



## Worms and welfare: Behavioural and physiological changes associated with gastrointestinal nematode parasitism in lambs

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### ABSTRACT

Parasitism with gastrointestinal nematodes (GIN) is a worldwide issue impacting negatively on animal production, health, and welfare. Therefore, early diagnostic signs of parasitism are required to allow for timely interventions. The objective of this study was to evaluate the behavioural and physiological changes in lambs associated with GIN infection. We used 30, 8-month-old Romney-cross wethers, that were administered anthelmintics until faecal egg counts (FEC) were zero and housed in an indoor facility. The study lasted 9 weeks, which comprised a 3-week pre-treatment, and a 6-week treatment phase. Lambs were randomly assigned to one of two treatments ( $n = 15/\text{treatment}$ ) trickle-dosed with: 1) 1500 infective third stage larvae (L3) three days/week for 6 weeks (27,000 total L3; challenged), or 2) water 3 days/week for 6 weeks (control). Within each pen there were 5 pairs of lambs (balanced for liveweight), with each pair comprising a challenged and control lamb. Blood, faecal, and saliva samples were collected 1 week pre-treatment and weekly for 6 weeks of treatment. Behaviour was observed (e.g., feeding, lying, standing) from video-camera recordings using scan sampling every 5 min for 8 h, 1 day pre-treatment and on the day immediately prior to physiological sampling across the 6-week treatment phase (7 days in total). Accelerometers were attached to each lamb to continuously monitor behaviour from 3 weeks pre-treatment and for the remainder of the study. Liveweight, body condition, faecal soiling and faecal consistency scoring were performed weekly as was lipidomic analysis of plasma samples. From week 2 of treatment, challenged lambs spent less time feeding and more time lying than control lambs until week 5 of treatment ( $P \leq 0.01$ ). At week 3 of treatment, elevated lipids (mainly triglycerides and phospholipids), loose faeces and faecal soiling around the anus were observed in challenged lambs compared with controls ( $P \leq 0.05$ ). From week 4 of treatment, FEC were elevated in the challenged compared to control lambs ( $P \leq 0.05$ ). There was also lower liveweight gain at 4 and 5 weeks of treatment in the challenged lambs compared with control lambs ( $P \leq 0.05$ ). These results show a clear timeline of changes in behaviour (e.g., feeding and lying), lipids such as triglycerides, and digestive function (e.g., faecal soiling) suggestive of GIN subclinical disease, which show promise for use in future studies on early identification of subclinical GIN parasitism in lambs.

### 1. Introduction

The negative effects associated with gastrointestinal nematode (GIN) parasitism on the production, health and welfare of livestock worldwide are well known (Fox, 1997; Mavrot et al., 2015; Craig, 2018). Depending

on the species and species composition, parasitic infection with GIN can cause anorexia (reduced appetite), diarrhoea, anaemia, nutritional deficiencies and parasitic gastroenteritis (Fox, 1997; Craig, 2018; Jacobson et al., 2020), which can result in reduced liveweight gain, milk yield, or wool production (Mavrot et al., 2015). At worst, high burdens of GIN

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can lead to mortality (Craig et al., 2006; Kelly et al., 2014). In turn, these negative impacts contribute to financial losses estimated to be tens-of-billions of dollars for sheep, goat, cattle and pig production globally (Roeber et al., 2013; Charlier et al., 2018).

Certain behaviours have long been considered to indicate illness and poor health in animals, as well as being useful for early disease detection (Weary et al., 2009). Behaviours displayed by sick animals commonly include anorexia, depression, and reduced grooming and play (Hart, 1988), which are often accompanied by fever; combined, these responses are part of the adaptive response to infection (Hart, 1988; Dantzer, 2004). Anorexia in sheep infected with GIN is common (Fox, 1997; Greer et al., 2005; Zaralis et al., 2008), and is part of a coordinated response to stimulate immunity and eliminate pathogens (Exton, 1997). Lambs infected with GIN show a reduction in activity, including grazing and walking after infection (Ikurion et al., 2020; Högberg et al., 2021). Further, sheep infected with GIN exhibited less complex (i.e. lower stochasticity) activity sequences than parasitized sheep treated with anthelmintic drugs, suggesting temporal organisation of sheep behavioural patterns changes with GIN infection (Burgunder et al., 2018). Collectively, these observations of reduced activity in GIN infected animals indicate sickness-induced depression or anorexia, which may function to conserve energy to mount an immune response.

As GIN reside in the gastrointestinal mucosa, it would be reasonable to expect that changes in the metabolome may indicate infection (Athanasidou and Huntley, 2008). Metabolomics, which concerns the identification and quantification of metabolites (e.g., amino acids, lipids), has useful application in identifying metabolic biomarkers that may change in response to a disease (Dunn and Ellis, 2005). Metabolic changes appear to precede physiological changes (Goldansaz et al., 2017) and identification of biomarkers of disease states can allow for early intervention prior to clinical signs of disease. Metabolomic studies in livestock species are mainly conducted in cattle, with comparatively few studies in sheep, focusing on metabolic growth and neurodegenerative disorders (Goldansaz et al., 2017). To our knowledge, studies utilising metabolomics on sheep with GIN have not yet been performed and may help to identify potential biomarkers of early-stage disease associated with GIN.

Clearly, GIN must be managed effectively in order to reduce the negative effects on animal health and welfare; however, the emergence of widespread anthelmintic resistance in GIN of livestock over the last 50 years is a significant global issue, particularly where GIN are multi-drug resistant (Gilleard et al., 2021). Therefore, diagnostic tools are needed to detect early signs of GIN parasitism in lambs to reduce the negative effects on production, health, and welfare. To this end, the objectives of this study are to i) evaluate the behavioural and physiological changes in lambs associated with a mixed GIN infection compared with non-infected lambs and ii) identify potential indicators (or biomarkers) of GIN parasitism to be validated in future studies on early detection of disease associated with GIN.

## 2. Materials and methods

This study was approved by the Ruakura Animal Ethics Committee under the New Zealand Animal Welfare Act 1999 (Protocol number 15315) and was conducted in Palmerston North, New Zealand from May to July 2021. The 9-week study comprised a 3-week pre-treatment and 6-week treatment phase.

### 2.1. Animals and housing

Prior to data collection, 40, 8-month-old Romney or Romney-cross wethers were tested for liver damage associated with facial eczema. Thirty-five healthy lambs were selected for inclusion in the study and transported to the animal facility. Over a 2-week period, the lambs were habituated to their pens, indoor diet, and environment. The five lambs that showed the least adaption to the indoor conditions (e.g., running

into pen walls, jumping fences) or presented any clinical signs of ill health, were returned to the source farm before data collection began.

Thirty lambs were used in this study and were housed in pens (5.0 m x 6.1 m) within a custom-built animal shed. Artificial light was used only during daylight hours so as to not disrupt circadian rhythms that may alter behaviour. Lambs were randomly allocated to one of three pens (n = 10 lambs/pen; balanced for treatment, described below) and remained with the same pen-mates for the duration of the study. Each pen contained a water trough with a continuous supply of fresh water and three feed troughs hung on the pen walls.

Fresh cut parasite-free ryegrass/clover mix was provided twice daily during the first week the animals were at the facility as they transitioned to an indoor diet. The indoor diet (also provided twice daily) was fed at 1.5 times maintenance (CSIRO, 2007), and consisted of Fibre Mix (Fibre Fresh Feeds, Reporoa, NZ), lucerne chaff, and pellets (Wenham Grain and Seed, Palmerston North, NZ) at a ratio of 50:25:25, respectively. Two hay nets, containing heat-sterilised pasture hay, were also hung on the wall of each pen. The pens and troughs were cleaned with a pressure washer daily and steam cleaned weekly to reduce risk of parasite transmission between animals.

Two weeks prior to data collection (during the habituation phase after the removal of fresh feed), all animals were drenched (once weekly) to the heaviest in the group with Startect® (15 mL/animal; Zoetis New Zealand Limited, Auckland, NZ) and Zolvix™ Plus (5 mL/animal; Elanco New Zealand, Auckland, NZ) until a zero faecal egg count (FEC) was achieved.

