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Confirmation of *Galba truncatula* as an intermediate host snail for *Calicophoron daubneyi* in Great Britain, with evidence of alternative snail species hosting *Fasciola hepatica*

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Abstract

Background: *Fasciola hepatica* is a highly prevalent parasite infecting livestock in Great Britain, while *Calicophoron daubneyi* is an emerging parasite within the GB livestock industry. Both *F. hepatica* and *C. daubneyi* require an intermediate host snail to complete their life-cycles and infect ruminants; however, there has been no confirmation of the intermediate host of *C. daubneyi* in GB, while there are questions regarding alternative host snails to *Galba truncatula* for *F. hepatica*. In this study, PCR was used to identify *C. daubneyi* hosting snail species on Welsh pastures and to identify any alternative snail species hosting *F. hepatica*.

Findings: Two hundred and sixty four snails were collected between May-September 2015 from six farms in mid-Wales known to have livestock infected with *C. daubneyi* and *F. hepatica*. Fifteen out of 134 *G. truncatula* were found positive for *C. daubneyi*, one of which was also positive for *F. hepatica*. Three snail species were found positive for *F. hepatica* [18/134 *G. truncatula*, 13/52 *Radix balthica*, and 3/78 *Potamopyrgus antipodarum* (New Zealand mud snail)], but no evidence of *C. daubneyi* infection in the latter two species was found.

Conclusion: This study indicates that *G. truncatula* is a host for *C. daubneyi* in GB. *Galba truncatula* is also an established host of *F. hepatica*, and interactions between both species at intermediate host level could potentially occur. *Radix balthica* and *P. antipodarum* were found positive for *F. hepatica* but not *C. daubneyi*. This could indicate a role for alternative snail species other than *G. truncatula* in infecting pastures with *F. hepatica* in GB.

Keywords: *Calicophoron daubneyi*, *Fasciola hepatica*, *Galba truncatula*, *Radix balthica*, *Potamopyrgus antipodarum*, Paramphistomosis, Fasciolosis, Great Britain

Background

Liver fluke (*Fasciola hepatica*) and rumen flukes (Paramphistomatidae spp.) are parasitic trematodes prevalent in GB livestock. Liver fluke disease (Fasciolosis) causes an estimated yearly loss of £300 million for the UK agriculture industry [1], with a study showing that 76 % of the dairy herds in England and Wales are infected [2]. Despite rumen flukes being present in GB for at least half a century [3], it is only in the past decade that these have been regarded as potentially pathogenic parasites, with increasing reports of disease (paramphistomosis) occurrence [4]. This increase may be due to the establishment of *Calicophoron daubneyi* as the prominent paramphistome species in GB, replacing *Paramphistomum cervi* [5]. How *C. daubneyi* arrived and why it spread across GB has not been confirmed, but increasing animal movements from mainland Europe, where *C. daubneyi* has been present for decades [6], and/or climate change may have facilitated its recent appearance as a parasite of significance.

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Both *F. hepatica* and *C. daubneyi* require a snail as an intermediate host in order to complete their life-cycle, a process in which the parasites exploit their host to develop and multiply rapidly. The main intermediate host of *F. hepatica* in GB is *Galba truncatula* (O. F. Müller) [7], however, reports from other countries in Europe have shown that other snail species such as *Radix* spp. [8, 9], *Succinidea* spp. [8], *Omphiscola glabra* (O. F. Müller) [10], and *Lymnaea palustris* (O. F. Müller) [9] can also act as intermediate hosts for *F. hepatica*. Nevertheless, fundamental questions remain regarding the capabilities of these species to support the development of *F. hepatica* from mother sporocyst to cercariae released into the natural environment. In GB, there has been no confirmation of the intermediate snail host of *C. daubneyi*. *Galba truncatula* has been shown to be the prominent host of *C. daubneyi* in Spain [11] and France, where *O. glabra*, *L. palustris*, *Physa acuta* (Draparnaud), and *R. balthica* (L.) have also been shown to host *C. daubneyi* [12]. Other paramphistome species such as *P. cervi* and *C. calicophoron* are known to infect aquatic snails of the family Planorbidae [13] which are present in the freshwater ecosystems of GB.

This lack of clarity regarding the intermediate host of *C. daubneyi* in GB may have a negative impact on farmers and veterinarians who wish to implement grazing strategies to reduce the burden of *C. daubneyi* in their livestock. There are also questions regarding alternative host species for *F. hepatica* in GB, including whether any shed significant numbers of cercariae onto pasture. In this case study, a panel of snail species found on pastures grazed by ruminants infected with both *F. hepatica* and *C. daubneyi* were screened using PCR assay to detect the presence of infection with these parasites in potential intermediate host snails. The goal was to reveal any *C. daubneyi* transmitting snail species on Welsh pastures and to identify any alternative snail species hosting *F. hepatica*.

