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LIFE CYCLE, HOST UTILIZATION, AND ECOLOGICAL FITTING FOR INVASIVE LANCET LIVER FLUKE, *DICROCOELIUM DENDRITICUM*, EMERGING IN SOUTHERN ALBERTA, CANADA

Bradley J. van Paridon, John S. Gilleard*, Douglas D. Colwell†, and Cameron P. Goater

Department of Biological Sciences, University of Lethbridge, Lethbridge, Alberta, Canada T1K 3M4. Correspondence should be sent to Bradley J. van Paridon at: *bradley.vanparidon@uleth.ca*

ABSTRACT: The expansion of parasite distributions outside of their native host and geographical ranges has occurred repeatedly over evolutionary time. Contemporary examples include emerging infectious diseases (EIDs), many of which pose threats to human, domestic animal, and wildlife populations. Theory predicts that parasites with complex life cycles will be rare as EIDs due to constraints imposed by host specialization at each life-cycle stage. In contrast to predictions of this theory, we report 2 new intermediate hosts in the 3-host life cycle of the liver fluke *Dicrocoelium dendriticum* in Cypress Hills Provincial Park, Alberta, Canada. Results of sequence analysis of the cytochrome oxidase 1 (*cox1*) mitochondrial gene identified the terrestrial snail *Oreohelix subrudis* and the ant *Formica aserva* as first and second intermediate hosts, respectively, in the region. Neither of these intermediate hosts, nor their suite of domestic and wild mammalian grazers used in the life cycle, occurs within the native range of *D. dendriticum* in Europe. Our results from host surveys show that the prevalence of *D. dendriticum* in samples of *O. subrudis* varied between 4% and 10%, whereas mean metacercariae intensity in *F. aserva* varied between 33 and 41 ($n = 163$, mean $\pm$ SD $= 38 \pm 35$). These results are the first to describe the complete life cycle of emerging lancet fluke in western North America. The process of multi-level ecological fitting, in which the lancet fluke possesses pre-existing traits to utilize host resources, rather than host species, at each life-cycle stage provides a mechanism for the establishment of this complex life cycle in a novel habitat and in novel hosts.

Emerging infectious diseases (EIDs) are pathogens and parasites that are currently undergoing geographical or host-species range extensions, or are currently increasing their patterns of occurrence within their host populations (reviews by Daszak et al., 2000; Tompkins et al., 2015). There is general consensus among ecologists, parasitologists, and epidemiologists that parasite and pathogen range extensions have increased over the past several decades due to factors such as climate change, wildlife encroachment, and the anthropogenic movement of livestock and wildlife (Daszak et al., 2000). Emerging parasites can pose risks to human, domestic animal, and wildlife populations. To understand and mitigate these risks within regions of emergence, an understanding of patterns of hosts-species utilization at each life-cycle stage is a fundamental requirement. In the absence of this key life-cycle information, predictions regarding the potential for further expansion into new areas and into new hosts are exceedingly difficult to make.

Most instances of EIDs involve microparasites such as viruses, protozoa, and bacteria. Cleaveland et al. (2001) noted that of the more than 200 species of emerging pathogens or parasites reported from humans and domestic animals, only 6% to 7% were macroparasites such as helminths. These authors reasoned that helminths are unlikely to emerge, in part, because they often have complex life cycles that require specific host species at each level. For example, emergence of a typical trematode with an obligate 3-host life cycle should be rare because, conceivably, transmission would require establishment into new molluscan first intermediate hosts, new second intermediate hosts, and new definitive hosts. If we hold to the traditional viewpoint that host-parasite associations are intimate, highly specialized interactions between 2 coevolving partners, then the host-switching events required for establishment would presumably be rare.

Yet many parasites that have complex life cycles have expanded their geographic and host-species distributions over evolutionary and ecological time scales (review by Hoberg and Brooks, 2015). Contemporary examples include the cestode *Echinococcus granulosis* that emerged in Australia from Europe (Thompson et al., 2010), the trematode *Schistosoma mansoni* that emerged from its homeland in Asia and Africa into the South American continent (Crellen et al., 2016), the emergence of the North American lung fluke *Haematolechus floedae* in leopard frogs in Costa Rica (Brooks et al., 2006), and the emergence of the liver fluke *Fascioloides magna* into Europe from western North America (Malcicka et al., 2015). In each of these cases, establishment in new hosts was not constrained by the time required for intimate coevolutionary interactions to occur within each life-cycle stage. This is not to say that coevolutionary interactions are not important in the development of novel host-parasite associations, only that they are not required for their initial establishment.

