Development and use of a nucleic acid-based assay to determine the natural and experimental prevalence of Fasciola hepatica in lymnaeid intermediate hosts
by Kristi Leigh Dimke

A thesis submitted in partial fulfillment of the requirements for the degree Of Master of Science in Veterinary Molecular Biology
Montana State University


Abstract:
Fasciola hepatica is an increasing problem for livestock producers in Montana and elsewhere. A thorough understanding of the interaction of the fluke with its intermediate snail host is necessary to implement pest control measures and reduce risk to livestock. To this end a sensitive, specific nucleic acid based assay was developed which detects fluke material in snails immediately after they become infected and throughout the course of infection in the snail. The assay involves isolating and purifying RNA from snails, making cDNA using reverse transcriptase, and using a fluke specific primer to amplify a fluke sequence on the small ribosomal subunit. After separation, by gel electrophoresis and Southern blotting, an end-labeled fluke specific probe is used to detect the fluke sequence. The assay was then used to determine the natural prevalence of F. hepatica infection in an enzootic area in Montana over a three year period. One Lymnaea modicella snail was found naturally infected during the study in the month of August, supporting the hypothesis that disease transmission occurs in the fall in this region of the U.S., however, the presence of fluke-positive snails at a neighboring thermal spring indicate that disease transmission may occur over a longer season at these areas. Experimental infections of several species of lymnaeid snails reveal that L. bulimoides and L. modicella are the most important intermediate host snails in Montana. Although all other species tested acquire the infection in large numbers, most do not routinely carry the infection to patency; in fact, a large percentage overcome the infection by 5 days post exposure; which may suggest the presence of a molluscan internal defense mechanism.

# DEVELOPMENT AND USE OF A NUCLEIC ACID-BASED ASSAY TO DETERMINE THE NATURAL AND EXPERIMENTAL PREVALENCE OF *FASCIOLA HEPATICA* IN LYMNAEID INTERMEDIATE HOSTS

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A thesis submitted in partial fulfillment
of the requirements for the degree

of

Master of Science

in

Veterinary Molecular Biology

MONTANA STATE UNIVERSITY-BOZEMAN
Bozeman, Montana

September 1996

APPROVAL

of a thesis submitted by

Kristi Leigh Dimke

This thesis has been read by each member of the thesis committee and has been found to be satisfactory regarding content, English usage, format, citations, bibliographic style, and consistency, and is ready for submission to the College of Graduate Studies.

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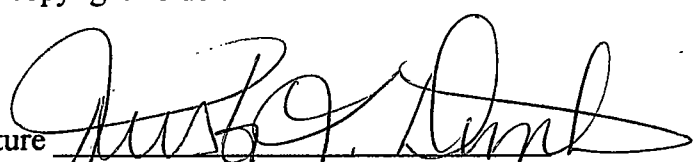
Signature 

Date 9/16/96

# ACKNOWLEDGEMENTS

I would like to thank Dr. Stuart E. Knapp for his guidance throughout my graduate career, for his sense of humor, and for his commitment to my personal and professional growth. I would also like to thank the members of my committee, Dr. Larry Jackson, Dr. C. A. Speer, and Dr. Michael White for helping me to bring my project into focus. Lastly, thanks to all those in the department who have encouraged me and assisted me in ways too numerous to mention, but in particular to Matthew Rognlie and Robert Potts, without whom research would have been tedious indeed.

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# ABSTRACT

Fasciola hepatica is an increasing problem for livestock producers in Montana and elsewhere. A thorough understanding of the interaction of the fluke with its intermediate snail host is necessary to implement pest control measures and reduce risk to livestock. To this end a sensitive, specific nucleic acid based assay was developed which detects fluke material in snails immediately after they become infected and throughout the course of infection in the snail. The assay involves isolating and purifying RNA from snails, making cDNA using reverse transcriptase, and using a fluke specific primer to amplify a fluke sequence on the small ribosomal subunit. After separation by gel electrophoresis and Southern blotting, an end-labeled fluke specific probe is used to detect the fluke sequence. The assay was then used to determine the natural prevalence of F. hepatica infection in an enzootic area in Montana over a three year period. One Lymnaea modicella snail was found naturally infected during the study in the month of August, supporting the hypothesis that disease transmission occurs in the fall in this region of the U.S., however, the presence of fluke-positive snails at a neighboring thermal spring indicate that disease transmission may occur over a longer season at these areas. Experimental infections of several species of lymnaeid snails reveal that L. bulimoides and L. modicella are the most important intermediate host snails in Montana. Although all other species tested acquire the infection in large numbers, most do not routinely carry the infection to patency; in fact, a large percentage overcome the infection by 5 days post exposure; which may suggest the presence of a molluscan internal defense mechanism.

# INTRODUCTION

## Historical and Economic Significance

Fasciola hepatica, the common liver fluke, is a parasite of worldwide importance, affecting cattle and sheep most commonly, but also goats, non-domestic animals, and humans. These digenetic trematodes are acquired by the definitive host by ingestion of the infective stage, the metacercaria, which is encysted on some type of vegetation. Excystment occurs in the digestive tract of the host, and immature flukes penetrate through the wall of the small intestine, find their way to the liver and penetrate the liver capsule. For the next six weeks the flukes migrate through the liver and finally come to rest in the bile ducts where they begin to produce eggs. The eggs, which are liberated in the feces of the infected animal, hatch in two weeks under ideal circumstances and the free-swimming miracidia must then find a suitable snail intermediate host (snail of the genus Lymnaea) before their energy reserve is exhausted. When a suitable lymnaeid snail is encountered, the miracidia penetrate into the snail and form sporocysts, which then give rise asexually to many rediae. These rediae give rise to tailed cercariae which leave the snail to encyst on vegetation and begin the cycle anew. The disease, fasciolosis, takes a chronic course in the bovine host, causing production loss and condemnation of the liver at slaughter, while in the ovine host the disease is often acute, resulting in death if not treated promptly. Since the original description of the fluke by Jean de Brie in 1379, (Taylor, 1964), records show that epidemics have plagued Europe regularly, wiping out

millions of sheep. The disease has now spread to nearly every continent, and there is potential for disease transmission wherever infected animals come into close proximity with an appropriate snail vector (Knapp et al., 1992). Worldwide losses due to fasciolosis are estimated at 2 billion dollars annually, and the economic impact will continue to be felt as the growing world population puts additional demands on agriculture to produce more high quality food products, such as animal protein (Boray, 1994). Additionally, in recent times fasciolosis has been recognized as an important world human health problem, especially in France and South American countries like Bolivia, where it has been reported that 40% of the population has been exposed (Hillyer, 1994).

In the United States, the disease is present in the West-Coast and Rocky Mountain states, and the Southwestern, Midwestern, and Gulf-Coast states. Krull (1934) reported that there were suitable snail vectors present in every state in the U.S., and concluded that the spread of disease to every state was possible. In addition, the incidence of the disease has been rising, from a widely accepted figure of about 5% reported in the early 1970's (Foreyt and Todd, 1976) to 17-19% in the 1990's, with some areas reporting almost 50% prevalence in slaughter cattle (Knapp et al., 1992, Briskey et al., 1994). Since the U.S. is a major world food producer, this disease is likely to have increasing economic impact in the future.

With half a million sheep and over 2.4 million cattle, Montana is one state likely to feel a heavy economic impact from this disease. A survey done in 1992 by Knapp et al. reported the incidence of disease among Montana slaughter cattle to be 17.24%, up from 5% in 1976 (Foreyt and Todd, 1976). A survey of lymnaeid snail distribution in Montana

done between 1990 and 1992 found that the snails are present in at least 28 of the 56 counties in Montana, and so the spread of the disease to these 28 counties is a distinct possibility (Dunkel et al., 1996).

## Conventional Methods of Control

Previously, chemical control methods including treatment of the definitive host with anthelmintics and eradication of the intermediate host with molluscicides were heavily relied upon to keep fasciolosis in check. Hexachloroethane and carbon tetrachloride were widely used as anthelmintics until the 1970's, when concern over the buildup of carcinogenic agents in meat intended for human consumption led to restrictions on their use (Malone et al., 1982). Anthelmintics in use today which are effective against mature flukes include the salicylanilides (rafoxanide, closantel), halogenated phenols (nitrooxynil, bithionol), and benzimidazoles (triclabendazole, albendazole, mebendazole) (Boray, 1994). The only anthelmintic which is highly effective against both mature and immature flukes is Clorsulon. In some areas reports indicate that flukes are becoming resistant to these agents, and treatment with two different anthelmintics is recommended (Boray, 1994). Unfortunately, the prospects for development of new anthelmintics are limited (Boray, 1994), necessitating the judicious use of the ones available. Several molluscicides, including copper sulfate, niclosamide, and n-trityl morpholine (Frescon) have been widely used to control the snail intermediate host and therefore the spread of the disease (Chroustova and Willometzer, 1974, Lam et al., 1989, and Pantelouris, 1965). These

efforts have been fairly successful, especially when combined with anthelmintic treatment of the definitive host (Chroustova and Willometzer, 1974). It has been suggested, however, that the risk of resistance developing in the target species is present whenever control involves long term use of pesticide, and indeed, increasing resistance to molluscicides has been reported among some trematode intermediate host snail species (Lam et al., 1989). In addition, environmental regulations have ended the use of molluscicides in the U.S., and other countries are reevaluating the risks associated with their continued use. Recently, the isolation from plants of natural products with molluscicidal properties has received attention, however, this research is still in the early stages (Singh et al., 1993, and Appleton et al., 1992).

Other options for control of the snail intermediate host include drainage of snail habitat and introduction of snail predators and competitor snails. With regard to drainage of snail habitat, snails are capable of aestivating for at least a year (Kendall, 1950), and so may seem to disappear only to appear again with the return of moisture. For this reason, drainage of snail habitat is impractical as a means of controlling snail populations, especially on farmland where irrigation is necessary. Most of the research done on the use of snail predators and competitor snails has been done to try to control the snail intermediate hosts of Schistosoma mansoni and other medically important trematodes. For schistosome snails, several potential competitor/predator organisms have been investigated, including North American crayfish, sciomyzid flies, and several species of snails including Melanoides tuberculata and Heliosoma spp. (Hofkin et al., 1991, Appleton et al., 1993, and Kruatrachue and Upatham, 1993,). Efforts to find a similar means of

control for F. hepatica intermediate hosts have not been as promising. The remarkable reproductive potential and hermaphroditic nature of these snails makes their eradication virtually impossible.

In the future, we need to be increasingly broad-based and innovative about developing control measures for fasciolosis. In his 1993 address to the American Society of Parasitologists, K. Darwin Murrell outlined an integrated pest control plan, which included the strategic use of anthelmintics based on an epidemiological understanding of parasite transmission, enhancement of host resistance (either the intermediate or definitive host) through breeding or vaccines, biological control, and the implementation of management practices designed to reduce the risk of parasite transmission (Murrell, 1994). All of these options necessitate a thorough understanding of parasite transmission, in which the snail intermediate host plays a pivotal role. Indeed, any of the above mentioned control measures could be applied to the intermediate host. It is therefore necessary to give attention to identifying the intermediate host snails in given areas, factors which influence their life cycle, and factors which influence the snail-parasite interaction in order to develop new management and control practices.

## Seasonal Transmission Studies in the U.S.

To identify potential intermediate host snail species, high risk areas, and peak seasons for disease transmission, snail and seasonal transmission studies have been done in various parts of the U.S. Five species of lymnaeid snails have been identified in Oregon,

and Lymnaea bulimoides has been present in every fluke infested area (Shaw and Simms, 1929). Lymnaea bulimoides has been reported from Texas (Olsen, 1944), and a study done in 1971 by Wilson and Samson found L. bulimoides, L. palustris, and L. modicella present at the junction between Colorado, New Mexico and Arizona; where the infection rate among cattle and sheep was 30% and 49% respectively. In Louisiana, where the infection rate in cattle reaches 80%, L. bulimoides is the principal lymnaeid snail present (Lindsay, 1979). Further study in Louisiana has shown that disease transmission occurs primarily in spring and early summer, and furthermore, that the risk of disease transmission can be predicted based on the numbers of L. bulimoides, which can be correlated to microclimatic conditions (Malone et al., 1982 and Malone et al., 1984). Disease transmission studies conducted in Florida using tracer sheep indicate that transmission peaks in February and April, ceases in the summer, and resumes again in November, December and January (Boyce and Courtney, 1990). In Oklahoma, which was not thought to have any incidence of indigenous infections, the presence of lymnaeid snails was confirmed, as were several cases of fasciolosis in native cattle, indicating the occurrence of disease transmission in the state (Cheriuyot and Jordan, 1990). In Western Montana, where up to 90% incidence of liver fluke infection has been reported in slaughter cattle (Marley et al., 1994), L. modicella is the predominant intermediate host snail species present (Dunkel et al., 1996).

