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Paramphistomosis of ruminants: An emerging parasitic disease in Europe

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Abstract

Whilst historically regarded as being of minor importance in European livestock, recent evidence suggests that the prevalence of paramphistomosis is greater than that of fasciolosis in parts of the UK. In order to address this emerging threat to ruminant farming systems, and associated risks for food security posed by rumen fluke infection, it is imperative that we develop a better understanding of the basic biology of this parasite and how it interacts with its ruminant host. In this opinion article we will review recent progress in tracking the spread of rumen fluke infection in Europe, and propose some research questions that should be addressed if we are to develop tools to diagnose and treat paramphistomosis more effectively in the future.

The paramphistomes

Paramphistomes (see Glossary), commonly known as rumen or stomach flukes, are digenean parasites that infect ruminant animals across a wide geographical range of countries as far apart as Mexico, India, Finland and Australia [1–4]. Paramphistome parasites belong to several different genera within the family Paramphistomidae, and all require an intermediate snail host and a definitive ruminant host within their lifecycle. Within the intermediate host the paramphistomes develop through three larval stages (sporocysts, rediae and cercariae) and undergo asexual reproduction before cercariae emerge from the snail and encyst as metacercariae. Infection of the definitive host occurs upon ingestion of vegetation harbouring dormant encysted metacercariae. The metacercariae then excyst in the small intestine (duodenum) before migrating through the upper gastro-intestinal tract (GIT) to the rumen-reticulum, where the mature parasites reside. Figure 1 illustrates this lifecycle for the species *Calicophoron daubneyi*.

Mature paramphistomes are pink pear-shaped organisms, typically 0.5-1.0 cm long, that are firmly attached to the host rumen wall by a posterior muscular acetabulum (Figure 2). They have a blind-ended, bifurcated gut with an oral opening protruding into the rumen at their narrower anterior end [5]. Immature stages of the parasite are much smaller at less than a millimetre in length and appear within the small intestine, as early as 9 days after exposure to infected pasture, as small nodules/dark spots, in association with haemorrhagic lesions of the superficial mucosal tissue [6,7]. Small rumen flukes, which are bright red in colour and around 2-3 mm in length but with similar external morphology to mature specimens, have been described within the rumen from day 38 post-exposure to infected pasture [6] as they complete their migration from the duodenum.

It is the newly excysted and migratory stages of the parasite that are thought to be responsible for clinical paramphistomosis; a condition regarded as a leading cause of livestock morbidity which results in economic loss in tropical and sub-tropical regions [1,4,8]. Clinical symptoms include lethargy/recumbency, dehydration, severe scour and sub-mandibular oedema, which occur as immature parasites excyst and penetrate the duodenal mucosa causing significant damage to the tissues [9]. In cases with large burdens of immature rumen fluke, mortality may occur as a result of the damage caused to host intestinal tissue and symptoms of haemorrhagic enteritis including anaemia and hypoproteinaemia [7,10]. Mature paramphistome infections have been associated with ruminal papillae atrophy and ulceration, at the point of fluke attachment [11,12], and negative impacts on production measures such as milk yields and growth rates have also been reported [13,14].

In this article we will highlight the growing prevalence of paramphistome infections in Western Europe, and the sparsity of data on the basic biology of these parasites. Key areas for future research will be proposed that support the development of diagnostic tools and

treatment options to enable the sustainable control of this emerging parasitic infection in the future.

