingestion via contaminated drinking water or wet mud, but the relative contribution of these pathways to transmission remains poorly understood [5].

A further question arising from this study is the reasons for low cercarial shedding seen in some forage treatments. In particular, *F. hepatica* metacercariae recovery was markedly reduced in the presence of Plantain, a bioactive herb with known anthelmintic properties [42]. It is therefore possible that water-soluble plant secondary metabolites influenced cercarial emergence here. However, this interpretation should be treated cautiously. Total metacercariae recovery was higher in other bioactive forage treatments, including Chicory and Sainfoin, and substantial *C. daubneyi* shedding occurred in the presence of Plantain. Moreover, the concentration of any plant-derived compounds released into the assay water was not measured, and total metacercariae recovery was highly variable throughout the study. Cercarial emergence is known to be sensitive to abiotic conditions such as temperature, light, and water conditions, as well as the physiological state of the snail host [43–45]. Consequently, although plant-mediated suppression of cercarial emergence is an intriguing possibility, the present data cannot distinguish this from either natural variation in snail shedding or potentially indirect effects of the plant on the assay environment or on snail behaviour. More broadly, the influence of pasture botanical composition on *G. truncatula* habitat use, behaviour and cercarial shedding remains poorly studied, and should be considered in future work examining plant-mediated effects on fluke transmission.

The main limitation of this study is that the assays were conducted under laboratory conditions and therefore cannot fully reproduce the complexity of natural fluke-risk habitats. Future work should test these findings under more realistic environmental conditions, although developing such systems is likely to be challenging. *G. truncatula* has been reared, maintained and experimentally infected under controlled laboratory conditions, with previous work describing its life history, experimental infection, culture methods and the influence of factors such as temperature, nutrition, substrate, water quality and food availability on snail survival and parasite development [46–49]. However, maintaining suitable conditions for *G. truncatula* outside the laboratory is likely to be more difficult as snail activity, reproduction, survival and cercarial shedding are strongly influenced by moisture, temperature and habitat structure. Historic field-based work demonstrates that snail populations can be followed under natural conditions, but these approaches were primarily observational rather than controlled manipulations of habitats [50]. Controlled semi-natural assays, such as outdoor mesocosms or enclosed wet-sward systems containing intact vegetation, managed water levels and infected snails, would therefore represent an important intermediate step between simplified laboratory assays and observational field studies. Developing such systems would unlock substantial research potential by allowing the effects of plant species, leaf orientation, water depth and temperature variation to be tested under more realistic conditions while retaining greater experimental control than fully natural fluke-risk habitats. Such systems could also be used to investigate broader habitat-level factors, including the effects of soil properties on *G. truncatula* populations and the potential use of soil amendments as a control strategy [51]. When combined with field studies incorporating botanical surveys, snail mapping and direct or molecular detection of metacercariae on individual plant species, these approaches could help build a more integrated understanding of fluke transmission and support the development of more sustainable *F. hepatica* control strategies.

In conclusion, this study demonstrates that forage species can influence the encystment of *F. hepatica* and *C. daubneyi* metacercariae on plant material, and that these effects differ between fluke species. The within-leaf distribution of *F. hepatica* and qualitative SEM observations further suggest that fine-scale leaf-surface characteristics may contribute to these effects. These findings identify plant–parasite interactions during the free-living metacercarial stage as a plausible but underexplored determinant of trematode transmission risk. While further field validation is required, the results suggest that sward composition may influence the spatial availability of infective trematode stages on pasture and should be considered in future studies of fluke ecology and sustainable parasite control.

Declarations

Ethics approval

The study was approved by the Aberystwyth University ethics committee (29512Sa).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Author Contribution

Conceptualisation: RJ, CJ. Formal analysis: RJ. Funding acquisition: RJ, CJ. Investigation: SE, SG. Methodology: RJ, SE, SG, CJ. Project administration: RJ, SE. Supervision: RJ. Visualisation: RJ. Writing—original draft: RJ. Writing—review & editing: SE and CJ.

