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SPECIAL ISSUE ARTICLE

Identification of factors associated with *Fasciola hepatica* infection risk areas on pastures via an environmental DNA survey of *Galba truncatula* distribution using droplet digital and quantitative real-time PCR assays

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Abstract

Environmental DNA (eDNA) is a powerful tool for identifying the spatial and temporal presence and density of species in a range of aquatic habitats. The analysis of eDNA has a wide range of application, one of which may be to inform of *Fasciola hepatica* infection risk on pastures based on the detection of its eDNA as well as that of its intermediate snail host, *Galba truncatula* eDNA. Here, droplet digital PCR (ddPCR) and quantitative real-time PCR (qPCR) assays were developed to detect the eDNA of *F. hepatica*, and its intermediate snail host, *G. truncatula* in water samples collected from pastures grazed by cattle and/or sheep. Environmental factors associated with species presence, as detected via an eDNA survey, were identified using zero-inflated linear mixed models. Sixty-four habitats were sampled across six farms in Ceredigion, Wales, UK, with ddPCR and qPCR identifying 42 and 33 habitats to be positive for *G. truncatula* eDNA, respectively. *G. truncatula* eDNA was significantly less likely to be detected in habitats fully shaded by trees, those that contained black or dark brown soils and habitats that contained deep water pools ($p < 0.05$). Significantly higher *G. truncatula* eDNA concentrations were observed in habitats that tend to dry up during Summer (i.e., temporary habitats) ($p < 0.05$). ddPCR also identified five habitats to be positive for *F. hepatica* eDNA; however, questions remain regarding the utility of *F. hepatica* eDNA detection due to a lack of specificity toward infective *F. hepatica* larval stages. The results of this study inform of factors which influences *G. truncatula* distribution and ecology on pastures and also provided practical information for farmers to aid *F. hepatica* control in their flocks and herds.

KEYWORDS
ddPCR, environmental DNA, *Fasciola hepatica*, *Galba truncatula*, qPCR

1 | INTRODUCTION

The detection and analysis of environmental DNA (eDNA) is recognized as a powerful methodology for evaluating species presence across space and time (Thomsen & Willerslev, 2015). Significant progress has been achieved over the past decade in optimizing analysis of water samples for eDNA of rare, invasive, and pathogenic species (Cristescu & Hebert, 2018). Vast surface areas can now be screened both quickly and efficiently for evidence of target species presence and to estimate population densities and biomass based on eDNA presence and concentration (Thomsen & Willerslev, 2015). Surprisingly, despite the successful application of eDNA analysis of environmental water in human health (Larsen & Wigginton, 2020) and ecology (Beng & Corlett, 2020), it is yet to be widely applied in veterinary health despite multiple possibilities for aiding disease control.

One of the areas within the veterinary field that has shown promise for the successful application of eDNA analysis is parasite control, in particular the management of trematode parasites whose larval stages are found in water at key periods of their lifecycle (Jones et al., 2018). Trematodes are also dependent on an intermediate host to complete their lifecycle, many of which are either aquatic or amphibious snails. One of the main trematodes of concern for livestock producers is the liver fluke, *Fasciola hepatica*, whose infections are estimated to cost the European livestock industry € 635 million annually due to decreased milk yields and growth rates, effects which will also increase the carbon footprint of livestock production (Jonsson et al., 2022), and increase in mortality and treatment costs (Charlier et al., 2020). *F. hepatica* also infects humans, with its disease, fasciolosis regarded as a neglected tropical disease by the WHO (Mas-Coma et al., 2018). This trematode uses *Galba truncatula* as its primary intermediate host, an amphibious freshwater snail which also hosts the rumen fluke *Calicophoron daubneyi* (Jones et al., 2017), an emerging parasite in western Europe which has the potential to cause clinical disease and mortality in ruminants (Jones et al., 2022). Controlling these trematode parasites is becoming extremely challenging globally, due firstly to the impacts of climate change, which has allowed *G. truncatula* snails to thrive in temperate climates (Fox et al., 2011) and secondly due to the emergence of anthelmintic resistance which limits treatments options post-infection (Castro-Hermida et al., 2021). These challenges highlight the importance of an integrated control strategy for *F. hepatica* and *C. daubneyi* in livestock, whereby infection risk areas on farmland are managed proactively to limit infection opportunities alongside responsible treatment of infected animals (Crilly et al., 2015).

One of the key measures required to effectively assess current infection risk is the presence of *G. truncatula* snails on pastureland, with inclusion of spatial information on habitats known to significantly improve the strength of predictive fasciolosis risk models (Charlier et al., 2011). Challenges are associated with accurately mapping *G. truncatula* snail distribution, with the snails difficult to find and identify and may not always be present in seemingly suitable habitats (Charlier et al., 2011). Furthermore, our understanding of microenvironmental factors that influence *G. truncatula* presence and population densities is limited which further restricts the effectiveness of wide scale remote sensing spatial models of *G. truncatula* distribution (Charlier et al., 2014).

In recent years, in an attempt to overcome these technical restraints, eDNA assays have been developed to detect the presence of *G. truncatula* in a range of water saturated habitat types, including boggy areas, ponds, streams, and ditches, as well as the detection of eDNA of both *F. hepatica* and *C. daubneyi* in these areas (Jones et al., 2018). Conventional PCR and LAMP assays have previously been designed and optimized for *G. truncatula* eDNA detection (Davis et al., 2020; Jones et al., 2018). However, these tools, despite their simplicity, low cost, and practicality, can lack sensitivity and tolerance to inhibitors, and may not be capable of detecting the minute quantities of eDNA that is commonly observed in environmental water (Fediajevaite et al., 2021). These methods are also incapable of quantifying eDNA levels within samples which may be beneficial to estimate species biomass (Yates et al., 2019). New *G. truncatula* eDNA amplification methods, such as quantitative real-time PCR (qPCR) and droplet digital PCR (ddPCR), may therefore further enhance previously designed eDNA protocols to deliver superior target detection (Capo et al., 2021; Rathinasamy et al., 2018; Wood et al., 2019) that will subsequently support efficient mapping and modeling of *G. truncatula* distribution and trematode infection risk on farmland (Jones et al., 2018).