Paramphistomosis is an emerging infection in European livestock

Historically, rumen fluke infection was rarely seen in the temperate climates of Western Europe. However, morphological identifications in the 1950s suggested that *Paramphistomum cervi* (or species later considered synonymous with *P. cervi*) was resident in Great Britain and Ireland [15] and Italy [16]. Since then, rumen fluke have persisted as a little acknowledged infection that many livestock producers remain unaware of. However, in recent years, significant increases in the prevalence of rumen fluke infections have been noted in ruminant livestock populations across western European countries. Using molecular sequence data, the species *Calicophoron daubneyi* has been clearly identified as the primary rumen fluke parasite infecting cattle, sheep and goats in Europe [17–20]. Prevalence levelsas high as 29-36% in Spain [21,22], 20% in France [23], and 59% in Wales [24] in cattle, and up to 77% in Ireland [18] and 42% in Wales [24] in sheep have now been reported (Table 1). The factors driving these increased levels are not fully understood, but the introduction of *C. daubneyi* to Western Europe during the movement of livestock [5], the presence of a suitable snail intermediate host [25,26], and climate change (milder winters and higher rainfall) favouring the completion of the parasite lifecycle [27] are all thought to contribute.

Although clinical disease and mortality owing to rumen fluke infections remain infrequent in temperate regions, fatal disease outbreaks, linked to significant immature parasite burdens, have been reported in both sheep and cattle [7,10]. Additional anecdotal reports from veterinarians and farmers indicate that paramphistomosis may be a rising problem in some areas. With a growing geographical range, and a demonstrable potential to

cause significant clinical disease, interest and concern is growing among both veterinarians and livestock producers regarding the potential impact of this parasite on animal health, productivity and welfare, and how this threat should be tackled.

Highly prevalent but poorly understood: how much do we know?

Despite their ubiquitous presence, our current knowledge of the fundamental molecular and developmental biology of rumen fluke is limited, particularly in comparison to other trematodes of veterinary significance such as the liver fluke, *Fasciola hepatica*. For instance, histochemical staining, as well as morphological and ultrastructural studies, have detailed the anatomy of several paramphistome species [3,5,28,29], but such work has largely focused on larval stages within the intermediate host or adult rumen-dwelling flukes that are easier to collect, mostly belonging to tropical and sub-tropical species of paramphistome to date. To our knowledge, the development of various organ systems (e.g. the tegument, gut, and reproductive structures) in the newly excysted and migratory stages has not yet been described for *C. daubneyi*, and only a few studies have been carried out which describe the development of other paramphistomes [30]. Uncertainty also exists regarding the feeding mechanisms used by the various life-cycle stages within the ruminant host. Based on a transcriptome analysis, Choudary *et al.* [31] proposed that mature *P. cervi* rumen flukes may obtain nutrition via damaged capillaries in the rumen wall allowing uptake of glucose and other nutrients directly from the bloodstream. However, the orientation of the parasite within the host – anchored by a blind-ended muscular acetabulum with their oral opening (which leads to the parasite gut) protruding away from the rumen wall - would indicate that the rumen contents are the primary source of nutrition for the parasite. Indeed, research dating back to the 1950s [32] has shown that rumen fluke can be sustained for a prolonged period

outside the host, with a supply of rumen fluid. Microscopic examination of mature rumen fluke showed rumen material, including Protozoa, within the parasite oesophagus, also suggesting that paramphistomes may feed on components of the rumen fluid digesta or microbiome [3]. Absorption of nutrients directly across the tegument is thought to be unlikely given its thickness in paramphistomes and a lack of mitochondria in the outer tegumental syncytium required for active transport mechanisms [28]. In contrast, ingested host tissue has been observed within the gut of an immature rumen fluke collected from the small intestine of a calf at post mortem [10]. The proposed switch from feeding on host tissue, in the small intestine, to digesta (or microbial contents) in the rumen as these parasites develop poses interesting questions about how they interact with their host during their migration along the GIT.

