AN INTRODUCED SNAIL AND SPREAD OF LIVER FLUKES IN OREGON

Sarah J. D'Atri

Advisors: Dr. Justin Sanders and Dr. Michael Kent

ABSTRACT

- Objective: Investigate the invasive snail *Lymnaea auricularia* as a potential intermediate host of *Fasciola hepatica* in Oregon
- Sample: 330 *Lymnaea auricularia* snails collected from a farm in Eugene, Oregon from May 2018 – August 2018
- Procedures: *L. auricularia* snails were plated in petri dishes, left overnight, and examined the next day under a dissecting scope for emergence of cercariae. Cercariae that were found were examined under a microscope for morphologic features consistent with *Fasciola hepatica*, photographed, and preserved in ethanol. Snails were then euthanized via freezing, homogenized, and analyzed for *Fasciola hepatica* DNA using PCR
- Results: *Fasciola hepatica* cercariae were not identified in *Lymnaea auricularia* snails with either microscopy or PCR.
- Conclusions and Clinical Relevance: This project did not identify *Lymnaea auricularia* as a carrier of *Fasciola hepatica* in Oregon. However, complications such as timing of cercarial shedding and presence of uninvestigated water sources on the farm may have precluded discovery of fluke cercariae in these snails. Further investigation of *L. auricularia* as a potential carrier of *Fasciola hepatica* will help address the broader goal of tracking intermediate hosts of *Fasciola hepatica* and enabling better management of Fascioliasis.

The liver fluke *Fasciola hepatica* has a complex life cycle involving a variety of different hosts. These include an aquatic snail as its first intermediate host, an aquatic plant as its second intermediate host, and a ruminant such as cattle as its definitive host. Tracking the intermediate

hosts of this fluke is of significant interest to cattle producers both due to its potential to cause disease and due to its economic impact.

Fasciola hepatica infections in cattle are typically characterized by a subclinical, chronic disease (Howell et al., 2015). Occasionally, formation of anaerobic tracts in the liver due to migration of *Fasciola hepatica* results in the proliferation of *Clostridium haemolyticum* (AKA *Clostridium novyi* type D) and subsequent development of acute bacillary hemoglobinuria (AKA red water disease) (Tagaki et al., 2016). However, the most common concern regarding *Fasciola hepatica* in cattle is its long-term economic impact. Migration of flukes through the liver creates significant hepatic damage, causing the liver to be condemned at slaughter. This can lead to significant economic strain, especially in smaller beef producers. For example, the USDA National Monthly Grass Fed Beef Report for November 2019 (https://www.ams.usda.gov/market-news/weekly-and-monthly-beef-reports) indicates that the average retail price of a grass fed beef liver is $7.00/lb; if one estimates that the average beef liver weighs up to about 15 pounds, this equates to a loss of over $100 per bovid. Given these considerations, it is clear that the study of *Fasciola hepatica*'s transmission amongst cattle herds is of significant interest to beef producers.

An understanding of *Fasciola hepatica*'s life cycle and seasonality of infection in different hosts is crucial to assessing its ability to spread amongst ruminant populations. *Fasciola hepatica* eggs typically begin to be shed in ruminant feces in the fall, and depending on climate conditions they may overwinter in the United States. After reaching water, the fluke's miracidium form hatches from the egg after about ten days depending on temperature. It then swims until encountering an