The aims of this study were to (1) develop qPCR and ddPCR assays to detect and quantify *G. truncatula* eDNA in environmental water samples collected across the pasture environment, and to (2) evaluate factors associated with the spatial distribution of *G. truncatula* snails on pastures in a temperate environment as identified using eDNA analysis.

2 | METHODS

2.1 | Primer and probe design

Primers and probes targeting a 90-bp amplicon of the *G. truncatula* ITS2 gene (GenBank accession no. AJ296271.1) were designed in silico using the PrimerQuest™ Tool (Integrated DNA Technologies, Coralville, USA). The forward 5′-CAAAGCATTCGTGTCCTTGC-3′ and reverse: 5′-CAGGGCGTATCCAACCATC-3′ primers and FAM/BHQ1 labeled probe 5′-AAACCGAAGCCTTGCGGCGT-3′ were tested for their specificity in silico using Genious Prime 2019 (https://www.geneious.com) and NCBI Blast (NCBI, Bethesda, USA). *F. hepatica* primers and probes designed and validated for qPCR by Rathinasamy et al. (2018), which target a 108-bp amplicon of the *F. hepatica* ITS2 gene, were also used in the study to analyze field samples. These primers and probe were incorporated into the *G. truncatula* ddPCR assay at concentrations of 900 and 250 nM, respectively. The probe was dual labeled with HEX and BHQ1, which allowed for multiplexing alongside the FAM labeled *G. truncatula* probe.

2.2 | qPCR protocol

The Q-qPCR machine (Quantabio, Beverly, USA) was used for qPCR analysis. The qPCR master mix composition and thermocycling profile of the qPCR assay were optimized within the manufacturer's recommended range. The PCR master mix was made in a final volume of 10 μl, and included 1× PerfeCTa® MultiPlex qPCR ToughMix®, 500 nM of each primer, 250 nM of Probe, and 2 μl of DNA extract. The optimal primer and probe concentration was determined via a primer/probe concentration titration experiment (500/250, 250/125, 200/100, and 100/50 nM primer/probe concentrations were tested) where the lowest cycle threshold (CT) value for *G. truncatula* DNA observed would indicate optimal concentrations. qPCR was run with an initial denaturation at 95°C for 3 min, followed by 45 cycles of denaturation at 95°C for 15 s and an annealing/extensions step of 60°C for 45 s. The optimal annealing/extension temperature was determined via an annealing temperature increment experiment (56, 58, 60, 62, and 64°C) where the lowest CT value for *G. truncatula* DNA would indicate optimal conditions.

2.3 | ddPCR protocol

The QX200 Droplet Digital PCR System (BioRad, Hercules, USA) was used for ddPCR analysis. The ddPCR master mix composition and thermocycling profile of the ddPCR assay followed the manufacturer's recommended protocols, with the exception that the number of cycles was increased to 45. The PCR master mix was made in a final volume of 20 μl, and included 1× supermix for probes (BioRad), 900 nM of each primer, 250 nM of Probe, and 4 μl of DNA extract. The master mix was placed in sample wells of the droplet generation cartridge, with 70 μl of droplet generation oil (Bio-Rad) added to each of the oil wells. These cartridges were placed in a QX200 Droplet Generator (Bio-Rad) which generated microdroplets, which were transferred into a 96-well PCR plate (Bio-Rad), heat-sealed with foil using the PX1 Plate Sealer (Bio-Rad). PCR was run with an initial denaturation at 95°C for 10 min, followed by 45 cycles of denaturation at 94°C for 30 s, annealing/extensions step of 60°C for 60s, and a final extension step at 72°C for 5 min. The optimal annealing/extension temperature was determined via an annealing temperature increment experiment (57.4, 58.5, 60, 61.8, and 63.3°C) where the greatest separation between positive and negative droplets and the highest copy number of *G. truncatula* DNA detected would indicate optimal conditions. The droplets were analyzed individually using the QX200 Droplet Reader (Bio-Rad), with the data analyzed using QuantaSoft software (Bio-Rad).

2.4 | Assay optimization, sensitivity, and specificity

To test the sensitivity of both assays, a series of 10-fold dilutions of DNA extracted from *G. truncatula* snails was analyzed. This DNA was extracted following Jones et al. (2017), with the addition of a final DNA purification step using AMPure XP magnetic beads (Beckman Coulter, Brea, USA) following standard protocols. The DNA within each assay ranged from 2 $\log_{10}$ ng to −5 $\log_{10}$ ng (as measured by NanoDrop™ 2000 Spectrophotometer) across x8 ten-fold dilutions. This analysis was conducted in technical triplicates, with the limit of detection (LOD) of each assay determined by the lowest detectable DNA concentration where all three replicates were positive. To determine the limit of quantification (LOQ) of the qPCR and ddPCR assays, the coefficient of variation among the three replicates at each dilution was calculated, with the LOQ determined as the last dilution where the LOQ was below 35% (Forootan et al., 2017). To further refine the LOD/LOQ estimates, a 2-fold dilution of initially calculated LOD DNA concentrations was analyzed in triplicate. To test the specificity of both assays, DNA extracted from *Omphiscola glabra*, *Stagnicola palustris*, *Stagnicola fuscus*, *Lymnaea stagnalis*, and *Radix balthica* snails (Davis et al., 2020) were run in biological duplicates for both assays, as was five environmental DNA extracts from farmland habitats known not to contain *G. truncatula* snails.

2.5 | Field eDNA sample collection

During April and May 2021 and May 2022, ten 50-ml samples of water were collected from 64 water saturated areas (500 ml in total) on six beef and/or sheep farms in Ceredigion, Wales, UK, with known cases of *F. hepatica* and *C. daubneyi* in their flocks/herds. The number of habitats sampled on each farm ranged from 8 to 16 (mean = 11). These habitats were either watercourses, poached areas on pasture (wet areas where soil has been mechanically disturbed by animals and/or vehicles), or ponds and were present in fields grazed by livestock. The environmental and physical nature of each habitat was recorded (Table 1). These data were later used for statistical analysis of factors associated with *G. truncatula* eDNA detection. The pH of water in each habitat was also measured using an electronic pH meter (Hanna Instruments, Rhode Island, USA). During water collection, a concurrent search for *G. truncatula* snails was completed where snails within 50 cm radius of each 10 collection spots were counted. All visits, sample, and data collection were conducted by the same individual on each occasion.