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Data Availability

The data that support the findings of this study has been deposited on PURE (awaiting DOI)

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Figures

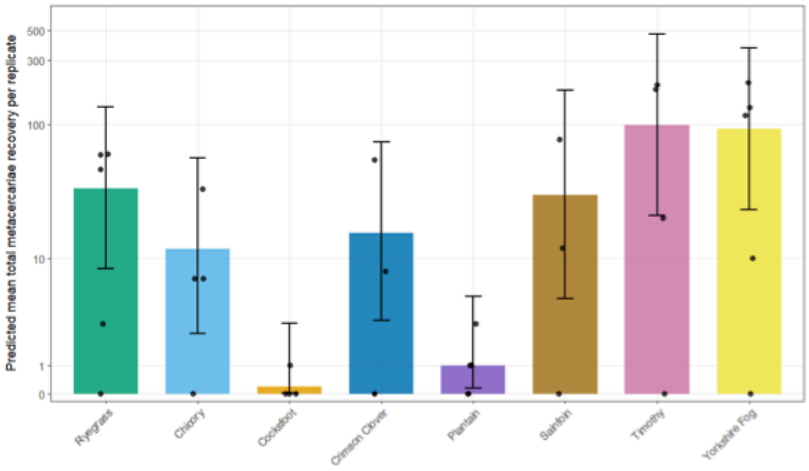

Figure 1

Effect of forage species on total *F. hepatica* metacercariae recovery. Bars show model-predicted means, points show raw replicate-level total metacercariae recovery counts, and error bars represent 95% confidence intervals.

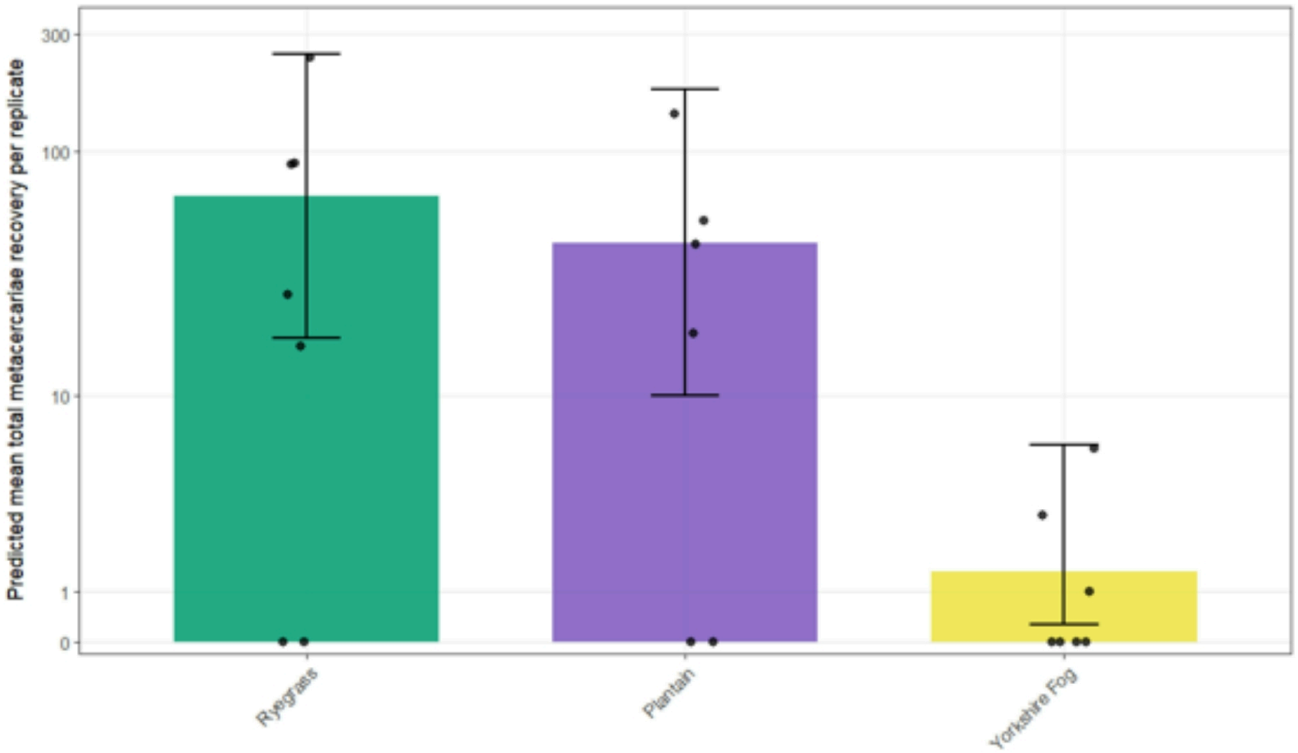

Figure 2

Effect of forage species on total *C. daubneyi* metacercariae recovery. Bars show model-predicted means, points show raw replicate-level total metacercariae recovery counts, and error bars represent 95% confidence intervals.