Perhaps due to their lack of recognition and poorly understood pathogenicity, no anthelmintic drugs (asides from a single formulation of oxyclozanide licensed only in France: Douvistome) are currently available with a label claim for rumen fluke control in Western Europe. Numerous studies, both *in vitro* and *in vivo*, have attempted to verify the efficacy of existing anthelmintics against a range of rumen fluke species [33–35]. Currently, oxyclozanide (normally marketed as a treatment for liver fluke) is the drug of choice to control both immature and mature paramphistome infections, although this drug does not appear to have been tested against immature *C. daubneyi* specifically [36]. Additionally, research into diagnostic tools for paramphistomosis has been very limited [37,38], and currently no diagnostic test has been developed for the identification of pre-patent infections, leaving faecal egg counts (FEC) or post mortem as the only options to identify chronic or acute disease respectively. Research on other trematodes has benefited from significant transcriptome and genome [39] datasets that facilitate comparative studies between life-cycle stages and species, as well as proteomics investigations to identify putative drug or vaccine

targets [40–42]. For the paramphistomes, only a single transcriptome assembly for adult *P. cervi* has been published, but the assembled contigs have not been made publically available [31]. Similarly, proteomic data for paramphistomes are sparse. Due to the lack of transcriptome/genome datasets most studies so far have simply described the proteome profile of excretory/secretory or somatic proteins (visualised by SDS-PAGE) and identified antigenic components by Western blot using sera from paramphistome infected animals [38,43–45]. These studies have focussed on tropical paramphistome species and, to date, there are no published studies on the proteome of *C. daubneyi* found in Europe.

New developments and discoveries

The renewed interest in paramphistomes in temperate climates has sparked several new studies and publications in the last few years. Since Gordon *et al.* [20] proposed *C. daubneyi* as the primary rumen fluke species infecting livestock in Britain in 2013, a number of studies have been conducted in the UK and Ireland, all of which have confirmed this finding using molecular approaches to species identification [46–48]. Studies across Western Europe, in the Netherlands [49], Belgium [50], Italy [51] and Spain [17] have also confirmed, using molecular tools, the predominant rumen fluke species identified in livestock to be *C. daubneyi*. A study in Ireland [18] confirmed a high prevalence of *C. daubneyi* in Irish sheep flocks, but also identified infection with another rumen fluke species, *Paramphistomum leydeni*, in a single animal. One case of *P. leydeni* infection in cattle has also been reported in the Netherlands [49]. This suggests that occasional infection from ruminant wildlife reservoir hosts, where *P. leydeni* has been identified previously [52], may also occur in livestock.

Looking for possible interaction between endemic *F. hepatica* and *C. daubneyi* on Welsh farms, Jones *et al.* [24] reported a significant negative correlation between infection

levels based on FECs for each parasite; lower egg counts were seen for *C. daubneyi* in animals with a high FEC result for *F. hepatica*, and *vice versa*. Furthermore, it has now been confirmed that *C. daubneyi* and *F. hepatica* share the same key host snail; *Galba truncatula* [26]. Studies of the interactions between these two species at the intermediate host level will be important to inform our understanding of the dynamics of trematode infection and possible co-infection within *G. truncatula* populations, and to better understand the ecology and epidemiology that underpin the risk factors for livestock infections [24]. It appears that successful co-infections (where cercariae and infective metacercarial cysts are produced) between these two species within an individual intermediate host are normally not seen under field conditions, even when both *C. daubneyi* and *F. hepatica* are present in the same snail population [53,54]. Successful co-infections producing metacercariae have been demonstrated in the laboratory [55,56], and reported from a small number of naturally infected lymnaeid snails in France [56]. However, co-infection prevalence in the snail population included in this study was <1%, further indicating the rarity of such co-infections. Transmission of the rumen fluke *P. leydeni* within wildlife, and occasionally livestock ruminant populations, is thought likely to occur via a snail other than *G. truncatula* [49]. These findings raise intriguing questions regarding the epidemiology of, and potential interaction between, liver fluke and rumen flukes in their common environment and shared host range. Such interactions may have significant implications for parasite control.