Each 500-ml water sample was transported in chilled conditions to the laboratory, where the samples were filtered through 2.7-μm micro-glass fiber filters (Whatman, Maidstone, UK) using a faucet vacuum suction pump, Büchner flask, and a funnel. Any soil sediments present at the bottom of the water sample were not filtered to limit the presence of inhibitors in the samples. DNA from half of each filter was extracted using an adapted DNeasy® PowerSoil® kit (Qiagen, Hilden, Germany) protocol, where the initial bead-beating step was undertaken in a 7-ml bijou tube. Extracts were eluted in 70 μl of elution buffer. Negative controls included double distilled water samples that were transported alongside field samples and processed following the same protocol to identify potential DNA contamination during the eDNA collection and filtering process, and blank filters to identify potential DNA contamination during the

TABLE 1 Environmental and physical factors recorded for each habitat during eDNA survey, along with the number of habitats that exhibited each factor

Measure	Category	Definition	N habitats
Habitat type 1	Watercourse	A channel of moving water	24
	Poached area	Area of stagnant water pooled on pasture	35
	Pond	A round or oval object of water	5
Habitat type 2	Permanent	A habitat that does not dry out for periods during average summer	43
	Temporary	A habitat that dries out for periods during average summer	21
Indicator Plants	Rushes	*Juncaceae* spp. present in habitat	56
	No rushes	No *Juncaceae* spp. present in habitat	8
Shade	Yes	Trees or bushes present within habitat	8
	Partial	Trees or bushes present adjacent to habitat	14
	No	No trees or bushes present within or adjacent to habitat	42
Soil color (Munsell, 1994)	Dark	Value <3 (Black or very dark brown soil)	23
	Other	Value ≥3 (dark brown, brown, or dark gray soil)	41
Grassland	Improved grassland	Modified agricultural grassland that has been Reseeded and/or regularly fertilized with chemical fertilizers	39
	Unimproved grassland	Areas that have never been plowed, reseeded, or heavily fertilized	25
Water depth	Deep	Sampling apparatus (50-mL falcon tube) could be submerged without touching soil when sampling	14
	Moderate	Sampling apparatus did not touch soil surface when half of the apparatus was submerged	25
	Shallow	Sampling apparatus touches soil surface when water was sampled	25
Water flow	Flowing	Water flowing across habitat	36
	Stagnant	No water flowing within habitat	28

DNA extraction process. Water filtering, DNA extraction, and assay setup/DNA amplification were completed in separate laboratories (Goldberg et al., 2016).

2.6 | eDNA analysis

Field samples were analyzed in technical duplicates using both the qPCR and ddPCR assays, respectively. The qPCR assay incorporated a standard curve run in duplicate in each run, where a 10-fold dilution series (1 $\log_{10}$ ng – −3 $\log_{10}$ ng) was used as a reference to quantify DNA concentration in each sample. For the second ddPCR replicate, the multiplex assay was used to enable detection of both *G. truncatula* and *F. hepatica* eDNA. This multiplex assay followed the same protocol that was optimized in this study for the singleplex *G. truncatula* assay. To test if significant differences in *G. truncatula* eDNA concentrations were identified whilst using the singleplex and multiplex assays, a paired t-test was used to compare the $\log_{10}$ transformed eDNA concentrations identified by both assays.

Each ddPCR and qPCR run incorporated a positive control (*G. truncatula* DNA extract) and non-template control samples. An automatic threshold was set in the Q-qPCR software to identify positive samples in the qPCR assay, whilst the ddPCR threshold for detection was set at 2000 for both *G. truncatula* and *F. hepatica* detection as these thresholds attained optimal separation of negative and positive droplets. For data reporting and visualization purposes, the concentration of eDNA in 1 L of field water per habitat was calculated following the equation described by Agersnap et al. (2017).

As the ddPCR assay identified more positive *G. truncatula* samples compared to the qPCR assay, all negative qPCR samples were reanalyzed, with an adaptation to the protocol, where 4 μl of DNA extract was added to each reaction. Furthermore, to test if inhibitors were impacting eDNA detection, all negative field samples were reanalyzed using the qPCR assay whilst spiked with −3 $\log_{10}$ ng of *G. truncatula* DNA extract.

2.7 | Statistical analysis

All statistical analyses were conducted in R (R Core team, 2019), primarily using the glmmTMB package (Brooks et al., 2017). Preliminary analysis was conducted to establish which generalized linear mixed model (GLMM) family would be most appropriate to analyze field eDNA data. Here, the deviance of simulated residuals of both normal and zero-inflated GLMMs of the Gaussian family, using $\log_{10}$

transformed copy concentration as the dependent variable, and the Poisson and negative binomial (nbinom1 and nbinom2) families, using raw copy count data as the dependent variable, were compared using the DHARMa package in R (Hartig, 2020). Zero-inflated GLMM of the Gaussian family was deemed to be superior to other models tested due to an observed lower deviance of simulated residuals, and thus models belonging to this family were created to identify factors associated with both the presence/absence of *G. truncatula* eDNA in surveyed habitats (zero-inflated component) and the concentration of *G. truncatula* eDNA ($\log_{10}$ copies/L) detected in positive samples (conditional component). Farm and field ID were included as random factors in the zero inflated and the conditional components of the model to account for potential between farm and field variance in *G. truncatula* eDNA detection. However, the removal of farm as a random factor in the conditional component of the model, and the removal of all random factors in the zero-inflated component of the model improved model fit and thus, the only random factor present in the final model was field in the conditional component of the model.