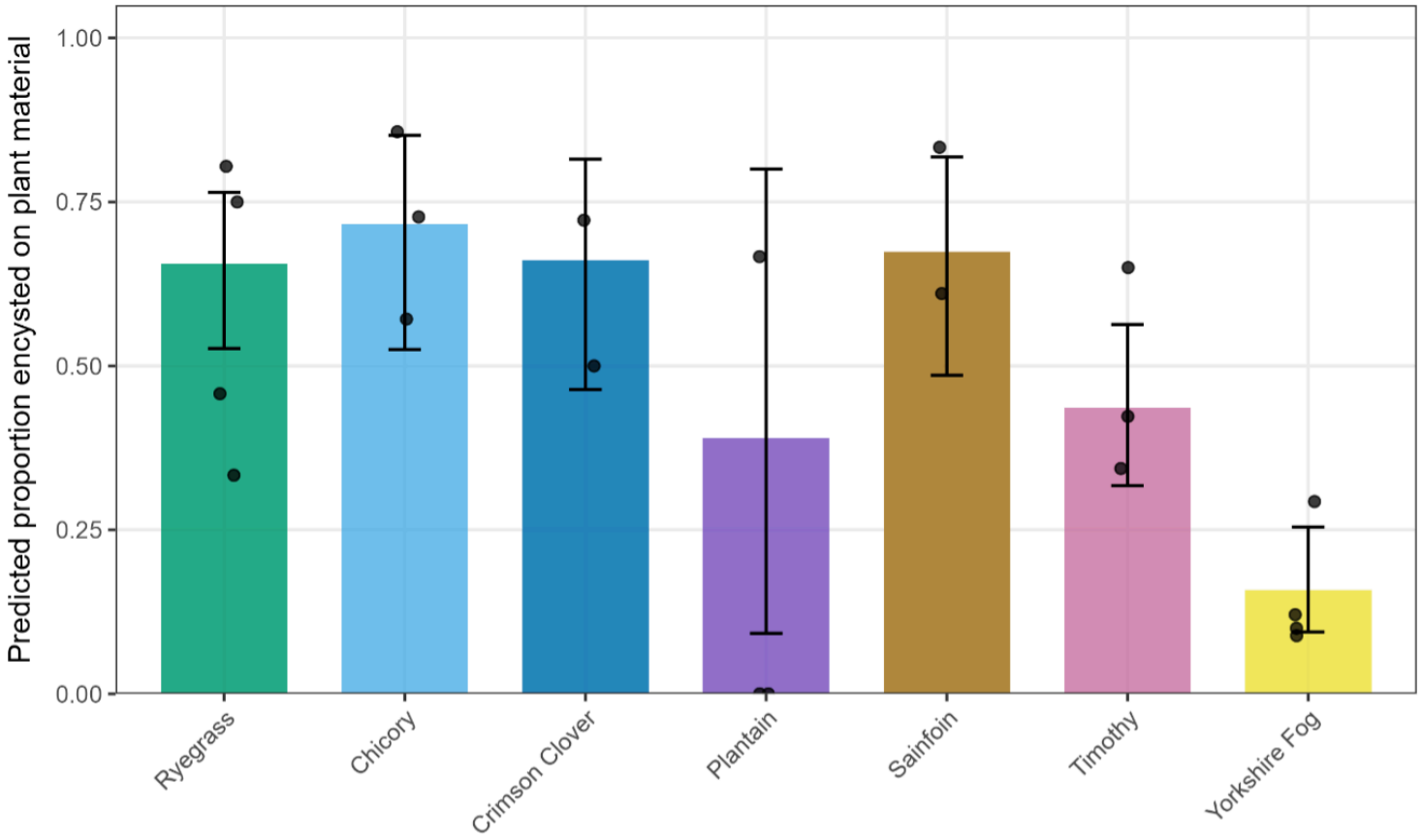

Figure 3

Effect of forage species on *F. hepatica* metacercarial encystment on plant material. Bars show model-predicted proportions from a beta-binomial model, points show raw replicate-level encystment proportions, and error bars represent 95% confidence intervals.