## Studies of Snail-Parasite Interaction

The last piece of the life cycle of F. hepatica to be elucidated was that a snail is the intermediate host. This was confirmed in 1857 by Wagener who observed the penetration of the miracidium into the snail and its development into rediae (Taylor, 1964). That a lymnaeid snail is the intermediate host for F. hepatica was proven in 1882 by Rudolf Leuckart (Taylor, 1964) and independently by Sir Algernon Thomas, who found naturally infected L. truncatula and produced fasciolosis in rabbits with metacercariae derived from these snails (Thomas, 1883). Lymnaea truncatula, which is not present in the U.S., has since been shown to be extremely susceptible and is the main intermediate host in Europe, as natural infection rates among these snails are 100% in some areas (Ross and O'Hagan, 1968). In the U.S., the first reports of naturally infected snails were of L. bulimoides in 1929 in Oregon (Shaw and Simms, 1929), and in Louisiana (Sinitsin, 1933). Since then, several more have reported finding naturally infected L. bulimoides in Texas, Colorado, Washington, and Louisiana respectively (Olsen, 1944, Wilson and Samson, 1971, Lang, 1977, and Lindsay, 1979), however, with extremely low infection rates, averaging less than 0.5%. In 1977, Lang also reported finding naturally infected L. palustris, L. proxima, L. stagnalis, and L. modicella in Eastern Washington, although the infection rate was not given. At the end of a three year survey in Louisiana, Malone et al., (1984) reported finding two naturally infected snails, both of the genus Lymnaea. Due to the difficulty in finding naturally infected snails, experimental exposure of snails to miracidia of F. hepatica

has become the method of choice to study the snail parasite interaction and to identify possible intermediate host species. Additional possible intermediate host species identified by successful experimental infection include L. columella, L. modicella, and L. traskii (Krull, 1933a, 1933b, 1934); and L. montanensis (Rowan et al., 1966) and L. cubensis (Cruz-Reyes and Malek, 1987).

Other attempts at experimental infection of snails have been less successful. Kendall (1949) exposed 101 L. stagnalis to F. hepatica miracidia, but only 13 of those became infected. Boray (1966) experimentally infected L. stagnalis, L. palustris, L. peregra, L. auricularia, L. truncatula, L. tomentosa, and L. lessoni, however, only L. truncatula and L. tomentosa were susceptible as adults, the rest were only susceptible when immature. Lymnaea lessoni and L. auricularia never produced cercariae. Experimental exposures done by Wilson and Samson (1971) on L. modicella and L. palustris indicated that the snails were susceptible only when less than 30 days old, and Rowan et al., (1966) found the same to be true for L. montanensis. Foreyt and Todd (1978) exposed L. bulimoides, L. caperata, L. modicella, L. umbrosa, L. palustris, and L. stagnalis to F. hepatica miracidia but failed to infect any but L. bulimoides. Bouix-Busson et al., (1984) found that 70% of juvenile and 95% of adult L. glabra develop abortive infections when exposed to F. hepatica, whereas the normal host, L. truncatula, develops evolutive infections 80% of the time, whether juvenile or adult. These results have led to the study of differential susceptibility among lymnaeid snail species.

Although many studies have been done to identify the external stimuli which attract miracidia to their snail hosts; the observation that infection rates and outcomes are

age dependent as well as species dependent has led to the search for a snail internal defense mechanism which could be responsible for terminating the development of the parasite. To address this issue, Boray (1966) transplanted F. hepatica rediae into adult L. stagnalis to see whether they would continue to develop, and found that they were destroyed by a host tissue reaction. Similarly, histological examination of juvenile L. palustris exposed to F. hepatica showed that all sporocysts were removed by a cellular encapsulation response in less than 14 days (McReath et al., 1982). Further, lymnaeid snails have been shown to have phagocytic cells, termed hemocytes, circulating in their hemolymph which are capable of encapsulating and destroying a variety of foreign particles. It remains to be discovered why some species of lymnaeid snails are able to destroy invading F. hepatica sporocysts through a hemocyte mediated encapsulation response and others, while possessing morphologically similar hemocytes, are not.

Although the range and distribution of the disease and the potential snail intermediate hosts in Montana have been identified (Knapp et al., 1992 and Dunkel et al., 1996, respectively), there is a lack of information regarding these snails and factors affecting the snail-parasite interaction which in turn have a crucial impact on the seasonal transmission cycle and ultimate risk to Montana livestock. It is therefore important that the snail intermediate host species be positively identified and the intermediate host potential of all lymnaeid snail species present in Montana be assessed. In order to accomplish this, new methods must be developed to detect naturally infected snails and to conveniently assess the susceptibility characteristics of each.

Our work on fasciolosis began in 1989 with a Montana Agriculture Experiment Station project designed to investigate the epizootiology of fasciolosis in Montana livestock and to determine ways in which the disease might be controlled. The work contained in this thesis is the result of two of the objectives outlined in the proposal, the first of which was to develop a DNA probe whereby minute quantities of the liver fluke may be detected in the snail intermediate host and thereby permit early detection of infected snail populations; and second, to determine the identification and distribution of the snail intermediate hosts for F. hepatica in Montana.

# DEVELOPMENT AND TESTING OF A NUCLEIC ACID-BASED ASSAY TO DETECT FASCIOLA HEPATICA INFECTED INTERMEDIATE HOSTS

## Introduction

The development of a better method of detection of infected snails was a necessary first step in the study of the snail host and the snail parasite interaction. Several procedures are commonly used to determine if a snail is infected with F. hepatica. Collected snails are observed microscopically to determine whether they are discharging cercariae or crushed and examined microscopically to determine whether there are larval forms of the parasite present in the snail tissue. Unfortunately, only mature infections can be detected in this manner, sporocysts are not visible unless the snail is sectioned and stained. Examination of individual snails in this way is time consuming and impractical when large numbers of snails need to be evaluated.

Nucleic acid probes have gained widespread acceptance as useful field diagnostic tools. Probes have been developed for several organisms including Aeromonas salmonicida, Plasmodium berghei, and Tritrichomonas foetus (Barry et al., 1990; Waters and McCutchan, 1990, and Severson, 1991). The approach taken by our group was to make an oligonucleotide probe derived from a hypervariable region of ribosomal RNA sequence from Fasciola hepatica (Shubkin et al., 1992). Because rRNA is present in multiple copies in each cell and contains hypervariable regions which can be used to

differentiate genera and species (Dams et al., 1988), it was a logical target for development of a probe. The initial oligonucleotide probe developed was used to detect a fluke specific sequence in a northern blot of total snail RNA (Shubkin et al., 1992). Subsequently, other groups have developed nucleic acid probes which detect fasciolid specific repetitive DNA sequences in preparations of snail DNA (Heussler et al., 1993; Kaplan et al., 1995). All of these methods, however, require preparation and testing of each snail individually. Our goal was to increase the sensitivity of our procedure so that snails could be processed in large batches, enabling one person to screen several thousand snails in a short time.

The first method developed in our lab used an oligonucleotide probe derived from an rRNA sequence to detect the fluke sequence in a northern blot of total snail RNA (Shubkin et al., 1992). The oligonucleotide sequence used for the probe was obtained by amplification from total RNA extracted from whole liver flukes. The primers used in the reverse transcriptase reaction (RT) and polymerase chain reaction (PCR) were oligonucleotide primers which hybridize to conserved regions of small subunit rRNAs. A region of approximately 650 base pairs was amplified, subcloned and sequenced. The sequence was analyzed and a region unique to Fasciola hepatica was identified (Figure 1.1.).

This sequence (Figure 1.1.) was used to develop the probe for F. hepatica. Testing of this probe indicated that it was specific to F. hepatica and that it could detect fluke material in preparations of total snail RNA; however, five miracidia were required to give a positive result. Since greater sensitivity was believed necessary to detect fluke

material in batches of snails, a modified method was developed involving PCR amplification of the fluke sequence before probing.

```
Human 18S          AATGAGCAAT AACAGGTCTG TGATGCCCTT AGATGTCCG      1515
F. hepatica        .......... .......... .......... .........
L. columella       .......... .......... .......... .........

Human 18S          GGC-TGCACG CGCGCTACAC TGACTGGCTC AGCGTG-TG     1552
F. hepatica        ...CC..... T........A ....G.TT.. ....A.TT.
L. columella       ...--..... .......... ...AG.AA.. ......GA.

Human 18S          CCTACCCTAC GCCGGCAGGC   1572
F. hepatica        GAAT..TGG. CTGA.....T
L. columella       ...-....GG C...AA...G
```

Figure 1.1. Sequence alignment of partial DNA sequences for small subunit rRNA in human, Fasciola hepatica and Lymnaea columella. Dashes represent deletions and dots represent sequence identity. Numbering of the human sequence is included for reference (Shubkin, et al., 1992).

This second method used a different region of sequence (Figure 1.2.) for the probe (called CS 6) and included RT and PCR reactions to amplify the region of sequence containing the probe site (Rognlie et al., 1994). The primer (called CS 9) for first strand cDNA synthesis was designed to hybridize to a semiconserved region downstream of the probe hybridization site. The primer (called CS 11) used in the PCR reaction was designed to hybridize to a variable sequence region unique to the Fasciolidae so that only amplification of the fluke sequence would occur.

```
Fh ACTTAAAGAAATTGACGGAAGGGCACCACCAGGAGTGGAGCCTGC-GCTTAATTCGACTCAACACGGGAA
Fb ........G.....................................G.....................-....
Sm ........G.....................................G.T...................
Lc .............................................-........T.............

Fh AACTCACCCGGCCCGGACACTGTGAGGATTGACAGATTGATAGCTCTTTCTTGATTCGGTGGTTGGTGGT
Fb ............................................................
Sm ............................................................G.......
Lc ..........T..........A.....................................G.......

Fh GCATGGCCGTTCTTAGTTGGTGGAGCGATTTGTCTGGTTAATTCCGATAACGAACGAGACTCTGGCCTGC
Fb ..........................................................T......T........
Sm ..........................................................T.AA.....
Lc ..........................................................A....AT
                                                        CS11
Fh TAAATAGTATGCCTGT-CTCTGTGCTCGTGCAGGTTGCGTTGTCCATTGCCTCCTCGTGGGGTAGTGGTT
Fb ................C.................T.....................................
Sm .........GA.TG..C...........TT...GC...GCT..T.....T..T.TA...A......TGG
Lc ........TC...---------------------.C.GTGGT..T......GA.G...------------
   ----

Fh ACGTTGACCGGCGGGTGCGGCGCAGGTACTTACTTCTTAGAGGGACAAGCGGT---ATTCAGTCGCACGA
Fb ............AA............A.....................C---C.............
Sm T...GAT............T..CA..TT.....................CACAC..A..........
Lc ----------------------------AC.............G..T..C---G..T..C.A.....

Fh AATTGAGCAATAACAGGTCTGTGATGCCCTTAGATGTCCGGGGCCGCACGTGCGCTACAATGACGGTTTC
Fb ..A.......................................................................
Sm ..........................................A.....................GC.
Lc .........................................-......C........C...A..AA..
   CS6
Fh AGCGAGTTTGGAATCCTGGCCTGAGCAGGTTGGGTAAACTGTATCATAACCGTCGTGACTGGGATCGGGG
Fb ............................C.............................................
Sm .......C.....A.......C..AAG.......C.......T....C.....................
Lc ....T.GA..CCTC.......C..AAG..C....A..C.C..TGA..CT..T.....CTA.....T....
                                                        CS9
Fh CTTGCAATTGTTCCCCGTGAACGAGGAATTCCTGGTAAGTGCAAGTCATAAGCTAGCTTGCGCTGATTAC
Fb ..........................................................----..............
Sm .........A................................................----..............
Lc ....T...........A..............CA.....C..G.......----..............
   ----

Fh GTCCCTGCCCTTTGTACACACCGCCCGTCG
Fb ............................
Sm ............................
Lc ............................
```

Figure 1.2. Multiple sequence alignment of small-subunit rRNAs (ssrRNA). CS11 is the upstream PCR primer hybridization site. CS6 is the fasciolid-specific probe hybridization site. CS9 is the RT and second downstream PCR primer hybridization site. Actual CS6 and CS9 sequences are complementary to sequences shown. Dots indicate bases identical to F. hepatica and dashes indicate gaps inserted for optimal alignment. *Fh*, partial Fasciola hepatica ssrRNA sequence (GenBank accession L24039); *Fb*, Fasciolopsis buski ssrRNA sequence bases 1,249-1,830 (Blair and Barker, 1993); *Sm*, Schistosoma mansoni ssrRNA sequence bases 1,249-1,834 (Ali et al., 1991); *Lc*, partial Lymnaea columella ssrRNA sequence (GenBank accession L24040). (Rognlie et al., 1994).

