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EPIDEMIOLOGY OF CATTLE AND SHEEP FASCIOLOSIS IN SELECTED ABATTOIRS OF ETHIOPIA AND ESTIMATION OF THE ASSOCIATAED ECONOMIC LOSSES DUE TO LVIER CONDEMNATION AND COPROLOGICAL STUDY IN AND AROUND DEBREBERHAN AND EVALUATION OF THE IMMUNE RESPONSE OF SHEEP AGAINST PRIMARY EXPERIENTIAL INFECTION WITH *Fasciola hepatica* METACERCARIAE

PhD dissertation

By

Abebayehu Tadesse Wazza

June, 2021

Bishoftu, Ethiopia

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A dissertation submitted to the College of Veterinary Medicine and Agriculture of Addis Ababa University in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Veterinary Parasitology

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June, 2021

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Department of Pathology and Parasitology

As memebers of the Examining Board of the final PhD open defense, we certify that we have read and evaluated the dissertation prepared by: Abebayehu Tadesse Wazza entitled Epidemiology of ruminant fasciolosis and its vectors in selected endemic areas of Ethiopia and evaluation of the immune responses of sheep against primary infection with *Fasciola hepatica* metacercarae and recommended that it be accepted as fulfilling the dissertation requirement for the degree of: Doctor of Philosophy in Parasitology.

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Final approval and acceptance of the dissertation is contingent upon the submission of its corrected copy to the graduate programs office through the concerned department. I hereby certify that I have read the revised version of this dissertation prepared under my direction and recommend that it be accepted as fulfilling the dissertation requirement.

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Department chair (Dr Bersissa Kumssa) Signature Date

STATEMENT OF AUTHOR

First, I declare that this thesis/dissertation is my bonafide work and that all sources of material used for this thesis have been duly acknowledged. This thesis has been submitted in partial fulfillment of the requirements for a PhD degree at Addis Ababa University, College of Veterinary Medicine and Agriculture and is deposited at the University/College library to be made available to borrowers under rules of the Library. I solemnly declare that this thesis is not submitted to any other institution anywhere for the award of any academic degree, diploma, or certificate. Brief quotations from this thesis are allowable without special permission provided that accurate acknowledgement of source is made. Requests for permission for extended quotation from or reproduction of this manuscript in whole or in part may be granted by the head of the major department or the Dean of the College when in his or her judgment the proposed use of the material is in the interests of scholarship. In all other instances, however permission must be obtained from the author.

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Date of Submission: _____

DEDICATION

To Freedom and Peace Loving People Accorss the Nations

To those striving and refuses to allow any of God's gifts to stagnate

BIBLIOGRAPHICAL SKETCH

I the author of this dissertation was born on October 29, 1974 in Wolaita zone, Southern Ethiopia. I attended my elementary education at Ligaba Beyene Abasebsib Elementary school (Wolita Soddo town), completed my junior grades (grade 7 and 8) at Soddober Junior Secondary school and high school education at Jinka Secondary School. I got my degree of Doctor of Veterinary Medicine (DVM) at Addis Ababa University, College of Veterinary Medicine and received MSc degree in Tropical Veterinary Medicine. Again, I joined Addis Ababa University, College of Veterinary Medicine and Agriculture and received the degree of Doctor of Philosophy (PhD) in Veterinary Parasitology. In my career, I have served in various governmental offices as field Veterinary Officer, head agricultre office and now I am working as an instructor at Hawassa University, Faculty of Veterinary Medicine in my capaity as Associate Professor.

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EPIDEMIOLOGY OF CATTLE AND SHEEP FASCIOLOSIS IN SELECTED ABATTOIRS OF ETHIOPIA AND ESTIMATION OF THE ASSOCIATAED ECONOMIC LOSSES DUE TO LVIER CONDEMNATION AND COPROLOGICAL STUDY IN AND AROUND DEBREBERHAN AND EVALUATION OF THE IMMUNE RESPONSE OF SHEEP AGAINST PRIMARY EXPERIENTIAL INFECTION WITH *Fasciola hepatica* METACERCARIAE

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PhD Thesis

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ABSTRACT

Fasciolosis is an economically important disease of ruminants which affects mainly sheep and cattle worldwide and causes important economic losses in the animal husbandry. These losses are attributable to mortality, morbidity and condemnation of livers at slaughter. In Ethiopian highlands, sheep and cattle production has remained as an important sector of the country's agricultural economy. However, their potential has been exploited far less than expected due to several constraints including shortage of forage, poor livestock management and diseases. In Ethiopia, fasciolosis is widespread encompassing the major productive highland plateaus except very limited areas in arid escarpments. The study of liver flukes in live animals depends on the detection of faecal eggs and the use of faecal egg counts. However, these detect only patent infections and their interpretation is constrained by the paucity of information about how they relate to parasite burdens and pathology. Moreover, abattoir-based studies have been used as a component of the study of the liver fluke and to describe various aspects of liver fluke infection.

Controlling fasciolosis by vaccination rather than chemotherapy would be a cheaper, more efficient and reliable long term solution for the prevention of infection and eradication of its transmission. Regardless of several attempts, vaccines against *Fasciola hepatica* were not yet produced to the point of commercialization. A variety of irradiation-attenuated parasite species have been used experimentally to induce protection in various host species. These studies have demonstrated that development of vaccine is potentially feasible. The present study entitled "Epidemiology of cattle and sheep fasciolosis in selected abattoirs of Ethiopia and estimation of the associated economic losses due to liver condemnation and coprological study in and around Debreberhan and evaluation of the immune response of sheep against primary experimental infection with *Fasciola hepatica* metacercariae" was undertaken in five abattoirs (Debreberhan, Addis Ababa, Bahrdar, HELMIX, ELFORA) while the experimental study was conducted at Aklilu Lemma Institute of Pathobiology. The objectives of the study were to assess the abattoir and coprological prevalence fasciolosis in sheep and cattle and assessment of the associated economic losses in three municipal and two export abattoirs as well as the vaccine trail on immune response of sheep to primary infection with attenuating irradiating dose of *Fasciola hepatica* metacercariae. The present findings on ruminants at five abattoirs had shown higher prevalence of fasciolosis (46.6% ± 0.059). However, this was much lower that observed in Debreberhan abattoir for sheep (84%) and cattle (77.8%). Overall fasciola infections were only diagnosed in 605 (53.8%) animals coprologically. The highest prevalence was for sheep (60.1%) and followed by cattle (49.2%). The overall herd level infection prevalence, as estimated from the egg-shedding index, was 50.9±29.3. In Debreberhan abattoir, *F. hepatica* was a dominant (87.9%) species identified followed by *F. gigantica* (6.3 %). However, this prevalence was much higher than that observed in five of the abattoirs altogether for *F. hepatica* (70.9%) and *F.*

gigantica (21.5%). The overall prevalence of fasciolosis observed in ruminants slaughtered in export abattoirs was 34.6% (877/2530) whereas it was significantly higher in ruminants slaughtered at municipal abattoirs 65.2% (1653/2530) as the whole. The mean annual financial loss recorded altogether in export and municipal abattoirs was 7, 049, 638 ETB / 335, 697.1 USD. The immune response to the infection was proved by the production of specific IgG1 antibodies to irradiated *F. hepatica*. The parasite viability was severely affected by doses of γ-irradiation of 120 Gy or 240 Gy. In the aforementioned doses relatively low numbers of mature flukes of about 60 (17.1%) and 38 (10.8%) were recovered than the control group, respectively. The sensitized lambs also showed less hepatic damage compared with the controls as indicated by lower liver lesions and lower levels of the serum enzyme glutamate dehydrogenase and γ-glutamyl transferase. The IgG1 antibody titers measured by ELISA vary with the dose of γ-irradiation. Sheep vaccinated with 240 Gy produced the highest antibody titre compared to the non sensitized positive controls. In conclusion, the present study plainly disclosed the high prevalence of fasciolosis both at herd and individual animal level and at abattoir survey. The eggs shedding index seemed to be useful approach in current epidemiological survey than the individual animal coprologic examination. The present findings on ruminants at abattoirs had shown higher prevalence of fasciolosis with significant annual financial loss. Vaccination of sheep with γ-irradiated metacercarae invariably yielded the specific immune response to different treatment groups. Irradiation of *F. hepatica* had resulted in reduced hepatic damage during migration of juveniles and a strong local immune response, represented by infiltration lymphocytes, eosinophils and macrophages and antifasciola IgG1 titers.

Keywords: Prevalence, Abattoir, Fasciolosis, γ-irradiation, immune response, *F. hepatica* metacercariae

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ABBREVIATIONS

AAMF	Alternatively Activated Macrophages
AME	Antmortem Examinations
APC	Antigen Presenting Cells
B cells	B Lymphocytes
CatL	Cathepsin L
CO1	Cytochrome C oxidase I
CsCp	Cysteine Protease
DC	Denderitic Cells
DNA	Deoxyribonucleic Acid
ELISA	Enzyme-Linked Immunosorbent Assays
FABP	Fatty Acid Binding Protein
FAO	Food and Agriculture Organization
EPG	Eggs Per Gram of Faeces
ESP	Excretory Secretory Product
FheCL	Fasciola hepatica Cathepsin L
FhLAP	Fasciola hepatica Leucine Aminopeptidase
FhSmIII	Fasciola hepatica Scistosoma Mansoni III
FLOTAC	Floatation Technique
FA	Freund's adjuvant

GGT	Gamma Glutamyl Transpeptidase
GLDH	Glutamate Dehydrogenase
GST	Glutathione S-Transferase
Hb	Haemoglobin
Ig	Immunoglobulins
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IgG1	Immunoglobulin G1
IL	Interleukin
INF- γ	Interferon Gamma
ITS1	Internal Transcribed Spacer 1
ITS2	Internal Transcribed Spacer 2
LAP	Leucine Aminopeptidases
LPS	Lipopolysaccharide
MHC	Major Histocompatibility Complex
ND1	Mitochondrial NADH Dehydrogenase I
NEJ	Newly Excysted Juveniles
NHmec	Leucine-7-Amino-4-Methylcoumarin
PCR	Polymerase Chain Reaction
PBL	Polyclonal B Lymphocyte
PME	Postmortem Examinations

RAPD	Randomly Amplified Polymorphic DNA
rDNA	Ribosomal DNA
T reg	T Regulatory cells
TCBZ	Triclabendazole
TCR	T Cell Receptors
Th	T Helper

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1. INTRODUCTION

Fasciolosis is a cosmopolitan parasitic infection caused by the digenetic trematodes *Fasciola hepatica* and *Fasciola gigantica*. *F. hepatica* has worldwide distribution due to its capacity to infect many different species and to the ability of the intermediate snail host to adapt to a wide range of ecological niches. *F. hepatica* mainly occurs in temperate areas whereas *F. gigantica* in tropical zones, and both species overlapping in subtropical areas. *F. gigantica* has a more restricted distribution due to the reduced ability of the aquatic snail intermediate hosts to invade new niches (Mas-Coma et al., 1999; Mas-Coma, 2004; 2005; Dorny et al., 2009).

Fasciolosis is one of the most important parasitic diseases in grazing animals with over 700 million production animals being at risk of infection (FAO, 1994; Dang and Nawa, 2005; Dorney et al., 2009; Nguyen et al., 2009). It is an economically important disease of sheep and cattle worldwide. The disease causes important economic losses in the animal husbandry, estimated at US$ 3 billion per year (FAO, 1994, Bennett and Ipjelaar, 2005; McGonigle et al., 2008; Spithill et al., 1999). These losses are attributable to mortality, reduction in milk and meat production, secondary bacterial infections, expensive anthelmintic treatment and condemnation of livers at slaughter. In some countries, up to 80-100% of ruminants are estimated to be infected with fasciola species, a significant constraint on productivity (Spithill et al., 1999a).

In Ethiopian highlands, sheep and cattle production has remained as an important sector of the country's agricultural economy where three fourth of the national sheep flock and a significant number of cattle are located in the highlands (Aleme and Lemma, 2015; Bekele et al., 1992;

Ngategize et al., 1993; Legese et al., 2014). However, their potential has been exploited far less than expected due to several constraints including shortage of forage, poor livestock management and diseases. Yilma and Malone (1988) suggested that fasciolosis is widespread in Ethiopia encompassing the major productive highland plateaus except very limited areas in arid escarpments. In affected areas lacking strategic and systematic parasite control and prevention measures, fasciolosis still continued as one of the most devastating animal diseases in sheep and cattle resulting in morbidity and associated mortality (Njau et al., 1988; Yilma and Mesfin, 2000; Legese et al., 2014). However, these have been exacerbated in highland regions particularly in sheep (Tekelye et al., 1992a; Tekelye et al., 1992b).

Apart from the altitudinal variations on the prevalence of fasciolosis, a study has demonstrated the differences in survival rate during fasciolosis among breeds of sheep. According to this study, the Ethiopian Menz sheep breed had shown superior survival rates, where other breeds have succumbed (Njau et al., 1989; Njau and Scholtens, 199). It is also recently recognized as important re-emerging zoonotic diseases of public health important (Mas-Coma et al., 1999; Mas-Coma, 2005). Despite the inconsistent reports of fasciolosis in central highland districts, the disease is considered as one of the most significant constraints on livestock production in Ethiopia (Bekele et al., 1992; Ngategize et al., 1993; Yilma and Malone, 1998; Biffa et al., 2006).