aquatic snail, which acts as its first intermediate host. The miracidium then transforms to sporocysts and redial forms, where it undergoes massive asexual reproduction and ultimately develops into a cercaria, which emerges from the snail after about one month (around May or June if the snail was infected in early spring). The cercaria swims to an aquatic plant, which is considered its second intermediate host, and encysts there as a metacercaria. A ruminant then eats the infected plant. After developing within the ruminant host, the adult *Fasciola hepatica* fluke resides within the animal's liver and bile ducts, where it lays eggs. These eggs are carried by bile into the intestines and are subsequently shed into the feces. Oregon has quite variable climates based on region (i.e. east of the Cascades versus coastal Oregon), and hence the seasonality of infection and development may vary between regions as well as in a given year. For example, *Fasciola hepatica* does not appear to survive over the winter in southern Idaho because mean monthly temperatures are consistently too low for activity of the snail intermediate host and fluke larval stages (it is estimated that mean temperatures of 10 degrees Celsius or higher are required for activity). This results in low transmission during the springtime but higher transmission in the fall, after warm summer temperatures have allowed snail and fluke activity (Hoover et al., 1984). A similar phenomenon was observed in Spain – harsh winters led to decreased overwintering of both *Fasciola hepatica* eggs and intermediate snail hosts, leading to minimal risk of infection the following spring (Pena et al., 1994). This indicates that regional climate as well as variation in weather from year to year may influence the timing of *F. hepatica*'s life cycle. Notably, the Eugene, OR region under study typically experiences winters with average temperatures below those required for *Fasciola hepatica* activity (average high of 7.6 degrees Celsius in December) (Rockey, 2018).

A key takeaway from *F. hepatica*'s life cycle is that the geographic distribution of the liver fluke depends on the geographic distribution of its intermediate hosts. *Fasciola hepatica* requires aquatic snails as first intermediate hosts; therefore, in order to infect the ruminants living in an area, the appropriate snails must be present. Knowledge of the snail species that can carry *Fasciola hepatica* is therefore integral to predicting where the liver fluke might cause disease. *Fasciola hepatica* is known to infect and develop in numerous species of aquatic snails such as *Lymnaea ovata, Lymnaea truncatula, Planorbis leucostoma*, and more (Dreyfus et al., 2002). Given this broad host range, it is likely that other snail species would be capable of carrying this parasite if given the opportunity.

The Oregon Veterinary Diagnostic Laboratory was contacted in 2018 by a small beef producer in the Eugene, OR area regarding recent outbreaks of *Fasciola hepatica* liver condemnations. The producer had noted the emergence of a new invasive snail species infesting his cattle's water trough around the same time frame as the fluke outbreaks, and he requested that Oregon State University investigate this snail as a potential source of *Fasciola hepatica* on his farm. The snail was identified as *Lymnaea auricularia*, which is documented as an endemic carrier of *Fasciola hepatica* in Europe and Asia. (Ngoc et al., 2015). However, *L. auricularia* is considered to be an invasive species in the United States and has not yet been documented as a host of *Fasciola hepatica* here. The cattle producer in Eugene suspected that the snail was brought onto his farm via aquatic plants that he had purchased from an overseas nursery. He had initially placed these plants into a pond on his property, then later moved the plants into the water trough in order to feed fish which he kept in the trough (the fish were kept in the trough to feed on any algal growth and thus keep the trough clean as well as control mosquitoes). Notably, this created a

microenvironment within the trough that was potentially capable of supporting *Fasciola hepatica*'s entire life cycle – an aquatic snail and aquatic plant were present to serve as intermediate hosts, and the producer reported that his cattle both drank from and defecated into the water trough. This means that if *Lymnaea auricularia* was capable of carrying *Fasciola hepatica*, the water trough could be the source of the producer's Fascioliasis. The following research project was therefore undertaken to determine if the invasive snail, *Lymnaea auricularia*, was a carrier of *Fasciola hepatica* on this farm.

MATERIALS AND METHODS

The research project was divided into three components: collection of *Lymnaea auricularia* snails from the Eugene farm, detection of cercariae emerging from the snails using dissection microscopes, identification of cercarial morphology using a compound microscope, and searching for *Fasciola hepatica* DNA in snails using PCR.