Ecological fitting can explain the rapid establishment of parasites within new geographical locations and new hosts (Araujo et al., 2015). First invoked in the context of free-living organisms, Janzen (1980) proposed that certain defense traits of plants could retain their effectiveness against attacks by new species of insects, even if the plants no longer co-occurred with the insects that selected for the defense. In the case of parasites, host shifts can occur rapidly within new hosts if parasites track resources that are similar to those in an ancestral host, and not necessarily a particular species. The probability that host shifts will occur will increase if the resource being tracked is widespread,

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* Department of Comparative Biology and Experimental Medicine, Faculty of Veterinary Medicine, University of Calgary, Alberta, Canada T2N 4Z6.

† Agriculture and Agri-Food Canada, Lethbridge Research Station, Lethbridge, Alberta, Canada T1J 4B1.

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or if a parasite is phenotypically plastic in its ability to utilize particular resources. For complex life-cycle parasites, shifts into new hosts can therefore occur via ecological fitting if larval and adult stages track ancestral resources when they become available.

Of the 4 known species of trematode in the genus *Dicrocoelium* (Trematoda: Dicrocoeliidae), the lancet fluke *Dicrocoelium dendriticum* has the widest geographical and host species range (review by Manga-Gonzalez et al., 2001). The obligate 3-host life cycle of *D. dendriticum* involving terrestrial snails as first intermediate host and Formicid ants as second intermediate host was first elucidated by Krull and Mapes (1952) for snails, ants, and grazing mammals in the northeastern United States. Since this initial life-cycle description, evidence from field surveys has indicated that putative *D. dendriticum* sporocysts are present in a wide range of land snails (Pulmonata, Stylommatophora), whereas metacercariae and adults have been reported from numerous species of Formicid ant (Manga-Gonzalez et al., 2001), and domestic and wild mammalian grazers (Otranto and Traversa, 2002), respectively.

The lancet fluke has extended its geographical and host-species range beyond its native range in Central Europe. However, little is known regarding its life cycle, epidemiology, or patterns of host utilization within regions of emergence. Krull and colleagues reported adults from sheep and white-tailed deer collected from pastures in central and northern New York State in the 1950s, reasoning that the worms originated from the importation of domestic sheep from central Europe (Mapes, 1951). Evidence regarding its subsequent spread within the continent has been limited to sporadic reports of adult flukes in livers of sheep, cattle, and wildlife from western North America (Lewis, 1974; Pybus, 1990). A survey of livers from >100 wild ungulates collected from western Canada in the 1990s showed that the prevalence of *D. dendriticum* was <1% (Pybus, 1990). The small numbers of infected animals reported in the latter study were found in samples of grazing mammals collected within Cypress Hills Interprovincial Park (CHP), in southeastern Alberta, Canada. Within this region, prevalence in final hosts increased to approximately 80% by the mid-1990s, with intensities frequently exceeding 1,000 worms/host (Goater and Colwell, 2007; Beck et al., 2014). This combination of results indicates that the introduction of *D. dendriticum* into CHP occurred prior to the 1980s, persisted within the region for an unknown period of time, then emerged over the last 20–30 yr. The life cycle of *D. dendriticum* at sites in North America is not known, other than at the sites originally studied by Krull and Mapes (1952) in New York State.

Here our focus is on the first description of the complete life cycle of this emerging parasite to establish proof of principle that the requirements for ecological fitting exist within these host communities. First, we use molecular barcodes of parasite DNA to verify the occurrence of larval *D. dendriticum* in snail and ant intermediate hosts in CHP. We also analyze host DNA barcodes to identify the species of snail and ant that are utilized in this site of emergence in North America. Last, we report the results of field surveys of samples of snail and ant intermediate hosts on general patterns of host utilization within this region.

MATERIALS AND METHODS

Cypress Hills Interprovincial Park (CHP)

CHP is situated on the southern Canadian plains (49°30′N, 110°W) covering an area of approximately 2,590 km^2 (Newsome and Dix, 1968). The hills have a maximum elevation of 1,420 m and comprise a plateau of pre-glacial bedrock that rises 430 m above the surrounding prairie (Kulig, 1996). The hills were surrounded by glaciers twice during the Quaternary period, but the plateau remained unglaciated (Westgate, 1968). Highland forest and grassland communities found in CHP are most similar to those of the Rocky Mountains located 300 km to the west and of the aspen parklands characteristic of the central regions of Canada's 3 Prairie Provinces (Newsome and Dix, 1968).