Ethiopia has possessed favorable climatic and ecological conditions for the development and spread of fasciolosis in various areas. The two species most commonly concerned as the etiological agents of fasciolosis in Ethiopia are *F. hepatica* and *F. gigantica* with their

distribution and prevalence associated with the presence of the snail intermediate host (Yilma and Malone, 1992; Yilma and Mesfin, 2000, Mas Coma, 2005)

In Ethiopia, the study of liver flukes in live animals depends on the detection of faecal eggs and the use of faecal egg counts. However, these detect only patent infections and their interpretation is constrained by the paucity of information about how they relate to parasite burdens and pathology. Information collected at abattoirs can be used for cross-sectional studies to compare animals' management and performance with enumeration of parasites and other indices of infection. Moreover, abattoir-based studies have been used as a component of the study of the liver fluke and to describe various aspects of liver fluke infection (Sargison et al., 2016).

Current recommendations for the control of liver flukes are based on strategically timed treatments with flukicidal drugs. The optimal timing of these treatments has been determined by studying the seasonal transmission dynamics of liver flukes in numerous locations throughout the world. Because of the long time required to complete the fluke life cycle, the window of opportunity to administer strategic treatments and still receive meaningful benefit is fairly large. To properly understand the rationale behind recommended control programs, reasonable deviations that can be made from these recommendations and issues pertaining to the economic impact of fluke infections in cattle, the complex life cycle of liver flukes needs to be appreciated (Kalpan, 2001).

Controlling fasciolosis by vaccination rather than chemotherapy would be a cheaper, more efficient and reliable long term solution for the prevention of infection and eradication of its transmission (Dalton and Mulcahy, 2001). A number of molecules including Cathepsin L, Glutathione-Stransferase (GST), Leucine aminopeptidase (LAP) and Fatty acid binding proteins (FABP) have the potency of inducing a protective response against fasciola species in laboratory and large animal models. The irradiation attenuated fasciola spp have been shown to induce protection to the host (Dalton et al., 1996; Dalton and Mulcahy, 2001; Jørgensen and Buchmann, 2011).

According to temperature and rainfall, the year in Ethiopia is divided into four seasons, namely long rainy (July–September), post-rainy (October–December), short rainy season (January–March) and dries (April–June). Studies carried out on fasciolosis around the world have determined that there exists a relationship between transmission and climatic factors (Malone et al., 1998). Microclimate can vary considerably from one region to another, from one farm to another or between neighboring open grasslands (Rangel-Ruiz et al., 1999). In addition, transmission of *F. gigantica* is known to depend on several factors related to the biology of the parasite, the vector and stock management (Malone et al., 1998).

Analysis of seasonal trends of *F. hepatica* infection in its final host is important since, this can establish the highest infection levels throughout the year. Most studies have been based on fecal examination, post-mortem surveys and immunological studies but were limited mainly to data on prevalence. This is important because consequences are not the same for a host parasitized by one parasite, a hundred or a thousand. In addition, *F. hepatica* can live for months or years in the

bile ducts producing eggs constantly. Since fasciola lives for more than 1 year in cattle, this complicates the determination of the major risk periods. Thus, new procedures have been developed or modified in order to obtain the necessary information to determine host infection intensity, its temporal variation and causal factors (Malone et al., 1998; Rangel-Ruiz et al., 1999).

Given the adverse impact of fasciola infection on animal and human health and its economic significance, rapid and accurate identification of fasciola species is necessary for successful clinical management of infection, and for epidemiological surveys (Mas-Coma et al., 1999; Mas-Coma et al., 2009). The occurrence of a disease is mainly affected among others by the presence of suitable snail intermediate hosts and seasonal variation on climatic factors. Despite persistent efforts, a vaccine with adequate protection against fasciolosis has not yet been developed to the point of commercialization (Dalton et al., 1996; Dalton and Mulcahy, 2001; Jørgensen and Buchmann, 2011). Moreover, there are few reports of irradiated vaccine trials on *F. hepatica* and the findings are not conclusive.

General objective

The general objective of the present study was to determine the epidemiology of ruminant fasciolosis and its vectors in selected endemic areas of Ethiopia and evaluate the immune response of sheep against primary infection with attenuating irradiating dose of *F. hepatica* metacercarae.

Specific objectives

- To establish the coprological prevalence of sheep and cattle fasciolosis and the associated risk factors (origin, age, sex, breed and body condition) in around Debreberhan, Central Ethiopia.
- To establish the abattoir prevalence of sheep and cattle fasciolosis and the associated risk factors (origin, age,sex, breed, body condition and altitude) in selected export and municipal abattoirs in Ethiopia
- To estiate the economic loss due to condemnation of fluke infected livers in selected export and municipal abattoirs
- To evaluate the immune response of sheep aginst primary infection with attenuating irradiating dose of *F. hepatica* metacercarae (assessment of parasitological, histopathological, enzymatic and immunological profiles)

2. LITERATURE REVIEW

2.1. General account on fasciolosis and fasciola

Fasciolosis is a parasitic trematode infection caused by liver flukes, *F. hepatica* (Linnaeus, 1758) and *F. gigantica* (Cobbold, 1856) belonging to the genus fasciola in the family fasciolidae (Dalton et al., 2003; Taylor et al., 2007). It affects mainly ruminants but also other animal species, such as horses and pigs. *F. hepatica* (also known as the common liver fluke or the sheep liver fluke) and *F. gigantica* are large liver flukes (*F. hepatica*: up to 30 mm by 15 mm; *F. gigantica*: up to 75 mm by 15 mm). According to the historical documents, *F. hepatica* has been known since 1379 (Reinhard, 1957; Grove, 1990). In addition to *F. hepatica* and *F. gigantica*, other minor species of the *Fasciola* have been reported, including *F. tragelaphi*, *F. jacksoni*, and *F. nyanzae* which are found in sitatunga-antelope, elephants and hippopotamus, respectively (Lofty et al., 2008). In tropical countries, fasciolosis is considered the most important helminth infection with reported prevalence of 30–90% (Dang and Nawa, 2005; Mas-Coma et al., 1999, 2005).

The disease causes important economic losses in the animal husbandry, estimated at US$ 3 billion per year, due to reduction in meat and milk production (FAO, 1994). It is considered as an economically important disease of sheep and cattle worldwide. In addition, the presence of hybrid and/or introgressed populations of liver flukes bearing genetic material from both *F. hepatica* and *F. gigantica* was demonstrated in both humans and animals. These “intermediate forms” that are thought to represent hybrids of the two species have been found in parts of Asia and Africa where both species are endemic. These forms usually have intermediate morphologic

characteristics (e.g. overall size, proportions), possess genetic elements from both species, exhibit unusual ploidy levels (often triploid), and do not produce sperm. Further research into the nature and origin of these forms is ongoing (Dang and Nawa, 2005; Le et al., 2007, 2008; Nguyen et al., 2009). Human infection is always associated with local endemic animal fasciolosis, although the distribution of human infection and level of prevalence may not always correlate with that observed in animals (Mas-Coma et al., 1999, 2005).

Inside a definitive host the site of infection may vary. Immature flukes undergo transient migration through the liver parenchyma and then settle as mature flukes in the bile ducts of their definitive hosts. In some (uncommon) hosts, aberrant flukes may be found encapsulated in lungs, skin or other organs. In snail intermediate hosts, several asexual multiplicative stages are formed; sporocysts first developing in tissues near the site of penetration (foot, antenna, gill), rediae then migrating to glandular tissue (hepatopancreas and gonads) and culminating in the release of tailed cercariae (Urquhart et al., 1996; Taylor et al., 2007).

2.2. Life cycle and infection

The life-cycle of fasciolosis is complex. It involves a final host (where the adult worm lives), an intermediate host (where the larval stages of the worm develop) and a carrier (entailing suitable aquatic plants). Adult flukes are hermaphrodites and reside in the liver and bile duct system of their definitive hosts and lay eggs that are passed out onto pasture in the faeces. A large proportion of an adult fluke's body consists of reproductive organs (Hanna et al., 2006) and each parasite has the potential to shed up to 25,000 eggs per day (Happich and Boray, 1969). These

are deposited in the host's faeces onto pasture and undergo embryonation in 9 to 10 days given warm (above 10^{o}C) and wet conditions (Schmidt and Roberts, 2005). After 2-3 weeks at these suitable conditions of temperature, humidity and oxygen tension, a free-living larval stage known as miracidium develops within the egg, hatches and migrates in thin films of moisture, actively seeking and penetrating an intermediate host, typically molluscs belonging to the genera Lymnaea (Galba), Pseudosuccinea, and Stagnicola (Rognlie et al., 1994; Moazeni and Ahmadi, 2016).

F. hepatica exhibits high intermediate host-specificity and will only develop in freshwater amphibious lymnaeid snails. These snails are pulmonate (with lungs), small (0.5-2.5cm long) and delicate; their shells being thin, fragile, lacking an operculum and the apertures located on the right-hand side (dextral). They live in freshwater and/or wet soils and survive dry periods by burrowing and aestivating. Various *Lymnaea* spp. are suitable intermediate hosts; the most common being *L. truncatula* in most continents, *L. tomentosa* in Australia, *L. viridis* in China, *L. columella* in the Americas, *L. viator* and *L. diaphena* in South America, and *L. bulimoides* in North America (Rognlie et al., 1994; Shubkin et al., 1992; Moazeni and Ahmadi, 2016). In Ethiopia, the intermediate host for *F. hepatica* stages has only been detected in *Galba* (*Lymnaea*) *truncatula* and *F. gigantica* only in *Galba* (*Radix) natalensis* (Yilma and Malone, 1998).

A ciliated miracidia can only survive for a few hours outside the snail. Miracidia actively seek snail hosts by chemotaxis, and must penetrate snail tissues within a few hours or die after 24 hours. Once the miracidia penetrate a snail, they form mother sporocysts that lack digestive organs but feed by absorption. Within the snail they undergo two further developmental stages,

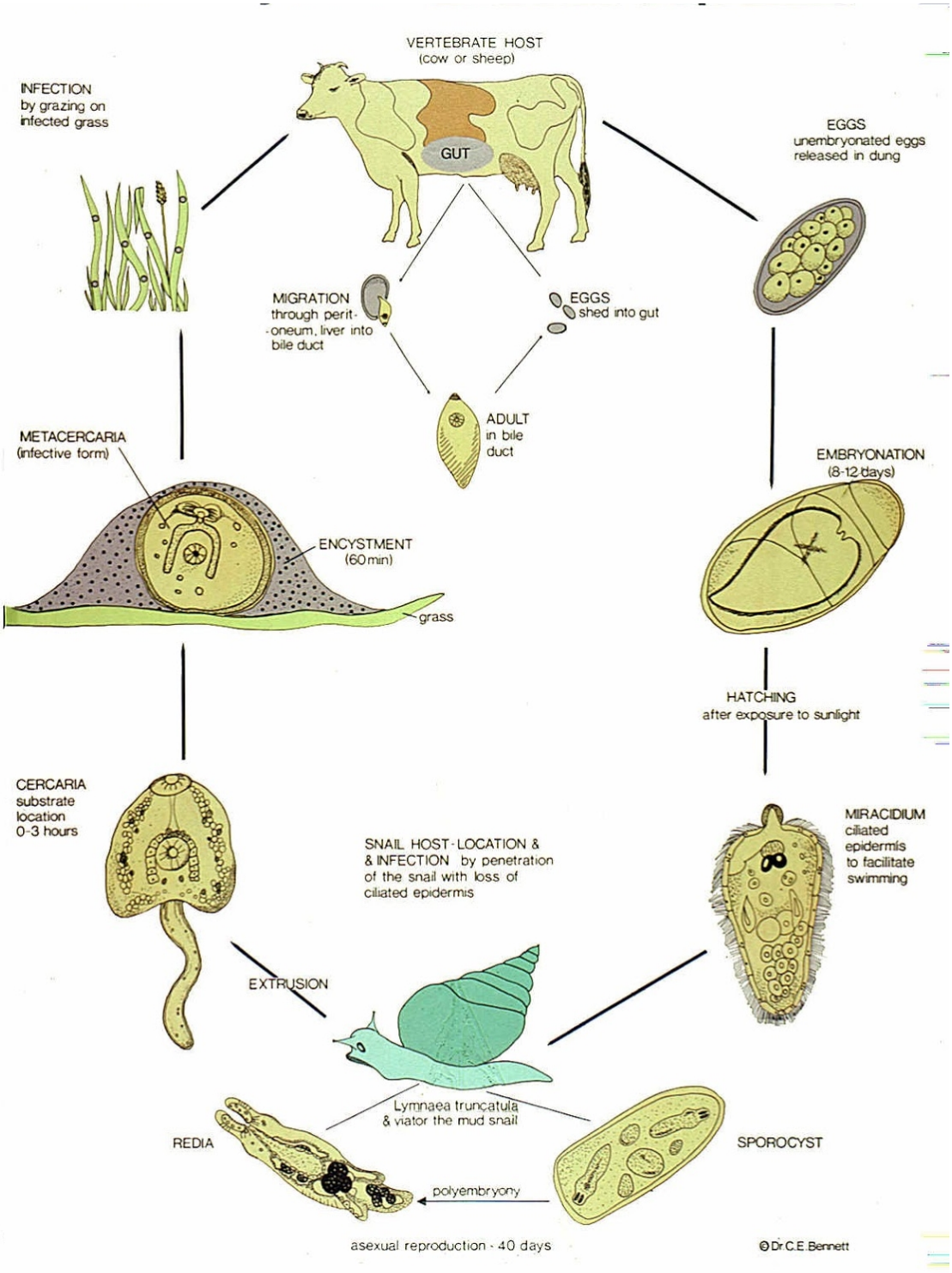
including multiplication, eventually becoming infective cercariae, which emerge from the snail when the temperature and moisture levels are suitable. The development and multiplication of the miracidia into sporocysts is followed by rediae, daughter rediae and finally cercariae. After penetration into the snail's body, the miracidium loses its ciliated covering, and forms a sporocyst. Sporocyst consists of a tightly packed mass of germinal cells within which each germinal cell multiply and produces a redia. Sporocysts multiply by asexual reproduction (an important amplification mechanism for all trematodes). Rediae have mouths and guts and feed on snail tissues, eventually maturing to single-tailed cercariae which bore their way out of the snail. Like the sporocyst, the redia is packed with germinal cells, which multiply and produce the final larval stage, the cercaria. The rediae grow until they burst the sporocyst wall and thus are liberated into the digestive gland (liver) of the snail (Graczyk and Fried, 1999; Moazeni and Ahmadi, 2016).