Recent studies have provided a valuable first look at the host immune response to chronic paramphistome infection. Histological examination demonstrated localised tissue damage and infiltration of inflammatory cell populations (mainly CD3+ T cells) at the site of attachment of mature rumen fluke to the ruminal papillae [12,57]. Cytokine analysis suggested a localised Th1 immune response with upregulation of IFNγ and IL-10 in response to paramphistome infection in the rumen [57]. This evidence of an active immune response

during chronic paramphistomosis is interesting in light of the supposed 'well-tolerated' status of mature rumen fluke by the host [48]. Whilst this tolerance is widely referred to, it is unclear what this really means for the host and any mechanism actively used by rumen fluke to promote this has not been characterised. Whilst the limited penetration by the adult parasites into host tissue will contribute to this, it is also likely that rumen flukes, like other helminth parasites, secrete immunomodulators to promote their survival in the mammalian host [58–61]. Alternatively, given the rich diversity of commensal gut microbes within the rumen fluid, these parasites may benefit from microbiome-induced immune modulation [62] but further research is required to investigate any such interactions. There is some evidence that the host immune response may confer a degree of protection against subsequent paramphistome infections [4,6], but it is not yet clear how long-lasting any effect may be, and if this is sufficient to prevent clinical disease upon repeat infections in the same or future grazing seasons.

Whilst production effects caused by tropical paramphistome species have been reported [1,13], it is not yet clear if sub-clinical disease, specifically due to chronic *C. daubneyi* infection, has a significant impact on animal productivity or welfare in temperate areas. Although limited by a small sample size (n=6), an abattoir study by Bellet *et al.* [47] did suggest a negative correlation between rumen fluke infection and cold carcass weights and fat coverage at slaughter in British cattle. Malrait *et al.* [50] also identified an association between rumen fluke infection and poor faecal consistency scores in Belgian cattle.

Based on FEC reduction tests in cattle, oxyclozanide was shown to be the most effective existing flukicide (compared with albendazole, netobimin and closantel) against *C. daubneyi* infection [35]. Closantel displayed a slightly lower efficacy against mature *C. daubneyi* but in other studies this drug was ineffective against paramphistomes [33,50]. To date, the efficacy of different anthelmintics against immature *C. daubneyi*, which are

responsible for most pathology in infected animals, has yet to be investigated. Conducting drug efficacy tests *in vivo* against immature paramphistomes is challenging, owing to the current lack of a diagnostic test for pre-patent infections or a suitable animal model for experimental infection. Some efforts towards diagnostic development have been made recently: Anuracpreeda *et al.* developed a diagnostic sandwich ELISA, based on detection of a 16 kDa protein from a *Paramphistomum gracile* whole worm extract [37,63,64]. This offers hope for the development of antibody-based diagnostic tools (e.g. for serum, bulk milk and saliva) for *C. daubneyi* in the future. Perhaps most desirable would be the development of a coproantigen test similar to that available for *F. hepatica* [65]. These tests are not invasive, and are able to identify current infections by direct detection of parasite antigens. This is a major advantage over traditional serology based diagnostics which detect specific host antibodies and therefore cannot discriminate between active infections and previous host exposure [65].

Research priorities: Playing catch-up

Rumen fluke have been much neglected in recent parasitological research efforts. However, there is now a pressing need to re-examine these parasites in order to understand how they interact with, and impact on, their ruminant hosts in the temperate climates and intensive farming practices of Western Europe (see Outstanding Questions). The success of much of this basic research ultimately hinges on the generation of large scale "omics" datasets for paramphistomes in general, but particularly for *C. daubneyi*. Transcriptomic and proteomic studies of *C. daubneyi* should reveal host-exposed molecules that are secreted by the parasite or presented on the tegumental surface. As seen with other trematodes [40,42], many of these molecules are likely to be antigenic and represent important diagnostic or vaccine candidates.

It is now recognised that many helminths also export a variety of protein and RNA molecules, including potential diagnostic/vaccine targets, packaged within extracellular vesicles [58,66–69]. Their characterisation in *C. daubneyi* may provide a further source of diagnostic antigens whilst revealing mechanisms of interaction between rumen flukes and their ruminant hosts.