Methods

Between May and September 2015, snails were collected from habitats grazed by animals identified as *C. daubneyi* and *F. hepatica* infected via sedimentation faecal egg count (FEC), using the 10 m transect method [14]. Collected snails were stored in 50 ml tubes and transported to the laboratory where they were identified using morphological characteristics [15]. The snail nomenclature used here follows Anderson [16]. Snails were placed in individual 0.5 ml tubes and crushed using a pellet mixer. Snail DNA was extracted using the Chelex® method [17], adapted with the inclusion of 20 μl of proteinase K (20 mg/ml, Fisher Scientific, Waltham, USA) prior to incubation at 56 °C. After extraction the sample was centrifuged at 15,000 rpm for 6 min with the supernatant collected and diluted ×10. Polymerase chain reaction (PCR) amplification was used to screen snails for infection of *F. hepatica* or/and *C. daubneyi* (Additional file 1: Table S1). In brief, snail DNA of the same species were pooled into groups of six, with each pool subjected to PCR on three occasions to detect *C. daubneyi* infection using primers to amplify a 167 bp strand from the cytochrome *c* oxidase subunit 1 (*cox*1) gene (GenBank JQ815200) and *F. hepatica* infection detected using primers to amplify 425 bp strand from the *cox*1 gene (GenBank AF216697) [11], and finally as a control amplifying 687 bp and 329 bp amplicons of Lymnaeidae spp. and *Potamopyrgus antipodarum* (J. E. Gray) 18S rRNA gene, respectively. Snails from positive groups were screened individually in identical manner to detect infection status. A subset of *C. daubneyi* and *F. hepatica cox*1 gene amplicons detected in infected snails, 18S gene amplicons for *G. truncatula* and *P. antipodarum*, and ITS2 amplicons for *R. balthica* (116 bp amplicon amplified using PCR for *Radix* species ID only), (Additional file 1: Table S1) were sequenced (ABI3100) and aligned to confirm species identity (Geneious Biomatters LTD).

Results

One hundred and thirty-four *G. truncatula* were sampled from six farms known to have animals infected with both fluke species (referred to as farms 1–6; Table 1). In total 15 were positive for *C. daubneyi*, and 18 were positive for *F. hepatica*. One *G. truncatula* was found positive for *C. daubneyi* and *F. hepatica*. A subset of *C. daubneyi* amplicons ($n = 5$) from positive *G. truncatula* were sequenced and aligned with the *C. daubneyi cox*1 sequence (GenBank JQ815200), and showed 100 % similarity (Additional file 2: Figure S1). Fifty-two *R. balthica* and 78 *P. antipodarum* were collected from farm 5, with *F. hepatica* DNA detected in 13 and 3 snails, respectively, but *C. daubneyi* DNA was not found in either species. A subset of *F. hepatica* amplicons from positive *G. truncatula* ($n = 2$), *R. balthica* ($n = 1$) and *P. antipodarum* ($n = 1$) were sequenced and aligned with the *F. hepatica cox*1 sequence (GenBank AF216697) and showed >99 % similarity (Additional file 2: Figure S2). A subset of *F. hepatica*-positive *G. truncatula* ($n = 2$) and *P. antipodarum* ($n = 2$) were sequenced and aligned with their respective 18S gene sequences (GenBank Z73985.1 and JF960455.1, respectively) with all showing 100 % similarity. *Radix balthica* ($n = 2$) were sequenced and aligned with their ITS2 sequences (GenBank AJ319633.1), and showed 100 % similarity.

Discussion

With *C. daubneyi* establishing as a prominent parasite within GB's livestock industry, further information is required on its epidemiology to allow veterinarians and

Table 1 Prevalence of *Calicophoron daubneyi* and *Fasciola hepatica* in *Galba truncatula* collected from six farms in Wales, Great Britain