Forested areas comprise roughly 20% of the landscape of the hills, while 70% is grassland and 10% mixed or aquatic (Newsome and Dix, 1968). Tree species found in CHP are predominantly *Pinus contorta* (lodgepole pine) and *Populus tremuloides* (trembling aspen) followed by *Picea glauca* (white spruce) and *Populus balsamifera* (balsam poplar), which are common but less abundant, with *Betula papyrifera* (white birch) and *Acer negundo* (Manitoba maple) being rare (Newsome and Dix, 1968). Grassland communities are dominated by *Festuca campestris* (rough fescue) and other grasses that characterize the mixed-grass natural sub-region (Downing and Pettapiece, 2006). Potential definitive hosts that graze within CHP include elk (*Cervus elaphus*), mule deer (*Odocoileus hemionus*), white-tailed deer (*Odocoileus virginianus*), moose (*Alces alces*), and beef cattle (Hegel et al., 2009).

Sampling and host surveys

Our sampling efforts for potential snail and ant intermediate hosts focused on a 50 × 50 m site in CHP (49°39′30.2″N, 110°15′40.1″W) where results from our previous surveys indicated infected ants and terrestrial snails were common. In a field survey of >100 randomly selected sites within CHP, Beck (2015) showed that ants at this site were consistently found clinging to vegetation, a behavior indicative of infection with metacercariae, between 2009 and 2013.

Samples of clinging ants and snails were collected on the same day each month between June and September 2013. Samples of the unknown *Oreohelix* spp. present at the site were collected by hand by walking the perimeter of the plot and collecting the first 50 snails encountered and placing them in 90% ethanol. These samples of snails were returned to the laboratory, dissected, and the presence of sporocysts and/or cercaria confirmed under a dissecting microscope. A sample of tissue and adjacent sporocysts/cercariae from a subset of the total infected samples was kept for use in molecular analysis to confirm the identity of the parasite as *D. dendriticum* as well as to identify the unknown species of *Oreohelix*. Concurrently, clinging ants were sampled by entering the plot each month and collecting the first 50 ants that were observed clinging to the vegetation. Ants were fixed in 90% ethanol, after which they were returned to the laboratory and evaluated for metacercariae counts under a dissecting microscope. An additional 5 clinging ants were collected during the June sampling period for DNA extraction of metacercariae and host tissue.

Statistics

A 2-tailed Fisher's exact test was used to evaluate significant differences in prevalence of infected snails between months. Kruskal-Wallis tests were used to test for significant differences in mean metacercariae intensity of infected ants between months.

DNA lysate preparation, PCR, and sequencing

Individual, 5 mg sections of snail/parasite tissue from the subset of total infected snails were rinsed twice in distilled water for 5 min and then were placed in lysis buffer and Proteinase K (10 mg/ml, New England Biolabs, Ontario, Canada). Lysis buffer contained 50 mM KCL, 10 mM Tris (pH 8.3), 2.5 mM $MgCl_2$, 0.045% Nonidet p-40, 0.45% Tween-20, 0.01% gelatin, and dH_2O in 50 ml volumes. Samples were then lysed in 50 µl for 98 min at 60 C followed by 15 min at 94 C. Lysates were stored at –80 C. The abdomens from the 5 additional ants collected in the June sampling period were removed, placed in lysis buffer, and crushed with a sterile plastic pestle to increase lysis efficiency of the ant exoskeleton and parasite material, after which lysis was completed under the same conditions as the snail tissue.

Three separate PCR reactions were carried out: One to amplify a species-specific region of ITS-2 rDNA from *D. dendriticum* found in both hosts and then 2 additional reactions to amplify the cytochrome oxidase-1 (*cox1*) genes from either the snails or ants. The first reaction was carried out on both the snail and ant lysates using previously validated species-specific primers DD_ITS2_SPEC (Bazsalovicsova et al., 2010) that amplify a 176 bp section of ITS-2 ribosomal DNA from *D. dendriticum*. Reactions were carried out in 25 µl volumes containing 1× Thermopol reaction buffer (New England Biolabs), 2 mM $MgSO_4$, 100 µM dNTPs, 0.1 µM forward primer and reverse primers, and 1.25 U Taq DNA polymerase at 5,000 U/ml (New England Biolabs). All reactions were carried out alongside negative and positive controls containing no DNA or DNA from adult *D. dendriticum* collected from cattle in CHP and prepared in the same manner. Thermocycler conditions were 95 C for 5 min followed by 35 cycles of 95 C for 30 sec, 56 C for 60 sec, and 72 C for 60 sec with a final extension of 72 C for 5 min. Products were visualized by electrophoresis using a 2% agarose gel stained with SYBER Green loading dye.