To test a snail, total RNA is extracted, RT-PCR is performed using sequence specific primers, and the resulting DNA is electrophoresed on an agarose gel, transferred to a nylon membrane using the Southern blotting technique, hybridized to the end-labeled oligonucleotide probe, and visualized by autoradiography. Cross-reactivity tests performed using F. hepatica (two strains), Fascioloides magna, Sphaeridiotrema pseudoglobulus, L. columella, and unidentified monostome cercariae demonstrated that the probe would detect RNA from F. hepatica and F. magna, but would not cross-react with RNA from the snail host or the other trematodes tested. Analysis of other trematode sequences aligned with the fasciolid sequence indicated that the probe probably would not cross react outside the Fasciolidae.

At this point it was necessary to test the sensitivity of the assay and the ability of the assay to detect fluke material in the snail host at each stage of fluke development. In addition, a modification of the procedure was needed which would enable large numbers of snails to be processed at once. Following are the experiments which address these points.

## Materials and Methods

### Parasite Materials and Experimental Infections

Adult F. hepatica were recovered from livers of infected Montana slaughter cattle, frozen in liquid nitrogen, and stored at -70 C. Fasciola hepatica ova were obtained from gall bladders of infected Montana slaughter cattle and cleaned by washing through sieves (75 um/40 um). Ova were incubated in total darkness in aerated distilled water at 28 C for

14 days. Every 3 days during the incubation period the ova were cleaned by sieving. Incubated ova were stored in total darkness at 4 C until needed. Miracidia hatched when ova were exposed to light at room temperature. Lymnaea modicella snails collected near Dillon, MT and laboratory reared L. columella snails were individually exposed to 3-5 active miracidia in 24 well tissue culture plates and then kept at 25 C with a 12-hr light:dark cycle. Snails were then frozen in liquid nitrogen every day or every other day post exposure and stored at -70 C.

RNA Preparation

Total RNA was purified using the procedure of Chomczynski and Sacchi (1987) from tissues prepared as described by Liou and Matragoon (1992). Snails were crushed in a 2 ml microfuge tube containing an amount of RNAse-free sand equal in volume to one snail. A lysis solution was then added (4.0M guanidine thiocyanate, 0.5% sarkosyl [w/v], 24mM sodium citrate) containing 8% [v/v] 2-mercaptoethanol added just prior to use, and the tube was agitated using a vortex mixer to homogenize the tissue. Debris was sedimented by centrifugation at 4,000g and the supernatant was transferred to a new tube. When groups of snails were processed together, snails were ground in a liquid nitrogen cooled mortar and pestle and transferred to a 15 ml tube containing 2 ml RNAse-free sand and 10 ml lysis buffer. The tube was agitated vigorously and one 650 ul aliquot was removed for analysis. Purification of RNA was accomplished by phenol/chloroform extraction and isopropanol and ethanol precipitation (Chomzynski and Sacchi, 1987). Purified RNA pellets were dissolved in RNAse-free water and then quantified by spectrophotometry prior to cDNA synthesis and amplification.

RT-PCR and Southern Analysis

First-strand cDNA synthesis was completed using M-MLV reverse transcriptase-RNAse H minus at 10u/μl with 3 μg of the total RNA preparation and the following reaction components: 1.0 uM CS9 primer, 0.75 mM dNTPs, 7.0mM MgCl, 100μg/ml bovine serum albumin, 10mM dithiothreitol, 50mM Tris (pH 8.3), and 40mM KCl. First-strand cDNAs were amplified from the entire RT reaction using Taq DNA polymerase and CS9 and CS11 primers under recommended conditions (Perkin Elmer Cetus, Norwalk, Connecticut). The reactions were carried out in a thermal cycler using the following cycle profile: 94 C for 1.5 min, 70 C for 1 min, and 72 C for 1 min, for 26 cycles. Ten or fifteen microliters of the 100-μl PCR reactions were electrophoresed on a 1.2% agarose gel. The gel was then stained with ethidium bromide and a 123-bp DNA standard was used to confirm the size of the amplification fragments. The DNA was then transferred to a 0.45-μm charged nylon filter using the method of Southern (1975), and fixed in a UV-crosslinker. Oligonucleotides used for hybridization were end-labeled with [$\gamma$-$^{32}$P]ATP with T4 polynucleotide kinase and purified on an Elutip-D column. Hybridizations were carried out using standard procedures (Sambrook et al., 1989) with a 65 C hybridization temperature and 58 C final wash. Specific activity of labeled oligonucleotides was typically 5 x $10^8$ cpm/μg and were used in the hybridization solution at 1 x $10^6$ cpm/ml.

Experimental Design

In order to be useful, the assay would have to detect the presence of the parasite at all stages of its intramolluscan development. To test this, L. columella were exposed to 5-10 active F. hepatica miracidia and tested at 23 time points between 1 and 37 days post exposure. Total RNA was prepared from two snails for each time point. To determine the reliability of the assay, 19 L. columella snails were exposed to 3-5 active miracidia each and tested at 6 hours post-exposure (Figure 1.5.). Nineteen additional snails were not exposed and used as controls to determine if the assay produced false positives. To determine whether snails could be processed in batches, L. columella snails were exposed to 5-10 active miracidia for one hour and then prepared in groups with other unexposed L. columella according to the following design: 5 exposed with 15 unexposed, 2 exposed with 18 unexposed, 1 exposed with 19 unexposed (Figure 1.6.). Two replicates of each were done and several aliquots of each sample were tested.

## Results

The sensitivity of the assay was determined using ten-fold dilutions of purified adult F. hepatica RNA in the presence of 5 μg each of purified L. columella RNA added as background. On an ethidium bromide-stained agarose gel, the detection limit is one pg (Rognlie et al., 1994). By continuing with the Southern blot and probe hybridization the detection limit is increased 100-fold to 10 fg (Rognlie et al., 1994).

Results of experimental exposures of L. columella indicate that the infection is detectable one day after exposure and throughout the infection period (Figure 1.3.).

Field-collected L. modicella were also tested in a similar fashion on days 1 through 21 post-exposure with similar results (Figure 1.4.).

Results of the reliability test indicate that the assay does not produce false positive results, since all of the unexposed snails tested negative. Seventeen of the nineteen exposed snails tested positive. This experiment was repeated with the same results.

Results of the batch test indicate that one infected snail is easily detectable in a batch of 20 total snails. A similar experiment was performed using one exposed snail with 49 unexposed snails (data not shown), and a positive result was obtained. All snails used in these experiments were between 4 and 8 mm in length.

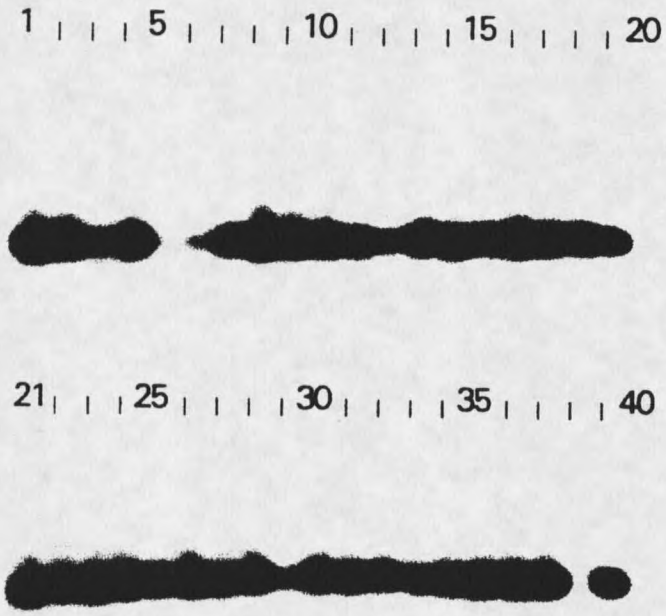


Figure 1.3. Ten microliters of a standard 100-ml RT-PCR reaction was separated on a 1.2% agarose gel and transferred to a nylon membrane. The blot was hybridized to CS 6 as described in the Materials and Methods and then exposed to x-ray film for 15 min at -70 C. Total RNA was prepared for use in the RT-PCR assay at various times after individual Lymnaea columella snails were exposed to 5-10 miracidia: 1 day (lanes 1,2), 2 days (lanes 3,4), 3 days (lanes 5,6), 4 days (lanes 7,8), 7 days (lanes 9,10), 8 days (lanes 11,12), 9 days (lane 13), 10 days (lanes 14,15), 11 days (lanes 16,17), 14 days (lanes 18,19), 15 days (lanes 21,22), 16 days (23,24), 17 days (lanes 25,26), 18 days (lanes 27,28), 21 days (lane 29), 22 days (lane 30), 23 days (lane 31), 24 days (lane 32), 25 days (lane 33), 29 days (lane 34), 30 days (lane 35), 36 days (lane 36), 37 days (lane 37). Lane 38: negative control with 5 μg snail RNA. Lane 39: positive control with 100 pg F. hepatica RNA. Lanes 20 and 40: DNA base pair standards. From Rognlie et al., (1994).