The cercaria has a long tail for swimming. It can also survive for months in aestivating snails buried in the soil during dry periods. The fully developed cercariae leave the snail 4-7 weeks after infection and several hundred (sometimes thousands) of cercariae may be produced. They swim freely in water and during a few minutes to 2 hour settle on various objects, mostly leaves of aquatic plants above or below the waterline. Subsequently each cercaria loses its tail and encysts to form a metacercaria, the highly resilient infective stage of the liver fluke which is almost immediately infective to the definitive hosts. Metacercariae are quiescent infective stages which can survive on aquatic vegetation or in water for several weeks. Emergent cercariae swim to suitable substrates and form encysted metacercariae by shedding their tails and producing thick cyst walls. Humans and other definitive hosts become infected after ingestion of infective

metacercariae on water plants and grass respectively or drinking water contaminated with the metacercariae (Andrews, 1999; Graczyk and Fried, 1999; Mas-Coma et al., 2014; Moazeni and Ahmadi, 2016).

When ingested, the metacercarial cyst wall is digested in the host's small intestine within an hour (Cheesbrough 2005). The Juvenile flukes penetrate the host's intestine wall, and appear in the abdominal cavity by about 2h. They then cross the peritoneal space and reach the liver within 4-6 days. The immature liver flukes migrate in the liver parenchyma between 5 and 6 weeks through which they tunnel, causing considerable tissue damage causing extensive haemorrhage and fibrosis. The flukes eventually reach the bile ducts, about 7 weeks after infection where they become sexually mature and begin laying eggs. From 8 weeks after infection, eggs are found in the bile and later in the faeces, thus completing the life cycle. In humans, at least 3-4 months are necessary for the flukes to attain sexual maturity. The lifespan of fasciola in humans may reach up to 13.5 years. They may remain for up to 1-2 years in cattle or as long as 20 years in sheep (Andrews, 1999; Mas-Coma et al., 2014; Moazeni and Ahmadi, 2016). The infection is patent about 10-12 weeks after the metacercariae are ingested. The whole cycle takes 18-20 weeks (Graczyk and Fried, 1999).

Figure 2.1. Life cycle of *Fasciola hepatica*



Adult flukes reproduce by cross and self-fertilisation in the bile ducts of the host whereas the immature stages are involved in asexual reproduction. The flukes survive for years in the liver and produce thousands of eggs that are passed out in faeces via the bile duct into intestinal tract and the cycle continues. It may take approximately 3 to 4 months for a fluke to develop into an adult and start producing eggs and lymnaeid snails act as the intermediate hosts (Miliotis and Bier, 2003).

The presence of *F. hepatica* depends on the factors that control the existence of intermediate host snails; the existence of appropriate habitats for the limnaeids and suitable environmental conditions, primarily moisture and temperature. To develop, the eggs must be separated from the fecal mass and need adequate temperature and humidity (Harris and Charleston, 1976).

2.3. Intermediate hosts

The intermediate hosts of fasciola are the amphibious freshwater snails of the family *Lymnaeidae*. *Fasciola* species have different affinities for different snail species, suggesting an old parasite-host relationship; *F. hepatica* with *Galba* (*Fossaria*) and *F. gigantica* with *Radix* (Bargues and Mas-Coma, 1997). This suggests a parallel evolution of each liver fluke species with different lymnaeid branches towards an increasing specificity (Bargues et al., 2001). The intermediate snail hosts of *F. hepatica* and *F. gigantica* differ in their environmental preferences, thus affecting their distribution and ultimately the distribution of the fasciola parasites.

2.3.1. Fasciola primary hosts

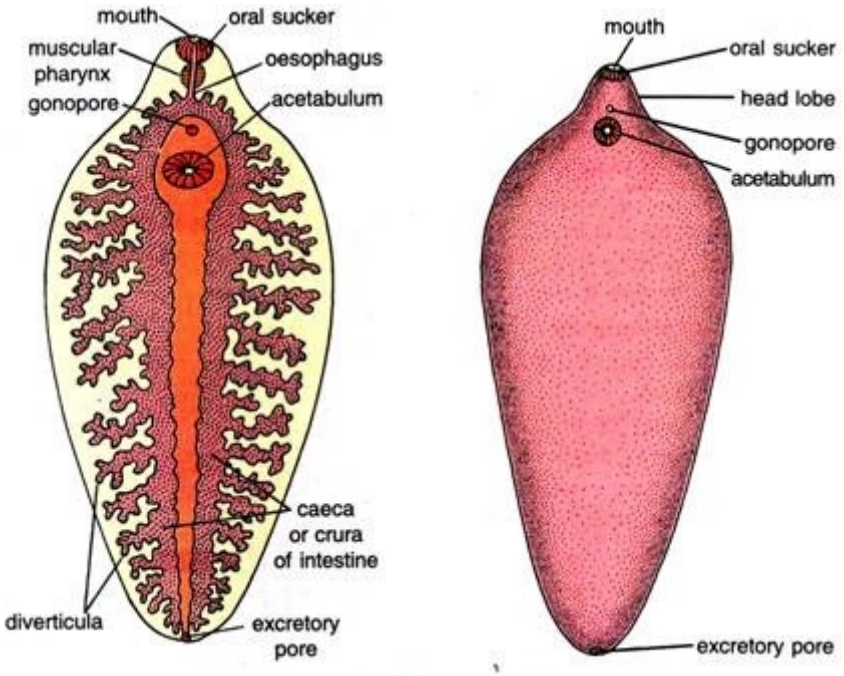
Fasciola has a wide range of main hosts. The principal hosts of *Fasciola* are generally ruminants such as cows, sheep and goats, however fasciola have shown an ability to expand from infecting important livestock to parasitising many animals indigenous. Principal hosts can also include various wild and domestic animals, including various marsupials, and humans (Mas-Coma, 1999; Spithill et al., 1999a; Torgerson and Claxton, 1999; Hurtrez-Bousses et al., 2001; Mas-Coma, 2005). *F. hepatica* has also been discovered in emus and rheas (Vaughan et al., 1997; Soares et al., 2007). Infestation levels of principal hosts vary among sites and among host species, as does the range and level of infection of host species in different regions.

2.3.2. Adult morphology and identification

These flatworms form seven different developmental stages: eggs, miracidia, sporocysts, rediae, cercariae, metacercariae, and adult flukes. The eggs are operculate (‘hatch’ at one end), brown and ovoid (130-150µm in length by 65-90µm in width). Miracidia are pyriform motile larval stages (150-200µm long) covered with cilia. Sporocysts are pleomorphic sac-like bodies (0.3-1.5mm in diameter) containing germinal cells which give rise to small rediae (embryos). Mature cercariae (0.5mm long) are free-swimming gymnocephalous stages with simple elongate club-shaped tails, which are subsequently shed when they encyst on vegetation to form membrane-bound metacercariae (0.2mm in diameter) (Urquhart et al., 1996, Fairweather et al., Fairweather and Boray,1999; 1999; Lotfy et al., 2002; Valero et al., 2005; Ashrafi et al., 2006).

The adult worm has a very characteristic leaf shape with the anterior end being broader than the posterior end and an anterior cone-shaped projection. Adult *F. hepatica* are similar to other flukes in that they possess a flat leaf-like body with a conical apex demarcated by wider 'shoulders', however they are one of the larger digenean parasites in the world. They generally measure 20-30 mm long by 8-13 mm wide and possess a tegument with backwardly directed spines. They are dorsoventrally flattened, the tegument is covered with scaly spines, and they have two suckers (distome arrangement with the oral sucker and acetabulum close together). The fluke possesses a powerful oral sucker at the end the anterior cone and a ventral sucker at the base of the cone which allow it to attach to the lining of the biliary ducts. The oral and ventral suckers are of approximately equal size, located on an anterior elongation known as a cephalic cone. Each worm possesses ovaries and testes which are highly branched and allow for individual flukes to produce eggs independently (Figure 2.1.2) (Urquhart et al., 1996, Fairweather et al., Fairweather and Boray,1999; 1999; Lotfy et al., 2002; Valero et al., 2005; Ashrafi et al., 2006).

Figure 2. 2. The digestive system of adult *Fasciola hepatica* and the lateral view



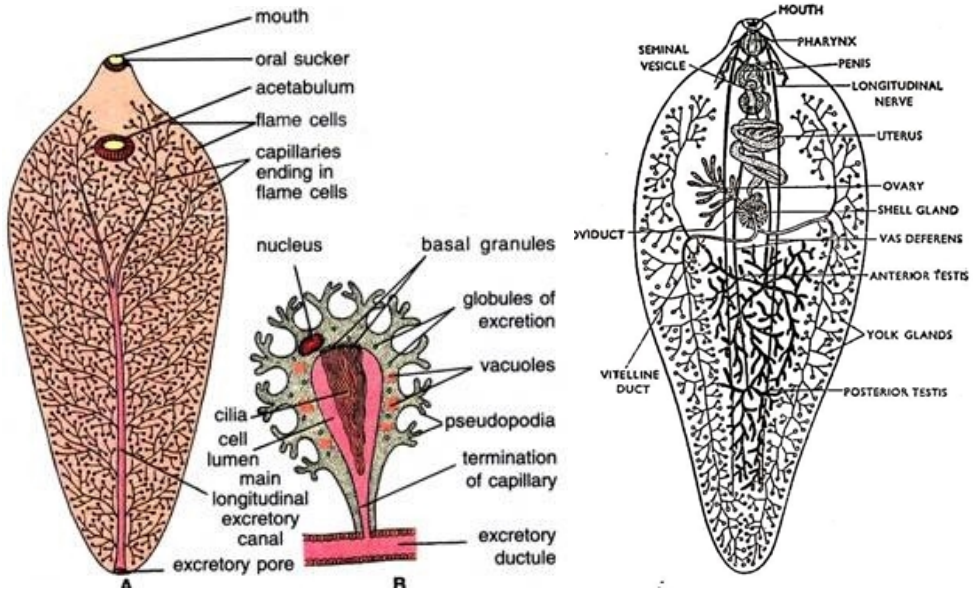
They have a bifurcate blind gut and each worm is hermaphroditic, possessing both male and female reproductive organs. The adult intestine is very highly branched, with numerous diverticulae extending from the extreme anterior to the extreme posterior of the parasite's body. Another distinguishing feature is their highly branched testes, located posteriorly and occupying almost the whole middle region of the body. The ovary, located above the testes, opens to a genital pore via a link to a short convoluted uterus. The genital pore is found above the ventral sucker. *F. hepatica* also possesses well developed vitellaria, highly diffuse glands that are

branched in the lateral and posterior region of the body (Fairweather et al., 1999; 1999; Lotfy et al., 2002; Valero et al., 2005; Ashrafi et al., 2006).

F. hepatica and F. gigantica are polymorphic with many factors affecting the morphology including age of the fluke, host species, and intensity of infection (higher intensity, smaller flukes), to mention just a few. Moreover, fixation of the specimen can have a profound effect not only on the absolute size of the fluke but also on the relative size of the various parts of the body that are used for identification (Stunkard, 1957; Kendall, 1965; Ternopolskaya, 1984, Lee and Zimmerman, 1993; Lofty and Hillyer, 2003).

Figure 2. 3. Fasciol hepatica, (A) Excretory system and flame cells (B) reproductive system

A B



2.3.3. Differentiation between species

There have been some taxonomical uncertainties regarding *Fasciola* species, particularly in Asia due to a range of morphological types (Kendall, 1965). Interspecific cross-hybridisation has been found within Korean (Agatsuma et al., 2000) and Japanese (Kendall, 1965) flukes, and molecular evidence of natural hybridisation between *F. hepatica* and *F. gigantica* has been reported (Agatsuma et al., 2000). This, along with the different intermediate and definitive hosts that *Fasciola* parasitise has led some researchers to suggest the possibility that subspecific or distinct evolutionary lineages may exist (Dosay-Akbulut et al., 2005) and has lead to certain species being described that are probably synonyms of *F. hepatica* and *F. gigantica*. As a result of problems associated with differentiation between *Fasciola* species using morphological, chromosomal and biochemical techniques a large component of the molecular research conducted on *Fasciola* has been applied to the problem of identification (Nolan and Cribb, 2005; Ai et al., 2011).