Further in-depth studies of these parasites within their intermediate snail host are also needed. These efforts may reveal opportunities to introduce parasite control measures to prevent transmission via the intermediate host [54], and inform understanding of epidemiological factors impacting lifecycle stages outside the definitive host. Knowledge of the dynamics of trematode infections within their intermediate hosts may further aid the identification of risk factors for both *F. hepatica* and *C. daubneyi* infections [24,53] and allow risk prediction models to be developed.

Because clinical paramphistomosis is invariably associated with burdens of immature parasites in the small intestine [7,10], it is imperative to study these juvenile and migratory life-cycle stages as well as the adult flukes that are more easily recovered from the rumen. Comparative proteomics studies may reveal how the antigenic profile of the parasite changes as it matures, and identify virulence factors involved in pathogenicity. Similar approaches, in other helminth species, have identified key molecules involved in parasite feeding/infectivity (such as the cathepsin L family of *F. hepatica* [70]) that are now being validated as targets for parasite control using RNA interference [71,72]. Understanding how *C. daubneyi* establishes infection within the small intestine, and how it feeds as it migrates along the GIT, may also present similar opportunities for rumen fluke control.

Characterising the host immune response as infection progresses from an acute to chronic stage will inform both vaccine and diagnostic development. For example, if *C.*

daubneyi secreted antigens are developmentally expressed, it may be possible to develop a diagnostic method capable of discriminating between acute and chronic stage infections [73,74]. Studying how the host immune system responds to infection will shed light on any immunomodulatory mechanisms employed by *C. daubneyi* and how these may impact on the ability of the host to respond to concurrent bacterial infections or mount an effective immune response against vaccine antigens. Thus, establishing suitable animal models of infection to study *C. daubneyi* immunology is a priority.

Given the foreseeable length of time and research effort required to develop a vaccine against *C. daubneyi* infections, the identification of effective anthelmintic treatments is critical. The current lack of a widely-licenced drug product for paramphistomosis, and the reliance on a single compound, oxyclozanide (often used off-licence), is far from desirable. With widespread drug resistance now affecting the control of a large range of parasitic and infectious diseases [75,76], reliance on a single drug compound raises the risk of untreatable isolates emerging, and is never good policy for sustainable parasite control. Future research may focus on the identification of novel compounds with efficacy against both juvenile and mature rumen fluke (or repositioning of existing drugs) and the selection and validation of new drug targets, using *in vitro* assays to assess anthelmintic efficacy against measurements such as fluke motility [77] or egg viability [78].

With paramphistomosis an established threat to sustainable agriculture in the tropics and sub-tropics [1,79], questions surrounding the exact sub-clinical, production or animal welfare impacts of chronic paramphistome infections in temperate climates are yet to be satisfactorily addressed. Comprehensive studies are now needed to establish the impact and importance of *C. daubneyi* infections within the ruminant livestock systems of Western Europe, in order to inform veterinarians and livestock producers of appropriate control

measures which may be necessary, or to alleviate concerns surrounding the emergence of this relatively unknown infection.

Concluding remarks

Based on recent survey work (Table 1), rumen fluke should now be considered an emerging parasitic infection of ruminant livestock in Europe. There are a number of significant challenges for the agricultural industries in the coming decades, including maintaining sustainable farming practices in the face of climate change and the need to feed an ever-growing world population. It will be essential to understand and manage all factors which may impact on livestock productivity and farming efficiency in order to overcome these hurdles. With paramphistomosis an emerging factor in European ruminant production systems, there is a clear need to quantify both production losses and the clinical threat from rumen fluke infection as well as to develop new diagnostic tools and therapeutic options. To gain a comprehensive understanding of this parasitic infection, large scale production trials, along with the development of 'omics' datasets, are urgently needed. Given the expansive data already available for other helminths, which pose a threat to livestock, a substantial effort is now required to bring our knowledge of paramphistome infections up to par.