Lymnaea auricularia snails were gathered from the Eugene farm during four monthly trips, from May through August 2018 (May 14th, June 21st, July 12th, and August 16th). All *Lymnaea auricularia* snails were gathered from the cattle's primary water trough located on the center of the property. Every snail that could be visually identified within the water trough was retrieved. They were then transported along with water from the trough within portable coolers for the 1-hour drive to Oregon State University, where they were transferred to fish tanks containing plants from the farm as well as kale for food. During the first visit to the farm in May 2018, a pond located on the property to which the cattle had access was also investigated for *Lymnaea auricularia*. A small number of a different unidentified snail species were found, but no

Lymnaea auricularia. It was noted that poor accessibility to the pond may have hindered the snail search as only some of the outer edges of the water could be safely accessed, and snails may have been located farther towards the center of the water. A creek and a second water trough were also present on the property, located on a pasture that was farther away from the main farm. The farmer did not mention these water sources to the researchers until their last visit in August. At that time, the sources were searched for *Lymnaea auricularia*, but none were found. However, it was late in the season to be seeking *L. auricularia* and associated *F. hepatica* cercariae at that point.

Each collection of snails was plated multiple times in order to encourage emergence of parasite cercariae. Snails were placed in a 6-well Petri dish, with one snail and a small amount of water from the snails' tank per well. They were then allowed to sit overnight because it is thought that the stress of being plated and left in the well overnight causes cercariae to emerge. The next day, the Petri dishes were examined under a dissecting microscope to search for cercariae in the water within each well. When cercariae were found, they were transferred to a microscope slide and examined for morphologic features consistent with *Fasciola hepatica* using a compound microscope. They were then preserved in ethanol.

After being plated for cercariae, snails were humanely euthanized by placing each specimen in a tube and freezing them. Snail bodies were then removed from their shells and were individually homogenized using an immersion blender. Conventional PCR with *Fasciola hepatica*-specific primers was performed on individual aliquots extracted from the homogenized snail bodies. Water from the snails' tank was also analyzed with PCR to detect *Fasciola hepatica* that may

have been migrating between intermediate hosts. This was done in Dr. Sanders' laboratory using a protocol described by Le et al. (2012). This protocol utilizes a forward primer specific to *Fasciola hepatica* (FHF) as well as a reverse primer common to *Fasciola hepatica* and *Fasciola gigantica* (FHGR) to target mitochondrial genes including cox1, yielding an amplicon of 1,031 bp for *Fasciola hepatica*.

During the last visit to the farm in August, four bovine fecal samples were collected from the pasture for analysis. Sugar floats and sedimentations were performed on these samples in an effort to identify *Fasciola hepatica* eggs. PCR analysis for *Fasciola hepatica* was also performed on the fecal samples.

RESULTS

Table 1: L. auricularia screenings and number of snails shedding cercariae from a water trough at a cattle ranch near Eugene, Oregon

Collection Group	Date of Screening	# Snails Screened	# Snails Shedding
May	5/18/18	12	2
May	6/1/18	24	1
June	6/22/18	24	1
June	6/27/18	24	0
May	7/3/18	24	1
May	7/6/18	12	0
June	7/10/18	12	0
July	7/13/18	24	1

July	7/16/18	12	0
July	7/18/18	18	1
July	7/27/18	18	1
June	7/27/18	30	0
July	7/31/18	12	0
July	8/3/18	6	0
July	8/14/18	6	0
July	8/16/18	6	0
August	8/17/18	18	0
August	8/21/18	12	0
August	8/24/18	12	0
August	8/28/18	12	0
August	8/30/18	12	0

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155 *Table 2: Cercariae shedding totals from Lymnaea auricularia collected from water through a cattle ranch near*

156 *Eugene, Oregon*

Collection Group	# Snails Screened	# Snails Shedding
May	72	4
June	90	1
July	102	3
August	66	0
TOTAL	**330**	**8**

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Overall, 330 snails were screened during the research project, and of these, 8 were shedding cercariae (Table 1, 2). None of the cercariae observed were morphologically consistent with *F. hepatica* cercariae.