A backward elimination method was used to create candidate best-fit models predicting *G. truncatula* eDNA presence and copy concentration, where independent variables were sequentially removed from each model based on their *p* values, with the independent variable with the highest P value removed before the model was run again. Candidate models were finalized when all variables were significant ($p < 0.05$). Further manual additions and subtractions of independent variables were made in an attempt to improve model fit further. The candidate models created were tested for their goodness of fit by analyzing model Akaike information corrected criterion (AICc) values (Burnham & Anderson, 2004), using the AICcmodavg package in R (Mazerolle & Mazerolle, 2017), with the model with the lowest AICc regarded as having the best fit. The best-fit model was then validated using DHARMa residual diagnostic tests (Hartig, 2020) to establish if this model was appropriately fitted. The tests conducted included a test for normality of scaled residuals, test of deviations between the scaled residuals and rank transformed predictions, spatial autocorrelation of residuals, and zero inflation of residuals. Independent variable multicollinearity and the adjusted intra-class correlation coefficient (ICC) of each model were also calculated using the performance package in R (Lüdecke et al., 2021).

The study also aimed to identify factors associated with *F. hepatica* eDNA detection using a binomial mixed model where farm and field were included as random factors. However, no significant associations between *F. hepatica* eDNA presence and any factor were detected ($p > 0.05$).

3 | RESULTS

3.1 | Assay optimization and performance

The optimal annealing/extension temperature for both qPCR and ddPCR assays was determined to be 60°C. For ddPCR, an annealing/extension step of 60°C yielded highest copy numbers and the greatest separation between negative and positive droplets (Figure 1). Lowest CT values were observed at 60°C during qPCR temperature gradient analysis, whilst a primer/probe concentration of 500/250 nM yielded lowest CT values.

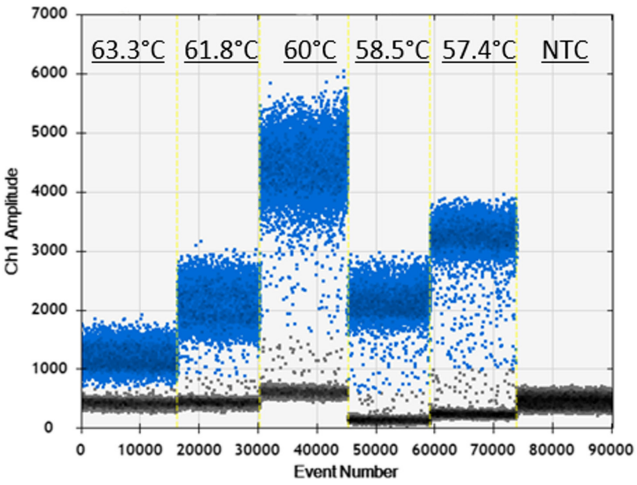

FIGURE 1 Optimization of the annealing temperature of the *G. truncatula* ddPCR assay. Lanes (split by yellow lines) represent the gradient of the five temperatures and a non-template control (run at 60°C).

Results of serial dilution analysis of ddPCR and qPCR assays can be seen in Figure 2. Analysis of 10-fold dilutions of *G. truncatula* DNA revealed qPCR assay efficiency of 99.7%, with an $R^2 > 0.99$ (Figure 2). ddPCR was 5-fold more sensitive in detecting *G. truncatula* DNA compared to qPCR, with the LOD and LOQ of the ddPCR observed to be −4 $\log_{10}$ ng (10 fg) per reaction with the qPCR LOD and LOQ identified as −3.3 $\log_{10}$ ng (50 fg) per reaction. The ddPCR assay and qPCR assay amplified −5 $\log_{10}$ ng (1 fg) and − 4 $\log_{10}$ ng (10 fg) per reaction in two of three replicates, respectively. Neither ddPCR or qPCR assays amplified DNA from any other Lymnaeid snails tested or from environmental DNA extracts from farmland habitats known not to contain *G. truncatula* snails.

3.2 | eDNA analysis

Water samples were collected and processed from 64 habitats, 42 of which were positive for *G. truncatula* eDNA when analyzed using ddPCR, with only 33 positives identified when analyzing using qPCR (Figure 3). No further qPCR positive samples were identified following the analysis of the third replicate and there was no evidence that inhibitors impacted eDNA detection, as all negative samples were successfully amplified by qPCR when spiked with *G. truncatula* DNA. Example 1D plots generated by QuantaSoft software during analysis of these samples via ddPCR can be seen in Figure 4. The mean copies of *G. truncatula* DNA measured by ddPCR to be present in positive sampled water was 3.35 $\log_{10}$ copies/L (Figure 5). *G. truncatula* snails were physically detected in only 3 of the 64 habitats, all of which were positive via ddPCR and qPCR analysis. In samples from these

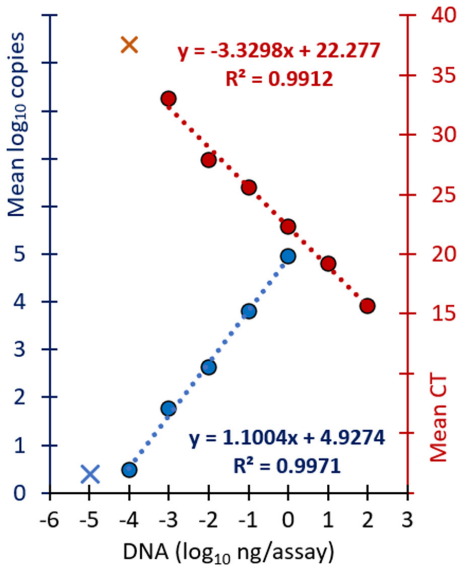

FIGURE 2 ddPCR and qPCR amplification of 10-fold dilutions of *G. truncatula* genomic DNA. Red: Mean CT values of three technical replicates plotted against *G. truncatula* genomic DNA concentrations (log_{10} ng/assay) used in the dilution series. Blue: Mean log_{10} target DNA copies detected three technical replicates via ddPCR are plotted against *G. truncatula* genomic DNA concentrations (log_{10} ng/assay) used in the dilution series. 1 and 2 log_{10} ng led to oversaturation of positive droplets in ddPCR. X markers indicate mean log_{10} copies/CT value of DNA concentration falling below LOD threshold as one replicate did not amplify in each respective assay.