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Figure 1: Life-cycle of the rumen fluke, *Calicophoron daubneyi*

Unembryonated eggs pass from the rumen, through the gastrointestinal tract and onto grazing pasture in the host's faeces (1). Eggs embryonate under favourable environmental conditions (adequate temperature and moisture) (2) and miracidia hatch from the egg and locate a suitable snail intermediate host (typically *Galba truncatula*) (3). Within the snail host (4), parasites reproduce asexually and develop through 3 larval stages (4a Sporocysts, 4b Rediae, 4c Cercariae), before the free-swimming cercariae are released from the snail and encyst on vegetation (5). Metacercariae remain dormant, resistant to moderate environmental changes, until they are ingested by the definitive ruminant host (6). Parasites then excyst in the duodenum of the small intestine (7) where immature flukes feed on sub-mucosa until they are ready to begin their migration to the rumen. In the rumen, mature fluke attach firmly to the rumen wall, or rumen papillae, via their muscular acetabulum, and release eggs into the rumen contents (8).

Figure 2: Images of rumen tissue recovered from a naturally infected cow at slaughter

The area marked 'A' shows an example of the raised nodules seen where parasites attach to the rumen wall. 'B' shows an area of papillae atrophy, commonly seen alongside rumen fluke infection. P, normal papilla.

515 **Table 1: Reported prevalence of rumen fluke across Western Europe**

516

Area	Host	Method[a]	Prevalence	Reference
France	Goat	FEC	58.1%	[34]
France	Cattle	PM	20%	[23]
Belgium	Cattle	PM	28%	[50]
		FEC	22%	
Ireland	Sheep	PM	14%	[80]
	Cattle	PM	52%	
Spain	Cattle	FEC	36%	[22]
Spain	Cattle	PM	6.2%	[17]
Wales	Sheep	FEC	42%	[24]
	Cattle	FEC	59%	
Ireland	Cattle	FEC[b]	57-100%	[48]
Ireland	Sheep	FEC	77%	[18]
England	Cattle	PM	25%	[47]

517 [a]Detected prevalence levels are detailed, along with the methods used to diagnose the
518 infections. FEC, faecal egg counts (farm level); PM, post mortem identification in the
519 slaughterhouse; [b], herd level.

520

Glossary

- **Omics:** Generally, the suffix '-omics' is used to describe large-scale studies which analyse the interactions of biological processes and specific molecule types within a particular cell, tissue type or organism, including (but not limited to) genes (genomics), transcripts (transcriptomics), proteins (proteomics) and small metabolites (metabolomics). Omics technologies encompass a range of tools and research platforms which enable a whole systems approach to studying molecular biology, and the analysis of omics data relies heavily on bioinformatics.

- **Digenean:** A subclass of organisms within the phylum Platyhelminthes and Class Trematoda, the digenea are obligate parasites with complex lifecycles, involving multiple hosts. Most commonly a molluscan first and vertebrate definitive host are required, with many species having 2^{nd} and 3^{rd} intermediate hosts also. Digenea are characterised by their syncytial tegument and usually two suckers – one oral, leading to the gut, and one muscular, which is blind-ended. Typically digenean parasites are hermaphroditic, with the Schistosomes a notable exception.

- **Paramphistome:** digenetic trematodes from the family Paramphistomidae that reside in the rumen of their definitive hosts, typically sheep and cattle. The term paramphistome refers to their two-mouthed appearance, with an anterior oral sucker and posterior muscular sucker which is used for attachment to the host.

- **Rumen:** The rumen is the second of the four stomach chambers described in ruminant mammals, although the term is commonly used to refer to the single organ formed by both the reticulum (first chamber) and rumen. The primary role of this chamber is the microbial degradation of ingested plant materials and for the uptake of volatile fatty acids produced by microbial fermentation.