The second PCR was carried out on snail lysates only and used universal primers HCO2198 and LCO1490 to amplify a 710 bp region of the snail *cox1* genes (Folmer et al., 1994). Reactions were carried out in 25 µl volumes containing 1× Thermopol reaction buffer (New England Biolabs), 2 mM $MgSO_4$, 100 µM dNTPs, 0.1 µM forward primer and reverse primers, and 1.25 U Taq DNA polymerase at 5,000 U/ml (New England Biolabs). All reactions contained DNA negatives as control. Thermocycler conditions were 96 C for 2 min, 28 cycles of 96 C for 40 sec, 48 C for 20 sec, and 72 C for 60 sec with a final extension of 72 C for 7 min.

Finally, LepF1/R1 primers were used with the ant lysates only to amplify an 800 bp region of the ant cytochrome oxidase 1 gene (*cox1*) from the ant abdominal lysates (Hebert et al., 2004). Reactions were carried out in 25 µl volumes containing 1× Thermopol reaction buffer (New England Biolabs), 2 mM $MgSO_4$, 100 µM dNTPs, 0.1 µM forward primer and reverse primers, and 1.25 U Taq DNA polymerase at 5,000 U/ml (New England Biolabs). All reactions contained DNA negatives as control. Thermocycler conditions were 94 C for 2 min was followed by 5 cycles of 94 C for 40 sec, 45 C for 40 sec, and 72 C for 60 sec. This was followed by 36 cycles of 94 C for 40 sec, 58 C for 40 sec, and 72 C for 60 sec with a final extension of 72 C for 5 min.

All PCR products for both snail and ant *cox1* genes were visualized by electrophoresis using a 2% agarose gel stained with SYBER Green loading dye. PCR amplicons of the *cox1* genes of both snails and ants were purified using Omega BioTek Micro Elute Cycle Pure Kit (D6293-02) and sequenced on both strands using the same amplification primers on an Applied Biosystems 3730xl genetic analyzer (Applied Biosystems, Foster City, California) through the University of Calgary Core DNA Services Sequencing Lab (http://www.ucalgary.ca/dnalab/sequencing).

DNA sequence analyses

Chromatograms from both strands of the *cox1* gene amplicons from snails or ants were imported into Geneious software (Kearse et al., 2012) and the primer sequences and poor quality 5′ and 3′ ends removed. Forward and reverse sequences for each product were then assembled into a single contig and the resulting consensus sequences used for subsequent analyses. Individual sequences that showed 100% sequence similarity were collapsed into 1 representative haplotype. The haplotypes were used in a BLAST search (http://blast.ncbi.nlm.nih.gov/) to find closely related sequences in the GenBank database and confirm identification.

RESULTS

Molecular identification of *D. dendriticum* larval stages from snails and ants

All 5 snail lysates and all 5 ant lysates produced the 176 bp fragments of *D. dendriticum* ITS-2 rDNA using the DD_ITS2_SPEC (Bazsalovicsova, 2010) PCR primers. Positive controls of *D. dendriticum* DNA previously sequenced from adult worms collected from cattle in the Cypress Hills during pilot studies also showed the 176 bp fragment in each of these reactions.

Molecular identification of snail intermediate hosts

Five fragments of the snail *cox1* gene sequences were obtained and sequenced from the infected snails (Accession nos. KX923809–KX923813). Sequences were imported into Geneious (Kearse et al., 2012), which produced a 640 bp alignment of 5 sequences that each showed 100% sequence identity. These 5 sequences were collapsed into a single haplotype that was used in a nucleotide BLAST search of GenBank. The top hit was a *cox1* sequence from *Oreohelix* sp. B (DQ858129.1) reported by Weaver et al. (2006). Query coverage was 100%, with an E value of 0.0. Sequence identity between the 2 sequences was 99% with only a single nucleotide change from G/A at position 321.

