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Original Article

The feeding of heather (*Calluna vulgaris*) to *Teladorsagia circumcincta* infected lambs reduces parasitism but can detrimentally impact performance

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Abstract

Gastrointestinal nematode (GIN) infections impact small ruminant health, welfare, and production across farming systems. Rising anthelmintic resistance and regulation of synthetic drug use in organic farming is driving research and development of sustainable alternatives for GIN control. One alternative is the feeding of plants that contain secondary metabolites (PSMs) e.g., proanthocyanidins (PA, syn. condensed tannins) that have shown anthelmintic potential. However, PSMs can potentially impair performance, arising from reduced palatability and thus intake, digestibility or even toxicity effects. In this study, we tested the trade-off between the antiparasitic and anti-nutritional effects of heather consumption by lambs. The impact of additional feeding of a nematophagous fungus (*Duddingtonia flagrans*) on larval development was also explored. Lambs infected with *Teladorsagia circumcincta* or uninfected controls, were offered *ad libitum* heather, or a control chopped hay for 22 days during the infection patent period. Eight days into the patent period, parasitised lambs were supplemented (or remained unsupplemented) with *D. flagrans* for a 5-day period. Performance and infection metrics were recorded, and polyphenol levels in the heather and control hay were measured to investigate their association with activity.

The lambs consumed heather at approximately 20% of their dry matter intake, which was sufficient to exhibit significant anthelmintic effects via a reduction in total egg output ($P = 0.007$), compared to hay-fed lambs; the magnitude of the reduction over time in heather fed lambs was almost 10-fold compared to control lambs. Negative effects on production were shown, as heather-fed lambs weighed 6% less than hay-fed lambs ($P < 0.001$), even though dry matter intake (DMI) of heather increased over time. *D. flagrans* supplementation lowered larval recovery in the faeces of infected lambs by 31.8% ($P = 0.003$), although no additive effects of feeding heather and *D. flagrans* were observed ($P = 0.337$). There was no

significant correlation between PA, or other polyphenol subgroups in the diet and egg output, which suggests that any association between heather feeding and anthelmintic effect is not simply and directly attributable to the measured polyphenols. The level of heather intake in this study showed no antagonistic effects on *D. flagrans*, demonstrating the methods can be used in combination, but provide no additive effect on overall anthelmintic efficacies. In conclusion, heather feeding can assist to reduce egg outputs in infected sheep, but at 20% of DMI negative effects on lamb performance can be expected which may outweigh any antiparasitic benefits.

Keywords: Anthelmintic; Anti-nutritional; *Calluna vulgaris*; gastrointestinal nematode; *Teladorsagia circumcincta*

Introduction

Gastrointestinal nematode (GIN) infections are a common cause of decreased productivity and welfare in ruminants around the world (Charlier et al., 2018). Anthelmintic drug use is a key component of GIN-related disease control (Kaplan, 2020), however, due to the rise in anthelmintic resistance (Sangster et al., 2018) and regulations on the use of anthelmintics in organic systems, sustainable alternatives are being investigated. These alternatives aim to compliment the use of anthelmintic drugs and consequently reduce the reliance on them, which is important to mitigate anthelmintic resistance and support low input farming systems (Willer et al., 2021). The use of bioactive plants i.e., plants rich in secondary (otherwise known as specialised) metabolites (PSMs) that exert biological activity, have shown anthelmintic activity in vitro and in vivo. In some cases, active PSMs identified include polyphenols e.g. proanthocyanidins (PAs, syn. condensed tannins) (Bahuaud et al., 2006; Hoste et al., 2006), and terpenes (Katiki et al., 2017; Peña-Espinoza et al., 2018).

Bioactive plants have been associated with direct anthelmintic activity which has been demonstrated in vitro, where various parasite stages are detrimentally affected when exposed to PSMs (Martínez-Ortíz-de-Montellano et al., 2013; Engström et al., 2016). Direct effects have also been shown in vivo during short-term feeding experiments, where PSM consumption resulted in an immediate decrease in worm numbers in sheep, prior to the development of immunity (Athanasiadou et al., 2000, 2001). The consumption of certain PSMs can have additional, indirect benefits; for example, PAs can bind to dietary protein, protect it from rumen degradation and consequently increase rumen by-pass protein availability (Lorenz et al., 2014). At times of protein scarcity, this additional protein supply leads to improved expression of immunity, as demonstrated via a reduction in worm burdens or an increase in immune cells in parasitised hosts (Athanasiadou et al., 2008). However,

some PSMs and in particular PAs, have been shown to also exhibit anti-nutritional properties when consumed above a threshold, demonstrated by reduced intake leading to weight loss, and even toxicity (Athanasiadou et al., 2001; Hervás et al., 2003). Marie-Magdalene et al (2010) showed that sheep offered a PA-rich cassava foliage had reduced *Haemonchus contortus* load compared to control animals, but penalties on intake and growth were also noted (Marie-Magdalene et al, 2010). Similarly, sheep infected with *H.contortus* showed a reduction in their parasite burden following consumption of a PA-rich foliage, but also reduced dry matter digestibility, which may have resulted in penalties in growth (Mendez-Ortiz et al, 2012). Solving this trade-off between the positive (anthelmintic) and the negative (anti-nutritional) effects of PSM and quantifying whether there is an overall cost or benefit for the animal is a key factor in the use of bioactive plants for parasite control (Athanasiadou and Kyriazakis, 2004).

The perennial shrub heather (*Calluna vulgaris*) is highly abundant across Europe and is rich in PAs (Tolera et al., 1997). Its antiparasitic effects have been shown in vitro (Moreno-Gonzalo et al., 2013a, 2013b), and in vivo in goats experimentally infected with *Trichostrongylus colubriformis* (Moreno-Gonzalo et al., 2014), *Teladorsagia circumcincta* (Moreno-Gonzalo et al., 2013c) and with natural infections (Osoro et al., 2007). In sheep, heather consumption at 11% of total DM intake resulted in a small reduction in faecal egg counts (FEC) in *Haemonchus contortus* infected lambs (Maurer et al., 2022). This impact on the FEC was short-lived but there were no penalties on the performance of parasitised sheep. This indicates that the inclusion level of heather in the diet of parasitised sheep could potentially be increased to improve anthelmintic efficacy, but it is not known whether this may have a negative impact on the performance of the sheep. This will be tested in the current study.

Due to these constraints in the use of bioactive plants, i.e. lower efficacy compared to anthelmintic drugs and potential anti-nutritional effects, it is unlikely they will be used in isolation (Houdijk et al., 2012). Nematophagous fungi naturally occur in the environment and target larval stages of nematodes in faeces. The fungi trap and kill nematodes with the use of hyphal trapping devices thus preventing ingestion and infection of hosts (Waghorn et al., 2003; Lopez-Llorca et al., 2007). The combined use of bioactive plants and nematophagous fungi could deliver additional benefits compared to their use in isolation. The two strategies have different mechanisms of activity, with bioactive plants and nematophagous fungi targeting the parasites within the animal and in the environment, respectively. Importantly, evidence on a potential additive impact of nematophagous fungi could mitigate possible anti-nutritional effects of heather by enabling the use of a lower level of plant consumption without compromising overall anthelmintic efficacy arising from their combined use.

The objective of our study was to investigate the trade-off between the anti-nutritional and the anthelmintic effects of heather consumption in lambs artificially infected with *T. circumcincta*. Additionally, the efficacy of using nematophagous fungi (*Duddingtonia flagrans*) on larval development in the presence of heather is investigated, as there is some evidence that PA may have anti-fungal properties (Peng et al, 2018). Our hypothesis was that heather administration at a level higher than previously incorporated in the diets of parasitised sheep (Maurer et al., 2022) will reduce the level of parasitism in infected lambs, but animals will suffer the penalties of anti-nutritional effects.