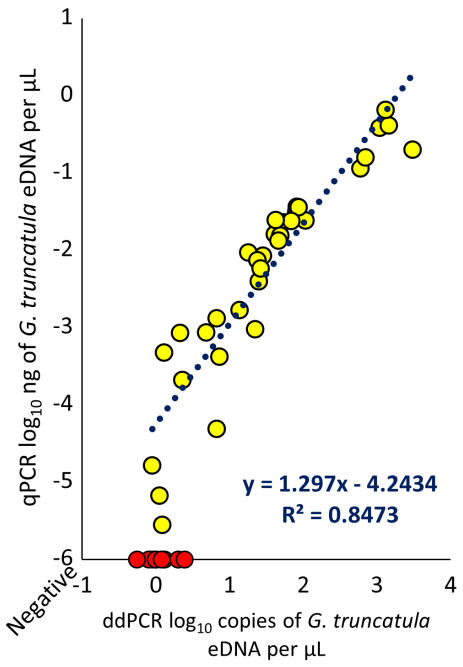

FIGURE 3 Association between eDNA concentrations of *G. truncatula* in eDNA extracts as measured using qPCR and ddPCR where a significant positive association was present ($p < 0.001$). Red markers indicate samples that were positive via ddPCR but negative via qPCR analysis.

habitats, a mean eDNA concentration of 4.76 log_{10} copies/L was detected by ddPCR analysis, a significantly higher eDNA concentration compared to positive habitats where *G. truncatula* snails were not physically detected (3.24 log_{10} copies/L) ($p = 0.013$). ddPCR also identified 5 of 64 samples to be positive for *F. hepatica* eDNA, with a mean eDNA concentration of 2.24 log_{10} copies/L. There was no significant difference between log_{10} transformed *G. truncatula* DNA concentrations observed in samples when analyzing using the singleplex ddPCR assay and the multiplex ddPCR assay ($p = 0.959$). Examples of habitats sampled and the eDNA concentrations detected can be seen in Figure 6.

Factors identified as being significantly associated with *G. truncatula* eDNA detection and the log_{10} number of *G. truncatula* eDNA copies per liter of field water in each habitat as identified by ddPCR can be seen in Table 2. Habitats with black or dark brown soil, those that contained deep water pools and those that were fully shaded were significantly less likely to contain *G. truncatula* eDNA ($p < 0.05$), whilst higher eDNA concentrations were found in temporary *G. truncatula* habitats ($p < 0.05$). The final *G. truncatula* eDNA mixed model had an ICC of 0.237, and no collinearity was detected between variables in the final model. Standardized residuals were normally distributed ($p = 0.99$), and there was no zero inflation ($p = 0.99$) or spatial autocorrelation of residuals ($p = 0.55$), and no significant deviations between the scaled residuals and rank transformed predictions were detected ($p > 0.05$).

4 | DISCUSSION

ddPCR and qPCR assays were successfully developed in this study to detect *G. truncatula* eDNA from water samples collected on pastureland. The ddPCR assay identified 42 *G. truncatula*-positive samples compared to the 33 habitats qPCR identified as being positive, highlighting its superior sensitivity, as has been demonstrated when targeting eDNA of other species (Capo et al., 2021; Wood et al., 2019). Despite its failure to match the sensitivity of ddPCR, the qPCR assay has superior sensitivity to previously designed PCR and LAMP assays when comparing with LOD values recorded by Davis et al. (2020) and outperformed a physical survey of *G. truncatula* in sampled habitats in this study. These assays, along with conventional PCR and LAMP assays previously designed (Davis et al., 2020; Jones et al., 2018), offer a variety of options to detect *G. truncatula* eDNA. Despite their inferior sensitivity, LAMP and PCR may be preferable in scenarios where limited technical expertise or budget is available, indeed all of these methods have their merits, with the assays selected for future research and application of eDNA analysis likely to vary depending on eDNA survey aims, budget, equipment availability, and the technical expertise of researchers.

Analysis revealed multiple environmental factors associated with the spatial detection of *G. truncatula* eDNA on farmland. Habitats fully shaded by trees were significantly less likely to contain detectable *G. truncatula* eDNA, a finding which concurs with the observations of Vareille-Morel et al. (1998) who found that *G. truncatula* egg masses were rarely found in shaded areas. Sunlight is a key driver