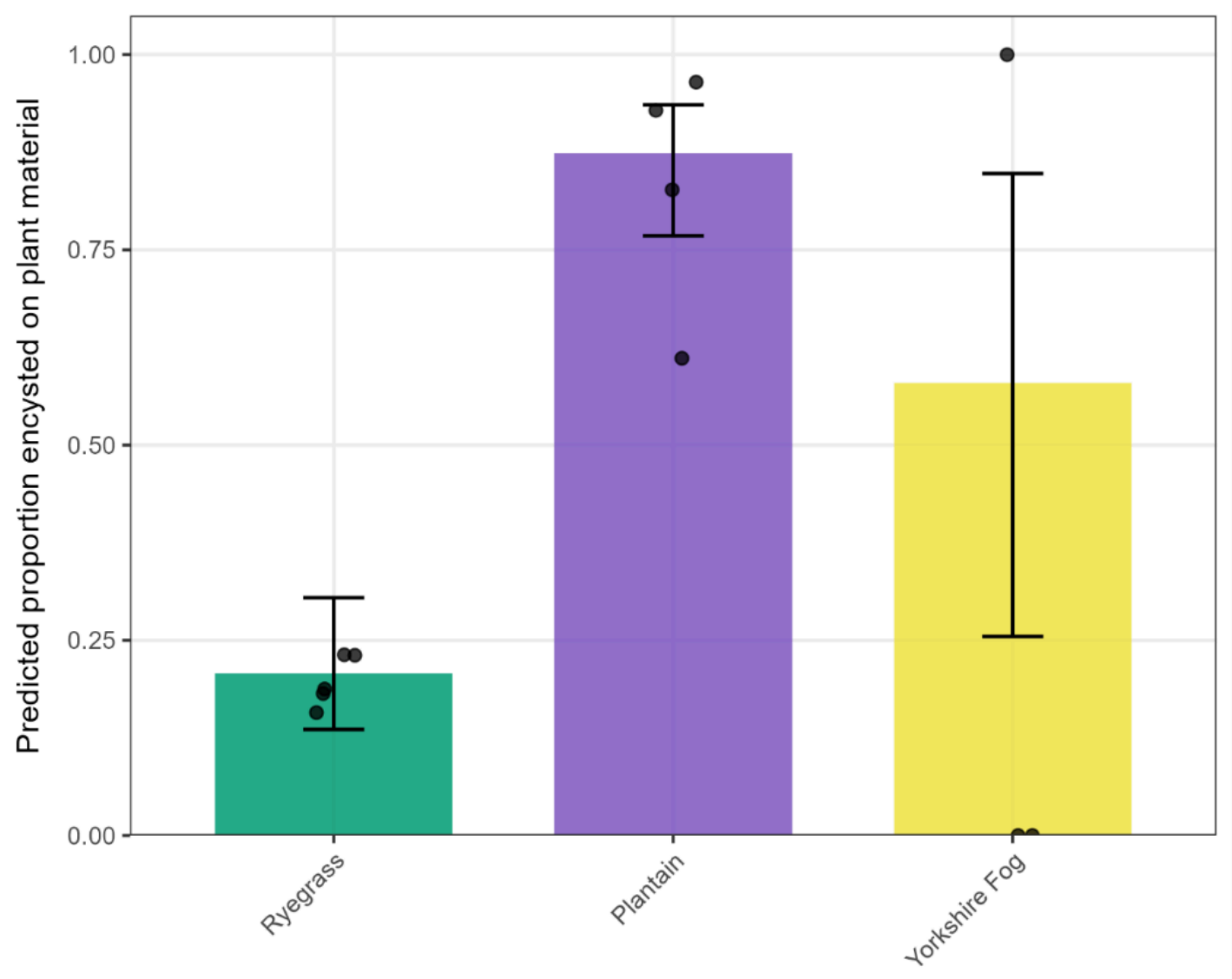

Figure 4

Effect of forage species on *C. daubneyi* metacercarial encystment on plant material. Bars show model-predicted proportions from a beta-binomial model, points show raw replicate-level encystment proportions, and error bars represent 95% confidence intervals.