Materials and methods

Animals

Sixty Texel-cross lambs, equal number of females and castrated males, were used in the trial. The experimental work was performed under UK Home Office licence (P1B4DFBA5) and maintained in accordance with the Animal (Scientific Procedures) Act 1986. The study was approved by SRUC's Animal Experiment Committee (Approval number, SHE AE 20-2020; Approval date, 24th June 2020). The lambs were reared indoors from birth to remain parasite naïve. At the start of the experiment the lambs were aged an average of 161.3 days (standard error [SE] 0.96) and weighed on average 34.6 kg (SE, 0.49).

Experimental design and housing

The experiment included six groups of ten lambs, all balanced for body weight (BW) and sex, and with standard deviation (SD) of BW as low as possible within groups (average group SD, 3.8 kg). Power calculations were performed to determine group size; based on our previous studies, coefficient of variation is higher for worm counts (20%) compared to faecal egg counts (15%). The results of the power calculations showed that to detect a difference in worm counts between the groups of animals feeding on heather or control hay of the magnitude of 40%, at a level of significance P=0.05, sample size should be set at 10 animals per group. As the variation previously described is lower for FEC, it was expected that this group size would be adequate to identify differences in FEC too, if there were any. The pen number for each lamb was assigned at random, and lambs were placed in individual pens measuring 1.45 m by 1.84 m on wood shavings in four rows of fifteen pens, and were able to view, touch and hear each other. After 24 days of acclimatisation to their housing conditions, four groups of lambs were artificially infected in their pens with a single dose of 12,000 *T. circumcincta* L_3 larvae in 5 mL of water (infected, I), and the two remaining groups were given 5 mL of water as a sham infection in their pens (uninfected, U). Before the

experimental diets commenced (day 21), eight uninfected lambs were found to be excreting a small number of eggs, probably through bedding contamination (<50 epg), so all the uninfected lambs were consequently dosed with 1 ml/ 10 kg of Monepantel (Zolvix, Elanco). Twenty-two days after infection, the dietary treatments were introduced, and the lambs were maintained on the experimental diets for a further 22 days.

Dietary treatments

During the acclimatisation (days -24- -1) and prepatent (days 0-21) periods, all lambs were given ad libitum access to long hay and were also offered 500 g grain-based coarse ration (GoldenBlend Lamb 18, I'Anson Brothers Ltd) and 500 g lucerne pellets (Tarff Valley Ltd). During the patent period (days 22-44) the amount of lucerne pellets offered was reduced to 100 g, to encourage all animals to increase the experimental diet intake. The experimental diet was either heather or chopped hay; two groups of infected lambs and one group of uninfected lambs were given ad libitum access to heather (H groups), and the remaining three groups, two infected and one uninfected were given ad libitum access to chopped hay (50mm length from10 year old ley) as a control (C group). Overnight, all lambs had access to a restricted amount of long hay which was fed at roughly 30% of dry matter (DM) intake as calculated during the prepatent period. Feeding protocols during the timeline of the experiment are shown in Fig. 1.

Figure 1 here

Eight days into the patent period, two groups of infected lambs (one receiving heather and one on the control diet) received 1 x 10^5 chlamydospores/ kg bodyweight of *D. flagrans* (D) (isolate FiBL-DF-P14; CBS 138751) once a day for a 5-day period (day 30 – 34). No spores

were given to uninfected lambs as they do not directly affect animal performance but are only active against parasites within the faeces (Braga and De Araújo, 2014). The pellets containing the spores (1 g /100 kg bodyweight) were mixed in with 10 g of coarse mix and placed in a separate pot to ensure the spores were consumed.

Heather collection and preparation

Heather was collected fresh in the morning on three occasions (day 15, 23 and 31) during the trial from Castlelaw, a site in the Pentland hills, Scotland (55.8705° N, 3.2391° W). A scythe cutter bar was used to cut the heather (set to 70mm) and it was picked up by hand. It was stored in wool bags within the trial shed and trimmed to ensure large woody parts of the plant were discarded and mostly green shoots were offered to the H lambs.

Feed analyses

Samples of coarse mix, lucerne pellets, and pooled samples of offered and refused heather and hay were analysed for nutrient content and predicted energy at Scotland's Rural College (SRUC) Analytical Services. A commercial method standardised by master near-infrared spectroscopy instruments and gold standard wet chemistry analysis was used. Metabolisable energy (ME) was calculated as per equations were taken from AFRC (1993).

Heather samples from each batch collected on days 15, 23 and 31, along with pooled refused heather from each batch, were analysed for their polyphenol profiles with ultraperformance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) as outlined by Malisch et al. (2016) and Salminen (2018). A sample of offered and refused hay was also analysed. Twenty mg of finely ground heather or hay powder was extracted with 2 x 1.4 mL acetone/ water (80:20, v/v) on a rotary shaker for 2 x 3 h (280 rpm), followed by

centrifugation for 10 min. The supernatant was transferred to a new micro centrifuge tube and evaporated to water phase in an Eppendorf concentrator (5301, Eppendorf AG). Aqueous samples were frozen at −20 °C and lyophilised. The freeze-dried phenolic extract was dissolved in 1 mL of Milli-Q purified water, vortexed for 5 min, and filtered with a 0.20 μm PTFE filter into UPLC vials. The sample was 1:5 diluted with water and analysed with group specific UHPLC-MS/MS methods according to Engström et al. (2014, 2015) on an Acquity UPLC system (Waters Corp., Milford, MA, USA), interfaced to a Xevo TQ triple-quadrupole mass spectrometer with electrospray ionisation (Waters Corp., Milford, MA, USA).

Animal measurements

Once animals were individually housed (day -24), refused feed was weighed every morning to determine individual daily feed intake; lambs were weighed weekly to determine body weight. From the first day of *T. circumcincta* egg shedding, which was 21 days post-infection, faecal samples were collected weekly; the last faecal sample was collected on the day of euthanasia for determination of FEC. Faecal samples were also collected for larval coprocultures the day before and on days 3 and 5 of *D. flagrans* dosing. Across 2 days at the end of the trial (day 45 and 46 of the experiment) the lambs were euthanised using intravenous barbiturates at 0.8ml/ kg (Euthatal, Boehringer Ingelheim Animal Health UK Ltd) and the abomasum was removed for parasite recovery. Thirty lambs were selected for post-mortem on each of the 2 days. The selection was blocked for treatment group and was random, using a number generator on Excel.

Faecal egg counts, faecal output and egg output

Faecal egg counts were carried out to determine the concentration of eggs in one g (EPG) of fresh matter (FM) faeces using an adapted egg flotation method (Christie and

Jackson (1982). Faecal samples were taken from the rectum, stored at 4℃ until the FEC was undertaken on the same day for all samples apart from the ones collected on the day of euthanasia which were carried out 4 days later.

As FEC is a concentration number, it can be affected by diet, the amount of food ingested, digestibility and composition, as well as feed consistency (Houdijk 2008); to account for some of this variation, estimates of FEC DM and total egg outputs were also calculated for three dates on which excess faeces was available: days 21, 28 and 42 post-infection. Faecal DM content was determined by weighing faecal samples before and after placing in a drying oven at 40℃ until dry weight reached, and then EPG of FM faeces was divided by dry matter proportion to get FEC DM. To calculate total egg output, an estimate of faecal output was determined using the equation taken from Garrett et al. (2020):

$$\text{Faecal output DM}\left(\frac{g}{d}\right) = \text{DMI}\left(\frac{g}{d}\right) \text{ x } \left(1 - \frac{\text{Digestibility \%}}{100}\right)$$

Firstly, dry matter intake (DMI) of the dietary components for each lamb was calculated for three consecutive days prior to FEC analysis. The digestibility (D-value) of each dietary component from the nutritional analysis (Table 1) was then used to determine the dry matter digestibility for the total feed ration. DMD has been reported to be a reliable marker of digestibility for heather (Milne et al, 1977). Then, using the equation above, the average DM faecal output could be calculated for the three consecutive days by inputting the DMI and digestibility values, assuming that rations offered and ingested had similar digestibility for each diet. The DM % of the faecal sample taken on the day of the FEC was used to convert the faecal output value into FM. The FM faecal output could then be multiplied by EPG from the corresponding FEC FM to estimate total daily egg output.