Trends

- Helminth parasites impact hugely on livestock productivity by affecting growth rates, fertility, meat quality, wool or milk production, and sometimes cause mortality.
- Levels of infection with the rumen fluke, *Calicophoron daubneyi*, have increased dramatically in the UK and Ireland in recent years and may exceed that of liver fluke (*Fasciola hepatica*) in some areas.
- There are a growing number of reports of animal morbidity and mortality associated with acute paramphistomosis.
- Current diagnostic and treatment options are very limited, and improving these will depend on answering basic questions about rumen fluke biology.

Outstanding questions

- What is the impact of chronic *C. daubneyi* infection on animal health, welfare and production?
- How do immature *C. daubneyi* contribute to the pathology of infected animals?
- What is the mechanism of feeding used by immature and mature *C. daubneyi* in the small intestine and rumen respectively?
- Adult *C. daubneyi* are often said to be "well tolerated" by their hosts. What does this mean for the host and what (if any) parasite-derived molecules drive it?
- Can we identify new drugs or drug targets to help control rumen fluke infection?
- Since pre-patent infections are the primary cause of clinical paramphistomosis, can we identify antigens specific to early stage rumen fluke infection, to enable timely diagnosis and treatment?
- What immune response does the host mount against rumen fluke and is this amenable to vaccine design?
- What is the outcome of potential competition between *C. daubneyi* and *F. hepatica* within an intermediate snail host population? What impact does this have on transmission to the definitive ruminant host?

577 **Figure 1**

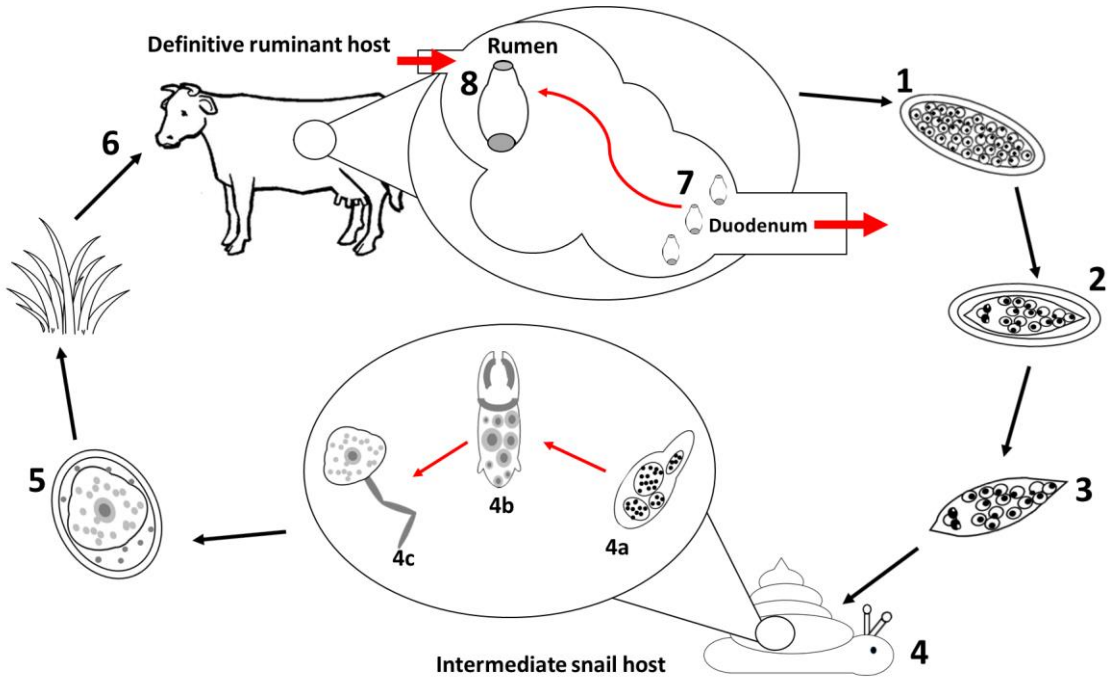

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Figure 2