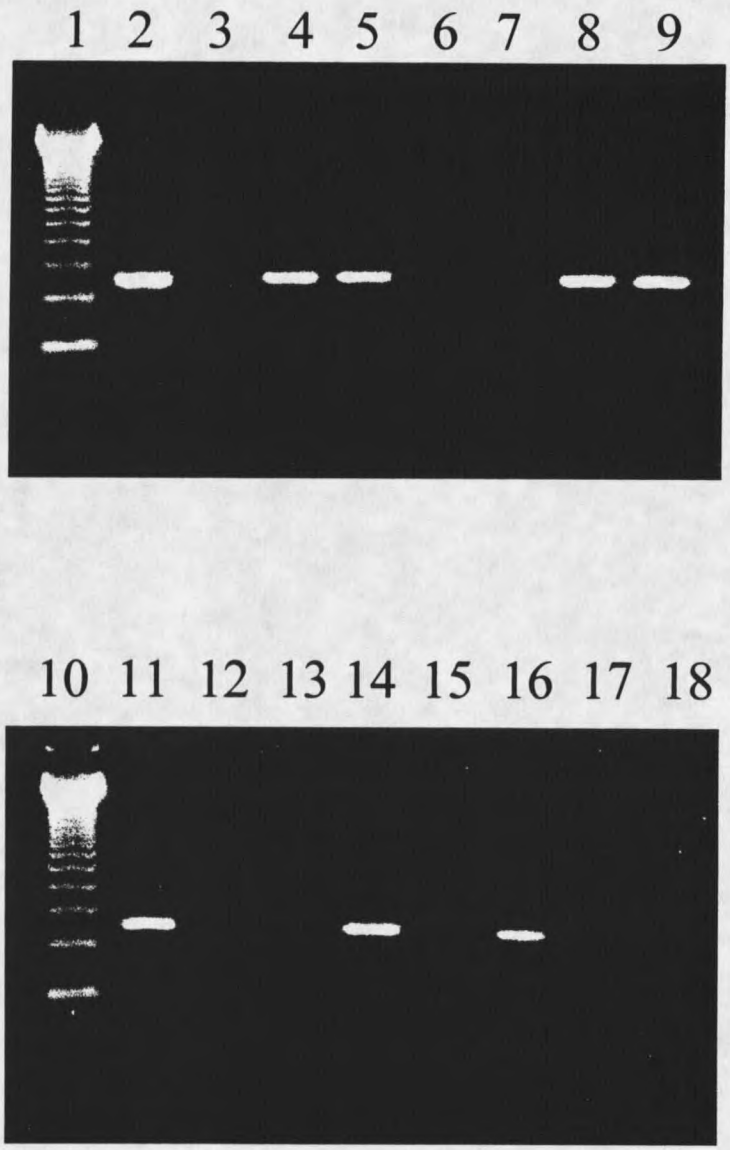


Figure 1.4. Ten microliters of a standard RT-PCR reaction was separated on a 1.2% agarose gel. Total RNA was prepared for use in the RT-PCR assay at various times after individual Lymnaea modicella snails were exposed to 5-10 miracidia. Top Row L to R: DNA standard, 100 pg fluke RNA (positive control), unexposed snail RNA (negative control), 1 day Post-Exposure (PE), 4 days PE, 5 days PE, 7 days PE, 8 days PE, 11 days PE. Bottom Row L to R: DNA standard, 13 days PE, 15 days PE, 18 days PE, 19 days PE, 21 days PE, 21 days PE.

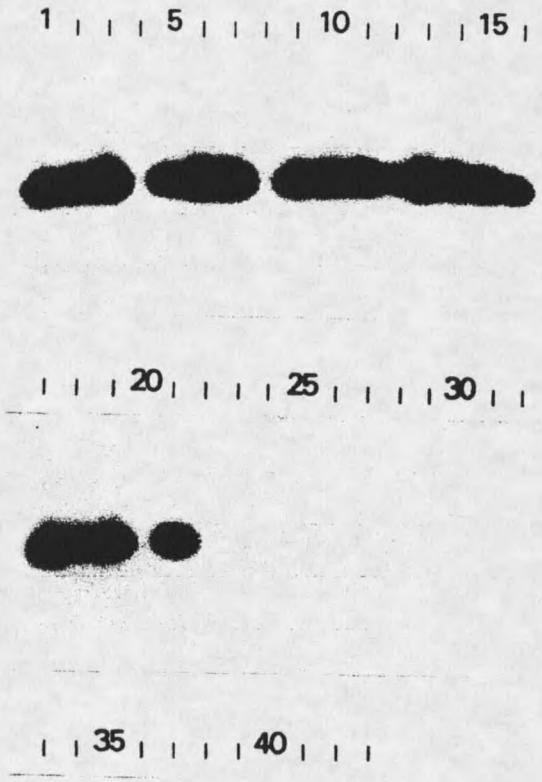


Figure 1.5. Ten microliters of a standard 100 ul RT-PCR reaction was separated on a 1.2% agarose gel and transferred to a nylon membrane. The blot was hybridized to CS 6 as described in the Materials and Methods and then exposed to x-ray film for 15 min at -70 C. Total RNA was prepared from L. columella snails exposed (lanes 1-7, 9-20) and unexposed (lanes 23-32, 34-37, 39-43) to F. hepatica miracidia. Snails were processed 6 hr post exposure. Lanes 8, 22, and 38: DNA base pair standards. Lane 21: positive control with 100 pg fluke RNA. Lane 33: negative control with 5 μg snail RNA.

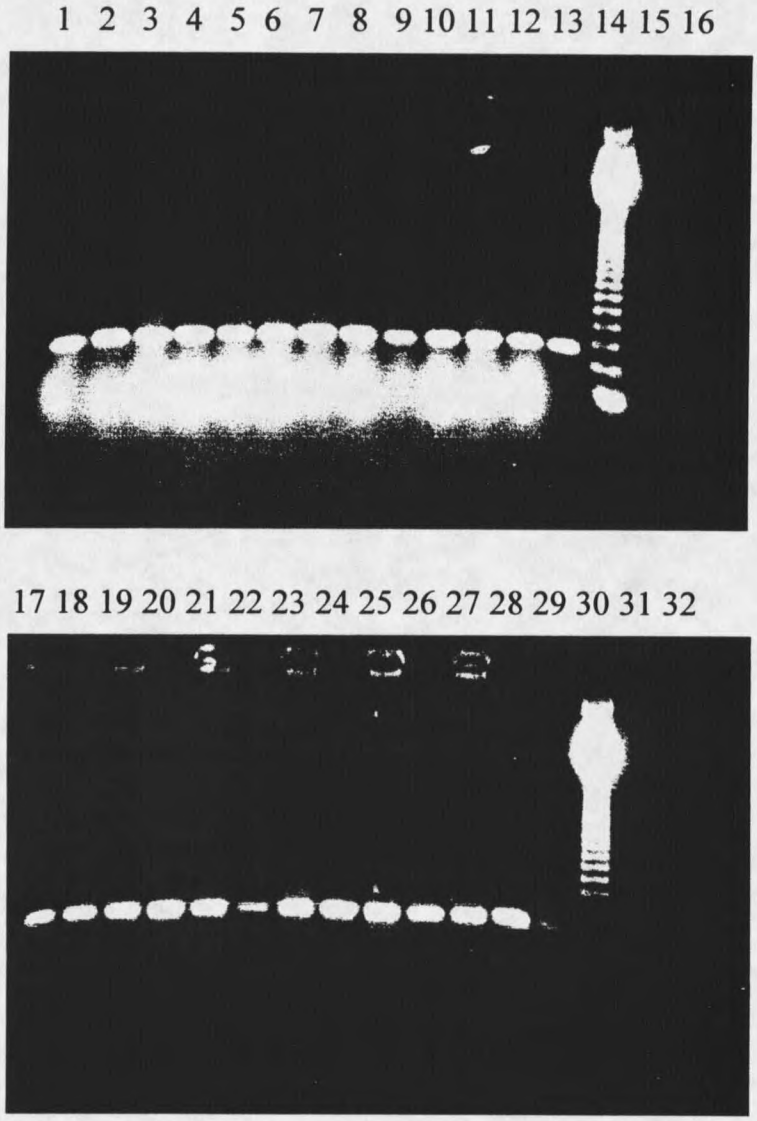


Figure 1.6. Fifteen microliters of a standard RT-PCR reaction was separated on a 1.2 % agarose gel and stained with ethidium bromide. RNA was prepared from 4 aliquots each of batch preparations containing Lymnaea columella snails exposed and unexposed to Fasciola hepatica. Lanes 1-4: 5 exposed and 15 unexposed, lanes 5-8: 5 exposed and 15 unexposed, lanes 9-12: 2 exposed and 18 unexposed, lane 13: 1 μg fluke RNA (positive control), lane 14: DNA standard, lane 15: blank, lane 16: 5 μg snail RNA (negative control), lanes 17-20, 2 exposed and 18 unexposed, lanes 21-24: 1 exposed and 19 unexposed, lanes 25-28: 1 exposed and 19 unexposed, lane 29: 1 μg fluke RNA (positive control),lane 30: DNA standard, lane 31: blank, lane 32: 5 μg snail RNA (negative control).

## Discussion

The reason for developing this assay was that a better method of detecting F. hepatica infections in snails was needed to study the parasite-snail interaction. The method of Shubkin et al., (1992) which used a nucleic acid probe to detect a fluke sequence on the small rRNA subunit has been improved by adding RT-PCR amplification of the fluke sequence before probing, giving much greater sensitivity. This RNA-based detection method is not as convenient for field use as the repetitive DNA squash blot technique of Heussler et al., (1993), since snails must be transported back to a fully equipped laboratory for assay, and processed or frozen in liquid nitrogen immediately after sacrifice to prevent degradation of the RNA. Although DNA is more stable and thus easier to work with in the field and in the lab, it is less abundant in cells than RNA; consequently, the RNA-based assay has greater potential for sensitivity than a DNA-based assay. The detection limit of the assay is 10 fg. Since the PCR primer allows only fasciolid-specific sequences to be amplified, results can be visualized on the agarose gel before Southern blotting and probe hybridization is performed, with a slight decrease in sensitivity. The detection limit at this point in the process is one pg, which has proven to be good enough for most applications, making this method even faster and more convenient. The reason for extending the sensitivity beyond detection of the minimum biologic unit (i.e. one miracidium) is to be able to process snails in large batches, where only a fraction of the total RNA would be used for RT-PCR. The batch approach has

been successful, since the assay yields a reliable, positive result when one exposed snail is prepared and tested with 19 unexposed snails. Preliminary testing using one exposed snail with 49 unexposed snails shows that the assay is sensitive enough to detect fluke material at this dilution, however one negative result was also obtained, and it remains to be seen whether this was a truly negative result (i.e. the snail was not harboring the parasite) or the assay is not reliable at this dilution. The ability to examine at least 20 snails in one batch will increase the number of field collected snails that can be assayed and thereby provide more accurate data regarding infection distribution and prevalence in the intermediate host. The main drawback of using the batch method to examine field collected snails is that there is no way to tell whether a positive result is due to only one or many infected snail(s). Although it is possible to quantitfy PCR products and relate this information to the amount of material originally present in the sample; this would not necessarily be useful, because it would be impossible to tell whether the amount of fluke material originally present was due to one heavily infected snail, for instance, or to multiple lightly infected snails. Since several reports have found the infection prevalence to be less than one percent,(Lindsay, 1979; Malone et al., 1984; Cruz-Reyes, 1987; and Olsen, 1944) a positive result in a batch of 20 to 50 snails is likely to be due to only one infected snail. If a situation arises where the infection prevalence among the intermediate host is sufficiently high that every batch is positive, the batch size can be scaled down accordingly, or a small number of snails can be tested individually to determine the optimal number of snails to prepare in each batch to yield approximately one infected snail per batch.

This new approach was tested for cross-reactivity against different trematode and snail RNA and it was found that there was no evidence of cross-reactivity outside the Fasciolidae (Rognlie et al., 1994). The assay does not distinguish between Fasciola hepatica and Fascioloides magna, however, an investigator should be able to determine which fluke is present in an area based on the proximity of the definitive host for each fluke and the species of intermediate host snail present, since F. hepatica and F. magna generally use different intermediate hosts within the Lymnaeidae (Foreyt and Todd, 1978). Both trematodes can, however, use L. columella (Krull, 1934) and L. bulimoides (Foreyt and Todd, 1978) as intermediate hosts, so that in areas where these snails are present with both flukes and their definitive hosts, further analysis must be done to determine which fluke species is being studied. To this end, the assay might be modified to distinguish between members of this family by including additional sequences in primer or probe hybridization sites. The advantage of the current method is that it is more versatile, since it can be used for experimental study of all members of the Fasciolidae.

By testing exposed and unexposed snails, it was determined that the assay does not produce false positives. Two of the exposed snails, however, tested negative (Figure 1.5.). Although the RT-PCR process involves many steps and failure could occur at any point, it is more likely that these two snails were truly negative for infection; since it is common to have a small percentage of L. columella snails which do not acquire the infection, even under ideal exposure conditions (Baldwin, pers. comm., 1995). Testing exposed snails every other day post exposure throughout intramolluscan development

confirms that the assay is capable of detecting the presence of the parasite in the snail immediately after exposure and at all stages of development. Since it has been shown in several parasite-snail systems that some snails have the ability to destroy an invading parasite, (Lie et al., 1987, Loker et al., 1982) the question arises whether the assay can distinguish between material from actively developing flukes and residual material from dead flukes. The advantage of using an assay which detects the presence of RNA is that RNA is highly susceptible to degradation, and once a parasite is killed its RNA would be rapidly degraded by endogenous RNases. It is reasonable to say that the assay would only detect RNA from a living fluke, however, the question of whether the infection will eventually reach patency cannot be answered without observation of cercarial release. It is possible that a snail could harbor the fluke in a state of arrested development for an extended period, however, careful experimental study of intermediate host species will reveal which are likely to carry the infection to patency and which are not.