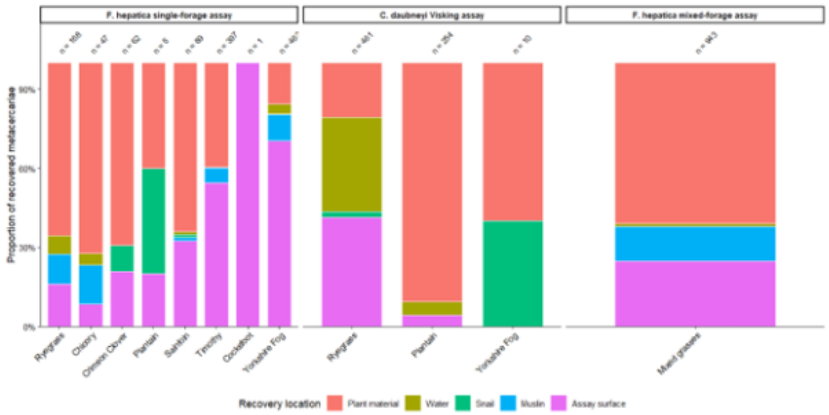

Figure 5

Proportional distribution of recovered *F. hepatica* and *C. daubneyi* metacercariae across assay locations.

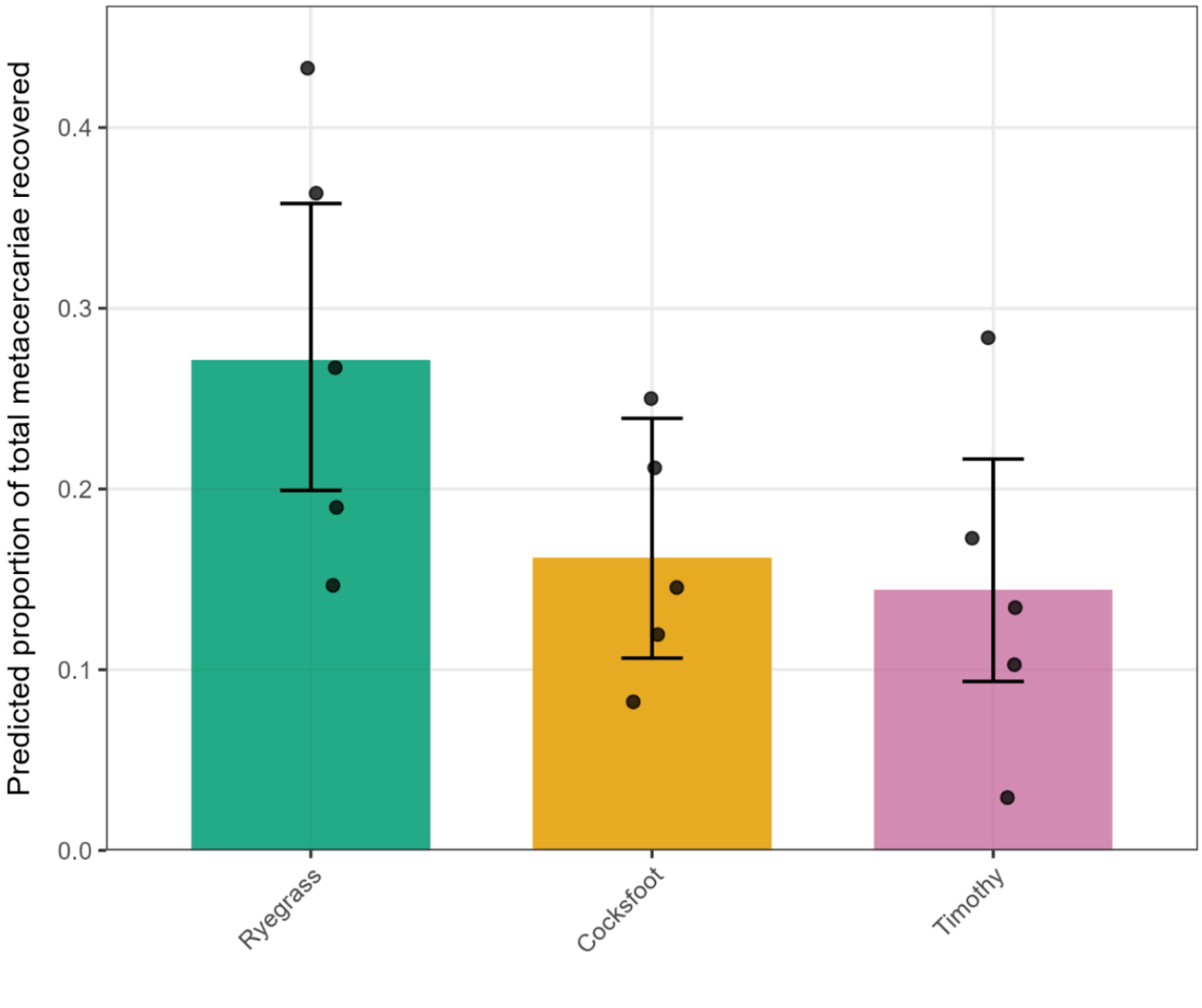

Figure 6

Distribution of *F. hepatica*metacercariae in a mixed-forage competition assay. Bars show model-predicted proportions, points show raw replicate-level proportions and error bars represent 95% confidence intervals.