Adult worm counts and per capita fecundity

The abomasum and its contents from infected lambs was removed and placed into 5 litres of saline (0.9% NaCl) at 25℃ for 4 h to allow adults worms to disperse into the solution. The abomasa were then rinsed and discarded, and a 500 mL sample was taken after vigorous mixing to give a homogenous sample. Samples were fixed with 10% paraformaldehyde and stored at room temperature. A 2% sub-sample of the abomasal content (20% subsample of the 500 ml samples) was used to count the number of male and female adult worms on a grided petri dish using a stereoscope. The number of worms recovered from the sub-sample was then multiplied by 50 to give total number per lamb. An estimate of per capita fecundity was calculated by dividing egg output calculated on day 42 by the number of female adult worms recovered at euthanasia.

Coproculture and larval recovery counts

Fresh faeces for the coprocultures were collected either by grab sample, or directly via the rectum. Ten g from each lamb were placed in covered petri dishes containing moist paper towels for 14 days at 25℃ to allow eggs to hatch and develop to L_3 larvae. Faeces were then placed over Baermann apparatus in liners within falcon tubes with a pore size that allowed L_3 larvae to migrate through and, after being left overnight, a drop of Lugol's reagent was added to kill and preserve the larvae. The number of L_3 larvae recovered were counted to determine the number of larvae per 10 g of faeces. The larvae development rate (%) was estimated by dividing the number of L_3 recovered from the larval cultures by the number of eggs counted in 10 g from the closest FEC FM and multiplying by 100.

Statistical analysis

All statistical analysis was carried out in Genstat 19th edn (VSN International, 2020). Data from two lambs in separate treatment groups were excluded from the analysis; one due to illness not related to the experiment (UH), and one due to a technical mistake (IC).

To assess the effect of time, treatment (6 treatments combinations of diet, infection and fungi supplementation) and their interactions on daily DM intake and weekly BW during the prepatent and patent periods, repeated measures ANOVA was used. The BW value before infection was included in the model as a covariate for both variables. Repeated measures analysis for BW was followed by fitting orthogonal contrasts (diet x infection status) for BW analysis on each week; this was done as the repeated measures ANOVA showed significant interaction between time and treatment, and so to locate the basis of the interaction. Daily heather intake and BW data for H lambs were associated and r^2 values, which describe the proportion of the variation in the data that can be explained by the model, were obtained. Average individual daily liveweight gain was calculated by linear regression at lamb level; all weight data points were included in the model. Daily liveweight gain was analysed by two-way ANOVA, with diet and infection status as factors and their interactions, for the overall experimental period. Animal/pen was included in the model as a block effect.

Adult worm counts and per capita fecundity were analysed using a two-way ANOVA with diet (H or C) and date of euthanasia (day 45 or 46) as the treatment factors, and FEC on day 21 as a covariate. Faecal egg counts on FM and DM basis, faecal output and daily egg output were analysed with a repeated measure ANOVA, with diet as a factor and the FEC value on day 21 (before the experimental diets commenced) as a covariate. Typically, FEC follow a negative bionomial distribution with large number of zeros. The Shapiro-Wilk test for Normality was used to test the distribution of the residuals for FEC, egg output and per

capita fecundity prior to analysis. The residuals of all parasitological parameters here were not normally distributed; the log-transformations (log (n) or log(n+1)) that are usually used to transform non normally distributed data did not work on FEC FM and adult worm count data, so these data were square root transformed prior to analysis. FEC DM, egg output and per capita fecundity data were log-transformed (log(n+1) to stabilise the variance. The use of these transformations achieved normality of the residuals in all parameters. Transformed means are reported as back transformed means with 95% confidence intervals. To associate heather and polyphenol subgroup intake data with egg output for IH lambs, r^2 and P values were obtained in Genstat. Linear regressions between daily egg output and heather (or polyphenol groups) intake, for infected heather-fed lambs were fitted. In the regressions we have included one point per animal (average egg output vs average polyphenol or heather intake). Heather or polyphenol intake was calculated from three days prior to daily egg output determination on the two occasions (days 28 and 42; the average intake was include in the regression analysis). The chemical analyses values of heather used to calculate polyphenol intake were the respective ones for each of the dates included in the analysis (for day 28 heather sampled on the 07/10/20 and for day 42 heather sampled on the 15/10/2020).

Larval recovery was measured in the faecal cultures of infected lambs and the development rate (%) was analysed using a two-way ANOVA, with diet (H or C) and fungi addition (D or not) used as treatment factors; larval development the day before the addition of *D. flagrans* was used as covariate.

Results

Nutritional and chemical analysis

The nutritional analysis of the feeds on trial are shown in Table 1. Dry matter values were 83.5% for offered hay and 46.5% for offered heather. CP was higher in offered hay

(89.6 g/kg DM) compared with offered heather (78.8 g/kg DM), however D-values and estimated ME were similar; refused forages had less CP compared with offered forages, which was more pronounced for heather than for hay.

Table 1 here

Chemical analysis of offered and refused hay and heather samples are shown in Table 2. The samples mainly contained proanthocyanidins, flavonols (kaempferol and quercetin derivatives) and quinic acid derivatives. Contents of total PA in offered heather ranged from 8.65 – 15.29 mg/g and were mainly composed of procyanidins (PCs) in all samples. Refused heather samples from each batch had lower contents of all polyphenol subgroups analysed. The content of the detected polyphenol subgroups increased across batches. In all hay samples no significant levels of PAs or any other polyphenols were detected.

Table 2 here

Feed intake, bodyweight and growth rates

Throughout the experiment, all lambs consumed all the coarse mix and lucerne pellets offered to them. Daily total DM intake for each treatment group across the prepatent and patent period are shown in Fig. 2. During the prepatent period there was a significant effect of time (P <0.001) on DM intake as overall intake decreased, while during the patent period there was an overall increase in DM intake (P <0.001). Furthermore, during the patent period there was a significant interaction between time and treatment group (P <0.001); the effect of diet was consistent as H lambs consumed less DM than C lambs, although this effect was

more pronounced until day 32, after which the effect reduced in size. There was no impact of infection on DM intake at any time point during the prepatent and patent period.

Figure 2 here

Average group DM intake and *P*-values for each day during the patent period, and overall period averages for the prepatent period, are shown in Supplementary Table 1. The average daily heather intake of lambs offered heather throughout the patent period was 200 g DM (SE, 11.0), which was in average 20% of their total DMI; daily heather intake increased over the patent period with an average increase of 94 g DM (SE, 15.1) from day 22 to 44 (Fig 3).

Figure 3 here

Repeated measures showed that overall, lambs were growing during both the prepatent and patent periods, as time had a significant effect on BW (P <0.001). During the prepatent period, treatment group and time had a significant interaction (P <0.001) as infected lambs started to become lighter than uninfected lambs from week 4 onwards. Orthogonal contrast analysis showed that this impact of infection continued up until week 7, as a time x treatment group interaction was also seen during the patent period (P = 0.014). Diet had a significant effect of lamb BW during the patent period as H lambs were lighter than C lambs throughout (P <0.001). Although there was no significant interaction between diet and infection during the patent period (P >0.150), there was a stronger negative association of heather intake and final BW in the UH lambs compared to IH lambs (Fig. 4). The outcome of the orthogonal contrasts analysis on weekly BW for each treatment group and *P*-values are shown in Table 3.

Figure 4 here

Table 3 here

The average daily liveweight gain during the overall experimental period was significantly affected by diet (P<0.001) with animals on heather growing significantly less compared to those in the control hay (144 vs 91 g/day in C vs H lambs; SED: 8.3). For the overall experimental period, infection status did not have a significant impact on average daily liveweight gain (115 vs 122 g/day in I vs U lambs; SED: 14.08; P=0.180). The diet x infection status interaction was not significant (P=0.396).