This assay has several advantages over conventional methods in that it is faster, more sensitive, and can be used to study prepatent infections. One disadvantage is that the assay costs more per test than other assays such as the DNA squash blots developed by Heussler et al., (1993) and Kaplan et al., (1995) due to the enzymes used in the RT-PCR; however, as many as 50 snails can be prepared in one batch which saves reagents, labor, and time. Combined with existing knowledge of the snail intermediate host and logical experimental design, this assay will be a valuable tool which will greatly simplify the gathering of seasonal transmission data and allow in-depth experimental analysis of the parasite-snail interaction.

## Summary

A better method than the customary microscopic examination was needed for detection of F. hepatica infection in snails. To this end, an assay was developed which uses an oligonucleotide probe to detect a unique fasciolid sequence on the small rRNA subunit in a preparation of total snail RNA. The assay involves extraction and purification of snail RNA, RT-PCR, gel electrophoresis, Southern blotting, and hybridization to the end-labeled probe. In practice, the fluke-specific primer used in the PCR permits only the specific fluke sequence to be amplified, so that a positive result can be seen on the ethidium bromide-stained agarose gel and further analysis is not necessary. The method is sensitive enough to detect fluke material in one snail immediately after miracidial penetration, prepared with 49 uninfected snails. It has been shown previously by Rognlie et al., (1994) that the probe does not cross-react with any other organisms outside of the Fasciolidae. Finally, this method is capable of detecting fluke material in the snail at all stages of development and produces no false positives. This assay provides a fast, reliable, and sensitive method for detecting F. hepatica infected snails as early as one hour post-exposure and throughout intramolluscan development.

# FIELD USE OF A NUCLEIC ACID BASED ASSAY TO DETECT NATURALLY INFECTED FASCIOLA HEPATICA INTERMEDIATE HOSTS AND TO STUDY SEASONAL TRANSMISSION OF FASCIOLOSIS

## Introduction

In March of 1992, Dr. Stuart Knapp was called to consult with the owners of a cashmere goat ranch in Dillon, MT. where several goats had died of acute fasciolosis. The ranch was divided into several paddocks, some entirely dry, some bordering natural water, and some with low-lying areas which contained standing water in certain seasons. During wet seasons, these areas were capable of supporting large numbers of lymnaeid snails. One paddock in particular appeared to be the main focus of transmission of the disease, since it contained the majority of the snail population and had housed each of the dead animals at some point in time. This ranch provided an excellent place to field test our assay and to study the seasonal transmission of Fasciola hepatica. In July of 1992, we began the first two phases of a three phase study. We visited the ranch regularly to collect snails and fecal samples. Fecal samples were examined for the presence of fluke eggs, and snails were tested for the presence of fluke infection using the assay developed by Rognlie et al., (1994). Additional snails were collected and used to start laboratory cultures for experimental work. In 1993 we began the last phase of the study, rotating three new

tracer sheep each month through the suspect paddock from September 1993 to September 1994 to determine when they acquired the parasite.

In conjunction with the collection and study of snails at the ranch, a study was begun to determine whether areas of thermal activity provide snail habitat throughout the year and whether they are important in transmission of F. hepatica. Previously, Dunkel et al., (1996) had done a survey of lymnaeid snails in the state and found snails at several thermal sites. In 1993 we began studying the dynamics of the snail populations at four thermal sites representing geographically distinct regions of the state. Snails were collected at each of these sites for identification and origination of laboratory cultures (Potts, 1995). Lymnaeid snails were found at three of these sites (Potts, 1995) and were collected and tested using the assay to determine whether they were infected with F. hepatica.

## Materials and Methods

### Study Site Descriptions

Green Springs is located in a cattle pasture in Sanders Co., Latitude 47D 26M 35S N, Longitude 114D 40M 7S W. The thermal spring continuously fills a marshy depression which covers approximately 0.2 ha to a depth of 0.6 m at the center (Potts, 1995). The average water temperature at the source was 21.7 C +/- 4.2 C (Potts, 1995).

New Biltmore Hot Spring is located in Beaverhead Co., Latitude 45D 27M 58S N, Longitude 112D 29M 1S W. A pipe carries the water from its reservoir to a ditch 1.5 m across with high vertical sides (Potts, 1995). The average water temperature at the source

was 34.6 C +/- 4.8 C (Potts, 1995).

Beaverhead Warm Springs is located in Beaverhead Co., Latitude 45D 22M 47S N, Longitude 112D 26M 33S W. The average water temperature at the source was 21.7 C +/- 6.0 C (Potts, 1995). The spring emerged in the middle of a stream 3.0-4.5 m wide with gently sloping sides, such that the water upstream was ice covered in the winter and the water downstream was clear (Potts, 1995).

Pioneer Mountain Farms is a private cashmere goat ranch located in Beaverhead Co., Latitude 45D 17M N, Longitude 112D 33M W. The 25 ha ranch is divided into 15 paddocks, housing a total of 100 goats and around 50 sheep. Two adjacent paddocks, designated 4 and 5, encompass the study site, which was a slough draining a marshy area between the ranch and the Beaverhead river, which borders the ranch. Water level in the 1 m deep slough is affected by seepage, river level, and irrigation activity (Rognlie et al., 1996). Since the water level in the slough is never very deep, it is expected that the water temperature will closely parallel the air temperature. The 30-year average daily temperature at the Dillon weather station (near the study site) shows an average low of between -5 C and -10 C in January and February and an average high of nearly 20 C in July and August. Additionally, the data show that for approximately 30 weeks per year (October to May) the temperature is below 10 C.

Snail Collections

Snails were collected by hand using forceps or by using a dip net when possible. Collections were done approximately every two weeks to one month from July 1992 to

October 1994 in paddocks 4 and 5 at the goat ranch and intermittently at the other locations. Snail collections were usually done in the afternoon and as many as possible were collected in the time allowed, usually one to four hours. Snails were transported back to the lab in a cooler in containers filled with water. Snails to be assayed were then frozen in liquid nitrogen and stored at -70 C. Snails to be identified were sent to Dr. Shei Kuei Wu, Curator of Molluscs, The Museum, University of Colorado, Boulder, CO.

Snail Testing

Snails were prepared in batches of 1-100. Those prepared in batches of 100 were 1-2 mm in length. The average batch size was 22 snails. The assay was performed as described previously, except that results were read directly from the ethidium bromide-stained agarose gel instead of continuing on with the Southern blot and probe hybridization.

## Results

Snail Collections

Over the three year period, a total of 3,497 lymnaeid snails were collected from the four sites for testing using the assay (See Appendix). From Pioneer Mountain Farms, paddock 4, 1,551 snails, identified as Lymnaea modicella, were collected (See Appendix). From paddock 5, 1,317 snails, identified as L. modicella and on one occasion as L. bulimoides, were collected (See Appendix). Three non-lymnaeid species, Oxyloma spp., Aplexa elongata, and Gyralus spp., were also present in paddocks 4 and 5 (See Appendix). Live L. modicella snails were found in paddocks 4 and/or 5 when the ground was not

frozen, usually April through December. Although no attempt was made to study snail population dynamics at this site, it was observed that the snail population density was much higher in the spring of 1992, shortly after the initial disease report, than at any other time during the survey, despite the fact that macroclimatological conditions appeared to be similar from year to year. During the course of the study, 132 batches of snails collected from paddocks 4 and 5 were tested with the assay, averaging 23 snails each (See Appendix). One batch containing 25 snails collected from paddock 4 on August 4, 1994 was found to contain fluke ribosomal RNA (Table 2.1).

From Beaverhead Warm Springs 590 snails were collected, mostly during the late summer months of 1994, and were identified as L. obrussa (See Appendix). Of these, 10 positive batches were found; two containing 10 snails each collected 4/14/94, and 8 containing an average of 25 snails each collected 7/14/94 (Table 2.1.). Snails collected at New Biltmore Hot Springs were identified as L. modicella and 39 snails were tested, all of them negative (See Appendix). Snails collected from the Green Spring as part of an earlier study (Dunkel et al., 1996) had been identified as L. caperata, but snails collected on 3/19/94 were identified as L. montanensis. From the Green Spring, four snails were collected on 3/19/94 and together tested positive for fluke rRNA (Table 2.1.). Potts (1995) reported that lymnaeid snails were present and active at all of these thermal locations throughout the year.

Table 2.1. Species Identity and Place and Date of Collection of All Snails Found Naturally Infected with Fasciola hepatica.

| Lot Number | Species | Location | Date | Number |
|---|---|---|---|---|
| 410 | L. montanensis | Green Spring | 03/19/94 | 04 |
| 413 | L. obrussa | BWS | 04/14/94 | 10 |
| 422 | L. obrussa | BWS | 04/14/94 | 10 |
| 463 | L. obrussa | BWS | 07/14/94 | 25 |
| 457 | L. obrussa | BWS | 07/14/94 | 25 |
| 458 | L. obrussa | BWS | 07/14/94 | 25 |
| 459 | L. obrussa | BWS | 07/14/94 | 25 |
| 468 | L. obrussa | BWS | 07/14/94 | 25 |
| 471 | L. obrussa | BWS | 07/14/94 | 21 |
| 474 | L. obrussa | BWS | 07/14/94 | 25 |
| 477 | L. obrussa | BWS | 07/14/94 | 25 |
| 490 | L. modicella | PMF | 08/04/94 | 25 |

BWS; Beaverhead Warm Spring
PMF; Pioneer Mountain Farms

Tracer Sheep and Fecal Examinations

Results of the tracer sheep experiment and fecal collections are reported in depth by Rognlie et al., (1996). Briefly, of the several groups of animals selected for monthly fecal examination at least one group of animals was fecal positive for F. hepatica in every collection during the 27 months of study (Rognlie et al., 1996). Prevalence of positive samples was highest (24.9%) for the animals housed in paddock 4 (Rognlie et al., 1996). Of the 36 tracer sheep housed in paddock 4 (three each month for 12 months), three sheep became infected with F. hepatica. One sheep from the September group and two sheep from the October group were found to be infected with F. hepatica by fecal examination and by the presence of adult flukes in the liver at necropsy.

## Discussion

In conjunction with conventional methods, this study uses a nucleic acid based assay which detects F. hepatica infections in snail intermediate hosts to characterize seasonal transmission of F. hepatica in an enzootic area, and to evaluate the potential for parasite transmission around thermal springs. In the southern U.S., disease transmission peaks twice in the year, once in the spring from snails which overwintered with an infection, and again in late fall from snails which became infected during the summer months (Malone et al., 1984, Boyce and Courtney, 1990). In contrast, results of the goat ranch study indicate that disease transmission peaks only once in Montana in the late fall, although results obtained from the study of thermal springs indicate that these sites may be foci for disease transmission in the spring as well.

Data from all three phases of the study support the conclusion that disease transmission occurs in the fall. Of the 36 tracer sheep that were pastured in paddock four only three sheep, pastured at the ranch in September and October, acquired the infection. Fecal examinations of animals in this paddock reveal chronically infected animals, shedding parasite eggs at a continuous rate throughout the year. Although lymnaeid snails are present and active for much of the year in paddock 4, the only batch of snails found to be positive for fluke material was collected in August. This positive result would be consistent with fall transmission of the parasite, since parasite development within the snail may have been in the intermediate stages. Results of this study are consistent with results reported by Hoover et al., (1984) who found that fluke transmission occurs once a year in Idaho, beginning in June and continuing through November. Since Idaho typically has milder weather it is reasonable that parasite transmission occurs over a larger time period, but still during the same season. It seems clear that F. hepatica transmission occurs only once a year in the northwestern U.S. as opposed to twice a year in the southern states, probably due to the fact that fluke/snail development only occurs above 10 C (Kendall, 1965), which, at the goat ranch, leaves a 20 week window between May and October for intramolluscan development of the fluke.