The differential diagnosis between *F. hepatica* and *F. gigantica* infection is very important because of their different transmission and epidemiological characteristics. *F. hepatica* and *F. gigantica* can generally be distinguished based on their morphology. However, it is usually difficult to discriminate accurately between *F. hepatica* and *F. gigantica* because of the high variations in their morphological characteristics. The two species can be discriminated by DNA sequences of nuclear ribosomal internal transcribed spacer 1 (ITS1), ITS2, and 28S rDNA regions and of mitochondrial NADH dehydrogenase I (ND1) and cytochrome C oxidase I (CO1)

genes. Intraspecific genetic variations among liver flukes may reflect differences in virulence, host specificity and drug susceptibility or resistance (Nolan and Cribb, 2005; Ai et al., 2011).

2.4. Pathogenesis

Infections have been associated with two types of liver disease in domestic animals: acute or subacute necrotic disease due to juvenile flukes; and chronic fibrotic disease due to adult flukes. Penetration of the liver capsule by immature flukes generally does not cause much damage, but their subsequent migration through the liver parenchyma may cause significant necrosis (liver rot). Mass migration of juveniles may produce extensive traumatic tissue damage, coagulative necrosis, haemorrhage, urticaria, eosinophilia, leukocytosis, pallor, anaemia, and can be fatal. Acute infections in sheep can also be complicated by secondary bacterial infection causing clostridial necrotic hepatitis ('black disease'). Chronic infections by the long-lived adults feeding on the lining of the bile ducts may result in progressive loss of condition, biliary epithelial hyperplasia, duct fibrosis, biliary obstruction and cholangitis, jaundice, and eventually a fibrotic hardened liver. Sheep may become anaemic and emaciated, developing submandibular oedema (bottle-jaw) and ascites. In cattle, the bile ducts often become calcified producing a 'clay-pipe' or 'pipe-stem' liver. Chronic fascioliasis causes significant economic losses to many animal industries through mortality, reduced meat, milk and fibre production, condemned livers, secondary infections and expensive treatments (Urquhart et al., 1996, Fairweather et al., 1999; 1999; Lotfy et al., 2002; Valero et al., 2005; Ashrafi et al., 2006; Rojo-Vázquez et al., 2012)

The clinical features and the lesions caused by *F. hepatica* are related to host species, the number of ingested metacercariae and the duration of challenge, nutritional status and metabolic

demands, such as advanced pregnancy. The damage to the host is due to mechanical and chemical effects and the host inflammatory and immune responses. The mechanical effects are associated to the migration of the juveniles through the liver and are accomplished with the aid of the sharp spines of the fluke tegument and protelytic enzymes secreted by the parasite. Some chemical products, such as cathepsins, also play an important role in the pathogenesis of the infection. The infection is associated with a strong Th2 response, which in turn is connected with the production of cytokines contributing to the overall pathophysiological pattern. The severity of the infection may be reduced in animals with previous experience, in which the result is a decrease in helminth size and egg production, a delayed onset of anaemia, an earlier increase in the number of eosinophils and lymphocyte infiltration into the liver (Rojo-Vázquez et al., 2012).

Many of the early studies on *F. gigantica* and *F. hepatica* assumed that infection with the former was essentially similar to that of *F. hepatica*. However, based on fluke biomass, average fluke size, degree of liver damage, plasma glutamate dehydrogenase (GLDH) levels and gamma glutamyl transpeptidase (GGT) responses during the first 10 weeks of infection, *F. hepatica* develops more rapidly than *F. gigantica*, resulting in increased plasma levels of GLDH, indicating greater damage to the liver parenchyma (Boyd, 1962; Meeusen et al., 1995) and an increase in the GGT levels at 10 weeks post-infection, indicating epithelial damage in the bile duct (Chauvin et al., 1995; Martínez-Valladares et al., 2010b).

The greater damage to the *F. hepatica* infected sheep was also evident by the subjective scoring of greater lesions in the livers and the compensatory hypertrophy of the liver, leading to a significant increase in the relative liver weights and the decrease in haemoglobin levels and

packed cell volume by 9 weeks post-infection (Chauvin et al., 1995; Martínez-Valladares et al., 2010b). *F. gigantica* remains in the liver for 12–14 weeks post-infection and only then migrates to the bile ducts (Spithill et al., 1999).

The eosinophilic response following *F. hepatica* infection is biphasic with an initial peak at 4 weeks post-infection (migratory phase) and a second peak 9 weeks post-infection and an increase of the GGT levels by 10 weeks post-infection (epithelial damage in the bile duct). This response is similar to that observed in sheep infected with *F. gigantica* (Roberts et al., 1997; Hansen et al., 1999; Zhang et al., 2005).

Few studies have been carried out to compare the impact of *F. gigantica* and *F. hepatica* parasitism on production, feed intake and host responses. Some authors showed that high doses of both *F. gigantica* and *F. hepatica* were able to induce similar effects on production, despite the greater damage caused by *F. hepatica* infection suggesting that production losses are not only related to fluke biomass and liver pathology; rather, there is a parasite threshold above which production losses are comparable between *F. gigantica* and *F. hepatica* (Rojo-Vázquez et al., 2012).

2.5. Clinical features

Clinical signs are closely associated with the severity of the disease which is determined by the level of infection, nutritional plane of the animal and also on the individual host species and breed (Behm and Sangster, 1999). Fasciolosis can be classified as acute, sub-acute or chronic

(Urquhart et al., 1996, Valero et al., 2005; Rojo-Vázquez et al., 2012; http://parasite.org.au/parasite/text/fasciola-text.html).

2.5.1. Acute Fasciolosis.

Acute fasciolosis is not common, but occurs in sheep. Animals with acute fasciolosis most often do not show any clinical signs and animals are found dead, even in good apparent bodily condition. Acute and subacute forms of fasciolosis develop in 2–3 weeks after massive infections of a large number of metacercariae over a short period of time, resulting in the massive invasion of the liver parenchyma and haemorrhaging due to migrating young flukes and signs include anorexia, abdominal pain, yellowish and pale conjunctivae, weight loss and sudden death. Heavy infestations by immature flukes may cause death in the stage of acute hepatitis. A close observation of the flock allows identification of some animals with pale mucosae, lethargic, dyspnoeic when moving, liver enlargement detected by abdomen palpation and ascites. The only means of diagnosis is by postmortem, parasitological and blood examination. This form of the infection is frequently complicated by simultaneous infection with Clostridium oedematiens B resulting in 'Black' disease (Boray, 1969; Haroun and Hillyer, 1986; Torgerson and Claxton, 1999; Piedrafita et al., 2004; Rojo-Vázquez et al., 2012).

2.5.2. Subacute fasciolosis

Subacute fasciolosis occurs after 6–10 weeks of the ingestion of lower doses of metacercariae over a longer period of time and reflects liver damage by the still migrating immature flukes and also adult flukes in the bile ducts where they cause cholangitis and it also appears in late autumn

and winter. It is not so rapidly fatal as the acute form, so many animals in the flock show clinical signs during 1–2 weeks before death, such as ill-thrift, lethargy and loss of condition. The examination of animals demonstrates anaemic mucosae and palpable liver enlargement. Elevated levels of the serum enzymes glutamate dehydrogenase and gamma glutamyl transpeptidase, are indicators of damage to the liver parenchyma and bile ducts, respectively, it is an expected outcome with migrating fluke. Moreover with protracted infection, anaemia would be indicated by a lowered packed cell volume (Valero, et al., 2008; Rojo-Vázquez et al., 2012)

2.5.3. Chronic Fasciolosis

The chronic fasciolosis is seen in late winter and early spring and is the most common form of the disease. It occurs after 4–5 months of ingesting a low/moderate number of metacercariae nearly throughout the whole year, but especially during autumn and winter. The infection is more severe in undernourished animals or when the animals' requirements are higher, as in late pregnancy or lactation and results in a progressive loss of condition. The main pathogenic effects are anaemia and hypoalbuminaemia, which can result in emaciation, paleness of the mucous membranes, submandibular oedema (bottle jaw) and ascites. Production losses can be economically significant even in relatively light fluke infections (Rojo-Vázquez et al., 2012). Naturally infected sheep may show decreased red blood cell counts and increased leucocyte counts. The gamma glutamyl transpeptidase activity is higher than the reference values, remaining high during even 4 weeks despite anthelmintic treatment (Martínez-Valladares et al., 2010a).

Specific hyperimmunoglobulinaemia is a common feature in fasciolosis (Nansen et al., 1975). From 2 weeks post-infection, serum IgG and IgA anti-*F. hepatica* titres reach maximum values at 8 weeks p.i., decreasing slowly thereafter (IgA levels were lower than IgG levels). Specific IgG and IgA titres in bile followed a similar pattern to serum immunoglobulins, but reaching maximum values at 14 weeks post-infection (Ferre et al., 1997); however, the levels of bile specific immunoglobulins were considerably lower than the serum titres during the whole experimental period. When the IgG/IgA ratio was calculated, the proportion of IgA relative to IgG was slightly higher in bile than in the serum (Ferre et al., 1997).

2.6. Distribution

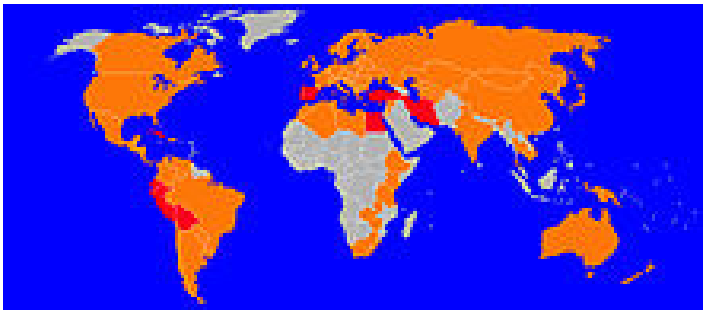
Fasciola are dependent on a consistent set of suitable environmental conditions to survive. They require acceptable moisture and temperature conditions both for the intermediate host and for their own growth and development. *F. hepatica* has a cosmopolitan distribution, while *F. gigantica* is largely confined to the tropics. While both species are endemic to many countries, *F. hepatica* often predominates at higher altitudes, due to its lower temperature requirements (Mas-Coma et al., 1999a; Mas-Coma et al., 1999b; Mas-Coma, 2005).

Fasciolosis has a great capacity to spread to new areas, due to the colonising ability and introduction of intermediate-host snail species, and the parasites' ability to infect a large range of primary hosts. Changes in climate can also introduce Fasciola to new areas (Fairweather and Boray, 1999).

There are human endemic areas where fasciola infection is regarded as a serious health problem, particularly in South America (Mas-Coma et al., 1999a; Nithiuthai et al., 2004). Ecologically, human infection occurs most frequently in sheep- and cattle-raising regions (Bogitsh et al., 2005). However, there is only a very basic correlation between human and livestock infection, and high or low prevalence do not seem to be related between humans and animals (Esteban et al., 1997; Mas-Coma et al., 1999a; Mas-Coma et al., 1999b; Mas-Coma, 2004).

F. hepatica is widely distributed in Europe, the Americas, Asia, Australia, New Zealand and some North African countries, as well as highlands and cooler zones of Ethiopia, Pakistan, Kenya and South Africa. *F. gigantica* is present in southern Europe, south and south-east Asia, South America and wide-spread in most of Africa, except for some arid zones.

Figure 1.4. *Fasciola hepatica* prevalence:



The countries in red are those with high prevalence, while those in orange have low-medium prevalence (Source: Cywińska, 2005; Mas-Coma, 2005; McManus and Dalton, 2006).

2.7. Transmission

In many areas of the world there are yearly cycles of livestock infection, with disease outbreaks usually relating to peak pasture contamination by metacercariae. This is not always the case however, and continuous contamination of pastures can occur. Both domestic and wild animals act as reservoir hosts, with the range of hosts infected differing strongly among regions. Sheep, cattle and goats are generally the predominant animal reservoirs. Humans can also play an important role in transmission of the parasite, at least in hyperendemic areas (Mas-Coma et al., 2005).

Human infections occur through ingestion of contaminated watercress and other plants associated with water and eaten raw in salads. Water has also been cited as a source of human infection through direct drinking or contamination of food or utensils (Mas-Coma et al., 2005). Experimental evidence in mice and pigs also suggests that people who consume raw dishes prepared from fresh livers infected with immature flukes can also become infected (Taira et al., 1997).

2.8. Diagnosis of fasciolosis

In general, the diagnosis of a disease is not an easy task; however, combining of signs, symptoms and test results, the clinician attempts to determine the correct diagnosis. Nevertheless, the diagnosis of fasciolosis can be performed by combining and analysing the observation of clinical signs and diagnostic imaging, biopathological studies, such as blood parametres and enzyme

tests, results of laboratory analysis of faeces (parasitological methods), immunodiagnosis, a post-mortem examination of the liver and PCR based molecular examination of samples.