Farm No.	Month of sampling	*G. truncatula* No. of collected snails	*C. daubneyi* No. of infected snails	*F. hepatica* No. of infected snails	No. of co-infected snails
Farm 1	May	35	0	10	0
Farm 2	June	1	1	0	0
Farm 3	June	24	1	3	0
Farm 4	July	28	0	2	0
Farm 5	August	26	12	1	1
Farm 6	September	20	1	2	0
Total		134	15	18	1

livestock producers to implement strategies to minimise its impact. This farm survey found that *G. truncatula* is a host for *C. daubneyi* in Wales, which reflects the situation in mainland Europe. If *C. daubneyi* was introduced to GB during the past decade from animals imported from mainland Europe, the fact that its intermediate host *G. truncatula* is abundant in GB is likely to have facilitated its establishment. *Galba truncatula* is already the prominent intermediate host of *F. hepatica* in GB [7]; however, it has been shown in Europe that other lymnaeid snail species can be infected with *F. hepatica*. It is unclear to what extent *F. hepatica* develops in the latter species and whether they may shed significant numbers of cercariae onto pastures. By dissecting a subset of *R. balthica* in our study, free cercariae were seen in snails which were later shown to be positive for *F. hepatica* infection. This would suggest that not only are alternative snail species in GB being infected by *F. hepatica*, but are also shedding cercariae, which could be significant on pastures where *G. truncatula* are absent [8]. Despite the difficulties recorded in experimental infections of *Radix* spp. with *F. hepatica* [18], studies have shown that snails infected at the juvenile stage [19] or persistently exposed to *F. hepatica* over successive generations, are more susceptible to infection and eventual shedding [20]. These results could also explain the *F. hepatica*-positive *P. antipodarum* recorded in our study, however, it must be stressed that these infected snails were not dissected, and thus no confirmation of the patency of infection can currently be made.

With *F. hepatica* and *C. daubneyi* now both present in GB there are unanswered questions regarding potential interactions between these parasites within their intermediate snail hosts. There is evidence to suggest that the presence of *C. daubneyi* within a snail population may facilitate infections with *F. hepatica* [21]; this could increase the susceptibility of alternate snail species to *F. hepatica*. However, co-infections with both parasites in *G. truncatula*, as seen in only one case in this study, have been shown to be rare [22]. This could be down to numerous factors including competition between the two digenean species. *Fasciola hepatica* has been shown to eliminate *C. daubneyi* within *G. truncatula* [23] and it has been suggested that co-infected *G. truncatula* suffer from increased mortality [24]. These two mechanisms could lead to a wide scale antagonism, where the presence of one digenean within a snail population supresses another. This has been hypothesised to be the reason for the absence of *F. hepatica* in populations of *G. truncatula* infected with *Haplometra cylindracea* [24, 25], and species of the Echinostomatidae [26]. Co-infections with *C. daubneyi* and *F. hepatica* have been successfully sustained to cercarial shedding within laboratory settings [23], while a high prevalence of digenean infection within a snail population is required for significant antagonism to occur [27]. Therefore, it could be disputed if any significant antagonism occurs between these two species.

Conclusion

Our study confirms for the first time that *C. daubneyi* is infecting *G. truncatula* in GB. With a high density of grazing ruminants, widespread populations of *G. truncatula*, endemic *F. hepatica* levels, newly established *C. daubneyi*, and favourable climate for both parasite and intermediate host, a situation may now arise in GB where significant interaction between *F. hepatica* and *C. daubneyi* occurs at intermediate host level. This could in theory impact positively or negatively on the number of viable cercariae shed on pastures due to a synergistic or antagonistic effect; however, it is unclear if this potential interaction would have any major effect on the prevalence of these parasites in livestock. The role of alternative intermediate host snails for *F. hepatica* and *C. daubneyi* should also not be underestimated, with our data concurring with other studies that *F. hepatica* is adaptable in infecting and developing in these species. Further research is required on the intermediate hosts of both *C. daubneyi* and *F. hepatica* and any potential interaction within GB, encompassing greater numbers of snails within a greater extent of snail habitats and farms across a longer period of time.

Additional files

Additional file 1: Table S1. Primers and PCR cycling conditions used for detection of snail infection status and confirmation of snail identification to the species level (DOCX 16 kb)

Additional file 2: *Calicophoron daubneyi* and *Fasciola hepatica* sequences amplified from infected snails and aligned with GenBank sequences. Figure S1. Sequences for *Calicophoron daubneyi* from infected *Galba truncatula* in farm 1 (GT CD 1), farm 2 (GT CD 4), farm 5 (GT CD 2, GT CD 3), farm 6 (GT CD 5) aligned with *C. daubneyi cox*1 gene sequence (GenBank JQ815200.1). **Figure S2.** Sequences for *Fasciola hepatica* from *Galba truncatula* co-infected with *Calicophoron daubneyi* (GT 2 FH), *Potamopyrgus antipodarum* (PA 1 FH) and *Radix balthica* (RB 1 FH; RB2 FH) aligned with *F. hepatica* cox1 gene sequence (GenBank AF216697.1). (DOCX 17 kb)

Competing interests
The authors declare that they have no competing interests.

Authors' contributions
RAJ, HWW and PMB conceived and designed the study; RAJ and HWW collected the samples; RAJ analysed the samples; all authors contributed to the final manuscript.

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