A photograph of a cercaria that was identified during the project is shown in the top image of Figure 1 and is morphologically similar to all cercariae that were recovered. This specimen is known as a xiphidiocercaria, which is characterized by having a stylet and lacking eye spots. The specimen's stylet is indicated with a red arrow in Figure 1. In contrast, the bottom image of Figure 1 shows a *Fasciola hepatica* cercaria, which does not feature a stylet. Thus, morphologic detection of a stylet within the cercariae eliminated *Fasciola hepatica* as a possible identity. The xiphidiocercariae that were found during the research project were not studied extensively because their stylet meant that they were not the specimen of interest, but brief morphologic identification efforts revealed that they may have *Paralecithodendrium* spp. or *Lecithodendrium* spp., which are parasites that are passed through aquatic insects as their second intermediate hosts and ultimately develop into adult worms in bats.

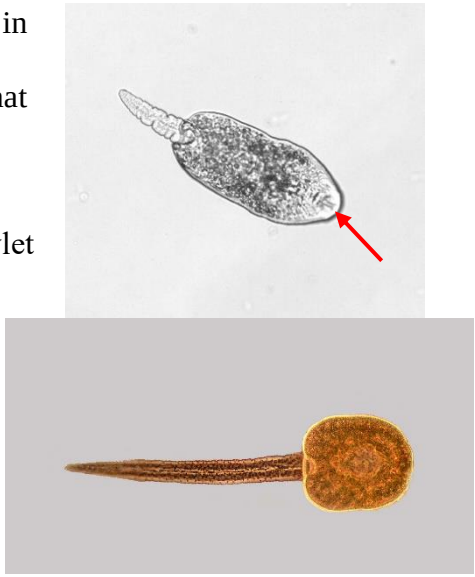

Figure 1: xiphidiocercaria (top) and Fasciola hepatica cercaria (bottom)

Efforts to detect *Fasciola hepatica* via PCR were also unsuccessful. Twelve *Lymnaea auricularia* specimens were chosen at random for DNA extraction and PCR analysis, and the results of all were negative. PCR performed on water from the snails' tank was inconclusive.

Sugar float and sedimentation of the bovine fecal samples yielded trichostrongyle-type eggs but no *Fasciola hepatica*, and PCR analysis of the feces was negative.

DISCUSSION

The relevance of tracking the ability of snail species to carry *Fasciola hepatica* should not be underestimated. *Fasciola hepatica* requires an aquatic snail as its first intermediate host; thus, the geographic spread of *Fasciola hepatica* mimics that of these gastropods. This means that managing the distribution of certain aquatic snails can help limit the spread of *Fasciola hepatica* within mammalian hosts. Similar efforts have yielded progress in the management of other snail-borne diseases. For example, efforts to control transmission of the parasitic trematode disease schistosomiasis in humans have involved research into the ecology of its snail intermediate host. This has enabled development of sophisticated predictive models of disease transmission based on the location and abundance of appropriate snails (Guarie et al., 2018). A thorough search of the available literature revealed that current information regarding the distribution of native *Fasciola hepatica* carriers in Oregon is disappointingly limited. Lymnaeid species including *Lymnaea columella*, *Lymnaea bulimoides*, *Lymnaea humilis*, and *Lymnaea palustris* have been documented as endemic snail species in Oregon that are capable of carrying *Fasciola hepatica* (Shaw, 1972). However, precise data regarding where in Oregon these snails are found is unavailable in the literature. This reveals an informational deficit that negatively impacts efforts to limit *Fasciola hepatica*'s spread. Better tracking of the snail species that can carry *Fasciola hepatica* would enable targeted efforts to isolate these species from ruminant populations and thus minimize contraction of liver flukes.

This was the overarching goal of documenting *Lymnaea auricularia* as a carrier of *Fasciola hepatica* in Oregon. If it had been determined that *L. auricularia* carries and thus spreads *Fasciola hepatica* in this state, this would have indicated a need to curtail invasive snail's spread within the region. It also would have emphasized the clinical relevance of preventing invasive species from becoming established in an area; these invasive species can facilitate spread of disease. However, while our project did not identify *Lymnaea auricularia* as a carrier of *Fasciola hepatica* in Oregon, a number of factors unrelated to the actual ability of the snail to serve as a carrier could have led to this result.