2.8.1. Blood parameters and enzyme tests

Levels of some hepatic enzymes are useful for diagnosis of the infection, but their value depends on the sensitivity, specificity and stability in the plasma (Rowlands and Clampitt, 1979). Diagnosis can be confirmed by serum parameters such as increase in the level of γ-GT and GLDH and the number of eosinophilic leukocytes. However, these parameters may vary according to the stage of the infection and are not always specific. In the subacute form of fasciolosis, there is an increase in liver enzyme concentrations, altered serum protein concentrations, and reduced liver weight gain of up to 15 kg over 40 weeks (Sykes et al., 1980b).

Blood samples reveal elevated concentrations of aspartate aminotransferase, glutamate dehydrogenase and γ -glutamyltransferase. There is a marked hypoalbuminaemia (15 g/L) and hyperglobulinaemia (68 g/L). The increase of the glutamate dehydrogenase is indicative of the liver damage by migrating flukes; when the parasites reach the bile ducts, the activity of the biliary epithelial enzyme γ-glutamyltransferase is at its highest level. In consequence, if no other parameters are available, the rise in these two enzymes (glutamate dehydrogenase and γ-glutamyltransferase) is indicative of acute and subacute/chronic fasciolosis, respectively. Some other tests, such as the liver function bromosulphoftalein and the plasmatic clearance of the antipyrine are also of value (Rowlands and Clampitt, 1979; Sykes et al., 1980b).

2.8.2. Parasitological examinations

The presence of eggs in faeces is the best way to know if a host is harbouring flukes. Coprological analyses are of limited value in newly infected animals. In the acute form of fasciolosis, faecal tests are negative and can also be negative in subacute infections. On the other hand, the detection of fluke eggs in the faeces of affected animals is a valuable diagnostic method in chronic fasciolosis. The pre-patent period is 8–10 weeks depending on the host species; hence, egg counts are only useful from about 8-week post-infection onwards. In addition, other factors such as host age, faecal water content and the number of aliquots tested per sample, can all affect the sensitivity of the faecal egg count (AlvarezRojas, et al., 2014). False positives may occur due to the retention of eggs in the gall bladder for at least 2 weeks after successful treatment (Flanagan et al., 2011).

Many techniques have been described from simple methods (direct faecal analysis) to sophisticated quantitative tests, trying to concentrate the eggs from a faecal sample by means of either sedimentation or floatation techniques. Floatation methods use high density solutions, like zinc sulphate and potasium iodomercuriate that allow the eggs to float, but collapse them, thus making the identification difficult. The sedimentation technique is the recommended and the most common technique, because fluke eggs are rather heavy compared with the eggs of gastrointestinal nematodes, and do not float in flotation media such as saturated NaCl. Fluke eggs can be recognized by their color and shape. Although the specificity of the sedimentation technique is high one drawback is its relatively low sensitivity, only about 70%. The fasciola eggs may confuse with the eggs of paramphistomes, although these eggs are generally slightly larger and greyish in color. Coprological sedimentation methods are well established in routine

diagnostic laboratories, and methods such as FLOTAC (Cringoli et al., 2010) and Flukefinder (Foreyt, 2001) are available.

The observation of the typical fluke eggs under the microscope is facilitated by adding a stain (methylen blue) to the concentrate, in which fasciola eggs have a yellowish colour. Egg counts from 100 to 200 epg (eggs per gram) are indicative of an active and severe infection requiring the use of a flukicide. The number of eggs decreases as intensity of infection increases but this density-dependent mechanism is lack of relationship above a given infection level (Rojo-Vázquez et al., 2012).

2.8.3. Immunodiagnosis

The most commonly used technique for immunodiagnosis of fasciolosis is an ELISA test using secretory/excretory antigens for antibody detection. Improved ELISA tests have a high sensitivity and specificity, but its usefulness is greater at the flock level in naturally infected animals than in individual diagnosis of the infection (Pfister, 1990). Both antibody- and antigen-based enzyme-linked immunosorbent assays (ELISA) are available to demonstrate the presence of liver fluke infection in serum/milk or feces, respectively. These ELISAs use a range of native, purified fractions, or recombinant antigens with sensitivities ranging from 86 to 100% and specificities from 83 to 96%. Antibodies can be detected from 2 to 4 weeks after infection, but antibodies do persist for some time after treatment, and therefore only indicate that the animal has been exposed to the parasite at some time point. Detection of *Fasciola* antibodies in bulk-tank or individual milk samples, if performed regularly, about 3–4 times a year, can provide

valuable information on infection level and the efficacy of control programs within a dairy herd. Some tests are commercially available for the diagnosis of fasciolosis during the prepatent period by means of the detection of coproantigen test, detecting the infection as early as 5 weeks post infection (copro-antigen ELISA detects *F. hepatica* antigens in feces about 2–4 weeks before eggs are detected in feces). The detection of *F. hepatica* infections during the prepatent phase of the infection can be carried out by means of a sandwich ELISA allowing the identification of active infection before egg excretion, but are also of value for epidemiological surveys (Sánchez-Andrade et al., 2000). However, some inconsistent results have been obtained that are difficult to explain, because the antigen is known to not cross react with nematodes, cestodes or other trematodes (Mezo et al., 2004).

2.8.4. Molecular diagnosis

The molecular detection of fluke infections can be achieved through the PCR-based amplification of specific DNA sequences. The identification of suitable target sequences for species specific PCR primers, as well as the molecular differentiation of fluke isolates from varying geographic regions has been achieved through the genetic characterisation of ribosomal gene sequences: the internal transcribed spacer (ITS) (Nguyen et al., 2009; Capuano et al., 2007) regions, and mitochondrial gene sequences: the cytochrome C oxidase subunit I (COI) (Farjallah et al., 2009). Alternatively, instead of sequencing distinct genes for development of species specific PCR primers, McGarry et al. (2007) used sequences information from Randomly Amplified Polymorphic DNA (RAPD)-PCR generated fragments to obtain a method reliable for the molecular differentiation of *F. hepatica* and *F. gigantica* DNA. The application of sequence-

related amplified polymorphism was shown to be suitable for detection of genetic variability in *F. hepatica* derived from different host species and geographic regions (Alasaad et al., 2008).

In an earlier study, also employing RAPD-PCR, inter- and intraspecies variations were demonstrated between different isolates of *F. hepatica* and *F. gigantica* obtained from cattle and sheep (Ramadan and Saber, 2004). Furthermore, by digestion through a suitable restriction enzyme PCR-amplified, ribosomal DNA was cut into fragments of characteristic length, allowing the unequivocal delineation of Fasciola isolates from different regions in China (Huang et al., 2004) or Iran (Rokni et al., 2010). In a similar approach, the 28 s ribosomal RNA gene sequence was amplified and restricted to develop a species specific assay for the differentiation of *F. hepatica* and *F. gigantica* (Marcilla et al., 2002). The use of mass spectrometry analysis allowed identification of *F. hepatica* infection associated biomarkers in sheep serum and the characterisation of their expression profile during different phases of the infection (Rioux et al., 2008).

It is hoped that this technological approach will contribute to an improved understanding of parasite-host interactions and diagnostics of fluke infections. Moreover, the sequencing and bioinformatic analysis of the liver fluke transcriptome (Young et al., 2010) will certainly foster the development of future diagnostic methods for the detection of liver fluke infections in sheep and the assessment of the clinical, immunological and pathological effects.

2.8.5. Postmortem examination

Infection can be also confirmed at necropsy and many farmers use abattoir returns to identify if *F. hepatica* is present in their livestock (Mazeri et al, 2016). Diagnosis of fasciolosis after slaughter is relatively easy. The lesions caused by the flukes are typical (fibrosis and cholangitis) and cannot be confused with other infections of the liver (larval tapeworms). Heavily affected livers will be condemned and are excluded for human consumption. Apart from the already mentioned weight loss and reduced milk yield, this forms another important source of economic loss. The acute fasciolosis can only be confirmed by necropsy. The affected animals show a focal hepatic fibrosis, enlargement of the liver with haemorrhagic tracks and numerous flukes in different developmental stages throughout the liver parenchyma (Murray and Rushton, 1975). Sporadically, flukes can also be found in the peritoneum and, less commonly, in the spleen, the pancreas and the lungs. Postmortem examination can confirm severe liver damage and widespread adhesions between liver, abdominal wall, diaphragm and small intestine with immature flukes in the liver (Murray and Rushton, 1975; Rojo-Vázquez, et al., 2012).

The subacute form is characterised by liver hypertrophy and bloody tracts and also a large number of flukes (500–1500), both juveniles and adult flukes. The liver shows irregular fibrosis extending from the capsule into the parenchyma, with prominent bile ducts and multifocal abscessation (Mazeri et al, 2016; Rojo-Vázquez, et al., 2012).

Animals suffering from chronic fasciolosis show a deterioration of the carcase, cholangitis, biliar occlusion and hepatic fibrosis, and the average number of flukes are around 200, most of them

found in bile ducts. Besides the liver, other organs and structures can be found damaged, such as periportal and mesenteric lymph nodes that are markedly enlarged and exhibit a brownish colour. Definitive diagnosis should consider other disorders. For instance, acute fasciolosis must be differentiated from clostridial disease (pulpy kidney disease, black-leg, black disease and braxy), pasteurellosis and other septicaemic diseases, poisoning and acidosis fed grain. Differential diagnosis of the chronic form include cobalt and copper deficiencies, poor nutrition, and other helminth infections of sheep such as gastrointestinal nematodes (haemonchosis), tuberculosis, scrapie and other chronic wasting conditions (Rojo-Vázquez, et al., 2012).

2.9. Control and treatment of Fasciolosis

Fasciolosis is difficult to control for a number of reasons, some of which include; amplification of the intermediate stages of the parasite within the snail host which rapidly creates high levels of infective cysts on pasture, a lack of natural immunity to infection in the definitive host and the presence of wildlife reservoirs (Andrews et al, 1999). However, it can be controlled by using a combination of strategies in order to reduce heavy pasture contamination and to an extent prevent definitive host contact with the infective stages (Brunsdon, 1980).

Much of the literature on the control of *F. gigantica* is based on successes and failures of methods used in temperate countries to control *F. hepatica*. These include strategic anthelmintic treatment, grazing management, application of molluscicides, and fencing off or draining swampy areas. The relevance of these approaches to control of *F. gigantica* in the tropics must be questioned and control options developed on the basis of a sound understanding of the

transmission of *F. gigantica* in tropical production systems. Relatively little is known about the epidemiology of *F. gigantica* and this hampers the development of control strategies for tropical production systems in areas where infection with *F. gigantica* is endemic. These are often areas where irrigated rice is cultivated intensively. Although regional recommendations have been made for some developing countries (FAO, 1994), in most areas of tropical Asia there are no routinely used control programs for fasciolosis (Spithill et al. 1999).

2.9.1. Pasture management

The intermediate stages of *F. hepatica* develop within the mud snail *G. trunculata*. These snails inhabit wet and marshy environments. These areas are likely to harbour high levels of the infective metacercariae after they exit the intermediate host. Wet areas of fields can be fenced off to prevent contact of the host with infective stages or drained to make them less habitable for the snails, thus reducing the number of infective metacercariae produced. However, this is a costly method and it is not always feasible or practical (Kendell, 1951; Wilson et al., 1982; Urquhart et al., 1996; Taylor et al., 2007).

2.9.2. Snail control

Molluscicides, aiming to kill the snail intermediate host, have provided successful short-term control of infective stages present on the pasture and have proven cost effective in the past (Crossland, 1976; Urquhart et al., 1996). However, they have gained little support and are now considered to be environmentally/ecologically unacceptable (Wilson et al., 1982). Despite being

very effective against the snails they are nonspecific and often kill other species including fish and crabs (Roberts and Suhardono, 1996; Taylor, et al., 2007). In addition, there are now regulations which restrict the use of chemicals such as molluscicides on pastures. Furthermore, their effect is often short lived as the snails, being hermaphrodite, have a huge potential for rapid repopulation once molluscicides clear from the environment and can therefore repopulate an area in a short time (Roberts and Suhardono, 1996).

Snails are naturally predated by arthropods, amphibians, reptiles, birds and rodents (Torgerson and Claxton, 1999). Normally they exist in equilibrium with their predators, rapidly increasing when conditions are favourable (Torgerson and Claxton, 1999). The intensive farming of ducks and geese has been shown to eradicate snails from pastures but this method is, again, not always feasible.

2.9.3. Treating infected animals

A series of chemotherapeutic agents are available for the treatment of animal fasciolosis, including closantal, clorsulan, rafoxanide, nitroxynil and triclabendazole (Urquhart, 1996; Dalton et al., 2003; Taylor et al., 2007; Fairweather & Boray, 1999). The major flukicide, triclabendazole is efficient against both juvenile flukes in the hepatic parenchyma and adult flukes in the bile ducts. However, triclabendazole resistant parasites have been reported in Europe and Australia also in many parts of North America and Africa. Most importantly, government and consumer concern about chemical and antibiotic residues in animal foods (milk and meat) and the environmental threat from chemical and pesticide (Molloscocide) use on

pastures. Chemicals runoff into water supplies will make the chemical control of fasciolosis more difficult in the future (Dalton et al., 2003).