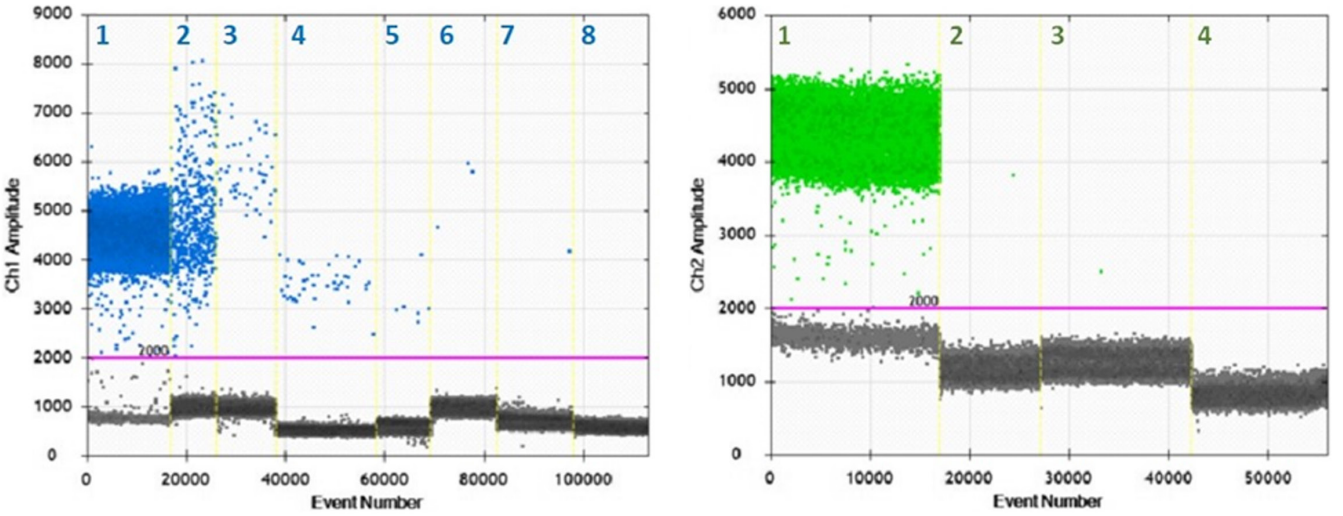

FIGURE 4 Example outputs of ddPCR analysis using Quantasoft software. (left) *G. truncatula* assay, lane 1 = positive control; lane 2 = positive field sample (3290 copies/µl reaction); lane 3 = positive field sample (4.9 copies/µl reaction); lane 4 = positive field sample (2.6 copies/µl reaction); lane 5 = positive field sample (9 copies/µl reaction); lane 6 = positive field sample (0.27 copies/µl reaction); lane 7 = positive field sample (0.08 copies/µl reaction); lane 8 = negative field sample (0 copies/µl reaction). (right) *F. hepatica* assay, lane 1 = positive field sample (2610 copies/µl reaction); lane 2 = positive field sample (0.12 copies/µl reaction); lane 3 = positive field sample (0.08 copies/µl reaction); lane 4 = negative field sample (0 copies/µl reaction).

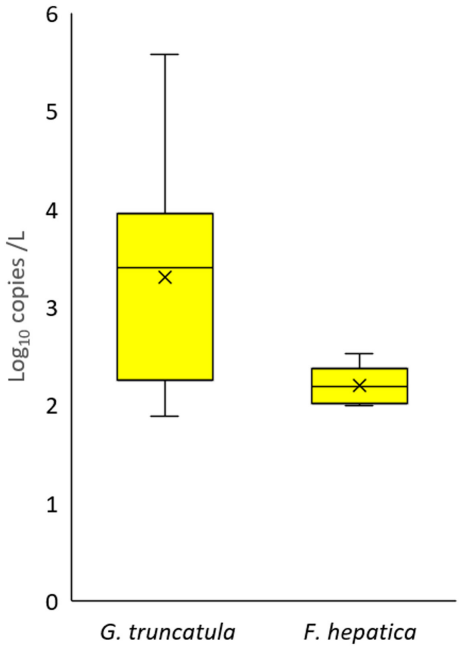

FIGURE 5 Boxplot of mean (×) and median (–) *G. truncatula* ($n = 42$) and *F. hepatica* ($n = 5$) eDNA concentrations across habitats sampled in study as measured by ddPCR.

for unicellular algae, the main food source of *G. truncatula*, which grows on bare mud surfaces (Dreyfuss et al., 2015). Shaded areas may, therefore, lack the capability of supporting populations of *G. truncatula* snails due to limited food (Petzold, 1989). Shaded areas are also likely to be cooler which may impact snail population size as *G. truncatula* snails are increasingly active, grow quicker, tend to live longer, and produce more offspring as temperatures increase in temperate conditions (Hodasi, 1976) and traits that may also lead to increased eDNA excretion (Jones et al., 2021). However, contradictory positive associations between trees and *G. truncatula* presence were identified by Roessler et al. (2022) in Switzerland. It was concluded there that the presence of single trees or bushes as well as a certain proximity to woodland is beneficial to the snails as trees offer protection from direct sunlight and drying out. In our work, no significant difference was observed between eDNA detection rates in habitats partially shaded and those not shaded. This suggests that some shading is not completely detrimental to *G. truncatula* populations, and when considering the findings of Roessler et al. (2022), some shading may be beneficial in certain circumstances to protect habitats from desiccation. Furthermore, a near significant difference in eDNA detection was also observed when comparing habitats partially shaded and those fully shaded which further emphasizes that the degree of shading is vital to determining habitat suitability to *G truncatula*. Currently, major drives are underway to plant trees on agricultural land with the aim of increasing carbon storage in efforts to reach net zero carbon emissions within the agriculture sector. Within this drive to plant more trees, it has been suggested that strategically planted trees could be employed to limit habitat suitability to *G. truncatula*, especially considering their ability to improve soil drainage (Crilly et al., 2015). However, questions remain regarding the impact of strategic planting of trees on *G. truncatula* populations and the subsequent dynamics of trematode transmission in those areas. To answer these questions, eDNA analysis could be employed in future studies to monitor changes in *G. truncatula* population dynamics over time as trees, or any other mitigations are employed.

Soil color was also associated with eDNA detection, with eDNA detection less likely in soils categorized as dark brown or black. Darker soils often contain high levels of organic matter and peat and