One possibility why *Fasciola hepatica* was not identified within *Lymnaea auricularia* specimens could have been that we simply were investigating the wrong water source on the Eugene farm. As was noted previously, a pond on the property to which the cattle had access could have been where the *Lymnaea auricularia* snails that possessed liver flukes were located. This is especially likely because the plants that are thought to have brought *Lymnaea auricularia* snails to the property were initially placed in the pond before being moved to the water trough. The pond was not surveyed to a reasonable extent because of difficulties accessing most of the pond and safety concerns, but a thorough search of the pond for *Lymnaea auricularia* snails may potentially reveal the source of the cattle's fluke infestations. Furthermore, the second water trough and creek on the upper pasture could also serve as a source of *Lymnaea auricularia* snails. These sources were not searched until August 2018. A search for the snails earlier in the year would be required in order to rule out the trough and creek as sources of *L. auricularia*.

Another possibility why we did not find *Fasciola hepatica* in the invasive snail is that there was an issue with seasonality of cercarial shedding. The timing of *Fasciola hepatica* cercarial emergence is highly dependent on climate conditions, including the availability of a moist environment and sufficiently warm temperatures over the winter to allow snail and fluke survival. We began searching for cercariae beginning in mid-May of 2018 and maintained the search through that August, but weather differences may have led to cercariae being shed at a different time of year. Notably, the spring of 2018 was particularly cold and wet in the Willamette Valley, which may have affected the timing of *Fasciola hepatica*'s life cycle. In the future we plan to search for *Fasciola hepatica* cercariae at variable times in the year in order to ensure that we catch the cercarial shedding window, whenever that may occur.

Issues with the timing of *Fasciola hepatica*'s life cycle are likely why no fluke eggs were recovered nor *Fasciola hepatica* DNA identified on the bovine fecal samples. These samples were collected during the last visit to the farm, which occurred in August of 2017. However, *Fasciola hepatica* eggs are not typically expected to be seen in host feces until about September. Furthermore, the cattle producer's herd contains animals who carry *Fasciola hepatica* because their livers continue to be condemned for flukes. As of August 2019, the producer had 19 out of 19 livers of cattle sent to slaughter condemned due to *Fasciola hepatica* migration. This is a clear indication that *Fasciola hepatica* is present in his herd, and addressing the issue via identification and control of the carrier snail is of immediate interest to the producer.

Notably, a very similar research project to ours was undertaken in Ecuador and was successful in identifying *Fasciola hepatica* within an invasive snail species. A paper by Caron et al. (2017)

details the researchers' efforts to document the invasive species *Galba schirazensis* as a natural carrier of *Fasciola hepatica*. *G. schirazensis* specimens were euthanized and then squished between two microscope slides and were examined at x10 magnification to identify larval forms of *Fasciola hepatica* within the snails' tissues. The snails were then homogenized using a pellet mixer, pooled into groups, and analyzed for liver fluke DNA using PCR. Using these methods, the researchers were able to identify a prevalence of *Fasciola hepatica* within *Galba schirazensis* at a rate of 6%. This indicates that the overall premise of our research methods (i.e. light microscopy followed by PCR) is unlikely to have played a role in our failure to identify *Fasciola hepatica*, although subtle differences in the two projects could be investigated as means of improving our chances for success. For example, investigating entire snail bodies for visual evidence of *Fasciola hepatica* larvae rather than encouraging cercariae to emerge may be a more sensitive microscopic identification method.

Given both the Eugene cattle producer's pressing *Fasciola hepatica* problem and the broader goal of effectively managing the spread of liver flukes, continuing our investigation of *Lymnaea auricularia* as a potential carrier of *Fasicola hepatica* is clearly of interest. Future efforts should target the pond, second water trough, and creek on the Eugene farm, and snails should be collected at variable times throughout the year to ensure that the cercarial emergence window is not missed. Understanding the role of invasive aquatic snails in the life cycle of *F. hepatica* will allow more effective management of *Fasciola hepatica*'s intermediate hosts to reduce liver fluke parasitism in Oregon cattle.

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