### 2.2. Experimental design

This study used a pair-wise comparison within a randomized block design comprising of three pens. Within each pen there were 5 pairs of lambs, and each pair comprised a challenged and control lamb (n = 5/ treatment/pen), which was determined a priori based on liveweight.

Lambs were randomly assigned to one of two treatments (n = 15/ treatment); either trickle-dosed with: 1) 1500 infective third stage larvae (L3) three days weekly for 6 weeks (27,000 total L3; challenged), or 2) water 3 days weekly for 6 weeks (control).

### 2.3. Parasitology

Parasites used in this study were cultured from a bulk collection of faeces from older ewes on a known drench-susceptible farm in the Manawatu. Challenged lambs received approximately 10 mL of water containing 1500 L3 dosed *per os* by syringe. Control lambs were dosed with approximately 10 mL of tap water.

Individual faecal samples were sampled *per rectum* and stored at 5 °C until processing within 2 days of collection. Samples were collected 1 week pre-treatment and then weekly for 6 weeks during treatment. The number of strongylid species eggs present in the samples was determined using an automated FEC system (Parasight®, Lexington, KY), which had a level of detection of 7 eggs per gram (epg) (Slusarewicz et al., 2021). Faecal egg counts were used as an indirect measure of parasite burden.

Additional faecal samples were collected from the floor of each pen (during the post-treatment phase) for culturing to assess GIN compositions within the animals. Pooled samples were analysed for species composition using the nemabiome (<https://www.nemabiome.ca/>). Challenged lambs had a mixed infection of *Haemonchus contortus* (58.3 %), *Cooperia curticei* (18.9 %), *Teladorsagia circumcincta* (8.2 %), *Bunostomum trigonocephalum* (5.4 %), *Trichostrongylus colubriformis* (4.2 %), *Trichostrongylus axei* (2.0 %) and other species (*Oesophagostomum venulosum*, 1.5 %; *Chabertia ovina*, 1.3 %; and *Trichostrongylus vitrinus*, 0.3 %).

## 2.4. Blood sampling

Blood samples were collected via jugular venipuncture at 1 week pre-treatment and weekly for 6 weeks (at the same time of day) during treatment. Each lamb was firmly restrained by an experienced handler while samples were collected using 20 G 2.54-cm needles (PrecisionGlide, Becton Dickinson, Franklin Lakes, NJ) and 10 mL of blood was collected into EDTA vacutainer tubes (Becton Dickinson). There was no difference in PCV between challenged and control lambs ( $32.5 \pm 1.3$  and  $34.1 \pm 1.3$  for challenged and control lambs, respectively;  $P = 0.23$ ). Immediately after collection, the tubes were inverted 10 times and placed on ice until processing within 2.5 h of sampling. Samples were centrifuged at  $2000 \times g$  for 15 min at  $4^\circ\text{C}$ . Plasma was immediately separated into Nunc™ cryogenic tubes (Thermo Fisher Scientific New Zealand Limited., Auckland, NZ) and then stored at  $-80^\circ\text{C}$  until analysis. Samples were transferred to the AgResearch Lincoln Research Centre for determination of metabolites.

## 2.5. Lipidomics analysis

### 2.5.1. Samples and randomisation

Plasma samples were shipped on dry ice and stored at  $-80^\circ\text{C}$  prior to analysis. A batch randomisation table was created where the order of the lambs was randomised, and then the order of the samples from each individual lamb was randomised. This ensured randomisation while avoiding undue impact of analytical variation on data from the same lamb across time. The paired structure of the design was not accounted for in this randomisation.

### 2.5.2. Sample extraction

Plasma lipids were extracted using the method of Huynh et al. (2019). Briefly, plasma samples were thawed at room temperature under low light conditions, mixed, and 10  $\mu\text{L}$  was then aliquoted into a 2 mL Eppendorf tube. Butanol/methanol with 5 mM ammonium formate (1:1, 95  $\mu\text{L}$ ) was added, along with 5  $\mu\text{L}$  of internal standard (Lipidomix Splash Mix, Avanti Polar Lipids Ltd., Alabaster, AL). Tubes were vortexed in a multitube vortex mixer for 3 min at 2500 rpm at room temperature and then sonicated for 60 min and centrifuged at  $16,000 \times g$  for 10 min at room temperature. The supernatant was transferred to amber chromatography vials with inserts for analysis.

### 2.5.3. LC-MS lipidomics analysis

Detection of lipids were carried out using a Shimadzu LCMS 9030 LC-qTOF mass spectrometer. Separation was on a Waters Acquity CSH C18 column (1.7  $\mu\text{m}$  particle size,  $2.1 \times 100$  mm ID) at  $65^\circ\text{C}$  and eluted over a 17 min gradient with a flow rate of  $400 \mu\text{L min}^{-1}$ . Mobile phase A was water:acetonitrile:isopropanol (50:30:20) with 20 mM ammonium formate, and mobile phase B was water:acetonitrile:isopropanol (1:9:90) with 20 mM ammonium formate. All solvents were LC-MS grade (Optima LCMS grade, Thermo Fisher Scientific, Auckland, NZ). The gradient elution programme was as follows: 10–45 % B (0–2.7 min), 45–53 % B (2.7–2.8 min), 53–65 % B (2.8–9 min), 65–89 % B (9–9.1 min), 89–92 % B (9.1–11 min), 92–100 % B (11–11.1 min), held at 100 % B (11.1–13.9 min), 100–10 % (13.9–14 min), held at 10 % B (14–17 min) (Su et al., 2019). The autosampler was held at  $20^\circ\text{C}$  and 2  $\mu\text{L}$  of extract was injected onto the column. The mass spectrometer acquired data in ‘full scan’ mode between 50 and 1200  $m/z$ , and in data independent acquisition MS/MS mode across the same range in 20  $m/z$  windows to acquire fragmentation data on all lipids, with a collision energy ramp from 6 to 23 eV. Loop time for the MS method was 0.5 s.

Data were converted into mzML format and processed using MS DIAL (Tsugawa et al., 2015). Data were normalized based on the LOWESS algorithm in MS DIAL and exported for manual curation (checking for duplicate identifications, merging adducts with the same feature ID, data quality checking). Identification was based on matching against the LipidBlast database (Kind et al., 2013) in MS DIAL and checking against

known retention time windows for individual lipid classes.

## 2.6. Saliva sampling

Saliva samples were collected at 1 week pre-treatment and weekly for 6 weeks during treatment to assess for anti-CarLA IgA. Samples were collected using the procedure described by Shaw et al. (2012). Briefly, saliva was collected using a swab attached to long-nosed surgical forceps that was manipulated in the lamb’s mouth (between gum and cheek) for approximately 10 s. The swab was then spun to extract the saliva and diluted to 1/20 for the assay of CarLA-specific IgA using the protocol described by Shaw et al. (2012).

## 2.7. Behavioural observations

To allow for individual identification, each lamb was marked with stock marker (Tell Tail aerosol, FIL, Mount Maunganui, NZ) using numbers along the back. Behaviour was continuously recorded using security cameras (GXV3674/FHD\_VF; Grandstream Networks Inc., Boston, MA) and recorded at 20 frames/s. Two cameras were positioned approximately 3 m above each pen (6 cameras in total) providing an aerial and side-on view of the lambs. Video-recordings were made during daylight hours, where the lambs could be clearly observed and were analysed using scan sampling, where the behaviour of each lamb at that point in time was recorded every 5 min for 8 h from 8:30 h to 16:30 h. Over the 9-week trial period, behaviour was assessed on one day during the 1-week pre-treatment period and on the day immediately prior to physiological sampling across the 6 weeks of treatment (7 days total). The ethogram of behaviours that were evaluated are presented in Table 1. Behaviours that could not be clearly observed from the video-recordings were not recorded. In addition, behaviour was not analysed when any person was in the pen. Feeding from the hay nets, self-grooming, allogrooming, head butting and rubbing of the head, rump and side were observed so seldom, that the data was excluded from analysis.