2.10. Immune response against *Fasciola hepatica*

2.10.1. The innate immune response against *Fasciola hepatica*

The innate immune system plays an important role in the defense against *F. hepatica* infection and also in priming the adaptive immune response. Innate effecter mechanisms elicited upon infection include rapid eosinophilia and macrophage activation. Eosinophilia is a pronounced, yet controversial, characteristic response to most helminth infections. Studies using murine hosts deficient in eosinophils fail to show overall differences in helminth parasite burdens (Betts and Else, 1999; Swartz et al., 2006). In cattle infected with *F. hepatica*, elevated eosinophil counts were found 4 weeks post infection of and persisted over a 16-week period (Bossaert et al., 2000), while the presence of biphasic eosinophilia has been demonstrated in sheep (Zhang et al., 2005), occurring at weeks 4 and again at 9–10 post-infection. Elevated eosinophil counts in the lamina propria of gut loops of immune rats have been correlated with protection in an ex vivo model (Van Milligen et al., 1999).

A recent review of this topic suggests that eosinophils may target larval Trichinella spiralis during secondary infection via antibody dependent cellular cytotoxicity (Bruschi et al., 2008). It is becoming increasingly apparent that the alternative activation of macrophages during helminth infection is a cornerstone of helminth immunology (Martinez et al., 2009). Studies have shown

that *F. hepatica* is capable of generating alternatively activated macrophages (AAMF) both in vitro and in vivo (Donnelly et al., 2005; Flynn et al., 2007a). However, they may be involved in priming Th2 cell differentiation or indeed they may act as suppressor type cells given their high IL-10 expression (Flynn and Mulcahy, 2008a). A strong correlation can be made between the presence of these cells, with suppressor capacity, and susceptibility during secondary infection (Flynn *et al*., 2007b). Evidence for their role in priming Th2 cell differentiation comes from a recent study whereby *F. hepatica* primed MF were used to preferentially drive Th2 cytokine secretion from CD3 stimulated naive T-cells (Donnelly et al., 2008). Recently a role for AAMF derived IL-10 during fibrosis and parasite attrition has been proposed (Haçariz et al., 2009). Other models of infection have already confirmed a role for AAMF in fibrosis (Loke et al., 2007) and protection from immunopathology (Herbert et al., 2004). Furthermore AAMF have been associated with a protective memory response (Anthony et al., 2006), and the recent results from Donnelly et al. (2008) might be indicative of a similar function within *F. hepatica* infection.

2.10.2. Cell-mediated immune response

T-cell activation: the precise nature of T-cell activation and the generation of an adaptive T-cell response while being paramount to effective containment of any infection are relatively unexplored in the context of helminths. Traditionally antigen presenting cells (APC) such as dendritic cells (DC) and MF have been thought to contribute to this process involving APC:T-cell interactions and antigen presentation via MHC Class II. Work on the involvement of DC in helminth infection has to date failed to show a definitive role for these cells in Th2 differentiation (Wynn, 2009).

Recent murine models of nematode infection have revealed a previously unappreciated role for basophils in the activation of CD4+ Th2 cells. Independently two groups demonstrated that basophils can present antigen and secrete the IL-4 necessary to drive Th2 cell priming (Wynn, 2009). While basophils are recognised in ruminants, their functions are unexplored. Interestingly a negative effect of *F. hepatica* extracts on DC in vitro DC's has been noted. Stimulation of DC with *F. hepatica* tegument antigen (TEG) has been shown to downregulate activation markers and cytokine production of lipopolysaccharide (LPS)-stimulated cells (Hamilton et al., 2009).

The involvement of pathogen associated molecular patterns in the activation of the immune system has only begun to be explored in *F. hepatica* infection. The parasite generates a large number of secreted molecules and these have a variety of effects within the host. However, specific molecule/receptor interactions have yet to be identified. Possible evidence for the involvement of Toll-like receptor 2 was described by using *F. hepatica* ES to inhibit the activation of macrophages by purified protein derivatives from Mycobacterium bovis (Flynn and Mulcahy, 2008a).

2.10.3. Humoral immune response

Within 4 weeks of infection an adaptive B cell response develops with the generation of parasite-specific antibodies. Studies have shown that antibody subclass involvement is dominated by IgG1 as serology conducted to date has shown poor IgG2a and IgM responses along with transient IgA in infected animals (Clery et al., 1996). This finding is supported by evidence from

studies using infected sheep where IgG1 was the dominant isotype (Raadsma et al., 2007). And as is typical in Th2-mediated immune responses studies have shown the generation of antibody responses to be reliant on IL-4 (O'Neill et al., 2000). Also of note is the existence of a correlation between IgG2a and evidence of protection against infection, this has been noted in a number of cases and situations. Using vaccination with native fluke derived antigens protection as judged by reduction in parasite burdens were correlated with increased parasite-specific IgG2a titers and avidity (Mulcahy et al., 1998; Raadsma et al., 2007).

2.11. Cytokine regulation of host immunity

Early IFN-g production has been reported in mice (O'Neill et al., 2000), cattle (Flynn and Mulcahy, 2008b), and sheep (Moreau et al., 1998). This early expression of IFN-g would suggest a Th1/Th0 type response changing to Th2 as infection establishes. Studies of lymphocyte proliferation also show that responsiveness is a feature of early infection and that this activity diminishes over time (Clery and Mulcahy, 1996; Flynn and Mulcahy, 2008b). In mice, there is a general increase in Th2 cytokine expression as infection progresses (O'Neill et al., 2000).

Parasite-specific IL-4 was generated in the hepatic LN, and mesenteric LN, but not spleen. Conversely IL-5 secretion showed a reverse pattern with MLN cells producing the greatest IL-5 response followed by the HLN and spleen cultures. Extremely low levels of IL-2 were found in highly infected animals, this may represent a mechanism accounting for poor lymphocyte proliferation responses (O'Neill et al., 2000). Infected cattle demonstrate early production of a strong IL-4 response in peripheral lymphocytes (Waldvogel et al., 2004). Post-mortem

examination of HLN samples revealed that IL-4 was highly expressed when stimulated. When left un-stimulated basal cytokine mRNA was detected for both IFN-g and IL-4, however, IL-4 levels were much greater than those of IFN-g. A definitive study of the kinetics of IL-4 was not fully possible in bovine models of the disease until recently due to the lack of a commercial IL-4 protein ELISA. Work in our group has shown that IL-4 rose to peak levels in PBMC within 4–6 weeks post-infection and waned thereafter (Flynn and Mulcahy, 2008a,b). Evidence from rodents suggests that CD4+ cells are the predominant source of IL-4 (Tliba et al., 2002).

An examination of the regulatory cytokines, IL-10 and TGF-b1, in cattle found that IL-10 levels rose over time and that TGF-b1 reached high levels early in infection and were maintained thereafter. This differential kinetics of these cytokines could indicate separate roles for them, and use of neutralising antibody confirmed this with IL-10 predominantly responsible for controlling IFN-g, and TGF-b1 was found to control IL-4 production (Flynn and Mulcahy, 2008b).

A recent study conducted using sheep has found that infected animals stratified on the basis of parasite burdens displayed differential levels of IL-10 and TGF-b1 mRNA levels within the HLN. Animals with low burdens had high levels of IL-10 and TGF-b1 expression while those undergoing infection with higher burdens had low levels of gene expression (Hacariz et al., 2009). In this case the authors hypothesise that IL-10 and TGF-b1 working together were responsible for fibrosis and hence parasite attrition. The potential for IL-10 and TGF-b1 having dual roles needs further examination before any conclusions can be made. The regulation of *F. hepatica* Immune responses are only being explored with little work to date published on this topic. However, the existence of parasite specific, IL-10 and TGF-b1 producing, T-regulatory

cells capable of suppressing parasite-specific Th1 and Th2 responses has recently been demonstrated in a murine model (Walsh et al., 2009).

Overall the cytokine kinetics described above would support the idea that a strong Th2 memory response, characterised by IL-4, may be generated both at the site of infection and peripheral lymph nodes during infection. Investigators have found a persistent Th2 memory response in *H. polygyrus* infection (Mohrs et al., 2005). These authors speculated that by maintaining a Th2 memory population in the peripheral immune organs a host would be better suited to combat a reoccurring infection, while continued expression of IL-10 would help to limit at the site of infection. Recent murine knock-out models suggest a major role for a group of cytokines that act up-stream of the classical Th2 molecules. IL-25 (Fallon et al., 2006), IL-33 (Humphreys et al., 2008), and TSLP (Ramalingam et al., 2009) have all been recently shown to play roles in the development of helminth immune responses. Identification of bovine homologues of these molecules has not been reported nor have roles for these in murine *F. hepatica* infection have not yet been examined.

2.12. Development of Liver Fluke Vaccines

Recent results from several laboratories have demonstrated that animals can be significantly protected against infection by vaccination with defined fasciola antigens. Apart from reducing fluke burdens, some vaccines can elicit a concurrent reduction in parasite egg production. The expectation of a commercially feasible vaccine that might also reduce parasite transmission in the field is now realistic, although major hurdles still exist. A number of defined proteins from *F. hepatica* and *F. gigantica* have shown promise as vaccines in ruminants; these include a fatty

acid-binding protein (FABP) termed Fh12, glutathione-*S*-transferase (GST), cathepsin L (CatL) proteinase and haemoglobin (Hb). The FABP antigen is a major component of the FhSmIII (M) complex, a protein fraction isolated from adult *F. hepatica* that is recognized by cross-reacting antibodies raised to *Schistosoma mansoni* adult worms. Cross-protection against *S. mansoni* in mice and hamsters with a worm complex from *F. hepatica* has been reported (Hillyer, 1977; Hillyer, 1979). Vaccination of calves with two doses of 500 mg of the FhSmIII (M) fraction in Freund's adjuvant (FA) induced a significant 55% reduction in mean worm burdens (Hillyer, 1987). High levels of protection were also observed in mice (Hillyer, 1985).

The Glutathione-*S*-transferase (GSTs) of *F. hepatica* (FhGSTs) were chosen as candidate vaccine antigens on the basis that homologous GST proteins from *S. mansoni* (Sm28) and *S. japonicum* (Sj26) were shown to protect laboratory animals21. GSTs comprise a large family of isoenzymes whose functions include the initial steps of detoxification of xenobiotics and endogenous toxic compounds. GSTs are proposed to play a major role in these reactions in helminths21–23. The GSTs of adult *F. hepatica* were purified and shown to consist of a mixture of at least five isoenzymes of size 23–26.5 kDa that showed N-terminal sequence heterogeneity24–26 (Hillyer, 1977; Hillyer, 1979).

Cysteine proteases in parasites are potent inducers of vertebrate host immune responses and may under certain circumstances take part in the pathogen's immune evasion strategies. These capacities place these parasite molecules as interesting candidate antigens in antiparasitic vaccines for use in vertebrates. Parasite cysteine proteases are able to skew the Th1/Th2 profile in mammals towards a response which allows sustainable parasite burdens in the host. DNA

vaccines are also able to skew the Th1/Th2 profile by different administration techniques and the use of cysteine proteases in these genetic immunizations open perspectives for manipulation of the host immune response towards higher protection (Jørgensen and Buchmann, 2011).

Dalton et al. (1996) investigated the effect of two cathepsin L vaccines in cattle, in combination with liver fluke haemoglobin against infection with *F. hepatica*. The vaccines induced protection levels up to 72.4% and they also reduced the viability of the fluke eggs recovered from the vaccinated groups. The adaptive immune response induced activated cell-mediated protection in addition to antibody production. Rats vaccinated against *F. hepatica* with a DNA vaccine encoding cathepsin L-like proteases resulted in a significant reduction of fluke load (Harmsen, 2004). Furthermore, a DNA vaccination trial with Sprague-Dawley rats immunized against Clonorchis sinensis with a C. sinensis cysteine protease (CsCp) induced a significant level of protection of 31.5% (Lee et al., 2006).

2.13. Irradiated vaccines

Attenuation can be achieved by exposure of parasites to radiation from an ultraviolet, X-ray, or gamma source. Currently, vaccines are available against only a few parasite species of veterinary importance and largely involve the administration of live attenuated organisms. Attenuated antiparasite vaccines enable the host to mount a protective immune response against the organism without the development of the pathological symptoms of infection. Despite the empirical nature of this strategy, several irradiation-attenuated parasite species have been used experimentally to induce protection in various host species (Taylor et al., 1976; Taylor et al., 1979; Smith et al., 1993), including cattle against infection with the liver fluke, *Fasciola*

hepatica, and sheep and cattle against infection with *F. gigantica* (Haroun and Hillyer, 1986). There is abundant evidence to indicate that the resistance displayed by mice vaccinated with irradiated cercariae is mediated by specific immune mechanisms, unlike that observed in chronically infected animals. For example, it is schistosome species-specific and can be transferred across a parabiotic union between vaccinated and naive animals. The dose of radiation applied to parasites is an important factor influencing the eventual level of protection, although opinions have differed regarding the optimum amount (Coulson, 1997).

Little is known about the mechanisms by which protection against parasite infection is induced by irradiation-attenuated vaccines, but several hypotheses have been proposed including disruption of protein synthesis (Wales et al., 1992), alteration in carbohydrate expression (Wales et al., 1993), reduction in protease levels (Wright et al., 1981; Baylis et al., 1992), prolonged exposure to specific antigens (Bickle and Andrews, 1985; Sher et al., 1991), modification of expressed antigens (Wales and Kusel, 1992), reduced parasite survival post irradiation, induction of specific cytokines (Sher et al., 1991), and altered migration of parasites (Richter et al., 1995).