The snails in paddock 4 were identified as L. modicella, which corroborate the observation made by Lang (1977), that L. modicella is a natural host for F. hepatica. Snails in paddock five were identified as L. modicella and once as L. bulimoides. Lymnaea bulimoides is the most commonly reported natural intermediate host for F. hepatica in the U.S., and although none were found infected, its presence in this valley

represents the possibility of increasing disease transmission in the future. Other non-lymnaeid snails present in paddocks 4 and 5 were occasionally examined for the presence of fluke material, however, none were found to contain any.

The low prevalence of infection in the intermediate host is a curious result, since with superior detection methods we expected to find a slightly higher percentage of infected snails than reported previously by those using conventional techniques. Although no attempt was made to study population dynamics of the lymnaeid snails at the ranch, it was observed that there were extremely high numbers of snails present during the first year of the study when the livestock fatalities occurred. During this time hundreds of snails could be collected with a dip net in a few minutes, whereas during the last two years of the study, snails had to be searched for and collected one at a time by hand, with only one thousand being collected in one whole day. These observations support the conclusion by Malone et al., (1984) that disease transmission is dependent upon the lymnaeid snail population, and the risk of disease transmission could be predicted for a given year based on snail populations which could be estimated using microclimatological data.

Thermal habitats provide a uniquely favorable environment for both snails and flukes, and therefore may provide foci for disease transmission over a longer period of time than non-thermal areas in Montana. Since a study of snail population dynamics at thermal areas was already underway, some of the collected snails were tested using the assay to see if the disease was present. Although no attempt was made to make a systematic study of disease transmission at these thermal sites, the results obtained from

random testing were quite interesting. Of the three thermal sites supporting lymnaeid snails, positive snails were found at two. Although New Biltmore Hot Springs supported a large population of L. modicella, a suitable host snail, no positive snails were found at this site. This is most likely due to the small number of snails which were actually tested from this site, and the lack of livestock of any kind to complete the transmission cycle. The potential still exists for New Biltmore Hot Springs to become a focus for F. hepatica transmission.

At the Green Spring, only four snails were tested with the assay, however, a positive result was obtained. These snails were identified as L. montanensis, which has been reported as an experimental host for F. hepatica by Rowan et al., (1966). Snails which were collected at this site as part of an earlier study (Dunkel et al., 1996) were identified as L. caperata; a known host for Fascioloides magna, a fluke which has been reported in Sanders County. Although limitations of the assay make it impossible to say which parasite is being detected at this site, it is interesting that either fluke should be detected in a snail collected in March. Results of the snail population survey conducted at this site indicate that snails are present and active throughout the year (Potts, 1995), so this snail was active, indicating that the fluke was probably also actively developing. Since the thermal spring is above 10 C throughout the year, development of flukes within snails and shedding of cercariae could occur continuously, creating a constant focus of parasite transmission. To test this further, experimentally infected snails could be marked and released at the spring throughout the year and recovered periodically to measure fluke development.

Beaverhead Warm Spring was the site of most intensive collection and testing of snails, since it lies closest to the goat ranch. Snails identified here were L. obrussa, which has never been reported as a natural or experimental host for F. hepatica. Testing of snails from this site showed a high prevalence of snails containing fluke material. We assume that fluke material in these snails is F. hepatica because of the presence of sheep at this site and because F. magna has never been reported in Beaverhead County. Whether the infections were abortive or evolutive is not known, but experimental analysis of the vector potential of these snails should answer that question. In addition to the higher incidence of infection found among the intermediate host at this site, the extended time frame that infected snails are active is further evidence that seasonal transmission dynamics at thermal sites are different from those for Montana in general. Results show infected snails collected in April and July, which indicate that snails could either be overwintering with an infection, acquiring the infection earlier, or harboring an actively progressing infection all year. More study will be needed to determine the dynamics of fluke transmission at thermal sites, to determine what conditions favor a spring/fall pattern and what conditions could allow a year round pattern of transmission. It is clear from these preliminary data that thermal sites which support populations of suitable lymnaeid snails can be foci for F. hepatica transmission over a longer season than is possible for non-thermal areas of Montana.

## Summary

A comprehensive three part study was conducted at a goat ranch in an area enzootic for fasciolosis near Dillon MT, involving fecal examinations of the goats, snail collection and testing, and tracer sheep studies. In conjunction with this study, snails from several thermal areas around the state were collected, identified, and tested for the presence of fluke material. Results of fecal examinations indicate that the animals are shedding fluke eggs all year, and lymnaeid snails, identified as L. bulimoides and L. modicella, are present and active for much of the year. Despite the presence of suitable intermediate host snails and fluke eggs for much of the year, tracer sheep became infected only in September and October, and only in August was a positive batch of snails collected, indicating that disease transmission occurs only in the fall in this area. In contrast, testing conducted on snails collected from thermal areas revealed fluke positive snails from collections made in March, April, and August. Additionally, infection prevalence among snails collected from thermal areas was approximately an order of magnitude higher than that at the goat ranch. Infected snails collected at the thermal sites were identified as L. obrussa and L. montanensis. Although L. obrussa has never been reported as an intermediate host for F. hepatica, L. montanensis has been reported to be a suitable intermediate host (Rowan et al., 1966), and it is clear that the presence of a suitable lymnaeid intermediate host species near a thermal spring may create a focus of disease transmission, posing a unique threat to livestock.

# USE OF A NUCLEIC ACID BASED ASSAY TO DETERMINE THE INTERMEDIATE HOST CAPABILITY OF LYMNAEID SNAIL SPECIES IN MONTANA

## Introduction

It is well known that Fasciola hepatica uses lymnaeid snails as intermediate hosts (Thomas, 1883), however, it is also well documented that not all lymnaeid species are equally adapted to carrying the fluke infection to patency (Boray, 1966, Foreyt and Todd, 1978). It has been shown that Lymnaea truncatula is the primary intermediate host snail for F. hepatica in Europe, despite the presence of many other species of lymnaeid snails, including L. peregra and L. auricularia (Kendall, 1950). Similarly, in the continental U. S. L. bulimoides appears to be one of the most commonly reported intermediate host snails (Foreyt and Todd, 1978, Olsen, 1944, Shaw and Simms, 1929, and Wilson and Samson, 1971), while others including L. stagnalis and L. auricularia apparently do not usually carry the infection to patency (Boray, 1966,). Since permissive and non-permissive snails frequently occupy the same microhabitat, they are likely equally exposed to host-seeking miracidia. Many studies have been done to determine what attracts miracidia (both Schistosoma and Fasciola) to snails and whether miracidia are attracted to some species of snails and not others or are attracted to all species equally (Chernin, 1970, MacInnis, 1965). Results of these studies indicate that there are chemicals excreted or secreted by snails which stimulate and attract miracidia, however, it appears that the attraction is non-

specific (Chernin, 1970). Additionally, it has been observed that miracidia are not prevented from attaching to non-permissive snails (Foreyt and Todd, 1978); indeed, they are able to penetrate even non-host species such as Aplexa and Physa (Preveraud-Sindou et al., 1994), so it would seem that the events limiting the ability of F. hepatica to use some lymnaeid species as intermediate hosts occur after the sporocyst is inside the host snail. The inability of F. hepatica to use some lymnaeids as intermediate hosts is either due to some incompatibility between the snail and the fluke or due to active destruction of the fluke by a snail internal defense mechanism (Lie et al., 1987). There has been considerable support for the latter hypothesis, both indirectly from other similar fluke-snail systems and directly from experimental evidence within the Fasciola-Lymnaea system (Boray, 1966, McReath et al., 1982, Noda and Loker, 1989, Sullivan and Spence, 1994).

Using the assay developed by Rognlie et al., (1994), we have a unique opportunity to study the course of the fluke infection in the snail, since the assay can determine whether the snail actually acquires the infection, and at what time fluke material is no longer present in the snail. Using this assay, we will be able to assess the intermediate host capability of the lymnaeid species found in Montana in order to better understand the dynamics of transmission of the disease.

## Materials and Methods

### Snail Collection and Rearing

Lymnaea modicella and L. bulimoides were collected from Pioneer Mountain Farms, Beaverhead Co., Latitude 47D 17M N, Longitude 112D 33M W. Additional L. modicella were collected from New Biltmore Hot Springs, Beaverhead Co., Latitude 45D 27M 58S N, Longitude 112D 29M 1S W. Lymnaea obrussa were collected from Beaverhead Warm Spring, Beaverhead Co., Latitude 45D 22M 47S N, Longitude 112D 26M 33S W. Lymnaea elodes was collected from a roadside pond, Beaverhead Co., Lat. Long. Lymnaea auricularia were collected from Rattlesnake Lake, Yellowstone Co., Lat, Long. All Lymnaea columella used in the experiments came from cultures maintained in our laboratory. The original stocks for these cultures came from western Oregon.

Three species, L. columella, L. elodes, and L. auricularia, were maintained in aquaria containing gravel and distilled water. The water was aerated continuously and snails were fed red leaf lettuce and calcium carbonate. All other lymnaeid snails were maintained according to the method of Taylor and Mozely (1948) with minor modifications. Briefly, unglazed clay dishes were soaked in distilled water for 24 hours to remove any chemical residue. A gentle slope of mud was formed in part of the dish bottom, using mud which had been collected from snail habitat and sterilized by autoclaving. Sterilized aquarium gravel was added to cover the rest of the dish bottom, and enough distilled water was added to cover the gravel and part of the mud slope.

Dishes were kept in plastic liners filled with water to keep the sides of the dish moist, and water levels were kept constant by daily misting and addition of water. Water in the dishes was aerated continuously and snails were fed red leaf lettuce and calcium carbonate. All snails were maintained at a temperature between 22 and 27 C with a 12 hour light:dark cycle.

Parasite Materials and Experimental Infections

Fasciola hepatica ova were obtained from livers and gall bladders of infected Montana slaughter cattle, cleaned by washing through sieves as described previously, and stored in total darkness at 4 C until needed. Prior to incubation, ova were washed in 1% antibiotic/antimycotic solution. Ova were incubated in aerated distilled water in total darkness for 14 days at 22 C and then returned to 4 C until needed for exposures. Snails between 1 and 6 mm in length were exposed individually in 24 well tissue culture plates to 5-20 active miracidia for 6 hours at room temperature. Some snails were then returned to sterile aquaria or freshly prepared sterile culture dishes and maintained for 50 days. Other snails were frozen in liquid nitrogen at either 6 hours or 5 days post exposure and stored at -70 C.

Evaluation of Snails for the Presence of Infection

Snails maintained for 50 days post exposure were examined microscopically for evidence of cercarial release, then crushed with a clean glass rod and examined for the presence of rediae and cercariae. When rediae were present without cercariae, the condition of the rediae was noted, with particular attention to motility and the presence or

absence of germinal balls. Snails which were frozen in liquid nitrogen at 6 hours or 5 days post exposure were tested individually for the presence of infection using the assay as described previously. Results were visualized on an ethidium bromide-stained agarose gel.

## Results

Of snails examined at 50 days post exposure, only one species, L. auricularia, was never positive for larval forms of F. hepatica (Table 3.5.). Of the other five species examined, a low percentage of L. elodes and L. obrussa harbored rediae, however, cercariae were never observed (Tables 3.4. and 3.3.). In addition, microscopic examination of rediae recovered from these two snails revealed motionless, immature rediae containing no germinal balls and giving no indication of development. Fifty percent of L. bulimoides and an average of 6.5% of L. modicella were positive at 50 days post exposure, and both rediae and cercariae were observed in each species of snail (Tables 3.6. and 3.2.). In the case of L. columella variable results were obtained, with between 15 and 93% of snails positive at 50 days post exposure (Table 3.1.), however, both rediae and cercariae were always observed.