One experienced observer analysed the video-recordings using Replayer (Version 2.0; Grandstream Networks Inc.). The observer remained blind to the treatment each lamb had received when watching the video-recordings. The behaviour of each lamb (Table 1) was recorded into a spreadsheet (Microsoft Excel, Redmond, WA). Intra-observer reliability was completed by scoring the behaviour of 6 lambs selected at

**Table 1**

Ethogram of behaviours monitored of lambs that were dosed 3 days weekly for 6 weeks with either 1500 infective L3 larvae (challenged) or water (control).

Behaviour	Description
Feeding	Head lowered directly over the feeder.
Drinking	Head lowered directly over the water trough.
Standing (with or without Ruminantion)	Weightbearing on all four feet in upright position without movement.
Lying (with or without Ruminantion)	Lamb is resting sternally or ventrally and not weightbearing on feet.
Ruminantion	Mouth moving in a rhythmic chewing movement (excluding feeding).
Self-grooming	Muzzle touching the lambs’ body with a rhythmic motion.
Allogrooming	Muzzle of lamb touching another lamb with a rhythmic motion.
Walking	Moving in any direction at a slow, rhythmic pace.
Head butting	Head contacting another lamb’s body including head, usually with force.
Rubbing	
Head	Moving head against pen structure, wall or another lamb in a rhythmic back and forth motion.
Side	Moving side of body against pen structure, wall or another lamb in a rhythmic back and forth motion.
Rump	Moving rump against pen structure, wall or another lamb in a rhythmic back and forth motion.

random; behaviour was recorded for 6 h using 5 min scans and then re-examined. Percentage agreement for these observations were as follows: 100 % for feeding, 92 % for drinking, 75 % for standing with ruminating and 61 % for standing without ruminating, 100 % for lying with ruminating and 100 % for lying without ruminating, and 100 % for walking.

## 2.8. Accelerometers

Lamb behaviour was monitored continuously over the study period using triaxial accelerometers (Actigraph wGT3X-BT® acceleration sensor, Actigraph, LLC, Pensacola, FL), which measure acceleration across 3 axes (i.e., vertical, horizontal, and longitudinal). We used a similar protocol to that of [Ikurior et al. \(2020\)](#), except the accelerometers were fitted on collars instead of mating harnesses. The use of accelerometry data to infer behaviours has been validated for sheep on pasture ([Ikurior et al., 2021](#)). The accelerometers were attached to the top side of a 2.54 cm-width weighted collar and positioned on the withers of the lamb ([Fig. 1](#)). The accelerometers measured  $46 \times 33 \times 15$  mm and weighed 19 g. The loggers were prescheduled to collect acceleration data at a sampling rate of 30 Hz (30 logs per second). The accelerometers collected data in 2-week periods at the end of which data was downloaded and the batteries recharged. With the exception of 1–2 days between accelerometer removal and being refitted, the devices were on the sheep for 9 weeks, comprising a 3-week pre-treatment period and 6 treatment weeks. Accelerometer orientation was the same on all sheep at each time of attachment.

### 2.8.1. Machine learning to predict behaviour

All data read out from the accelerometers were processed and analysed using R statistical software (R core Team 2017, version 3.5.2). Accelerometer data were provided as individual files for each lamb, spanning the entire duration of the trial, containing raw data (X, Y and Z axes coordinates) at a frequency of 30 Hz. The data were processed and summarised into 5-second epochs by computing the following accelerometry features over the entire epoch: the mean of X, Y and Z (where x is the x-axis acceleration, y is the y-axis acceleration, and z is the z-axis acceleration), the minimum and maximum of X, Y and Z, the standard deviation of X, Y and Z, the signal magnitude area:  $mean(|X| + |Y| + |Z|)$  -



**Fig. 1.** A lamb wearing the Actigraph accelerometer attached to a collar and positioned on the top of the withers region.

distinguishes between periods of activity and rest, the signal vector magnitude:  $mean(\sqrt{X^2 + Y^2 + Z^2})$ .

A labelled dataset was prepared to train and validate the machine learning algorithm. For that, video-recordings of four randomly selected lambs were observed continuously over a 2 h period. The behaviours from [Table 1](#) were evaluated for each one-second time steps and each lamb. The behaviours were then summarised into 5-second epochs, and only the epochs with the same behaviour throughout the entire epoch were kept. The corresponding subset of accelerometer data were cleaned to remove epochs affected by missing logs or variable frequencies of logging. The behaviour data and the accelerometry data were merged by lamb and time epoch, with the final dataset comprising 4617 labelled epochs of 5 s for the 4 lambs over the 2 h period.

A random forest algorithm (R package 'randomForest') was trained to predict the sheep behaviour using the accelerometry features, running 1000 iterations. This method implements out-of-bag error estimation for robust and unbiased inferences, using bootstrap aggregation to train and validate the algorithm, by comparing model predictions to the gold standard (video observations). A confusion matrix was used to evaluate the overall performances of the classifier. Two metrics were used for overall classifier performance across behaviours: the overall accuracy and overall misclassification rate.

To optimise the performance of the classifier, rare activities ( $n < 10$ ) were removed, namely head butting, rubbing head and scratching. Walking ( $n = 99$ ) was virtually undistinguishable from feeding or standing. We thus elicited to train the classifier without this behaviour, to improve the specificity of model predictions for other behaviours, and for parsimony. Based on model predictions, remaining behaviours were recategorized as follows: feeding (merging feeding in feeders, feeding in hay nets, and drinking), lying (merging lying and lying/ruminating) and standing (merging standing and standing/ruminating). The final optimised classifier had an overall error rate of 17.3 %, with 10.0 %, 7.5 % and 55.9 % for feeding, lying and standing behaviours, respectively.

The above algorithm was then used to predict behaviours using the accelerometry data for the entire trial. The predicted data was further summarised into activity budgets: daily proportion of time spent feeding, lying or standing.

## 2.9. Clinical health measurements

Liveweight was measured weekly  $\sim 2$  h after morning feed distribution (at approximately the same day of the week and time) throughout the duration of the study by animal facility staff using a sheep weigh crate (Prattley 3-way manual weigh crate; Temuka, NZ; with Tru-Test MP600 load bars; Datamars, Auckland, NZ).

Body condition, faecal soiling, and faecal consistency scoring was carried out once weekly by the same operator. Body condition scoring used a scale from 1 to 5 where 1 represented prominent and sharp spinous processes and 5 represented no detectable spinous process ([Kenyon et al., 2014](#)). Faecal soiling scoring used a scale from 0 to 5, where 0 represented no soiling and 5 represented  $>2/3$  of the breech area soiled ([Broughan and Wall, 2007](#)).

Faecal consistency scoring used a scale from 1 to 5 where 1 represented normal formed pellets and 5 represented watery faeces ([Le Jambre et al., 2007](#)).

## 2.10. Statistical analysis

For all analyses, residuals were inspected for departures from the model assumptions of normality and constant variance. No animals were excluded from the data set for any reason. Over the duration of the study, 4 faecal samples and 2 saliva samples from different lambs were not taken due to human error and therefore were treated as missing.

### 2.10.1. Lipidomics

Multivariate statistical analyses using SIMCA 16 (Umetrics, Umeå, Sweden) were carried out for lipidomics data. After removal of analytical outliers, data from 208 samples was processed, resulting in 1624 features found with MS2-level identification, and after clean-up for unlikely identifications based on retention time. Data were analysed based on comparison at each time point, after subtraction of baseline data, and comparison of differences from baseline between paired lambs. A principal component analysis (PCA), and orthogonal projection to latent structures discriminant analysis (OPLS-DA), were carried out to assess overall data structure and to focus on between group differences, respectively. Differences between groups for individual lipids and lipid classes were determined using two-tailed t-tests. Statistical analyses were done only for lipids with MS2 level identification. Interpretation of *P*-values is based on the proportion of likely false positive results, as well as overall model strength, acknowledging the difficulty with standard statistical paradigms in the context of exploratory methods such as lipidomics (where most of the detected features have nothing to do with the hypothesis being tested). As this experiment was not specifically powered for lipidomics, to reduce the risk of removing excess data, the level of significance for differences in triglycerides was set at  $P < 0.10$ . To avoid likely false positives, only features that differed at three or more time points were evaluated. JASP software (version 0.16.3) was used for univariate statistical analyses of lipidomics data.