In the case of trematodes, research on irradiation-attenuated vaccines against *Schistosoma* spp. has demonstrated the importance of the disruption of synthesis and expression of proteins (Wales et al., 1992) and carbohydrates (Wales et al., 1993) following irradiation in the induction of host protection. Partial inhibition of protein synthesis and alterations in schistosomula tegumental carbohydrate may convert normally weak antigens into abnormal and highly immunogenic conformations (Wales and Kusel 1992). Indeed, the irradiation dose necessary to induce maximum changes in carbohydrate expression correlated with the capacity of the irradiated

larvae to protect mice against non irradiated schistosomes (Wales et al., 1993). The tegument of *F. hepatica* is also surrounded by a highly antigenic, complex carbohydrate glycocalyx that is sloughed during the early stages of migration and has been implicated in immune evasion by this parasite (Hanna, 1980). Following irradiation the developmentally regulated synthesis of tegumental cell bodies which contribute to the tegumental glycocalyx in juvenile liver fluke is disrupted 3 and 4 weeks after infection of mice with irradiated metacercariae (Burden et al., 1983).

Another potential mechanism by which irradiation-attenuated anti-parasite vaccines induce protection is by affecting parasite proteases (Wright et al., 1981; Baylis et al., 1992). Proteolytic enzymes have been described in a number helminth species and are secreted by the invasive stage of the parasite during infection. While several proteolytic enzymes have been identified and characterised from adult *F. hepatica* (Knox et al., 1994), a 29-kDa cathepsin-B protease has only recently been identified in somatic extracts of juvenile *F. hepatica* as well as in the ES material of the invasive NEJ liver fluke. The surface tegument of the liver fluke Fasciola hepatica is a syncytial cytoplasmic layer bounded externally by a plasma membrane and covered by a glycocalyx, which constitutes the interface between the parasite and its ruminant host. The tegument's interaction with the immune system during the fluke's protracted migration from the gut lumen through the peritoneal cavity and liver parenchyma to the lumen of the bile duct, plays a key role in the fluke's establishment or elimination. However, little is known about proteins of the tegument surface or its secretions (Wilson et al., 2011).

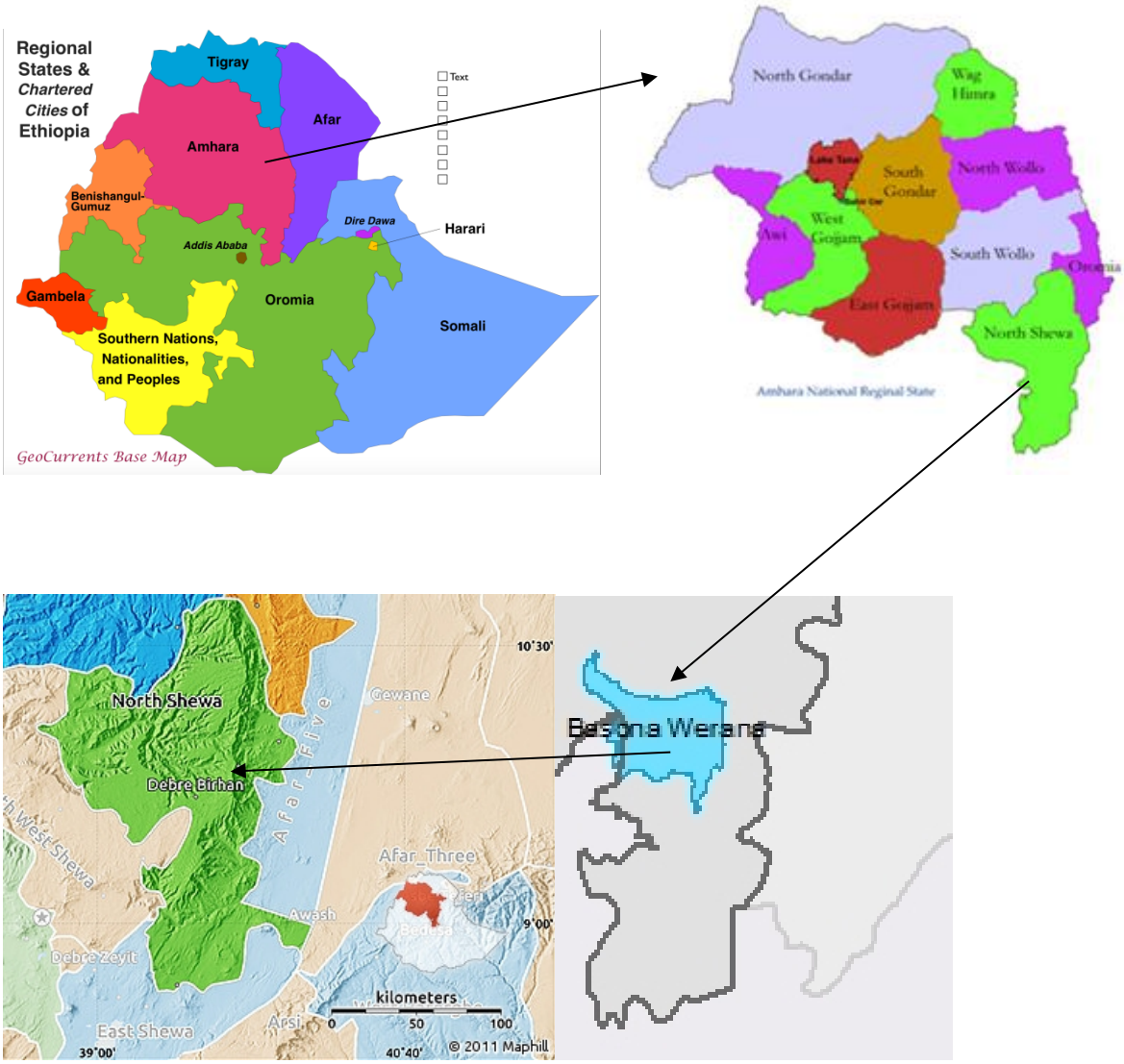
3. MATERIALS AND METHODS

3.1. The study area

3.1.1. Coprolgical study

The coprological study was conducted from November 2012 to April 2013 in and around Debere Berhan, Basona Worana, North shoa zone, Central Ethiopia. Debre Berhan lies at 09^0 31N latitude and $39^0$28E longitude with an altitude of 2780 m.a.s.l. located some 130 kms away in northeastern direction of Addis Ababa. The area is mountainous with large plain grazing land, dissected by rivers and streams. Five peasant Associations (PA's) namely Angolela, Birbirsa, Kormargefya, Weiniye and Goshbado located in close proximity of Debre Berhan were part of study. This area comprises large plain grazing land with extensive type of management system. The faecal samples and laboratory works for abattoir study were conducted at Debere Berhan Veterinary Clinic and Research Center.

Figure 3.1. Map of the study area showing Debre Berhan in Bosana district of North Shoa zone, Amhara region



3.1.2. The abattoir study

The abattoir study was conducted from November 2012 to April 2013 in two export abattoirs (Helimix and ELFORA) situated in the town of Debrezit, 50 kms away from the national capital (Addis Ababa) and three municipal abattoirs (Bahrdar, Debreberhan and Addis Ababa municipal abattoirs). Helimix has production capacity up to 2000 sheep/goat and 150 cattle per day and currently exports chilled meat to UAE, Saudi Arabia, Yemen and frozen meat to Egypt. The ELFORA export abattoir has branches in Metehara, Melge Wondo, Diredawa, Kombolcha and Gondar and it has similar export destination and meat production capacity as that of Helimix with possible potential to expand production (EMPEA, 2010). The municipal abattoirs used are located at Addis Ababa, Bahrdar (758Km Northwest of Addis Ababa) and Debreberhan towns (125km North of Addis Ababa). The laboratory works were carried out at College of Veterinary Medicine and Agriculture, the Veterinary Clinics residing in the respective cities (Bahr Dar, Debre Berhan, Addis Ababa). The laboratory works were carried out at College of Veterinary Medicine and Agriculture, the Veterinary Clinics residing in the respective cities (Bahr Dar, Debre Berhan, Addis Ababa).

3.1.3. The snail collection and the parasite

The populations of freshwater pulmonate snails were collected from different rivers, ponds and canals in Central and Southern Ethiopia. From river Angolela, only the population of *G. truncatula* was originated. The river was located at altitudes 2500 meters above sea level in Fasciola endemic areas around Debre Berhan city, in central Ethiopia. The city is located in the Semien Shewa Zone of the Amhara Region, about 125 kilometers north east of Addis Ababa on

the paved highway to Dessie. It has latitude of 9°41′N and longitude of 39°32′E and an elevation of 2,840 meters. However, the other populations of *G. truncatula* and *R. natalensis* were obtained from Central and Southern Rift Valley areas with an altitude ranges between 1500-2000masl (Guta river in central rift valley and Wondo-genet irrigation canals and ponds and Tikur wuha areas in Southern Ethiopia). Samples of 50 or 100 adult *G. truncatula* snails, measuring 6.8-7.2mm and *R. natalensis* 10–13 mm in height, were collected in April 2013 from the first population and in September–October 2013 from the other were progressively acclimatized for a 48-hour period to a constant temperature of 20 ^{0}C. The snails were collected in large plastic vessel containing river water and aquatic plants (blue green algae) with care to avoid shell damage and transported to the laboratory.

3.1.4. Experimental infection

The snail identifications and all the experimental infections involving the snails and the experimental animals were carriedout at Aklilu Lemma Institute of Pathobiology.

3.2. Study animals

3.2.1. Coprolgical and abattoir study

Local zebu cattle and their crosses with the Holestien-Freiesian cattle as well as Menz sheep and their crosses with Hawassi breed sheep were used for the coprological study in and around Debreberhan. Crosses of Holestien-Freiesian cattle and loal breed sheep were encountered in all study abattoirs. However, the significant number of animals used in this study were the local breed cattle and sheep managed extensively.

3.2.2. Experimental animals

The animals used in this experiment for testing the immune response were local breed of sheep. They were purchased from the market in Gojo district in central Ethiopia. Gojo is situated in West Shewa zone in Oromiya region of Ethiopia with geographical coordinates of 9° 16' 0" North, 38° 5' 0" East and with altitude of 2905 meters above sea level. The sheep were broght to the market from the surrounding areas of Gojo town. The area was supposed to be free or pose very low risk for fasciola species. The sheep breed were Arsi Bale type which has wider distribution in the central and southern parts of the country and is susceptible to Fasciola species (Alemu and Merkel, 2009).

3.3. The study design and sample size

The cross sectional study was taken place following the subsequent selection of district, PAs and herds. The sampling strategy involves purposive (accessibility) selection of a district and random selection of PAs within the district from the available list of PAs as sampling frame. This was followed by random selection of herds that will bring their animals for the examination and then selection of study animal for faecal sample collection using simple random sampling technique. Therefore, cattle and sheep from herds in different PA's were taken part in the investigation. The sample size was determined according to the formula given by Thrusfield (2007) with expected prevalence of 50% and precision of 5% with confidence level of 95%. However, to increase the precision, the sample size was made to 448 sheep and 667 cattle and this was allocated proportionally based on population number of animals in each PA. Accordingly, the PAs used in

this study were; Debre Berhan (199), Angolela (222), Birbirsa (211), Kormargefya (161), Weiniye (179), Goshabado (153).

On each herd animals were selected in different age groups as young (calves and heifers or lambs and weaned lambs), and adults. Age estimation was done by inspection of the incisor teeth according to a method by Yeates and Schmidt (1974) which is based on incisor teeth temporary teeth replacement and the degree of wear of permanent teeth. In addition sex of animals and their body condition status (poor and good) were recorded. The body conditions were estimated based on descriptions of Nicolson and Butterworth (1986) for zebu cattle and Thompson and Meyer (1986) for sheep.

Hence, attempt was made to determine the prevalence of fasciola species during the long dry season in slaughtered sheep and cattle with the help of both coprological examinations and abattoir survey at Debre Berhan Municipal Abattoir.

3.3.1. The coprologic study

A total of 1125 randomly selected animals comprising cattle (677) and sheep (448) managed under extensive traditional system were sampled. For the estimation of herd level prevalence fresh faeces were randomly collected, on a regular basis, directly from the rectum of all animals and transported to the laboratory in an airtight condition. The specimens were then subjected to qualitative coproscopic examination for the presence of characteristic *Fasciola* eggs by direct sedimentation technique employing a standard procedure (Urquhart et al., 1996). The mean

prevalence was then compiled on monthly basis and analyzed with respect to the different risk factors considered such as age, sex, breed, and body condition categories.

3.3.2. Egg output at herd level and the egg shedding index

The McMaster egg counting technique was used to determine the faecal egg count, herd egg output and subsequently the egg shedding index (Taylor et al., 2007; Malone, 1992). Accordingly, pooled faecal specimens from a herd of 6-10 animals belonging to different owners and from a total of 170 herds (80 sheep and 90 cattle herds) were examined. The pooling of samples was done regularly during the daily sampling of each animal. Prevalence and EP2G were used to calculate the herd shedding index, a parameter which estimates the herd-infection prevalence, using the following formula: HERD EGG-SHEDDING INDEX = PREVALENCE*EP2G (Malone et. al. 1992).