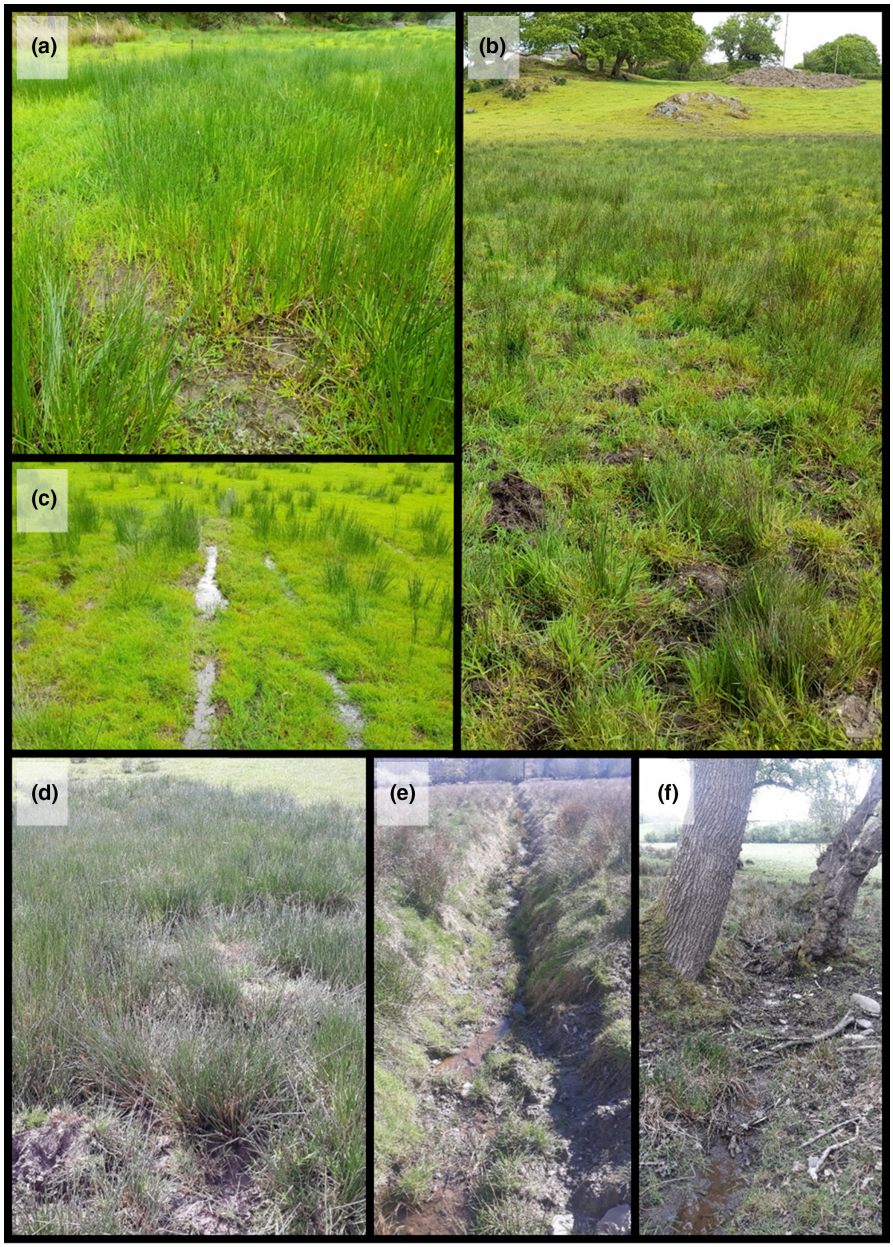

FIGURE 6 A selection of habitats surveyed in this study. (a) The habitat with highest *G. truncatula* eDNA concentration in study (424,000 copies/L); (b) a poached area on pasture (83,000 copies/L); (c) a Tire rut habitat (124 copies/L), which also had the highest concentration of *F. hepatica* eDNA in the study (373 copies/L); (d) a wet flush on pasture which had only recently appeared due to the failure of underground drains (6767copies/L); (e) drainage ditch habitat where *G. truncatula* snails were physically detected (98,000 copies/L); (f) a negative habitat, note the that this habitat was shaded by trees which were present within the habitat.

are often associated with lower pH values. Studies have shown that *G. truncatula* snails can tolerate a wide range of soil pH, although they tend to avoid highly acidic soils (pH <5.1) (Dreyfuss et al., 2015). Increasing *G. truncatula* population densities are often positively correlated with soil pH (Caron et al., 2014; Charlier et al., 2014) and this could explain the negative associations between these soils and the presence of detectable eDNA; however, there was no significant association between eDNA detection and sampled water pH. It has been suggested that peaty soils are less suited to *G. truncatula* snails due to an absence of suitable food sources (Heppleston, 1972) and its texture (Walton & Wright, 1926), rather than its acidity, and the darker soils observed in this study could be less suited to *G. truncatula* due to these alternative reasons. Furthermore, darker soils may have high concentrations of specific inorganic compounds such as manganese oxide (Dixon et al., 1990), yet the impact of soil mineral content on *G. truncatula* has yet to be studied in detail despite the detection of associations between soil mineral content and *F. hepatica* infection rates in livestock (McCann et al., 2010; Novobilský et al., 2015).

The analysis also revealed factors associated with increasing *G. truncatula* eDNA concentrations in positive samples. Habitats where snails were visually detected during sampling had significantly higher eDNA concentrations, which is expected and follows ample results showing a strong correlation between species density or biomass in a habitat and eDNA concentration (Yates et al., 2019). Significantly higher eDNA concentrations were also found in samples collected from temporary *G. truncatula* habitats (i.e., those that tend to dry up for periods in the summer). This finding contradicts the widespread notion that *G. truncatula* snails are found in higher densities in permanent habitats as snails in temporary habitats have increased mortality rates due to temporal variations in moisture levels which may vary from standing water to extremely dry ground

TABLE 2 Results of best-fit zero-inflated mixed model analysis which identified significant factors associated with absence and concentration of *G. truncatula* eDNA in samples as identified using ddPCR assay

Component	Variable	Estimate	*SE*	*Z*	*p*
Conditional component: Factors associated with increasing eDNA concentration (log_{10} copies per liter)	Intercept	3.004	0.203	14.82	<0.001
	Temporary habitat	0.656	0.300	2.189	0.029
	Permanent habitat	0	–	–	–
Zero-inflated component: Factors associated with absence of *G. truncatula* eDNA	Intercept	1.938	1.246	1.555	0.120
	Black or very dark brown soil	2.035	0.733	2.777	0.005
	Dark brown, brown, or dark gray soil	0	–	–	–
	Deep water depth	1.453	0.705	2.060	0.039
	Shallow or moderate depth	0	–	–	–
	Partial shade	−2.392	1.338	−1.789	0.074
	No shade	−2.929	1.198	−2.444	0.015
	Fully shaded	0	–	–	–

(Heppleston, 1972). Nevertheless, it has been found that *G. truncatula* snails inhabiting temporary habitats tend to be larger (Chapuis et al., 2007; Heppleston, 1972) which may cause an increase in eDNA levels expelled per individual snail which may explain the increased eDNA concentrations observed in this study in temporary habitats. Furthermore, fluctuations in habitat water volume may dilute or concentrate eDNA (Katz et al., 2021; Seeber et al., 2019), and the increased eDNA concentrations observed in temporary *G. truncatula* habitats may be, in part, caused by the drier than usual conditions observed during the study period. This hypothesis is supported by the fact that *G. truncatula* eDNA was less likely to be detected in habitats with deeper water pools, although in this instance it is widely accepted that *G. truncatula* snails avoid deeper pools due to their requirement to access air unlike other lymnaeid species (Skuce et al., 2014). This highlights a major challenge in quantifying and comparing eDNA concentrations in habitats of varying types, sizes, and water volume as is observed for *G. truncatula* habitats which may take the form of permanent wetlands, spring heads, ponds, streams, ditches, and poached soils on pastures in both temperate and arid climates (Dreyfuss et al., 2015).