### 2.10.2. Parasitology, saliva sampling, behavioural observations, and clinical health measurements

Data was analysed using Genstat software (version 21, VSN International, Hemel Hempstead, UK). Response variables including FEC, anti-CarLA IgA, frequencies for behaviours with more than 200 observed occurrences, liveweight, body condition score, faecal soiling, and faecal consistency data were analysed using a linear mixed model fitted by restricted maximum likelihood (REML). Fixed effects included treatment, date, and the treatment by date interaction. The random model included pen, lamb pair within pen, and lamb identification number. The correlation between data on the same lamb over time was modelled using a power model of order 1. Differences between and within treatments were compared using Unprotected Fisher's Least Significant Difference test. The level of significance was set at  $P \leq 0.05$ . Additionally, a PCA biplot (based on the sum of squares and products) was used to explore the relationships between challenged and control lambs with respect to behaviour.

### 2.10.3. Accelerometers

Accelerometer data was analysed using R statistical software (R core Team 2017, version 3.5.2). The daily activity budgets obtained from the predictions of the machine learning algorithm using accelerometry data were used to compute two outcomes of interest: 1) an iso-metric log-ratio transformation, resulting in one combined variable which looks at the ratio of time spent being either upright (i.e., feeding and standing) or lying down, and 2) a combined variable contrasted the time spent feeding versus standing when the animal was upright. Data with missing values for more than half the animals in any given pen were removed. The above outcomes of interest were analysed using separate linear mixed models fitted by REML. Fixed effects included treatment, number of days since beginning of trial, and the interaction between the two. The random model included pen and lamb pair within pen. The post-hoc pairwise comparisons were adjusted for using a Tukey adjustment. The level of significance was set at  $P \leq 0.05$ .

## 3. Results

### 3.1. Faecal egg counts

For FEC, there was a treatment by date interaction observed between challenged and control lambs ( $F_{7195} = 6.25$ ;  $P \leq 0.001$ ) and separate

effects of treatment ( $F_{1,26} = 25.74$ ;  $P \leq 0.001$ ) and date ( $F_{7195} = 6.19$ ;  $P \leq 0.001$ ; Fig. 2). Over the pre-treatment period and until week 3 of treatment, no differences between challenged and control lambs were detected ( $P > 0.05$ ). However, 4 weeks after treatment began, FEC were higher in the challenged than control lambs (155 egg and 1 egg for challenged and control, respectively; SED = 33 egg). At 6 weeks after treatment, FEC remained higher in challenged than control lambs (184 egg and 1 egg; SED = 33 egg) ( $P \leq 0.05$ ). Over the treatment period, raw FEC data ranged from 0 to 748 egg for challenged lambs and 0–20 egg for control lambs.

### 3.2. Lipidomics

Results are reported based on non-baseline corrected data. Differences between challenged and control lambs started to emerge in multivariate modelling 3 weeks after treatment began; however, a stronger response was detected after 4 weeks of treatment (Table 2). The majority of the lipid changes were for triglycerides, which were elevated in the challenged compared to control lambs. The feature-reduced model (removal of features that had no relevance to the treatment comparison) was strong, suggesting that there was less 'random noise'. As well as triglycerides, some phospholipids including ether phosphatidylcholines and sphingomyelins differed at week 3. Feature-reduced PCA plots for the pre-treatment and treatment weeks are presented in Fig. 3. While there is considerable inter-animal variability as would be expected, there is a clear trend for a difference between challenged and control lambs at most time points.

Several triglycerides were consistently different between challenged and control lambs at weeks 4, 5 and 6 of treatment. Although identified based on a combination of high-resolution mass for the overall triglyceride ion, and the fragments of that ion (MS2), and eluting within a known retention time window for triglycerides, some of the identifications are for triglycerides that contain rare fatty acids, while others that differed contain fatty acids that are commonly found in mammalian circulation. Results were divided into two sets, based on if at least two out of the three suggested fatty acids within the triglycerides have previously been reported in ovine plasma (Coleman et al., 2018) (Supplementary Table 1). The triglycerides that differed all had at least one fatty acid substituent that was highly unsaturated and corresponded to an n-3 fatty acid, with the exception of an ether-triglyceride, and the fatty acids that differed were all elevated in challenged lambs.

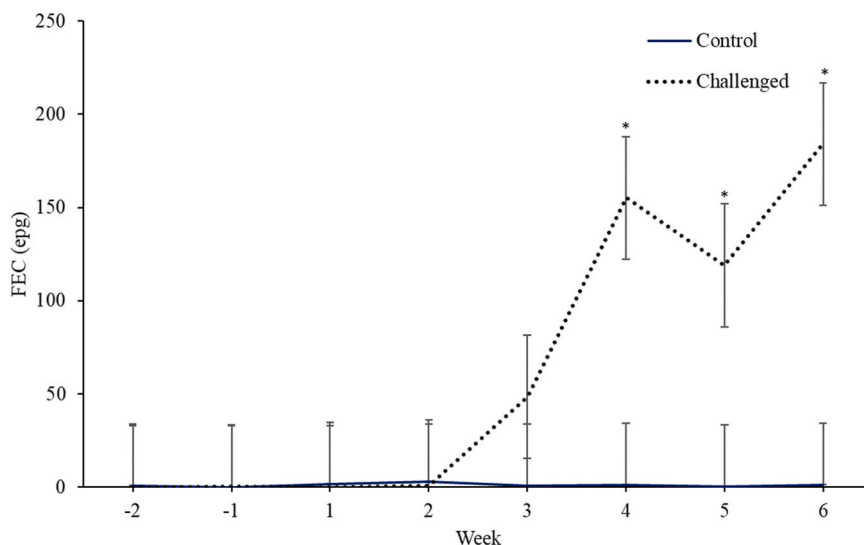
### 3.3. Anti-CarLA IgA

For anti-CarLA IgA, there was no treatment by date interaction ( $P = 0.54$ ) between challenged and control lambs over the course of the study. However, there were separate effects of treatment ( $F_{7,14} = 8.01$ ;  $P = 0.01$ ) and date on anti-CarLA IgA ( $F_{7152} = 3.4$ ;  $P = 0.002$ ). Anti-CarLA IgA peaked 2 weeks prior to treatment (1.7 IgA 1; SED: 0.3), which was higher than all other weeks over the study ( $\leq 0.6$  IgA 1; SED: 0.3;  $P \leq 0.05$ ). Anti-CarLA IgA were higher in challenged than control lambs over the study duration (0.97 IgA 1 and 0.16 IgA 1; SED: 0.3, for challenged and control lambs, respectively).

### 3.4. Behavioural observations

A PCA was carried out for all behaviours, which revealed no pronounced differences between challenged and control lambs following treatment (Fig. 4). The first two dimensions of the PCA (PC-1 and PC-2) explained 70.6 % of the variation.

For standing (without rumination), there was a treatment by time interaction ( $F_{6135} = 2.31$ ;  $P = 0.04$ ) and a separate effect of date ( $F_{6135} = 17.61$ ;  $P < 0.001$ ) but no overall effect of treatment ( $F_{1,28} = 0.55$ ;  $P = 0.46$ ). There was a tendency for challenged lambs to stand (without rumination) more often than controls at week 1 of treatment ( $P \leq 0.06$ ). Six weeks after treatment began, challenged lambs were observed



**Fig. 2.** Faecal egg counts (epg) of lambs that were either treated with gastrointestinal nematodes (challenged) or water (control) for 6 weeks. Asterisks (\*) indicate differences between challenged and control lambs at the 5 % significance level.