Faecal egg counts, faecal output and egg output

Faecal egg counts of H lambs were numerically lower than that of C lambs, but not statistically different for FEC FM (P = 0.668) or DM (P=0.134). For both FM and DM values a significant effect of date was seen (FM: P <0.001; DM: P = 0.001) as FEC decreased from day 28. The back transformed mean with 95% confidence intervals for FEC FM are shown in Table 4 and FEC DM are shown in Fig. 5 for infected lambs on each diet.

Table 4 here

Figure 5 here

IH lambs were found to have a significantly lower faecal output compared with IC lambs on day 28 (612.4 g, SE, 24.5; 1051.0 g, SE, 47.0 respectively) and day 42 (734.2 g, SE, 34.8; 944.8 g, SE, 43.3 respectively) during the patent period (P <0.001). There was a significant interaction seen with diet and date (P <0.001) as faecal outputs decreased for IC lambs and increased for IH lambs between day 28 and day 42. There was a significant effect

of covariate (faecal outputs on day 21; P <0.001) on the faecal outputs for infected lambs during the patent period.

All lambs on heather (IH and IHD) had significantly lower (P=0.035) daily egg output (for day 42: backtransformed means: 135,188 (97,446-187,548) eggs/day) during the heather feeding period compared with control lambs (IC+ICD) (for day 42: backtransformed means: 281,421 (222,563-355,845) eggs/day). For all infected lambs egg output on day 28 was greater than on day 42 (P = 0.003). During the patent period, the reduction in the egg output in IH lambs was almost 10-fold higher compared to IC lambs; the magnitude of the reduction in egg output was 6.7% vs 63% in IC and IH lambs respectively. The back-transformed data for egg output is shown in Table 5 and Fig. 6.

Table 5 here

Figure 6 here

Daily egg output of IH lambs showed a weak negative correlation with heather intake (r^2 = 0.059; P =0.300) and estimated daily intake of total tannins (r^2 = 0.057; P =0.310) (Fig. 7), flavonols (r^2 = 0.056; P =0.313) and quinic acid derivatives (r^2 = 0.053; P=0.328).

Figure 7 here

Adult worm counts and per capita fecundity

The number of total, male and female adult worms were not significant different between groups (total adults: P = 0.311, total males: P = 0.363, total females: P = 0.283; Fig. 8). IH lambs had female worms with 45% lower per capita fecundity (back transformed mean: 164

eggs/female; CI: 122-220) compared with IC lambs (back transformed mean: 291 eggs/female; CI: 250-339), but the difference was not significant (P = 0.097).

Figure 8 here

Larval cultures

Larval development was 31.8% lower in infected lambs receiving *D. flagrans* for a 5-day period, compared with infected lambs not receiving *D. flagrans* (P = 0.003). There was no effect of diet (P = 0.319) or any interaction with the diet on larval development (P = 0.337), and the fungi had no impact on larval development after 3 days of *D. flagrans* addition (P = 0.223).

Discussion

In this study we investigated the trade-off between the anti-nutritional and the anthelmintic effects of heather consumption in lambs infected with *T. circumcincta*. Our hypothesis was that heather administration will reduce the level of parasitism in infected lambs, but animals may experience anti-nutritional effects of PSM consumption in heather, reflected in performance. In addition, the use of *D. flagrans* in the diet of infected lambs should reduce larval recovery in faeces, with the potential for additive anthelmintic effects with heather consumption. Our results showed that heather consumption at approximately 20% of the total DM intake resulted in a significant reduction in daily egg output in infected lambs, compared to controls. However, BW of infected lambs on heather was significantly lower at the end of the patent period compared to those offered the control hay in spite them having significantly lower egg output. Average daily gain of all lambs offered heather during the patent period was 40% lower compared to those offered the control hay. *D. flagrans*

administration resulted in lower larval recovery in the faeces overall. The combined heather and *D. flagrans* consumption did not result in any additive effect on larval development, which would have been demonstrated by lower larval recovery in coprocultures of the lambs subjected to both strategies. There was also no evidence of any negative impact of heather consumption at 20% of DM intake on the trapping ability of *D. flagrans*.

Many papers attribute antiparasitic activity of bioactive plants to tannins (PAs and hydrolysable). Indeed, tannin consumption has been shown to lower larval viability and inhibit egg hatching in vitro, and to lower egg outputs and worm burdens in vivo (Athanasiadou et al., 2001; Iqbal et al., 200 7). Our data show that although heather consumption resulted in a reduction in total egg output, estimated tannin (majority PA) intake in the heather fed lambs did not significantly correlate with the decrease in egg output on days 28 and 42 (Fig. 7), an outcome also shown in previous studies (Niezen et al., 1998) where there was a lack of direct association of anthelmintic activity with PAs alone. Previous in vitro research revealed associations of anthelmintic activity with flavonols, such as kaempferol and quercetin derivatives (Mengistu et al., 2017; Shepherd et al. 2022) which were also found to be present in heather offered here. In fact, these flavonoid compounds have been shown to have higher bioavailability in mammalian tissues in comparison with PAs (Li and Hagerman, 2013) and therefore may be interacting with parasite stages. However, as the content of flavonols and quinic acid derivatives also showed no significant association with egg output reduction (data not shown), it is possible that a combination of polyphenols act synergistically to enhance the anthelmintic effect observed (Klongsiriwet et al., 2015; Malisch et al., 2016). Maurer et al. (2022) showed that parasitised lambs had higher estimated intake of PA in their diet (2.9 PA/ lamb/ day) compared to those in our study (2.2g PA/ lamb/ day), which was due to higher PA content in the heather samples rather than

higher heather intake. Despite the higher PA intake in that study, the extent of the anthelmintic effect observed was lower compared to the one observed here. This is in support of the hypothesis that PA concentration is likely not the only driver for the anthelmintic effects observed following heather consumption.

The significant effect of heather consumption observed on egg output could be attributed to an impact on per capita fecundity and a small (although not significant) impact on worm burdens. Other studies have shown effect on GIN fecundity of heather supplementation in sheep (Maurer et al., 2022) and goats (Frutos et al., 2008; Moreno-Gonzalo et al., 2013c, 2014), and supplementation of sheep with other bioactive plants (Minho et al., 2008; Werne et al., 2013). The significant effect on fecundity but not worm burdens could be due the PSMs in heather interacting with reproductive organs of the female worms, as it has been previously shown for *H. contortus* (Martínez-Ortíz-de-Montellano et al., 2013). The lack of a significant effect on faecal egg counts, despite the significant reduction in daily egg output in our study, was likely attributed to the reduction in faecal output in heather fed lambs, due to the drop in their feed intake when heather was introduced. It has previously been shown that variation in feed intake and/or dry matter digestibility can affect FEC, as FEC is a concentration number (eggs/g faeces) (Houdijk et al, 2008).

As well as effects on fecundity, polyphenol-rich extracts from several species of heather have previously been shown to act on *T. circumcincta* eggs and L_3 in vitro (Moreno-Gonzalo et al., 2013a; Shepherd et al., in press). In vivo studies have also shown bioactive forage supplementation decreased larval recovery in faeces (Shaik et al., 2006; Marume et al., 2012; Moreno et al., 2012), suggesting the antiparasitic effects of heather can act on several GIN stages. In our study, heather consumption alone had no impact on the numbers of L_3

recovered in the faeces, indicating there may have been insufficient PSMs present in faeces to influence development of eggs to L_3. The presence of PSM in faeces and the possible impact on egg development or larvae viability can have significant epidemiological benefit and requires further consideration.