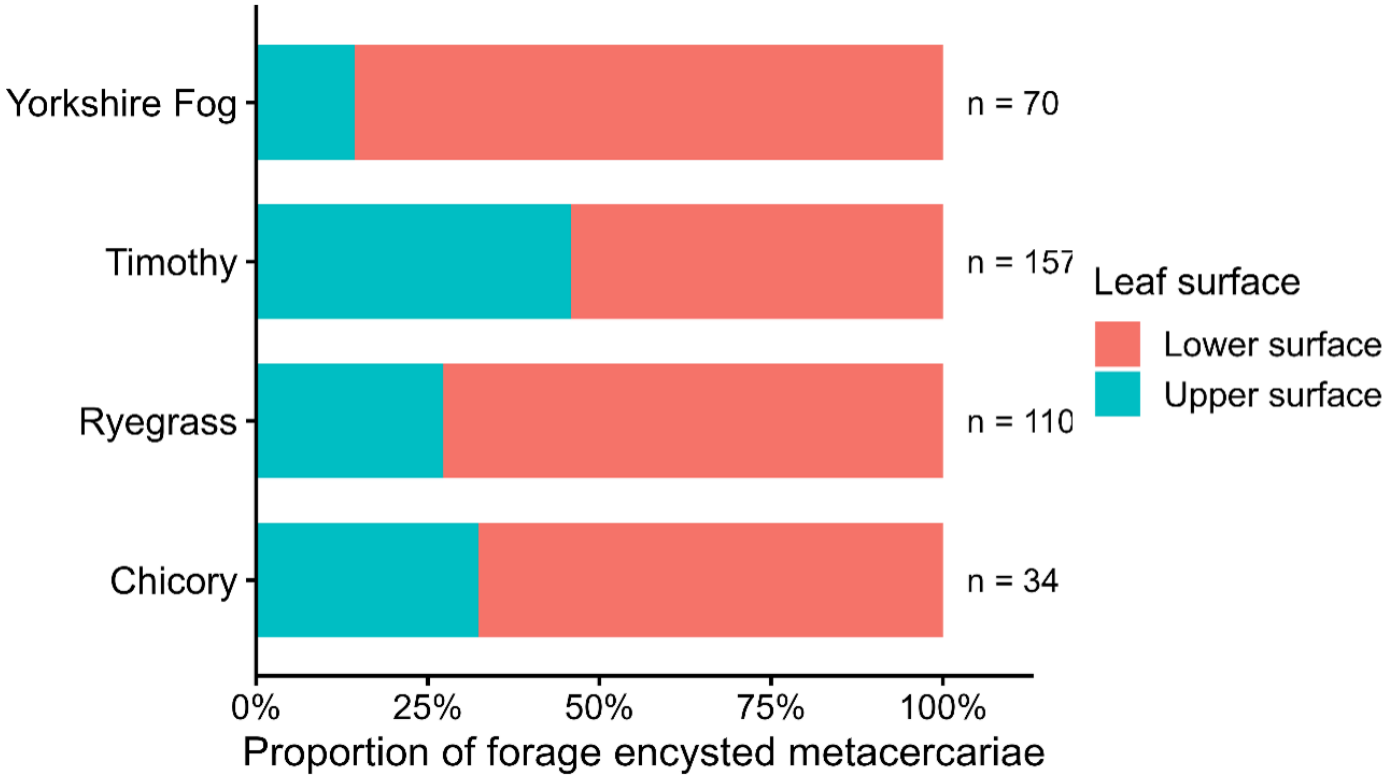

Figure 7

Distribution of *F. hepatica* metacercariae between upper and lower leaf surfaces.