**Table 2**

R2X and Q2 values for differences between lambs that were either treated with gastrointestinal nematodes (challenged) or water (control) for 6 weeks. Native model refers to all detected lipids being included, while feature-reduced model refers to the model that included only lipids that had a variable importance for the projection score > 1.5 included. R2X is the amount of variance due to the treatment explained by the OPLS-DA model and Q2 is a measure of the model validity.

Week	Native model		Feature-reduced model		Features P < 0.01
	R2X	Q2	R2X	Q2	
Uncorrected data					
-1	0.28	-0.36	0.30	0.08	3
1	0.50	-0.27	0.45	0.32	7
2	0.69	-0.83	0.37	0.31	2
3	0.31	-0.03	0.27	0.21	8
4	0.27	-0.03	0.38	0.26	8
5	0.28	0.05	0.30	0.27	26
6	0.31	0.11	0.46	0.40	70
Baseline-Corrected Data					
1	0.74	-0.69	0.60	0.40	5
2	0.44	-0.39	0.39	0.14	0
3	0.56	-0.40	0.24	0.17	1
4	0.25	-0.03	0.36	0.28	12
5	0.30	0.00	0.28	0.17	17
6	0.25	0.03	0.30	0.23	18

standing (without rumination) less often compared with baseline values ( $P \leq 0.05$ ), whereas control lambs showed no difference in standing between these dates ( $P > 0.05$ ).

For standing whilst ruminating, there was no treatment by time interaction ( $F_{6134} = 0.36$ ;  $P = 0.90$ ) or an effect of treatment ( $F_{1,26} < 0.01$ ;  $P = 0.99$ ), but there was a date effect ( $F_{6134} = 13.83$ ;  $P < 0.001$ ). Regardless of treatment, lambs stood more often during pre-treatment and week 1 of treatment than remaining treatment weeks ( $P \leq 0.05$ ).

For lying (without rumination), there was no treatment by date interaction ( $F_{6132} = 1.41$ ;  $P = 0.22$ ) or a separate effect of treatment ( $F_{1,28} = 0.28$ ;  $P = 0.60$ ), but there was an effect of date ( $F_{6132} = 35.54$ ;  $P < 0.001$ ). Lambs were observed lying (without rumination) more often at weeks 2 and 6 of treatment than pre-treatment ( $P \leq 0.05$ ).

For lying whilst ruminating, there was no interaction between treatment and date ( $F_{6131} = 0.77$ ;  $P = 0.59$ ), but there were separate effects of treatment ( $F_{1,14} = 7.05$ ;  $P = 0.02$ ) and date ( $F_{6131} = 2.65$ ;  $P = 0.02$ ). Control lambs were observed lying whilst ruminating more

often than challenged lambs (18.2 and 16.0; SED: 0.83;  $P \leq 0.05$ ). Lying whilst ruminating was performed more often pre-treatment and week 6 of treatment compared with weeks 2 and 3 of treatment ( $P \leq 0.05$ ).

For feeding, there was no treatment by date interaction ( $F_{6168} = 0.64$ ;  $P = 0.70$ ), or a separate effect of treatment ( $F_{1,26} = 0.19$ ;  $P = 0.67$ ), but there was an effect of date ( $F_{6168} = 9.10$ ;  $P < 0.001$ ). Lambs were observed feeding more often pre-treatment than during all treatment weeks ( $P \leq 0.05$ ).

For walking, there was no treatment by date interaction ( $F_{6168} = 1.03$ ;  $P = 0.41$ ) or a separate effect of treatment ( $F_{1,28} = 0.73$ ;  $P = 0.40$ ), but there was an effect of date ( $F_{6168} = 6.24$ ;  $P < 0.001$ ). Walking was observed more often during week 1 of treatment when compared with pre-treatment ( $P \leq 0.05$ ) but returned to pre-treatment levels thereafter ( $P > 0.05$ ).

For drinking, there was no treatment by date interaction ( $F_{6129} = 0.63$ ;  $P = 0.71$ ), or separate effects of treatment ( $F_{1,28} = 2.00$ ;  $P = 0.17$ ) or date ( $F_{6129} = 1.6$ ;  $P = 0.15$ ).

### 3.5. Accelerometers

The proportion of time spent in an upright position (i.e., feeding or standing) over the study period is presented in Fig. 5. From 3 weeks pre-treatment to week 1 of treatment, there were no differences between groups in the amount of time spent feeding or standing ( $P > 0.19$ ); however, from week 2 of treatment, challenged lambs spent less time feeding (or standing) than control lambs until week 5 of treatment ( $P \leq 0.01$ ). At week 6 of treatment, there was no difference in the amount of time spent feeding or standing between treatments ( $P > 0.61$ ).

The proportion of time spent lying (versus standing or feeding) over the study period is presented in Fig. 6. There was no difference between treatments in the amount of time spent lying at week 3 pre-treatment ( $P > 0.08$ ), but from week 2 pre-treatment until week 1 of treatment, challenged lambs spent less time lying (and proportionally more time either standing or feeding) than control lambs ( $P \leq 0.04$ ). At week 3 to week 5 of treatment this pattern reversed, and the challenged lambs spent more time lying than control lambs ( $P \leq 0.01$ ). There was no difference between challenged and control lambs in amount of time spent lying at 6 week of treatment ( $P > 0.06$ ).

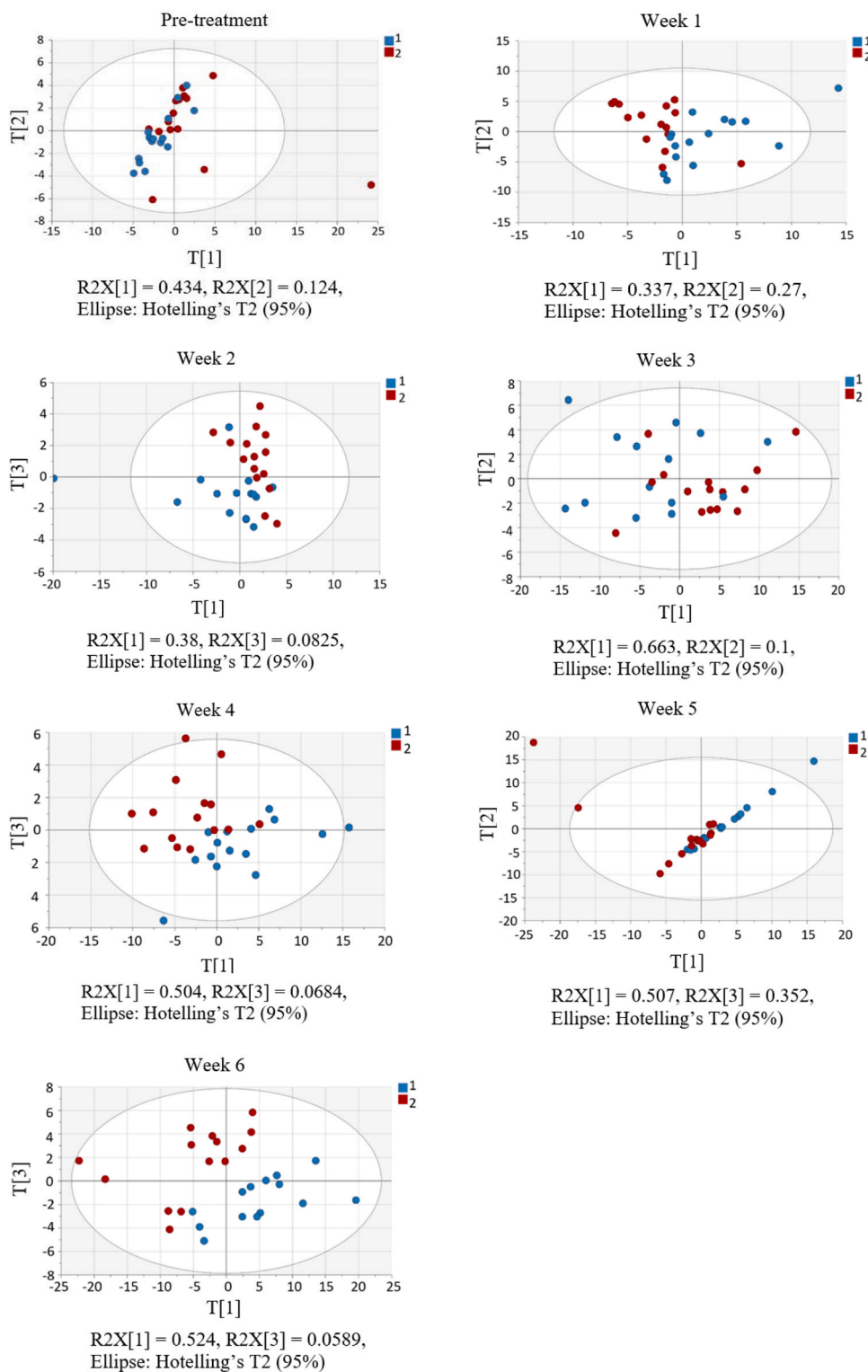


Fig. 3. PCA scores plots on feature-reduced lipidomics data. Blue (or light grey) dots represent challenged lambs (1), and red (or dark grey) dots represent control lambs (2). Where there are more than two components, the components have been selected to show the maximum separation between the two groups.