Molecular identification of ant intermediate hosts

A total of 4 ant *cox1* gene sequences were obtained and sequenced from the infected ants; 1 individual failed to amplify (Accession no. KX923805–KX923808). Sequences were imported into Geneious (Kearse et al., 2012), which produced a 639 bp alignment of 4 sequences, all having 100% sequence identity. Results of the nucleotide BLAST search between this haplotype and the GenBank database produced a top hit with the *cox1* sequence from *Formica aserva* (KR924623.1). Query coverage was 100%, with an E score of 0.0. Sequence identity between the 2 sequences was 99%, with only a single nucleotide change of G/A at position 316.

TABLE I. Patterns of larval *Dicrocoelium dendriticum* infection in samples of the terrestrial snail *Oreohelix subrudis* and the ant *Formica aserva* in Cypress Hills Park, Alberta. Data are presented as prevalence in snails and mean intensity in ants.

Month	Snails (n)	Ants (n)	Prevalence in snails (±CI%)	Mean intensity in ants (±SD)
June	25	49	8 ± 0.11	41 ± 35
July	50	43	6 ± 0.06	39 ± 40
August	50	54	4 ± 0.04	33 ± 26
September	50	17	10 ± 0.08	38 ± 45
Total	175	163	7 ± 0.04	38 ± 35

Patterns of infection snails and ants

The prevalence of larval *D. dendriticum* within the total sample of 175 snails collected at the Ski Hill site in 2013 was 7 ± 0.04% (range = 4–10%; Table I). There were no significant differences in prevalence between monthly samples ($P = 0.677$). Metacercariae intensity was highly variable between individual ants collected at this site, ranging between 1 and 182 metacercariae per ant (overall mean = 38 ± 35; Table I). There were no significant differences in metacercariae intensity between monthly samples ($H = 1.533$, $P = 0.675$).

DISCUSSION

Species-level diagnosis of adult *D. dendriticum* is straightforward based upon standard morphological characters and their location within the bile ducts of grazing mammals. Identification of metacercariae is also straightforward based on morphological characteristics of the cyst and their location within the ant hemocoel and brain. In contrast, the identification of larval stages in terrestrial snails is challenging due to their morphological similarity to sympatric species of trematode. Our molecular-based diagnoses, based upon *D. dendriticum*–specific primers, confirmed that the sporocysts we recovered from snail *Oreohelix subrudis* and the metacercariae that we recovered from ants *Formica aserva* were *D. dendriticum*, and that these matched the adult worms we recovered from beef cattle and elk that had grazed previously within CHP. These results are the first to confirm the sequence of intermediate and definitive hosts used in the life cycle of this emerging parasite in western North America, and they indicate that possession of a complex life cycle, in this case involving 3 life-cycle stages, is not a barrier to establishment.

The distributions of at least 5 confirmed hosts of *D. dendriticum*—the snail *O. subrudis*, the ant *F. aserva*, and the ungulates, mule deer, white-tailed deer, and elk—are confined to North America. In the case of the former 3 species, their distributions are centered in the western regions of the continent (Pilsbury, 1939; Gregg, 1972). Thus, the establishment of *D. dendriticum* within western North America had to involve colonization from outside the region, followed by establishment within new species of host at each of the 3 main life-cycle stages. Presumably, failure to establish within any one of these life-cycle stages during the colonization process would lead to local extirpation within just a few generations. The implication is that following the introduction of *D. dendriticum* into the Cypress Hills region, it rapidly became established, and persisted, within snail, ant, and mammalian hosts.

Snails in the genus *Oreohelix* are in the Order Stylommatophora, a taxa that includes all other known snail hosts of *D. dendriticum* (Manga-Gonzalez et al., 2001). These snails are endemic to mountainous regions in western North America, from southern Alberta, the eastern Sierra Nevadas, to northern Mexico, and as far east as the Black Hills of South Dakota (Pilsbury, 1939). Taxonomically, the classification of species within the genus is unresolved and complex (Turgeon et al., 1998). Numerous sub-species and alterations to species designations have been proposed (Weaver et al., 2006). The species of *Oreohelix* that we collected showed high sequence similarity to "*Oreohelix* sp. B" collected from various sites in Wyoming and Montana (Weaver et al., 2006). This species was denoted *O. subrudis* by Chak (2007) from collections in Montana, Wyoming, and South Dakota based upon the morphology of the radula and reproductive structures, and also DNA sequence similarities using numerous nuclear markers. Ongoing studies in our laboratory involving several genetic markers have documented the occurrence of this species at several sites surrounding Elkwater Lake in the westernmost regions of CHP. Two other *Oreohelix* spp. occur within CHP, both of which also contain *D. dendriticum* sporocysts (Dempsey, Burg, and Goater, unpubl. data). These results suggest that several species of *Oreohelix* land snail are compatible hosts for *D. dendriticum* within this region. Beck (2015) surveyed several other genera of land snail collected from numerous sites in CHP, including the Ski Hill site, but none of these were found to contain sporocysts. It is premature to conclude that no other species of snail is compatible in this region, given that so many other species of snail have been documented as first intermediate host in Europe. Considering that the distribution of snails in the genus *Oreohelix* is confined to the Rocky Mountains in western North America, yet adult *D. dendriticum* have been reported in Ontario and as far east as Newfoundland (B. J. van Paridon, unpubl. obs.), non-oreohelid terrestrial snails must be utilized in the life cycle within other regions of establishment within North America.