The field analysis conducted in this study revealed *G. truncatula* habitat locations on six farms. This information was shared with each individual farmer who, in conjunction with their veterinarians, adapted their *F. hepatica* control plans based on the information provided. For example, cattle/sheep were tested for fluke infection in Spring/early Summer before being moved to graze fields containing risk areas as identified via eDNA analysis. This practice identified groups of animals shedding fluke eggs that were then treated prior to accessing *G. truncatula* habitats in the Spring, thus limiting pasture contamination of *F. hepatica* eggs which can have major benefits in limiting future fluke infections (Parr & Gray, 2000). Further long-term solutions were also identified for future application which will aim to limit *G. truncatula* habitat suitability. For example, on two of the farms, *G. truncatula* habitats were identified in wet flushes where field drainage systems had failed (Figure 6) and fixing these drains would likely make these habitats uninhabitable to *G. truncatula* snails (Dreyfuss et al., 2015). Furthermore, multiple watercourses were identified across the farms (Figure 6) and fencing to limit livestock access could be a viable option in some of these areas. However, our knowledge of *G. truncatula* ecology and behavior is still limited, and it remains unclear how alternative *F. hepatica* control strategies may be optimized and applied. For example, limiting livestock access to specific *G. truncatula* habitats, such as watercourses, may not be successful if significant movement and migration of *G. truncatula* snails occur between these areas and surrounding pastures. It has been found that *G. truncatula* snails can travel 100s of meters and contaminate pastures free from livestock with *F. hepatica* metacercariae (Rondelaud, 1983; Rondelaud et al., 2005), a trait which may limit the effectiveness of grazing management practices aimed at limiting *F. hepatica* infections. Our lack of understanding of these movements and the factors that drive the colonization of specific temporary habitats by *G. truncatula* hinders our ability to control *F. hepatica*, yet our eDNA assay may offer significant insight into *G. truncatula* ecology, movements, and general spatial distribution over time, which may lead to the design and implementation of optimal measures to limit the *F. hepatica* lifecycle on farms. Furthermore, insight gained into *G. truncatula* ecology from eDNA surveys can be used to build high-resolution spatial models of *G. truncatula* distribution, population size, and overall *F. hepatica* infection risk at habitat level.

Our understanding of *F. hepatica* infection ecology may also benefit from environmental monitoring of *F. hepatica* eDNA. The multiplex ddPCR assay identified *F. hepatica* eDNA, although the detection of this species was rare with only five positive samples recorded. The rarity of *F. hepatica* eDNA in this study corresponds with the findings of Jones et al. (2018) who only recorded 6 of 57 farmland water samples to be positive for *F. hepatica* eDNA, albeit using a less sensitive conventional PCR amplification method. eDNA surveys may not be as successful in detecting the presence of species that expel low levels of DNA (Mauvisseau et al., 2019) which may well be the case for *F. hepatica*, especially considering lower eDNA concentrations recorded here in comparison with *G. truncatula*. Similarly, the maximum *F. hepatica* eDNA concentrations detected by

Rathinasamy et al. (2021) on an Australian farm were 95% lower than the maximum eDNA concentrations of its intermediate snail host, *Austropeplea tomentosa*, within the same habitats. The utility of identifying *F. hepatica* eDNA in farmland water has not been established, with questions surrounding the usefulness of identifying *F. hepatica* eDNA presence when its source, either infective cercariae/metacercariae or stages not infective to the final host, that is, miracidia/eggs, cannot be established (Jones et al., 2018; Rathinasamy et al., 2021). For some trematodes, including *Schistosoma* spp., the detection of eDNA, regardless of life stage origin, may be of great value to inform eradication programs of parasite reservoirs that require targeting (Sengupta et al., 2019), yet for *F. hepatica*, eradication is not considered a viable option at present. Furthermore, the rarity of detecting *F. hepatica* eDNA and its presence in only low concentrations, even on farms with current *F. hepatica* infections in grazing livestock, suggests that at present this methodology focusing on water may have limited value, especially considering that infective metacercariae will often be present on grass above water. Screening other environmental matrices for *F. hepatica* eDNA, in particular grass, may have greater value for evaluating current *F. hepatica* infection risk on pastures (Cuthill, 2020). However, major practical challenges are associated with screening non-liquid matrices such as plants and soil for eDNA, which include limited sample volume capacity per extraction and the presence of PCR inhibitors (Cuthill, 2020; Thomsen & Willerslev, 2015). Considering that water is the optimal screening matrix for eDNA, other molecular markers also present in water and that may be specific for distinct *F. hepatica* life stages, such as protein or RNA (Pawluk et al., 2018; Veilleux et al., 2021), could be targeted in future environmental water-based surveys, although substantial research would need to be undertaken initially to characterize proteins and/or RNA markers that are specific to each *F. hepatica* life cycle stage (Cwiklinski & Dalton, 2018).

In conclusion, this study developed novel ddPCR and qPCR assays to detect *G. truncatula* eDNA in water from pastureland. These assays can be utilized to map *F. hepatica* infection risk areas on farmland which can guide disease control strategies in flock and herds. Surveillance of *G. truncatula* eDNA and potentially *F. hepatica* eDNA can also be used in future to further our understanding about *G. truncatula* ecology and the *F. hepatica* lifecycle, and to monitor the effectiveness of management strategies aimed at limiting habitat suitability for *G. truncatula* or to limit contact between these snail and grazing livestock. The tools developed may also be applied in a human health context, specifically in regions of the world where *F. hepatica* commonly infects humans.

AUTHOR CONTRIBUTIONS

Rhys Aled Jones conceptualized and designed the study, collected field samples, concluded analysis of data, and wrote original manuscript. Rhys Aled Jones, Chelsea N. Davis, Justyna Nalepa-Grajcar, and Hayley Woodruff completed laboratory work. Rhys Aled Jones, Emma Jones, and Peter M. Brophy contributed to funding acquisition. Rhys Aled Jones, Peter M. Brophy, Hefin Wyn Williams, and Chelsea N. Davis contributed to revision and editing of manuscript.

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CONFLICT OF INTEREST

The authors declared that they have no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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