3.6. Liveweight

There was a treatment by date interaction on liveweight ( $F_{11,267} = 1.95; P = 0.03$ ; Fig. 7). There was also a separate date effect ( $F_{11,267} = 125.25; P < 0.001$ ), but no separate treatment effect on liveweight ( $F_{1,14} = 3.9; P = 0.07$ ). At week 2 of treatment the liveweight of challenged lambs tended to be lower than that of controls ( $P \leq 0.06$ ). Liveweight

was lower in challenged lambs at week 4 and 5 of treatment than controls ( $P \leq 0.05$ ).

3.7. Body condition score

For BCS, there was no evidence of a treatment by date interaction ( $F_{9209} = 0.63; P = 0.77$ ), or separate effect of treatment ( $F_{1,28} = 0.53$ ;

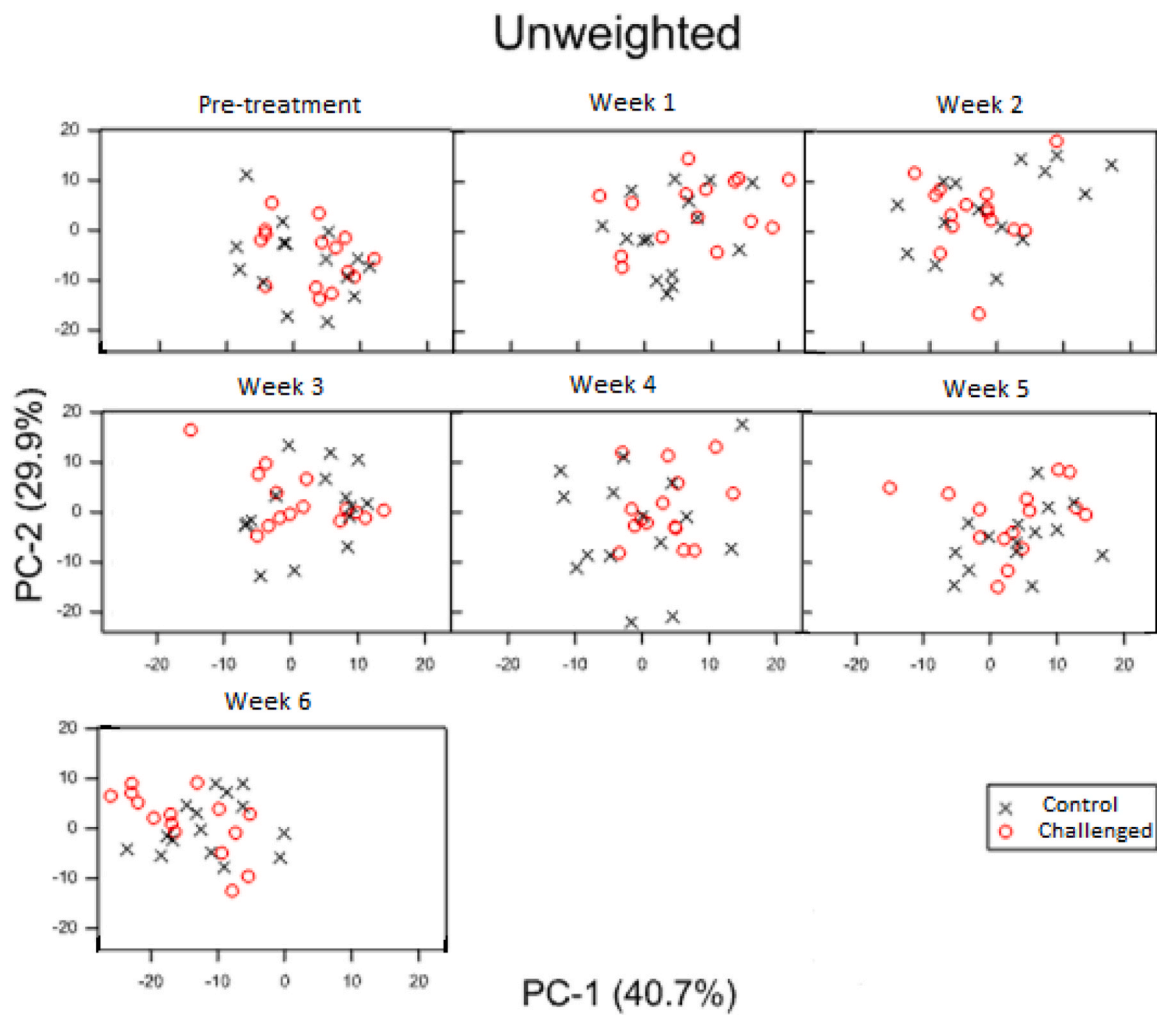


Fig. 4. Principal components analysis biplot of total behaviour occurrences of lambs that were either treated with gastrointestinal nematodes (challenged) or water (control) for 6 weeks.

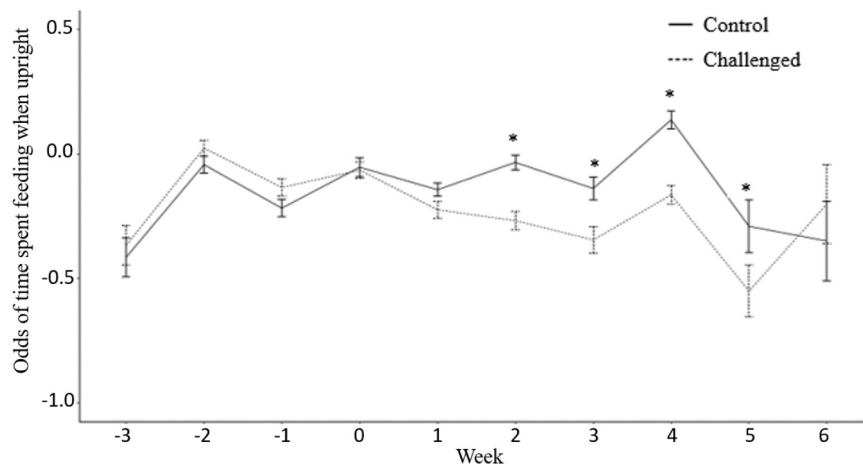


Fig. 5. Odds ratio of time spent feeding (when upright) of lambs that were either treated with gastrointestinal nematodes (challenged) or water (control) for 6 weeks. Asterisks (\*) indicate differences between challenged and control lambs at each week at the 1 % significance level.

$P = 0.47$ ) but there was an effect of date ( $F_{9209} = 8.72; P < 0.001$ ). From week 3 of treatment, BCS was higher than pre-treatment values (2.8, 2.8 and  $> 2.9$  for weeks 1 and 2 pre-treatment and week 3 of treatment, respectively; SED: 0.08;  $P \leq 0.05$ ).