Of snails examined at six hours and five days post exposure, 75-100% of all snails regardless of species tested positive for fluke material at six hours post exposure. At five days, however, only L. columella snails retained the infection in the same numbers. All L. elodes were negative for fluke material at five days post exposure (Table 3.4.). Only 8%, 25%, and 30% (ave.) respectively of L. modicella, L. auricularia, and L. obrussa harbored fluke material at five days post exposure (Tables 3.2., 3.5., and 3.3.).

Table 3.1. Results of Experimental Exposures of Lymnaea columella Snails to Fasciola hepatica Miracidia.

| Time PE | Trial | # Tested | # Positive | % Positive |
|---|---|---|---|---|
| Six Hours | 1 | 12 | 11 | 92 |
| | 2 | 12 | 11 | 92 |
| Five Days | 1 | 12 | 12 | 100 |
| | 2 | 12 | 11 | 92 |
| 50 Days | 1 | 92 | 14 | 15 |
| | 2 | 42 | 39 | 93 |

Table 3.2. Results of Experimental Exposures of Lymnaea modicella Snails to Fasciola hepatica Miracidia.

| Time PE | Trial | # Tested | # Positive | % Positive |
|---|---|---|---|---|
| Six Hours | 1 | 12 | 12 | 100 |
| | 2 | - | - | - |
| Five Days | 1 | 12 | 1 | 8 |
| | 2 | - | - | - |
| 50 Days | 1 | 66 | 4 | 6 |
| | 2 | 69 | 5 | 7 |

Table 3.3. Results of Experimental Exposures of Lymnaea obrussa Snails to Fasciola hepatica Miracidia.

| Time PE | Trial | # Tested | # Positive | % Positive |
|---|---|---|---|---|
| Six Hours | 1 | 12 | 8 | 75 |
| | 2 | 12 | 12 | 100 |
| Five Days | 1 | 8 | 1 | 12 |
| | 2 | 12 | 5 | 42 |
| 50 Days | 1 | 72 | 0 | 0 |
| | 2 | 59 | 7* | 12 |

PE; post exposure
*Cercariae were not observed, only rediae were present.

Table 3.4. Results of Experimental Exposures of Lymnaea elodes Snails to Fasciola hepatica Miracidia.

| Time PE | Trial | # Tested | # Positive | % Positive |
|---|---|---|---|---|
| Six Hours | 1 | 12 | 11 | 92 |
| | 2 | - | - | - |
| Five Days | 1 | 12 | 0 | 0 |
| | 2 | - | - | - |
| 50 Days | 1 | 28 | 1* | 4 |
| | 2 | - | - | - |

Table 3.5. Results of Experimental Exposures of Lymnaea auricularia Snails to Fasciola hepatica Miracidia.

| Time PE | Trial | # Tested | # Positive | % Positive |
|---|---|---|---|---|
| Six Hours | 1 | 12 | 12 | 100 |
| | 2 | - | - | - |
| Five Days | 1 | 12 | 3 | 25 |
| | 2 | - | - | - |
| 50 Days | 1 | 28 | 0 | 0 |
| | 2 | - | - | - |

Table 3.6. Results of Experimental Exposures of Lymnaea bulimoides Snails to Fasciola hepatica Miracidia.

| Time PE | Trial | # Tested | # Positive | % Positive |
|---|---|---|---|---|
| 50 Days | 1 | 30 | 15 | 50 |
| | 2 | - | - | - |

PE; post exposure
*Cercariae were not observed, only rediae were present.

## Discussion

The assay developed by Rognlie et al., (1994) was a valuable tool in this study. Previously, in order to study fluke development within the snail intermediate host, one would have had to fix the snail tissues at several time points post-exposure, section, stain, and examine them microscopically. This assay provides a fast and easy method of screening snails for fluke infection and is sensitive enough to detect one miracidium in a snail immediately after penetration. Other assays based on detection of fluke repetitive DNA sequences (Heussler et al., 1993, Kaplan et al., 1995) could be used to evaluate snails for the presence of infection, however, since DNA is more stable, a snail may test positive due to fluke DNA which has not been degraded when, in fact, the fluke has been destroyed. Since RNA is quickly degraded by endogenous RNases released by dying cells, it is unlikely that a snail would test positive with the assay after the fluke is destroyed.

Results of experimental infections indicate that L. bulimoides is the most permissive host of the indigenous snail species tested, followed by L. modicella. Lymnaea columella is also a permissive host, but it is not found in Montana. Lymnaea montanensis was not tested experimentally, but was successfully infected by Rowan et al. (1966). In addition, one sample of L. montanensis collected at the Green Spring tested positive for fluke material, unfortunately, not enough snails were found to produce sufficient numbers in laboratory culture to do experimental exposures. Of the other snails experimentally exposed, none were ever observed to be shedding cercariae, nor were cercariae observed

upon microscopical examination, although a small percentage contained rediae. These results are consistent with the observation that L. bulimoides is the most commonly reported intermediate host snail in the U.S. (Malone et al., 1982, Cruz-Reyes and Malek, 1987), and that L. modicella can also serve as an intermediate host (Lang, 1977). Clearly, there exists quite a range of susceptibility to F. hepatica within the Lymnaeidae.

It has long been argued whether variance in susceptibility to parasite infection in this and other snail-parasite systems is the result of innate host resistance or host unsuitability (Lie et al., 1987), however, there is considerable evidence that there is a cell-mediated Snail Internal Defense system (IDS) (Loker,1994), and that whether a snail becomes infected or not is determined by the strength of the host resistance and the capacity of the parasite to evade or interfere with the IDS (Lie et al., 1987). Lymnaea stagnalis has probably been the most widely studied snail in the search for an IDS among lymnaeids, and it has been shown that the amoebocytes of this snail are highly phagocytic (Sminia, 1972, and Sminia et al., 1974), and that it will encapsulate and destroy transplanted rediae (Boray, 1966). Kendall (1949) and Boray (1966) have reported experimentally infecting L. stagnalis with F. hepatica, and Lang (1977) has reported finding naturally infected snails; however, this species does not, as a rule, carry the infection to patency and produce cercariae.

To further explore the dynamics of the parasite-snail interaction, the RT-PCR assay was used to determine what percentage of snails of each species actually became infected with the parasite, and roughly the duration of the life of the parasite after infection. The observation that over 75% of all snails regardless of species acquire the

infection suggests that most snails will initially become infected with the parasite. Although theoretically a positive result at six hours post exposure could be caused by a miracidium still attached to a snail but unable to penetrate, this is unlikely. It has been shown that when a miracidium attaches to a snail the attachment is tenuous at best, and if it fails in the attempt to penetrate, it quickly detaches and dies (Koie et al., 1976 and Lie et al., 1987). In addition, we have observed that the miracidia placed in a well with a snail will locate the snail and attach in a matter of minutes, and since penetration of the miracidium into the snail typically takes less than 45 minutes (Koie et al., 1976), by six hours post exposure it is likely that all of the miracidia have penetrated or are no longer attached to the snails. Using the Biomphalaria/Schistosoma system as a model, the snails were tested again at five days post exposure. By this time, Biomphalaria snails which exhibit the fast-acting type of response have completely encapsulated, killed, and phagocytosed all invading parasites, so that no traces of even the capsules are left (Lie et al., 1987). Highly resistant snails may destroy the parasite in less than one day (Lie et al., 1987). Snails which exhibit the slow-reacting pattern quickly encapsulate the parasite, but retain it for a week or more before it degenerates, dies, and is phagocytosed (Lie et al., 1987). The results indicate that L. elodes probably exhibits the fast acting pattern, since none of the snails exposed tested positive at five days post exposure. One redia was found in one of the L. elodes at 50 days post exposure, however, it was immature and sessile, and probably not likely to develop further. Of course, the hypothesis that these snails may simply be unsuitable hosts which do not provide an environment conducive to parasite development cannot be ruled out. The rapid and complete destruction of the parasites in

these snails, however, suggests that the snail is actively and aggressively attacking the parasite.

It was interesting that an average of 30% of L. obrussa snails did not overcome the infection in five days and that 12% carried the infection for 50 days but contained stunted, immature rediae and no cercariae; and that 25% of L. auricularia still retained fluke material at five days post exposure, but never contained identifiable rediae or cercariae at 50 days post exposure. This may be indicative of the slow acting response, or may point to another type of resistance mechanism or simply the inability of the snail to provide the parasite with the basic requirements for development. In the Biomphalaria/schistosome system, snails which exhibit the slow acting response are not necessarily considered less resistant, in fact, they may be highly resistant; however, the response appears to be mediated by some other mechanism in which the cellular reaction has a secondary role (Lie et al., 1987).

With respect to the relative importance of these species of lymnaeid snails in completing the F. hepatica transmission cycle in Montana, these results indicate that L. bulimoides is probably the most important intermediate host snail, followed by L. modicella and L. montanensis (not experimentally infected but found naturally infected in this study and experimentally infected (Rowan et al., 1966)). Lymnaea elodes is probably not a factor in transmission of the disease, and although several L. obrussa were found to be naturally infected in this study and occasionally contain later larval stages, the inability of the parasite to complete its life cycle inside this species makes it unimportant in the transmission of the disease. However, it is important to note that the levels of natural

resistance to flukes found in some snail strains are not constant, and may be increased by selection and self-fertilization or may spontaneously decrease, as in the Biomphalaria/Schistosoma system (Lie et al., 1987). It may therefore be necessary to reevaluate snail populations periodically and to evaluate geographically distinct populations of lymnaeid snails in order to detect any such shift in susceptibility.

## Summary

To determine the intermediate host potential of several species of lymnaeid snails indigenous to Montana, experimental exposures of laboratory raised lymnaeid snails were done. Six species were exposed and kept for 50 days to determine what percentage of each would carry the infection to patency. It was found that 50% of L. bulimoides tested and an average of 6.5% of L. modicella tested carried the infection to patency. Twelve percent of L. obrussa and 4% of L. elodes (one snail) harbored immature, immobile rediae but no cercariae. Lymnaea auricularia showed no evidence of larval forms of fluke at 50 days post exposure. To determine whether a defense mechanism analogous to the IDS seen in some schistosome-resistant Biomphalaria strains is operating in any of these snail species, a series of exposures were done and snails were tested at 6 hours and 5 days post exposure using the assay developed by Rognlie et al., (1994). Results showed that most of the snails initially tested positive for fluke material, regardless of species, but in many species, the number still positive at 5 days post exposure was considerably less. This is consistent with the fast acting IDS response seen in Biomphalaria snails, and is evidence that lymnaeid snails may possess a similar defense system. Further, the assay used provides a fast and convenient method to assess the intermediate host capability of lymnaeid snails in any given area.