Previous studies have shown that ingestion of rich PA plant extracts, such as Quebracho, by parasitised ruminants, has an anthelmintic but also anti-nutritional impact (recently reviewed by Shepherd and Athanasiadou, 2023). To the best of our knowledge, this is the first paper where the anthelmintic effect of heather feeding on *T. circumcincta* infected lambs was accompanied with anti-nutritional effects. Heather-fed lambs showed reduced productivity compared to those on the control diet, as shown by lower BW during the patent period. Although these changes in BW could be attributed to changes in intestinal content due to the change in the diet (e.g. different fibre content between the two diets), part of this lower BW appears to be attributed to reduced feed intake initially, since heather as an unfamiliar forage resulted in a reduction in feed intake of lambs at the start of the heather feeding period. This reduction in feed intake diminished as the experiment progressed, suggesting the animals had the ability to adapt to the heather diet. Furthermore, heather refusals had lower levels of PA than heather offered, suggesting that lambs were not actively selecting against a high level of PA intake. Osoro et al. (2013) observed sheep grazing for a 5-year period and found heather intake to increase every year, which may suggest that as sheep become familiar with the forage, they will select it more often even when grass available. Additionally, our data showed an increase in polyphenol contents of heather over time, which coincided with the increase in heather intake of H lambs, supporting the view that it was not polyphenol content that deterred lambs from eating heather at the start of the experiment, but rather the novelty of the feed (Wallis et al., 2014). However, while the effect of heather consumption on

feed intake was reduced during the patent period, performance was penalised in H lambs throughout and this was reflected in the final bodyweights between the two groups (H lambs: 41kg, C lambs: 44kg). This indicates that the lambs may be experiencing direct negative effects from the PSM consumption in heather affecting their growth. For example, consumption of tannin-rich forages has been shown to interfere with digestion, compared to non-tannin forages (Barry and Manley, 1985); confirmation of this argument would require the use of a binding agent of CTs, such as polyethylene glycol (Hoste et al, 2022). Importantly, Fig. 4 shows that uninfected lambs may have been penalised more from the negative effects of heather consumption compared to the infected lambs. This may indicate that heather consumption can have a positive impact on host resilience, i.e., host's ability to withstand the consequences of GIN infection; this requires further investigation.

In our study lambs chose to consume heather at approximately 20% of their DMI, which appeared to have resulted in negative effects on performance. Maurer et al (2022) found when feeding cut heather to lambs they consumed heather at 11% of their diet, but no negative effects on production were observed. Other studies recording the DM proportion of heather in the diet of grazing sheep found average intake was also lower (Fraser et al. (2009); <10%), or similar (Osoro et al. (2013); 21%), to the proportion of heather in the diet recorded here. These proportions and impact on production factors varied depending on the season, breed of sheep and availability of other forage, rather than on the amount of heather consumed, and as these trials were free grazing, parasite infection levels may not have been considered. The higher level of heather in the diet did not impact on the trapping ability of *D. flagrans*, meaning the two methods could be used together without adverse effects, further lowering potential parasite burdens on pasture.

Conclusions

Heather consumption at 20% of DMI resulted in a significant anthelmintic activity on the daily egg output of *T. circumcincta* but also led to penalties on the performance of parasitised lambs, which led to an overall cost for these animals. Longer term access to heather may help lambs to adapt to the new diet and mitigate the possible negative consequences on their performance. This level of heather inclusion in the diet of infected lambs did not have a negative effect on the trapping ability of nematophagous fungi, which leaves open the possibility of using fungi supplementation to mitigate the anti-nutritional effects of heather. Indeed, if these findings are confirmed under grazing conditions, *D. flagrans* supplementation could compliment lower heather intake for parasite control, which may mitigate the anti-nutritional effects of heather and reduce the costs associated with those.

Conflict of interest statement

None of the authors has any financial or personal relationships that could inappropriately influence or bias the content of the paper.

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Table 1

Nutritional analysis of pooled samples of the feed components used in the trial.

Determination	Unit	Coarse mix	Lucerne pellets	Offered hay	Refused hay	Offered heather	Refused heather
Dry matter (DM)	g/kg	875	947	835	734	465	698
Crude protein	g/kg DM	174	129	89.6	81.4	78.8	53
D value	%	76.4	59.2	53.34	52.1	55	53.3
Metabolisable energy	MJ/kg DM	12.22	9.47	8.53	8.34	8.80	8.53
Chloride	% by Wt DM	0.496	0.72	0.8	0.641	0.133	<0.1
Phosphorus	g/kg DM	4.19	1.49	2.35	1.9	0.691	0.552
Potassium	g/kg DM	11	15.4	20.2	12.9	3.81	3.08
Magnesium	g/kg DM	1.73	2.08	0.965	0.864	1.09	0.745
Calcium	g/kg DM	8.53	13.2	2.81	3.19	3.14	2.44
Sodium	g/kg DM	2.67	1.17	0.65	1.42	0.333	0.348
Sulphur	g/kg DM	2.48	4.84	1.39	1.11	0.849	0.713
Molybdenum	mg/kg DM	1.75	2.18	1.13	1.36	0.454	0.436
Copper	mg/kg DM	9.28	7.42	4.96	7.07	9.47	7.93
Zinc	mg/kg DM	67.3	21.3	22.8	37.8	20	37.8
Boron	mg/kg DM	7.97	12.6	2.74	2.76	13.2	9.34
Iron	mg/kg DM	205	825	150	280	104	119
Manganese	mg/kg DM	71.7	42.3	36.1	70.7	545	475
Cobalt	mg/kg DM	0.52	0.339	0.083	0.155	0.081	0.101

Selenium	mg/kg DM	0.556	0.024	0.037	0.053	0.092	0.085

Table 2

Average polyphenol subgroup concentrations (mg/g) and size of PAs (mDP) with SE of pooled chopped hay samples (offered and refused) and pooled samples from the three collected batches of heather (offered and refused) (n = 2). HHDP and myricetin derivative levels were undetectable in analysis. Procyanidin subunits + prodelphinidin subunits = total proanthocyanidins.

Sample	Heather collection date	Galloyl deriv. (mg/g)	Quinic acid deriv. (mg/g)	Kaempferol deriv. (mg/g)	Quercetin deriv. (mg/g)	Procyanidin subunits of PAs (mg/g)	Prodelphinidin subunits of PAs (mg/g)	Total proanthocyanidins (mg/g)	mDP (PAs)
Offered hay	-	0.00 (0.00)	0.00 (0.00)	0.11 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)
Refused hay	-	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)
Heather offered	29/09/20	0.00 (0.00)	1.48 (0.11)	0.66 (0.05)	2.82 (0.19)	8.34 (0.28)	0.31 (0.02)	8.65 (0.31)	4.28 (0.01)
Heather refused		0.00 (0.00)	0.17 (0.01)	0.15 (0.01)	0.71 (0.02)	4.35 (0.33)	0.11 (0.00)	4.46 (0.33)	3.92 (0.67)
Heather offered	07/10/20	0.11 (0.00)	1.65 (0.04)	0.80 (0.02)	3.79 (0.02)	12.10 (0.33)	0.40 (0.02)	12.50 (0.31)	4.61 (0.02)
Heather refused		0.00 (0.00)	1.69 (0.07)	0.69 (0.00)	3.31 (0.13)	10.08 (0.67)	0.31 (0.03)	10.38 (0.69)	4.40 (0.05)
Heather offered	15/10/20	0.15 (0.01)	2.67 (0.15)	1.06 (0.01)	4.86 (0.05)	14.80 (0.46)	0.49 (0.02)	15.29 (0.45)	4.42 (0.01)
Heather refused		0.00 (0.00)	0.78 (0.01)	0.33 (0.00)	1.76 (0.00)	6.01 (0.21)	0.16 (0.01)	6.17 (0.22)	4.25 (0.01)

PAs, proanthocyanidins; mDP, mean degree of polymerisation; HHDP, hexahydroxydiphenoyl.

Table 3

Weekly average bodyweight (kg) per treatment group (n = 10) during the prepatent and patent periods as analysed with orthogonal contrasts. Covariate was bodyweight at week 2.