Our results indicate that in this region of emergence, *D. dendriticum* utilizes the North American ant *Formica aserva* as second intermediate host. This result is consistent with the results of field surveys in Europe that report *D. dendriticum* metacercariae in more than 20 species of ant belonging to the family Formicidae, mainly within the genus *Formica* (Manga-Gonzalez et al. 2001). According to the most recent survey of ant biodiversity in Alberta, 39 species of the genus *Formica* are found in the province (Glasier et al., 2013). Ants of the genus *Formica* are widespread and locally common across the northern hemisphere (Wheeler, 1913). Since multiple species of Formicid ant are likely to be compatible intermediate hosts in North America, including in CHP, it is unlikely that establishment within second intermediate hosts is a barrier to emergence at other sites.

Our limited host survey results involving larval *D. dendriticum* in the snail *O. subrudis* and in the ant *F. aserva* are consistent with the highly variable patterns reported at sites in Europe, Russia, and several Middle Eastern countries. Manga-Gonzalez et al. (2001) showed that the prevalence of *D. dendriticum* in samples of several species of terrestrial snail varied between approximately 0.5% and 5%, with rare extremes that exceeded 50%. The prevalence of larval *D. dendriticum* in *O. subrudis* at the Ski Hill

site ranged between 4% and 10% and there was no evidence for seasonality between early summer and fall. Our survey results involving *F. aserva* sampled from the same site showed that 100% of ants found attached to vegetation harbored metacercariae in the hemocoel. Overall mean intensity (38 ± 35) was also highly variable, but it was consistent with reports involving samples of Formicid ants collected from sites in Europe (review by Manga-Ganzalez et al., 2001). Following the results of this study that confirm the complete life cycle of *D. dendriticum* in this region of emergence, more spatially and temporally targeted sampling procedures can be developed to better understand spatio-temporal patterns of infection in these intermediate hosts.

Evidence for host switching at each life cycle stage is consistent with the process of multiple-level ecological fitting (Aroujo et al., 2015). In another example involving a 3-host life cycle, the North American lung fluke *Haematolaechus floedae* has been reported from Costa Rican leopard frogs, local species of snails, and local species of odonates in regions where none of its native hosts have been reported (Brooks et al., 2006). Similarly, the liver fluke *Fascioloides magna* has become established in Europe from its homeland in western North America by utilizing a new species of snail as first intermediate host and a new definitive host (Malcicka et al., 2015). In these examples, rapid and simultaneous establishment within all life-cycle stage following range expansion is considered inconsistent with the requirement for intimate co-evolutionary interactions. Instead, host shifts were made possible into new hosts by certain pre-existing traits that were present at the time of colonization that enabled the recognition and utilization of ancestral resources. We do not know the nature of the pre-existing traits that may have been involved in the establishment of *D. dendriticum* within the sequence of new hosts in CHP, but these traits are likely to be associated with its ability, at each life-cycle stage, to utilize a wide range of host resources within their native ranges.

Our results show that intermediate hosts native to western North America are utilized by emerging *D. dendriticum*. If the process of multi-level ecological fitting is generalizable at other localities, then regions where the distributions of land snails, Formicid ants, and wild and/or mammalian grazers overlap have the potential to support the establishment of this life cycle. Given the sporadic reports of *D. dendriticum* in Canada (Lewis, 1974; Pybus, 1990) and new reports from Vancouver and Salt Spring Islands on the west coast as well as Ontario and Newfoundland (B. J. van Paridon, unpubl. data), this potential appears to be realized. We suggest that increased monitoring of livers from grazing mammals, as well as surveillance of snail and ant species for larval stages, will lead to the discovery of additional pockets of establishment and emergence.

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