### 3.8. Faecal soiling

For faecal soiling, there was a treatment by date interaction ( $F_{8190} = 2.19; P = 0.03$ ) and a separate effect of date ( $F_{8190} = 2.55; P = 0.01$ ), but not of treatment ( $F_{1,28} = 3.27; P = 0.08$ ). One week pre-treatment,

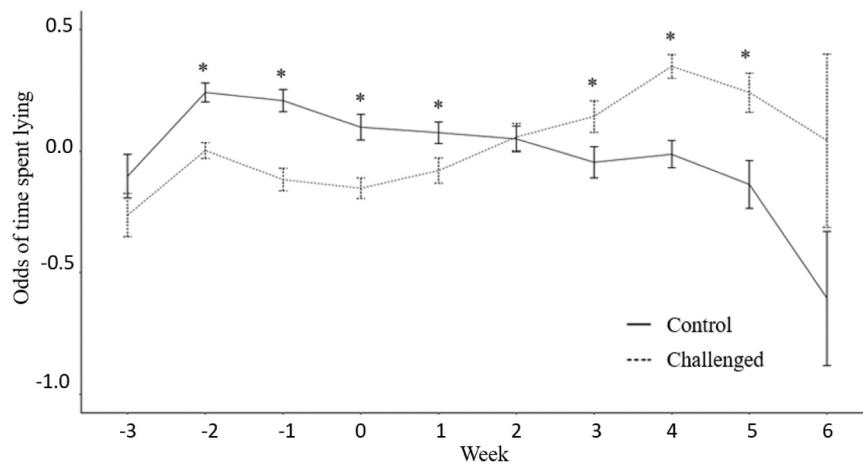


Fig. 6. Odds ratio of time spent lying of lambs that were either treated with gastrointestinal nematodes (challenged) or water (control) for 6 weeks. Asterisks (\*) indicate differences between challenged and control lambs at each week at the 1 % significance level.

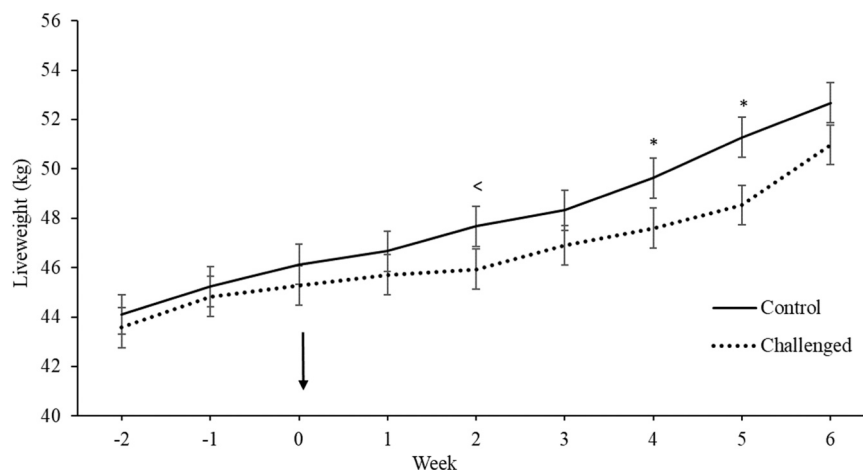


Fig. 7. Liveweight (kg) of lambs that were either treated with gastrointestinal nematodes (challenged) or water (control) for 6 weeks. Asterisks (\*) indicate differences between challenged and control lambs at the 5 % significance level. < indicates differences between challenged and control lambs at the 6 % significance level. Arrow denotes when treatment began, which continued until week 6.

challenged lambs had higher faecal soiling scores than controls (0.5 and 0.1 for challenged and control lambs, respectively; SED: 0.15;  $P \leq 0.05$ ). During week 3 of treatment, challenged lambs had higher faecal soiling scores than controls (0.3 and 0.0; SED: 0.15;  $P \leq 0.05$ ). For all remaining time points, there were no differences between groups ( $P > 0.05$ ).

### 3.9. Faecal consistency

For faecal consistency, there was a treatment by date interaction ( $F_{8197} = 2.74$ ;  $P = 0.007$ ) and a separate effect of treatment ( $F_{1,78} = 26.9$ ;  $P < 0.001$ ) and date ( $F_{8197} = 4.83$ ;  $P < 0.001$ ). There were no differences between challenged and control lambs during the pre-treatment weeks or weeks 1 and 2 of treatment ( $P > 0.05$ ); however, at weeks 3, 5, and 6 of treatment, challenged lambs had greater faecal consistency (i.e. wet faeces, not normal pellets) than controls ( $P \leq 0.05$ ).

## 4. Discussion

The objectives of this study were to evaluate the behavioural and physiological changes in lambs associated with a mixed GIN infection compared with non-infected lambs, and identify potential indicators (or biomarkers) of GIN parasitism to be validated in future studies on early detection of disease associated with GIN. Behaviours including feeding

or standing decreased in challenged lambs compared to control lambs from week 2 of treatment, and lying increased from week 3 of treatment. At week 3 of treatment, challenged lambs had elevated triglycerides containing polyunsaturated fatty acids, and phospholipids, and wetter faeces compared with controls. From week 4 of treatment, FEC was higher along with lower weight gains in challenged lambs than controls. Anti-CarLA IgA were higher in challenged lambs over the study duration. This set of comprehensive results show the behavioural and physiological impacts of GIN on lambs housed indoors.

The PCA of the behavioural observations showed no distinct clustering between challenged and control lambs, likely as the differences in behaviour between groups were subtle. For the analysis of individual behaviours however, control lambs were observed to ruminate whilst lying more over the study than challenged lambs, which indicates more comfort than the parasitised lambs. The accelerometer data detected altered standing, feeding and lying behaviour in challenged lambs at specific time points: challenged lambs spent less time feeding or standing from 2 weeks of treatment and more time lying from 3 weeks of treatment compared to controls. Although the behaviour data across the different collection methods was complementary, the accelerometer data provided a much clearer picture of the response of challenged lambs to the infection. This is likely because the accelerometers record behaviour continuously, whereas scan sampling behaviour every 5 min

may not be sensitive enough to detect the level of changes between challenged lambs with a relatively mild infection, and control lambs, that the accelerometers could detect.

Increased lying in sheep is likely an attempt to conserve energy during early stages of infection, and is common in animals parasitised with *H. contortus* or *B. trigonocephalum* that can cause anaemia, lethargy and weakness (Flay et al., 2022); both species were present in the lambs used in this study. A recent study of naturally infected lambs reared on pasture showed parasitised lambs will spend more time lying 7 days after weaning than 7 days prior to weaning (Högberg et al., 2021); although there is likely an effect of weaning stress on the behavioural response to infection. Högberg et al. (2021) also reported that lying time was lower in lambs with high- than low-level parasitism. Sick calves will lie down for longer periods (and with fewer bouts) than healthy calves 2 days prior to clinical signs of neonatal calf diarrhoea (Sutherland et al., 2018). Overall, it appears challenged lambs have altered standing and lying behaviour 2 weeks post-infection, and inclusion of these behavioural measures is warranted in future studies.

In the present study, challenged lambs fed less often than controls at week 2 of treatment. Reductions in feeding behaviour in lambs with GIN has previously been reported, whereby overall activity (including grazing, movement) was reduced in naturally parasitised lambs measured using triaxial accelerometers over a 4-day period compared to lambs treated with an anthelmintic (Ikurior et al., 2020). A voluntary reduction in feed intake, termed anorexia (Coop et al., 1982; Coop and Kyriazakis, 1999) associated with GIN parasitism, is a major negative impact on lamb health and welfare. *C. curticei* and *T. circumcincta* often cause anorexia in lambs (Niezen and Waghorn, 1995) and were present in low numbers in the lambs of the present study. Through the use of modelling, anorexia has been demonstrated to increase with increasing challenge, and anorexia can be ameliorated by increasing the nutritional content of feeds (e.g., high protein) (Laurenson et al., 2011). Previous reports of anorexia in lambs are not until 3–4 weeks after larval ingestion (*T. colubriformis*), which generally correlates with establishment of mature GIN in the small intestine (Kyriazakis et al., 1998). In the present study, the accelerometers used could detect apparently subtle changes in ingestive behaviour 2 weeks after challenge with GIN, and 2 weeks prior to traditional clinical signs of parasitism (e.g., FEC).