	Prepatent			Patent			
Group	Week 3	Week 4	Week 5	Week 6	Week 7	Week 8	Week 9
UC	39.12	40.67	41.85	41.37	43.93	44.05	44.47
UH	38.45	40.28	41.57	39.15	40.74	41.29	40.88
IC	38.48	39.43	40.52	40.40	42.70	43.25	43.58
ICD	38.61	39.66	41.12	41.29	43.41	43.84	44.36
IH	38.78	39.58	40.47	38.87	40.62	41.13	40.99
IHD	38.39	38.84	40.39	38.81	40.35	41.34	40.99
SED	0.376	0.458	0.432	0.378	0.349	0.379	0.426
P-values							
Covariate	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Infection	0.348	<0.001	<0.001	0.079	0.010	0.230	0.463
Diet	-	-	-	<0.001	<0.001	<0.001	<0.001
Interaction	-	-	-	0.658	0.150	0.334	0.244

SED, standard error of difference; UC, uninfected control; UH, uninfected heather; IC, infected control; ICD, infected control *D. flagrans*; IH, infected heather; IHD, infected heather *D. flagrans*.

Table 4.

Back-transformed means (low and upper 95% CI) of faecal egg counts (fresh matter) of lambs infected with T.circumcincta and offered heather (n=20; IH+IHD) or control hay (n=20; IC+ICD) *ad libitum* during the patent experimental period. Day 21 FEC were used as a covariate (before the experimental diets commenced). Data were analysed following square root transformation with a repeated measures ANOVA in Genstat.

	Days post infection				
Group	21	28	35	42	46
IH	298 (257-342)	407 (315-511)	178 (139-213)	195 (149-248)	184 (139-236)
IC	257 (227-287)	345 (285-411)	289 (249-332)	257 (212-307)	225 (178-278)

P-values	
Covariate	0.180
Diet	0.668
Time	0.001
Time x Diet	0.134

Table 5.

Backtransformed means (low and upper 95% CI) of daily egg output of lambs infected with *T.circumcincta* and offered heather (n=20; IH+IHD) or control hay (n=20; IC+ICD) *ad libitum* during the patent experimental period. Day 21 FEC were used as a covariate (before the experimental diets commenced). Data were analysed following square root transformation with a repeated measures ANOVA in Genstat.

	Days post infection		
	21	28	42
IH+IHD	366,448 (321,174-418,104)	226,743 (169,921-302,567)	135,188 (97,446-187,548)
IC+ICD	301,657 (257,445-345,869)	398,604 (327,059-485,800)	281,421 (222,563-355,845)

P-values	
Covariate	0.137
Diet	0.035
Time	0.003
Time x Diet	0.613

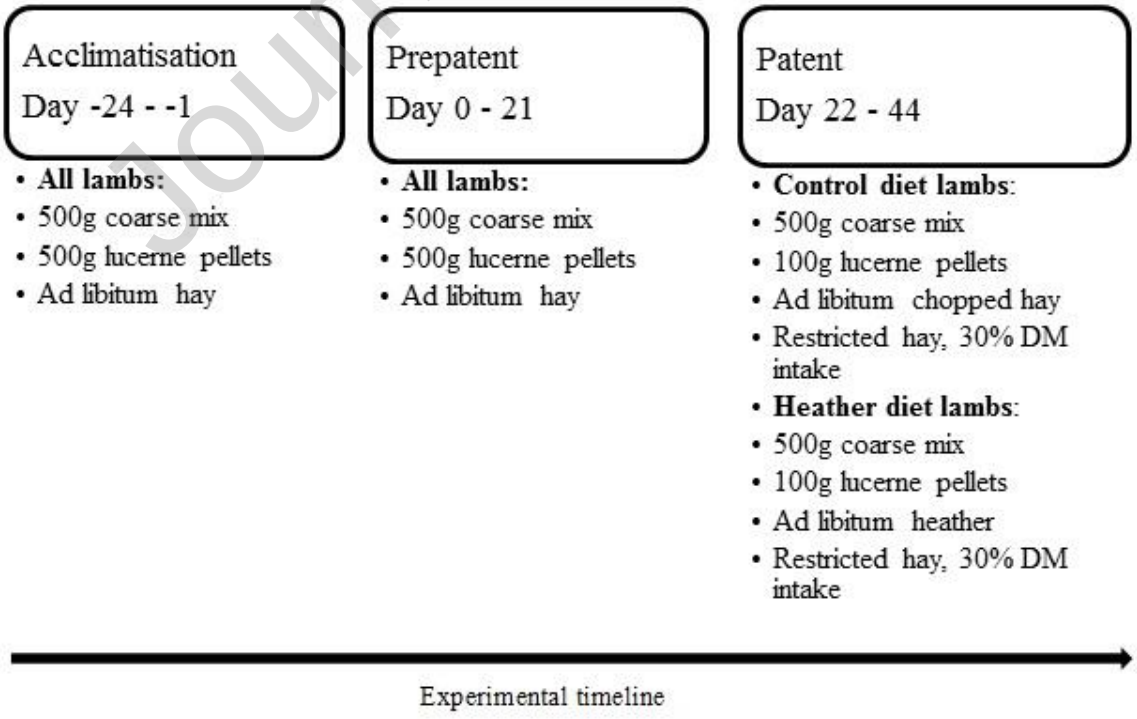

Fig. 1. Diet components during the acclimatisation, prepatent and patent periods. An average of 400g of heather were offered to each animal per day. The average amount of restricted hay (30% DM intake) consumed was approximately 300 g per day.

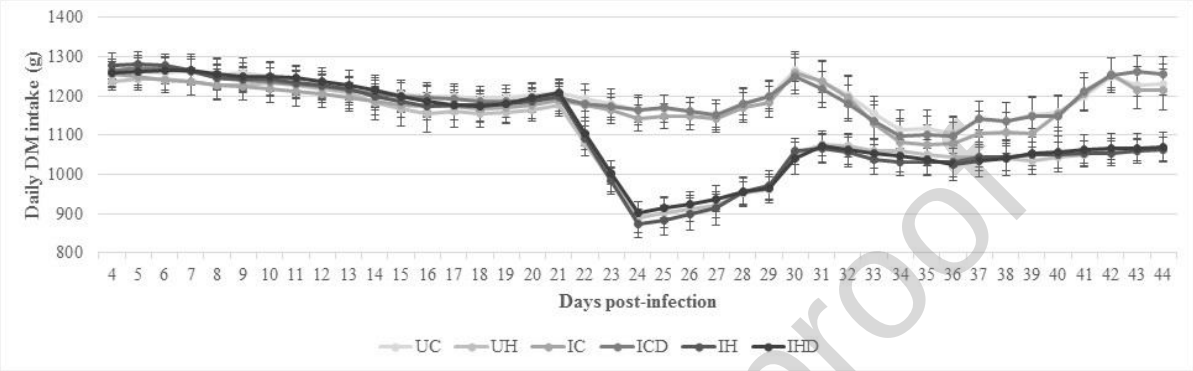

Fig. 2. Three day rolling average and SE of daily DM intake (g) for each treatment group (*n* = 10) during the prepatent and patent periods. UC, uninfected control; UH, uninfected heather; IC, infected control; ICD, infected control *D. flagrans*; IH, infected heather; IHD, infected heather *D. flagrans*.