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# APPENDIX

Table A.1. Lymnaeid Snails Collected in 1992 and Tested for Fluke rRNA

| Lot Number | Location | # Snails in Batch | Date Collected |
|---|---|---|---|
| F-21 | PMF 4 | 20 | 7/21 |
| G-22 | PMF 4 | 20 | 7/21 |
| Z36-23 | PMF 4 | 25 | 7/31 |
| Z37-24 | PMF 4 | 20 | 7/31 |
| Z38-25 | PMF 4 | 25 | 7/31 |
| C-26 | PMF 4 | 20 | 8/18 |
| D-27 | PMF 4 | 20 | 8/18 |
| E-28 | PMF 4 | 20 | 8/18 |
| Z19-29 | PMF 4 | 7 | 9/1 |
| Z18-210 | PMF 4 | 7 | 9/15 |
| Z1-211 | PMF 5 | 4 | 9/20 |
| Z10-212 | PMF 4 | 25 | 9/30 |
| Z15-213 | PMF 5 | 13 | 10/14 |
| Z32-214 | PMF 4 | 10 | 10/14 |
| Z17-215 | PMF 4 | 23 | 10/27 |
| Z3-216 | PMF 5 | 25 | 10/27 |
| Z20-217 | PMF 4 | 4 | 11/11 |
| Z6-218 | PMF 5 | 2 | 11/11 |
| Z4-219 | PMF 4 | 5 | 11/25 |
| Z9-220 | PMF 5 | 5 | 11/25 |

*Pioneer Mountain Farms (PMF)

Table A.2. Lymnaeid Snails Collected in 1993 and Tested for Fluke rRNA.

| Lot Number | Location | # Snails in Batch | Date Collected |
|---|---|---|---|
| 1A-31 | PMF 4 | 3 | 5/9 |
| 1B-32 | PMF 4 | 3 | 5/9 |
| 1C-33 | PMF 4 | 3 | 5/9 |
| 1D-34 | PMF 4 | 2 | 5/9 |
| 1-35 | PMF 4 | 1 | 5/18 |
| 426C-36 | BWS | 34 | 6/28 |
| Z6-37 | PMF 4 | 10 | 6/28 |
| Z13-38 | BWS | 13 | 6/28 |
| Z7-39 | BWS | 11 | 7/8 |
| Z5-310 | PMF 4 | 15 | 7/15 |
| B-311 | PMF 4 | 35 | 8/5 |
| 426-314 | PMF 4 | 26 | 11/12 |

*Pioneer Mountain Farms (PMF); Beaverhead Warm Spring (BWS)

Table A.3. Lymnaeid Snails Collected in 1994 and Tested for Fluke rRNA

| Lot Number | Location | # Snails in Batch | Date Collected |
|---|---|---|---|
| 426E-41 | PMF 4 | 55 | 1/7 |
| Z8-42 | PMF 4 | 7 | 3/11 |
| 11-43 | BWS | 4 | 3/11 |
| 2A-44 | PMF 4 | 3 | 3/11 |
| 2B-45 | PMF 4 | 3 | 3/11 |
| 2C-46 | PMF 4 | 3 | 3/11 |
| 2D-47 | PMF 4 | 3 | 3/11 |
| 6-48 | NBHS | 10 | 3/11 |
| 426B-49 | NBHS | 11 | 3/11 |
| Z2-410 | Green Spring | 4 | 3/19** |
| A-411 | PMF 4 | 30 | 4/14 |
| Z23-412 | NBHS | 3 | 4/14 |
| Z24-413 | BWS | 10 | 4/14** |
| Z25-414 | BWS | 10 | 4/14 |
| Z26-415 | NBHS | 6 | 4/14 |
| Z27-416 | BWS | 10 | 4/14 |
| Z28-417 | NBHS | 4 | 4/14 |
| Z29-418 | BWS | 5 | 4/14 |
| Z30-419 | BWS | 10 | 4/14 |
| Z31-420 | BWS | 10 | 4/14 |
| Z33-421 | BWS | 7 | 4/14 |
| Z34-422 | BWS | 10 | 4/14** |
| Z35-423 | PMF 4 | 1 | 4/14 |
| Z39-424 | PMF 4 | 20 | 4/14 |
| Z40-425 | PMF 4 | 20 | 4/14 |
| Z41-426 | BWS | 10 | 4/14 |
| Z42-427 | NBHS | 5 | 4/14 |
| Z45-428 | PMF 4 | 10 | 4/14 |
| H-429 | PMF 4 | 19 | 5/12 |
| Z11-430 | PMF 4 | 18 | 6/9 |
| Z12-431 | PMF 4 | 23 | 6/9 |
| Z14-432 | PMF 4 | 23 | 6/9 |
| Z21-433 | PMF 4 | 18 | 6/9 |
| Z22-434 | PMF 4 | 24 | 6/9 |

*Pioneer Mountain Farms (PMF); Beaverhead Warm Spring (BWS); New Biltmore Hot Springs (NBHS)

**Batches which tested positive for fluke rRNA

Table A.4. Lymnaeid Snails Collected in 1994 and Tested for Fluke rRNA cont'd.

| Lot Number | Location | # Snails in Batch | Date Collected |
|---|---|---|---|
| M23-435 | PMF 4 | 25 | 7/14 |
| M24-436 | PMF 4 | 25 | 7/14 |
| M25-437 | PMF 4 | 25 | 7/14 |
| M26-438 | PMF 4 | 25 | 7/14 |
| M27-439 | PMF 4 | 25 | 7/14 |
| M28-440 | PMF 4 | 25 | 7/14 |
| M29-441 | PMF 4 | 25 | 7/14 |
| M30-442 | PMF 4 | 25 | 7/14 |
| M31-443 | PMF 4 | 25 | 7/14 |
| M32-444 | PMF 4 | 25 | 7/14 |
| M33-445 | PMF 4 | 25 | 7/14 |
| M34-446 | PMF 4 | 25 | 7/14 |
| M35-447 | PMF 4 | 25 | 7/14 |
| M36-448 | PMF 4 | 25 | 7/14 |
| M37-449 | PMF 4 | 25 | 7/14 |
| M38-450 | PMF 4 | 25 | 7/14 |
| M39-451 | PMF 5 | 25 | 7/14 |
| M40-452 | PMF 5 | 25 | 7/14 |
| M41-453 | PMF 5 | 25 | 7/14 |
| M42-454 | PMF 5 | 25 | 7/14 |
| M43-455 | PMF 5 | 6 | 7/14 |
| M45-456 | BWS | 25 | 7/14 |
| M46-457 | BWS | 25 | 7/14** |
| M47-458 | BWS | 25 | 7/14** |
| M48-459 | BWS | 25 | 7/14** |
| M49-460 | BWS | 25 | 7/14 |
| M50-461 | BWS | 25 | 7/14 |
| M51-462 | BWS | 25 | 7/14 |
| M52-463 | BWS | 25 | 7/14** |
| M53-464 | BWS | 25 | 7/14 |
| M54-465 | BWS | 25 | 7/14 |
| M55-466 | PMF 4 | 25 | 7/14 |
| M62-467 | BWS | 25 | 7/14 |
| M63-468 | BWS | 25 | 7/14** |

*Pioneer Mountain Farms (PMF); Beaverhead Warm Springs (BWS); New Biltmore Hot Springs (NBHS)

**Batches which tested positive for fluke rRNA

Table A.5. Lymnaeid Snails Collected in 1994 and Tested for Fluke rRNA cont'd.

| Lot Number | Location | # Snails in Batch | Date Collected |
|---|---|---|---|
| M64-469 | BWS | 25 | 7/14 |
| M65-470 | PMF 5 | 25 | 7/14 |
| M66-471 | BWS | 21 | 7/14** |
| M68-472 | BWS | 25 | 7/14 |
| M69-473 | BWS | 25 | 7/14 |
| M71-474 | BWS | 25 | 7/14** |
| M72-475 | PMF 5 | 25 | 7/14 |
| M73-476 | PMF 5 | 25 | 7/14 |
| M74-477 | BWS | 25 | 7/14** |
| M85-478 | PMF 5 | 25 | 7/14 |
| M90-479 | PMF 5 | 25 | 7/14 |
| M91-480 | PMF 5 | 25 | 7/14 |
| M95-481 | PMF 5 | 25 | 7/14 |
| M96-482 | PMF 5 | 25 | 7/14 |
| M1-483 | PMF 5 | 25 | 8/4 |
| M2-484 | PMF 5 | 25 | 8/4 |
| M3-485 | PMF 5 | 25 | 8/4 |
| M4-486 | PMF 5 | 25 | 8/4 |
| M5-487 | PMF 5 | 25 | 8/4 |
| M6-488 | PMF 5 | 25 | 8/4 |
| M7-489 | PMF 5 | 25 | 8/4 |
| M8-490 | PMF 5 | 25 | 8/4** |
| M9-491 | PMF 5 | 25 | 8/4 |
| M10-492 | PMF 4 | 12 | 8/4 |
| M11-493 | PMF 4 | 25 | 8/4 |
| M12-494 | PMF 4 | 25 | 8/4 |
| M13-495 | PMF 4 | 25 | 8/4 |
| M14-496 | PMF 4 | 25 | 8/4 |
| M15-497 | PMF 4 | 25 | 8/4 |
| M16-498 | PMF 4 | 25 | 8/4 |
| M17-499 | PMF 4 | 25 | 8/4 |
| M18-4100 | PMF 5 | 25 | 8/4 |
| M19-4101 | PMF 4 | 25 | 8/4 |
| M20-4102 | PMF 4 | 25 | 8/4 |

*Pioneer Mountain Farms (PMF); Beaverhead Warm Springs (BWS); New Biltmore Hot Springs (NBHS)

**Batches which tested positive for fluke rRNA

Table A.6. Lymnaeid Snails Collected in 1994 and Tested for Fluke rRNA cont'd

| Lot Number | Location | # Snails in Batch | Date Collected |
|---|---|---|---|
| M21-4103 | PMF 4 | 25 | 8/4 |
| M22-4104 | PMF 4 | 25 | 8/4 |
| M56-4105 | PMF 4 | 25 | 8/4 |
| M57-4106 | PMF 4 | 25 | 8/4 |
| M58-4107 | PMF 4 | 25 | 8/4 |
| M59-4108 | PMF 4 | 25 | 8/4 |
| M60-4109 | PMF 5 | 23 | 8/4 |
| M61-4110 | PMF 5 | 25 | 8/4 |
| M67-4111 | PMF 4 | 25 | 8/4 |
| M70-4112 | PMF 4 | 25 | 8/4 |
| M88-4113 | PMF 4 | 25 | 8/4 |
| M93-4114 | PMF 4 | 25 | 8/4 |
| M75-4115 | PMF 5 | 25 | 8/18 |
| M76-4116 | PMF 5 | 25 | 8/18 |
| M77-4117 | PMF 5 | 25 | 8/18 |
| M78-4118 | PMF 5 | 25 | 8/18 |
| M79-4119 | PMF 5 | 25 | 8/18 |
| M80-4120 | PMF 5 | 25 | 8/18 |
| M81-4121 | PMF 5 | 25 | 8/18 |
| M82-4122 | PMF 5 | 25 | 8/18 |
| M83-4123 | PMF 5 | 25 | 8/18 |
| M84-4124 | PMF 5 | 29 | 8/18 |
| M86-4125 | PMF 5 | 25 | 8/18 |
| M89-4126 | PMF 5 | 25 | 8/18 |
| M92-4127 | PMF 5 | 25 | 8/18 |
| M94-4128 | PMF 5 | 25 | 8/18 |
| M97-4129 | PMF 5 | 25 | 8/18 |
| M98-4131 | PMF 5 | 35 | 9/15 |
| M99-4135 | PMF 4 | 10 | 10/25 |
| M103-4136 | PMF 5 | 50 | 10/25 |
| M104-4137 | PMF 5 | 100 | 10/25 |
| M105-4138 | PMF 5 | 100 | 10/25 |

*Pioneer Mountain Farms (PMF); Beaverhead Warm Spring (BWS); New Biltmore Hot Springs (NBHS)

**Batches which tested positive for fluke rRNA

Table A.7. Non-lymnaeid Snails Collected and Tested for Fluke rRNA

| Lot Number | Location | Snail Genus | # in Batch | Date Collected |
|---|---|---|---|---|
| 4 | PMF 4 | Zonitoides | 2 | 5/9/93 |
| 5 | PMF 4 | Zonitoides | 2 | 5/9/93 |
| 12 | PMF 4 | Gyralus | 5 | 5/9/93 |
| 3A | BWS | Physa | 1 | 7/15/93 |
| 3B | BWS | Physa | 1 | 7/15/93 |
| 3C | BWS | Physa | 1 | 7/15/93 |
| 13 | PMF 4 | Gyralus | 11 | 1/7/94 |
| 2 | PMF 4 | Gyralus | 1 | 3/11/94 |
| 3 | PMF 4 | Aplexa | 1 | 3/11/94 |
| 7 | PMF 4 | Aplexa | 1 | 3/11/94 |
| 8 | PMF 4 | Gyralus | 4 | 3/11/94 |
| 9 | PMF 4 | Gyralus | 2 | 3/11/94 |
| 10 | PMF 4 | Gyralus | 8 | 3/11/94 |
| 4A | PMF 4 | Aplexa | 1 | 3/11/94 |
| 4B | PMF 4 | Aplexa | 2 | 3/11/94 |
| 5 | NBHS | Physa | 10 | 3/11/94 |
| 426F | NBHS | Physa | 10 | 3/11/94 |

*Pioneer Mountain Farms (PMF); Beaverhead Warm Spring (BWS); New Biltmore Hot Springs (NBHS).