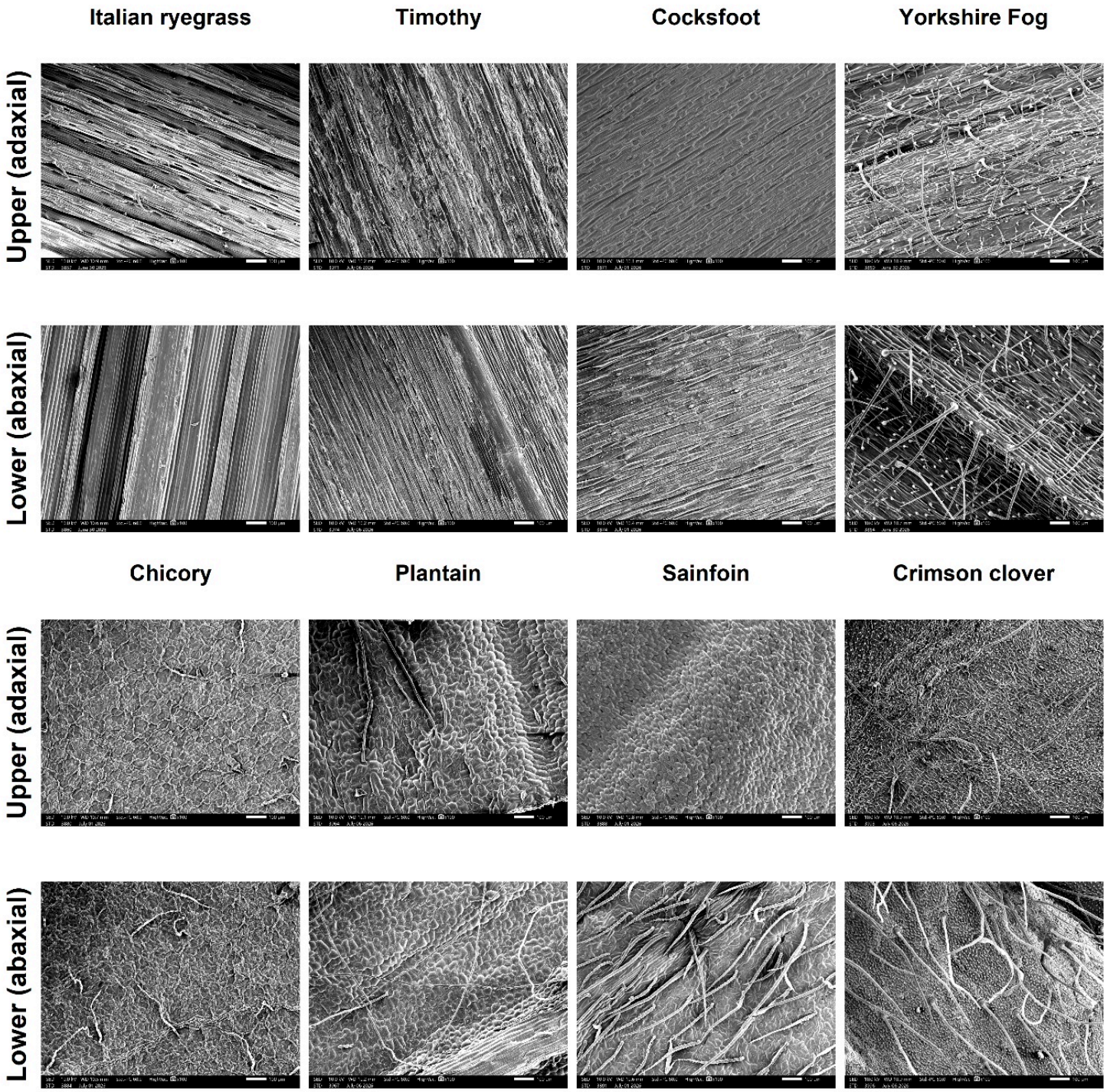

Figure 8

Scanning electron micrographs of the upper (adaxial) and lower (abaxial) leaf surfaces of forage species used in the metacercarial encystment assays. Images illustrate qualitative variation in leaf-surface structure, ridging and grooving, and the presence and arrangement of trichomes among forage species and between leaf surfaces. Scale bars = 100 µm.



Figure 9

Localised metacercarial encystment on Crimson Clover and Ribwort Plantain leaves. Left: *F. hepatica*metacercariae on Crimson Clover, with clustering observed in an area of relatively lower trichome density. Right: *C. daubneyi* metacercariae on Ribwort Plantain, with cysts aggregated along the longitudinal leaf veins.

Supplementary Files

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- floatimage1.jpeg