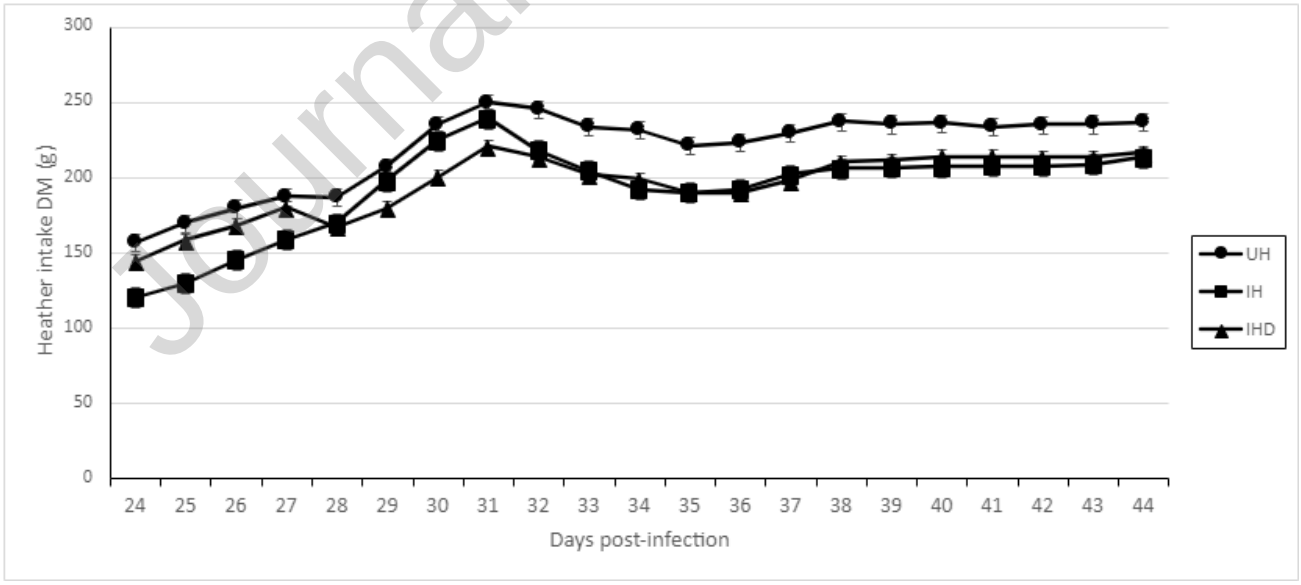

Fig. 3. Three day rolling average of daily DM heather intake for each treatment group (n=10) during the patent period. UH, uninfected heather; IH, infected heather; IHD, infected heather *D. flagrans*. Black bars represent the standard errors of the mean.

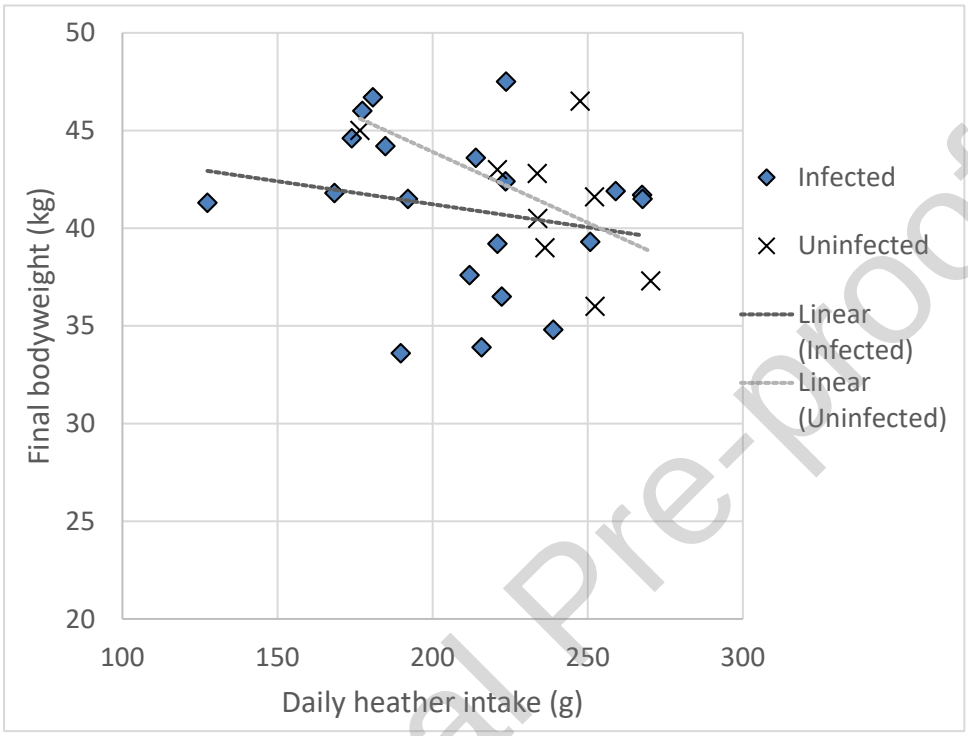

Fig. 4. Final bodyweight (kg) of H lambs (*n* = 30) and average daily heather intake (g) during the last 3 days of heather feeding.

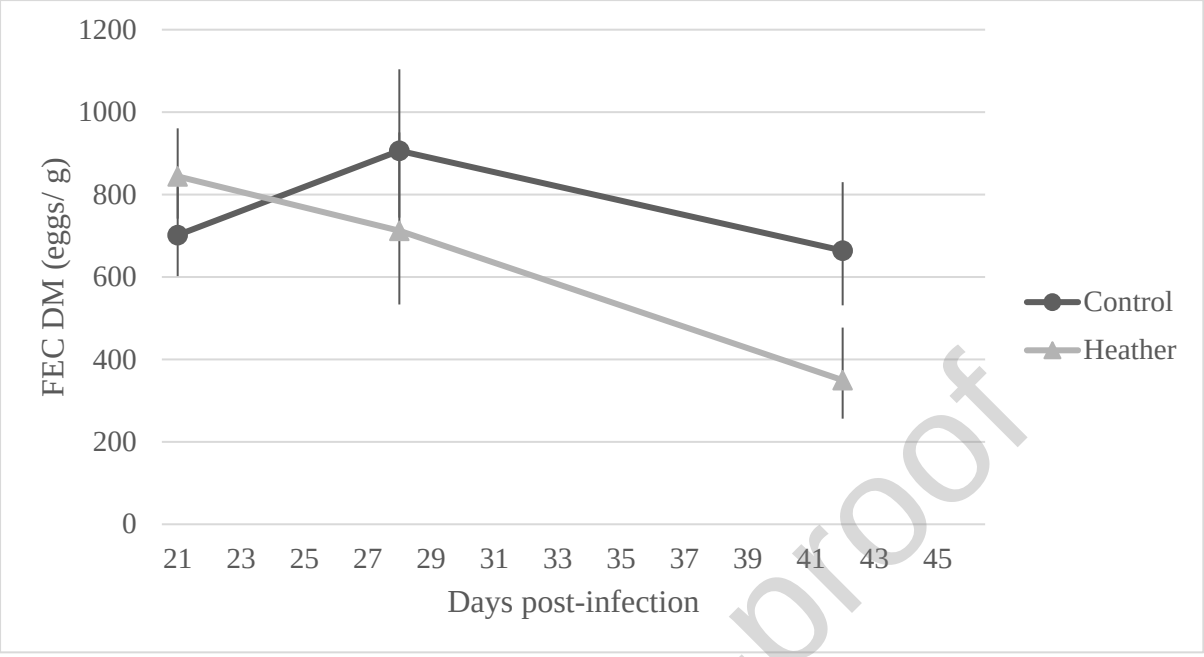

Fig. 5. Back transformed mean dry matter faecal egg count for two dates during patent period for infected lambs on control (*n* = 20) or heather diet (*n* = 20). Day 21 indicates faecal egg count before the experimental diets commenced. Black bars represent 95% confidence intervals.