The algorithm used to predict behaviour based on the accelerometer data had a relatively high level of error for standing behaviour (55.9%). This meant that the algorithm had difficulty differentiating between standing and feeding (due to similar head position), and that just over half of the time the algorithm classified animals that were actually standing as feeding. Therefore, we may have observed more instances of feeding than occurred, so some level of caution is required when interpreting the standing results. Feeding however, had a level of error of 17.3%, therefore lambs were more accurately predicted as feeding. To reduce the amount of error for standing, more annotated data to train the algorithm would be required, however this was not logistically possible in the current study.

From week 3 of treatment, triglycerides containing polyunsaturated fatty acids were elevated in challenged compared to control lambs. This may be the first data to show fluctuations in lipids associated with GIN, and highlight potential biomarkers to be validated in future studies. Most metabolomic studies of livestock have been carried out for disease detection (mainly for cattle), production and bioproduct assessment, feed efficiency and reproduction (Goldansaz et al., 2017). However, there appears to be no metabolomic studies on common diseases of sheep or goats (Goldansaz et al., 2017). In the present study, differences in lipids occurred in conjunction with increased rates of faecal soiling and looser faeces in challenged lambs, which is typical of *T. circumcincta* and *Trichostrongylus* spp. infection in lambs (Larsen et al., 1994). Several triglycerides in the present study were identified as having fatty acid 20:5 and 22:6 substitutions. Fatty acid 20:5, otherwise known as eicosapentaenoic acid (EPA) and fatty acid 22:6, commonly known as docosahexaenoic acid (DHA) are important in mammalian metabolism,

and provide the backbone for many lipid signalling mediators, especially in inflammation, which may be a consequence of GIN parasitism (Abbott, 2018). Although the mechanisms responsible for elevated triglycerides with these substitutions in lambs with GIN is not clear, EPA and DHA may have an important process in resolution of inflammation of ruminants (Calder, 2013). Whether EPA and DHA would be useful markers for inflammation related to GIN-associated tissue damage remains to be seen, and a comparison between free EPA and DHA, and EPA and DHA present in lipid species such as triglycerides is warranted. Differences in circulating triglycerides may also be an 'artefact' of differences in liveweight gain and feed efficiency due to parasite infection, although this requires further investigation and would not explain why triglycerides with a high degree of polyunsaturated fatty acids were those that differed. Major differences in the plasma lipidome of GIN-affected lambs did not occur until 5 weeks, based on the number of significantly perturbed lipids. This suggests that the lag-phase between inoculation and a broad impact on circulating lipids in response to parasitism is relatively delayed.

Metabolic changes do appear to be useful in early disease detection for cattle with periparturient disease states, such as metritis or mastitis (Hailemariam et al., 2014) and ketosis (Klein et al., 2012). Metabolic changes often precede physiological changes as metabolites can signal later stage physiological responses (Goldansaz et al., 2017). Metabolites can change depending on a host of factors including sex, time of sampling, and diet (Athanasiadou and Huntley, 2008; Jin et al., 2021), therefore it is important that these variables remain consistent throughout the study period. Compared with other metabolic studies (e.g., human or rat: n = 100 – 1000 individuals) (Goldansaz et al., 2017), the 30 lambs that were used in the present study is a relatively low number. Including a larger sample size in future studies, may allow for subtle changes in metabolites to be detected earlier in the timeline of GIN-associated disease and increase the statistical power. However, changes in circulating triglycerides appear to be useful indicators of GIN in lambs and should be validated in future biomarker studies.

Faecal egg counts were found in higher numbers in challenged lambs compared to controls at week 4 of treatment; prepatent periods of the main GIN species used in this study range from 2 to 3 weeks (Roeber et al., 2013). At least 9 species of GIN were detected, with the dominant species' including *H. contortus*, *C. curticei* and *T. circumcincta*. While *C. curticei* and *T. circumcincta* are mucosal feeders, which typically cause low-level pathology (e.g., diarrhoea), *H. contortus* is a blood feeder, and at high worm burdens can cause severe anaemia, and even mortalities in young lambs (Roeber et al., 2013). *B. trigonocephalum*, also a blood feeder, was found in low numbers (~5%) in the challenged lambs of the present study. *B. trigonocephalum* are not generally considered a parasite of importance in New Zealand (Vlassoff and McKenna, 1994), yet is present in older aged ewes in the central North Island, which is where the parasites for the present study were sourced. We did not observe any severe signs of parasitism in the lambs during this study; this may be attributed to a low worm burden indicated by low FEC. Generally, FEC will increase in young lambs that have greater potential for infection due to lower resilience, but from ~ 150 days of age, animals become more resilient to GIN infection due to developing immunity, and can manage or prevent parasitism effectively (Notter et al., 2017). Clinical signs of parasitism are generally only apparent in lambs up to 9–10 months of age (Vlassoff and McKenna, 1994). The lambs used in the present study were 8 months old and therefore presumably developing a degree of immunity to GIN.

Sheep with high levels of anti-CarLA IgA have been shown to have 20–30% lower FEC than animals with low or undetected anti-CarLA IgA, indicating higher immunity against GIN (Shaw et al., 2012). In the present study, anti-CarLA IgA were found at the highest concentration at 2 weeks pre-treatment for all lambs, despite having no detectable FEC; this may be due to residual anti-CarLA IgA associated with a previous worm infection. A longer habituation period (i.e., more than 2 weeks), where the lambs are drenched and kept off pasture is required in

future studies to reduce chances of residual pathologies from previous GIN infections.

Looser faeces were observed in challenged lambs compared to controls from weeks 3–5 of treatment. This is not an unusual finding on account of the presence of *T. colubriformis*, which in high numbers can cause watery faeces or ‘black scours’ (Levine, 1968). As the total worm burden in the challenged lambs was relatively low, black scours were not observed; only recording less pellet-like faeces compared to control lambs.

Faecal soiling around the breech area was observed post-treatment in the challenged lambs; however, it was also observed in the pre-treatment phase in the challenged lambs. This result may be associated with a previous infection prior to the beginning of the study. Similar to the elevated anti-CarLA IgA, in future studies, a longer habituation period is required.

## 5. Conclusions

This study took a combined behavioural and physiological approach to detecting GIN parasitism in lambs. We have provided a clear timeline of changes in behaviour (e.g., feeding, standing and lying), metabolites such as triglycerides with fatty acid substitutions (EPA and ADA), and digestive function (e.g., faecal soiling), which show some of the impact that GIN parasitism has on lambs. Further, the identified parameters show promise for use in future studies on early identification of sub-clinical GIN parasitism in lambs, which can provide tools for utilisation on farm to improve animal welfare outcomes and profitability.

## CRedit authorship contribution statement

**Ross Alastair B.:** Writing – review & editing, Visualization, Software, Resources, Methodology, Investigation. **Sauermann Christian W.:** Writing – review & editing, Supervision, Resources, Methodology, Investigation, Funding acquisition. **Sutherland Mhairi A.:** Writing – review & editing, Methodology, Conceptualization. **Cave Vanessa M.:** Writing – review & editing, Visualization, Formal analysis. **Hempstead Melissa N.:** Writing – review & editing, Writing – original draft, Visualization, Validation, Resources, Project administration, Methodology, Investigation, Formal analysis, Conceptualization. **Gibson Michaela J.:** Writing – review & editing, Methodology, Investigation. **Waghorn Tania S.:** Writing – review & editing, Resources, Methodology, Investigation. **Hannaford Rina:** Writing – review & editing, Methodology, Formal analysis. **Marquetoux Nelly:** Writing – review & editing, Software, Methodology, Formal analysis, Data curation. **Sutherland Ian A.:** Writing – review & editing, Supervision, Methodology, Funding acquisition, Conceptualization. **Corner-Thomas Rene A.:** Writing – review & editing, Resources, Methodology, Investigation.

## Declaration of Competing Interest

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

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## Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.vetpar.2023.110056.

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