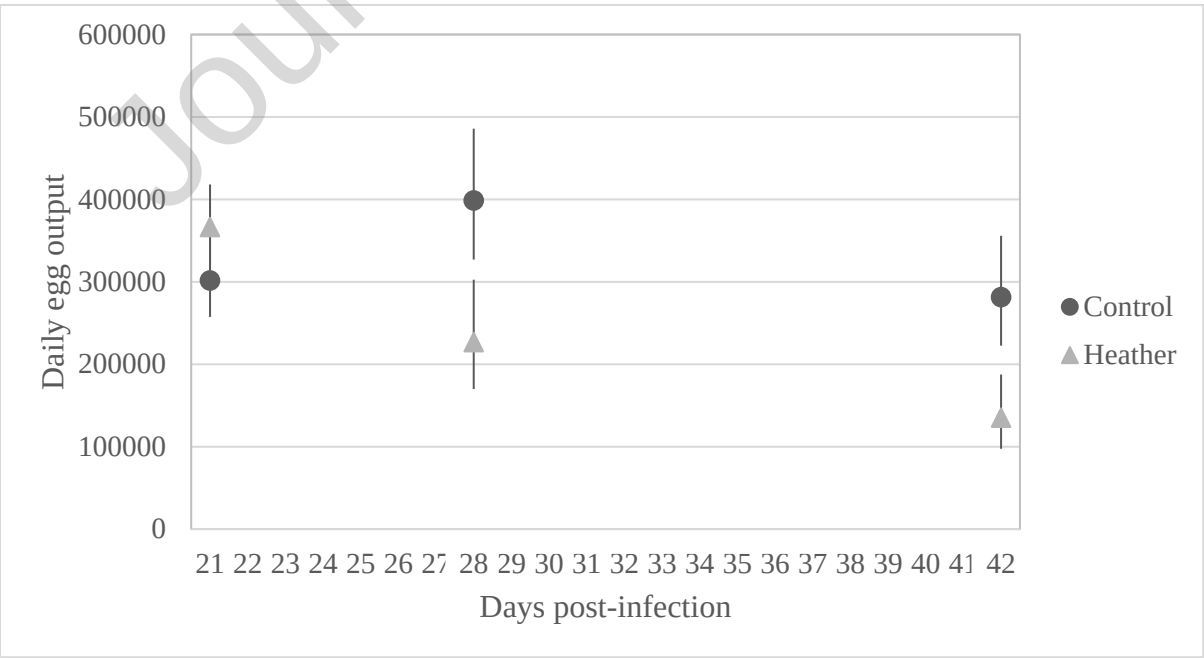

Fig. 6. Back transformed mean egg output on two dates during the patent period for infected lambs on control (n = 20) or heather diet (n = 20). Day 21 indicates egg output before experimental diets commenced. Black bars represent 95% confidence intervals.

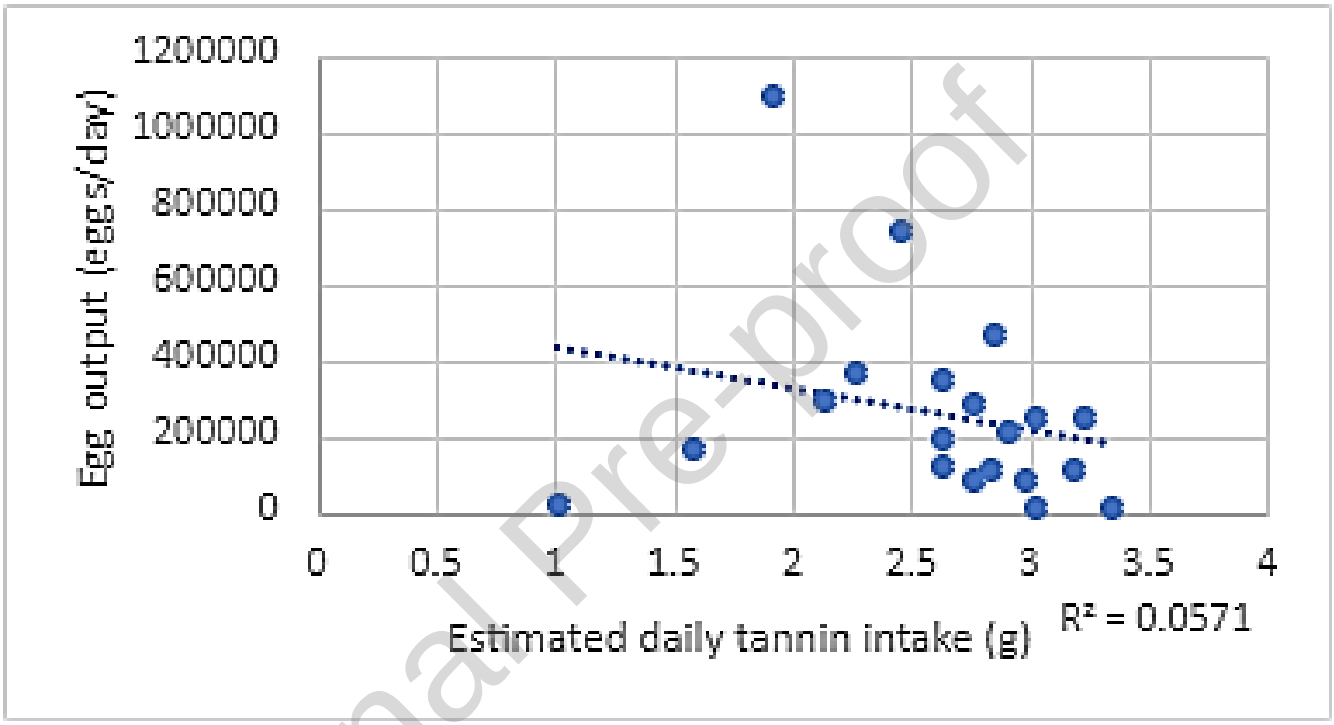

Fig. 7. Average daily egg output on two dates during the heather period (day 28 and 42) for infected H lambs (*n* = 20) and the average estimated daily tannin intake (g) from 3 days prior to egg output determination.

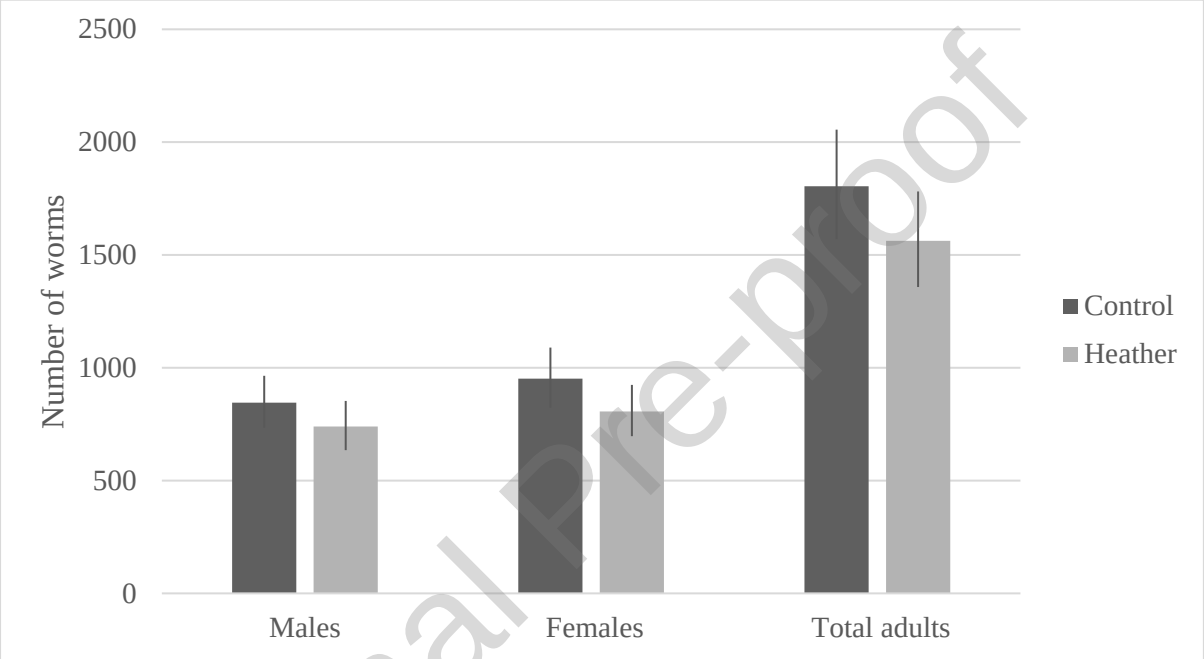

Fig. 8. Back transformed mean number of males, females, and total adult worms in the abomasum of infected lambs on control (*n* = 20) and heather diet (*n* =20). Black bars represent 95% confidence intervals.

Highlights

- Heather intake at 20% of DMI reduced gastrointestinal nematode daily egg output
- Adult worm counts were not affected by heather consumption in infected lambs
- Lambs on the heather diet suffered performance costs shown by lower bodyweight
- Supplementation with *Duddingtonia flagrans* reduced larval recovery in faeces