3.3.3. Postmortem examination

Adult fasciola parasites specimens were collected from condemned livers and associated gallbladder of cattle and sheep in Debre Berhan Municipal abattoir. Each liver was placed in a large basin and all the flukes in the gall bladder and the major bile ducts were collected into a small plastic container for subsequent counting. After visual observation and palpation of the liver, sharp incisions were made on the surface, through the major bile ducts into the parenchyma. The liver was then sliced into strips of about 1 cm in thickness and soaked in normal saline for about 5 h and washed extensively (incubated in physiological saline, 0.9% NaCl) in order to regurgitate the intestinal contents. Flukes emerging from the cut bile ducts

were put into the same jar and each sliced strip was thoroughly squeezed from end to end, washed in saline and discarded. The contents of the basin were sieved, put into a petridish and the adult, immature and cut pieces of flukes were added to the container (Foryet 2001, Urquhart et al., 1996). The abattoir prevalence was assessed with regards to different risk factors considered (age, sex, breed, bodycondition, altitude and origin of the sloughter animals).

3.3.3.1. Fluke identification

Individual flatworms were identified to species level according to existing keys and descriptions using their morphologic, morphoanatomic characters and morphometeric measurements (Urquhart et al., 1996; Lofty et al., 2002). Accordingly, they were classified as adult *F. hepatica*, *F. gigantica,* mixed and immature flukes. Adult *F. hepatica* are smaller than *F. gigantica* and have well developed 'shoulders' distal to the oral sucker whereas, the shape of *F. gigantica* is more streamlined without 'shoulders' (Urquhart et al., 1996). Counts of the heads of cut flukes was made and added to the appropriate count of adult flukes.

3.3.4. Snail survey

The snail survey was conducted in areas suspected of harboring the vector that include the water bodies (low-lying swamps, water lodged areas and slow flowing streams) and possible transmission sites (grazing and watering sites). Across the study district "six permanent and temporary" water bodies were selected based on their accessibility and animal-water contact frequency. Sample collections from these sites were made at monthly intervals from November 2012 to April 2012. A five meter by five meter quadrant was thrown on each sampling site.

Snail abundance was estimated from the number of snails collected per unit of time (30 min) using kitchen sieves and the counts extrapolate to give the number of snails per man per hour. The number of snails collected and the water pH were recorded. They were moved at frequent intervals to fresh vessels and were observed for cercaria shedding. The identification was carried out on the basis of the snail's shell morphology and classified into major categories as per the criteria described by Hansen and Perry (1994) and Malek (1985) and Frandsen and McCullough (1980).

3.3.5. Estimation of economic loss due to liver condemnation

The estimation of the economic loss due to the condemnation of fluke affected livers was done by considering all livers affected with fasciola as condemned. The annual loss from liver condemnation was assessed by considering the overall annually slaughtered animals in the abattoir and retail market price of an average ruminant liver. The annual loss from the liver condemnation was assessed by using the formula set by Ogunrinade and Adegoke (Ogunrinade et al., 1982).

ALC= CSR X LC X P

Where ALC= Annual loss from liver condemnation

CSR= mean annual cattle slaughtered at abattoirs

LC= mean cost of one liver

P= Prevalence of fasciolosis at the abattoir

3.3.6. Evaluation of the immune response of sheep to primary infection with attenuating irradiating dose of *fasciola hepatica* metacercarae

3.3.6.1. Experimental infections of snails

Experimental infections of the snail populations of *G. truncatula* originated from the River Angolela with cattle derived miracidia of *F. hepatica* was conducted to observe the characteristics of *F. hepatica* such as the prevalence, survival of infected snails at day 30 post exposure, patent and prepatent period, the number of cercraia shed by snails infected with single, three and five miracidia.

For examination of snail for cercarial shedding freshly collected snails were used. The snail species incriminated as intermediate hosts were examined using the methods described by (Frandsen and McCullough, 1980; Frandsen and Christensen, 1984; Malek, 1985; Hansen and Perry, 1994). The identification was carried out on the basis of the snail's shell morphology and classified into major categories. Aquatic Lymnaid snails, which act as intermediate hosts for *F. hepatica* and *F. gigantica* can be identified fairly easily as compared to other freshwater snails. The opening of the Lymnaid snails is on the right when the snail is held with the spires pointing away from the viewer. When the apex of the spire is facing the viewer the spires turn clockwise (Hansen and Perry, 1994).

For the examination of cercaria shedding of the snail, ten petri dishes (containing 30 ml of dechlorinated water and exposed to sunlight) were placed on a tray and exposed to artificial light

for 2 hours. At the end of the exposure period the water (15ml) from each vial containing exposed snails were examined using stereomicroscope. The procedures were repeated for another 2 hours; if no cercariae of fasciola were recovered before recording as negative. Snails that did not release cercariae on the first day of exposure were re-exposed every subsequent day until their death before being discarded. The cercariae of fasciola shed were identified based on the key given by Frandsen and Christensen (1984).

The collected snails were morphologically identified at Aklilu Lemma Institute of Pathology. Then the sanils were kept with continuous aeration until they were transferred to the clean new plastic containers provided dechlorinated tap water at 20^0C room temperatures. In addition, sterilized soil, substrate, and calcium carbonate (10%) were added to the water. They were fed algae and pH were monitored daily and the water was changed a week. To collect the eggs, uncolored plastic sheets were put into the aquaria (Olivier 1960). The laid egg mass seen in the plastic sheets or sides and bottom of the tanks were transparent and jelly. The faeces were removed regularly by siphon method (Prasad, 1989). To control predators such as oligochaetes worms and ostracods, which could have come from the field with the snails, acetic acid was used to wash the contaminated aquaria. After maturity (two months old snails), these new hatched baby snails were later used for rearing of generations of snails in the experiment as they were better adapted to the laboratory conditions to produce the sufficient metacercariae needed to the experiment.

3.3.6.2. Collection of Fasciola hepatica, a recovery of eggs

Fasciola hepatica eggs were obtained from gall bladders of infected cattle slaughtered at Debreberhan Municipal abattoir. After being thoroughly washed through a 60-mesh sieve in distilled water to remove the blood contaminants and the bile, the eggs were collected on a 400-mesh sieve. Then the eggs were incubated in double-distilled water in medium seized petri dishes at 18-24^{0}C for 16 days in the dark (Cruiz-Reyes and Malek, 1987). The excess eggs were kept in the refrigerator at 4^{0}C as egg stock for the future experiment. Embroynated eggs of *Fasciola hepatica* were induced to hatch by taking the container out of the dark, changing the water, and exposing the contents to the bright source of light. Matured adult snails were used to exposure to the miracidia which can move almost immediately after hatching to penetrate the snail. With a drawn pastuer pippet and using a dissecting mirosope the appropraite number of miraidia was placed with the snails in a volmue of water. A beam of plastic capsule was used to expose the snails individually. In all the trails the snails were exposed for two hours with a capsule full of spring water at room temperature (18-26^{0}C) using 1 to 5 miracidia per individual snail. In all the trails the snails were exposed for two hours with a capsule full of spring water at room temperature (18-26^{0}C) using 1 to 5 miracidia per individual snail. After exposure, all snails were removed from the individual test tubes (capsules) and the infection in these snails was confirmed 4 weeks after exposure in sunlight by checking for the shedding of cercariae (Prasad, 1989).

3.3.6. Snail infection and cercaria output

After infection, snails were kept in one culture vessel and were made to move at frequent intervals to fresh culture vessels, which contained growth of blue-green algae. The shedding of

cercariae post infection was recorded. The snails which did not shed cercariae within 2 h in the dishes were returned to the culture vessels. The snails which shed cercariae were maintained in individual dishes, allowed to feed with algae and were taken from culture vessels and receive fresh distilled water twice a week until they ceased cercarial shedding and died. Each dish was examined twice a week under a dissecting binocular microscope and the encysted cercariae (metacercariae) were counted. The viability of the metacercariae of *F. hepatica* was checked in vitro by stimulation of the metacercariae by artificial digestion prior to dosing of sheep based upon the motility of juvenile flukes within the inner cyst and clear observation of the excretory granules in some cysts under dissecting microscope (Wikerhauser, 1960).

3.3.7. Experimental animal selection for estimation of dosage

They were selected based on approximate similarity in estimated age (six months), body weight (25kg) and similar sex group (all are male) and Arsi bale breed sheep. Upon arrival all the sheep have been treated with Triclabendazole (Fasinex, 10-12 mg/kg) to preclude any fluke infection and Oxytetracycline (10mg/kg) to control opportunistic bacterial diseases. All of the animals were kept for about a month before the commencement of the actual experiment. Animals were tested for presence of internal parasites and Fasciola species before the beginning of experiment using simple floatation and sedimentation methods, respectively. These tests have been done throughout the adaptation period. The sheep were maintained with concentrate and well dried hay free of parasites. Each sheep were provided with 25gms of concentrate (75% wheat bran and 25% Nug cake) and with hay and water provided ad libitum. They were weighed twice before and every other week after the commencement of the experiment. The sheep were matched and randomly allocated into groups consisting of six animals each to begin the experiment. The trials

were carried out at animal ward of Aklilu Lemma Institute of Pathobiology of Addis Ababa University.

3.3.7.1. Experimental animal partition

The experimental groups consist of 36 six month old local breed sheep divided into six groups of animals each (GI, GII, GIII, GIV, PC and NC). The first four groups were vaccinated with 500 *F. hepatica* metacercariae per os with increasing level of irradiation dose. The fifth and sixth groups were served as infected control without irradiation of the metacercariae (positive control) and non vaccinated control (negative control) respectively. The irradiation dose used were 30, 60, 120, and 240 gray for groups I, II, III and IV respectively (Table 5.2.1). The 500 metacercariae were separately counted under a dissecting microscope. The counts were made five times for each sheep to determine the average 500 metacercariae. Administrations of the doses were done by giving in a gelatin capsule (Wikerhauser, 1960). At the time of infection, metacercariae were 2 month old and had 82% viability.

The experiment was lasted about 17 weeks. Animals were held every week for collection of blood samples and body weight measurement. Faecal samples were examined at weekly interval after 8 weeks of the experiment. This was due to the fact that flukes reach maturity after 8 weeks post infection.At the end of the experiment postmortem examination was carried out on all animals. The bile ducts and liver parenchyma was examined and the number and size of adult flukes inside was observed and counted. At the same time tissue samples were collected for histopathological examination.

Table 3.1. Experimental group partition to determine appropriate number of *Fasciola hepatica* metacercria in sheep and irradiating doses

Group	Number of sheep	Number of parasites (Metacercarae)	Irradiation dose (Killo radiance or Gray)
I	6	500	3 (30)
II	6	500	6 (60)
III	6	500	12 (120)
IV	6	500	24 (240)
Infected Control (PC)	6	500	-
Non Vaccinated	6	-	-

3.3.8. Serum collection and testing for anti fasciola antibody

The serum was separated from blood samples and collected into ephendrof tubes to be stored at -20°C before analysis. These were used to determine the serum antibody level and the assay of liver enzymes. The anti-fasciola IgG1antibodies in serum from infected sheep were determined using DRG ELISA kit (DRG International, Inc., USA) for bovine and ovine as well as for humans will be used for detection of antibodies of fasciola hepatica. The test uses 96-well microtitration plates sensitised by a monoclonal antibody specific to one protein of *Fasciola*

hepatica. This antibody is used to trap the protein as well as to purify it from lysate of the parasite. The plate's odd columns (1, 3, 5, 7, 9 and 11) contain the specific protein, whereas the even columns (2, 4, 6, 8, 10 and 12) contain only the monoclonal antibody. This is a genuine negative control to differentiate specific anti-Fasciola hepatica antibodies from non specific ones. The principles of the test procedures and interpretation of results are indicated in annex. Briefly the procedure is as follows. The test blood sera will be diluted in the dilution buffer. The plate will be incubated and washed, then the conjugate, a peroxidase-labelled anti-ruminant IgG1 monoclonal antibody, will be added to the wells. The plate will then incubated a second time at 21°C+/- 3°C, washed again and the enzyme's substrate (hydrogen peroxide) and the chromogen tetramethylbenzidine (TMB) are added. This chromogen has the advantages of being more sensitive than the other peroxidase chromogens and not being carcinogenic. If specific *Fasciola hepatica* immunoglobulins are present in the test sera the conjugate remains bound to the microwell that contains the antigen and the enzyme catalyses the transformation of the colorless chromogen into a pigmented compound. The intensity of the resulting blue colour is proportionate to the titer of specific antibody in the sample. The signal read off the negative control microwell is subtracted from that of the positive microwell sensitised by the antigen. The interpretation of the results is done by comparing the signals of the samples (serum) with those of the positive controls.

3.3.9. Serum Enzyme Assay

Blood samples for biochemical analysis were collected from the external jugular vein into marked vacuum tubes and transported at +4 °C to the laboratories similarly as have been done for ELISA IgG1 test (The same samples). The blood tubes were centrifuged at 3.000 rpm for 10

minutes for serum separation. Due to poor stability of enzymes, the samples were analyzed within 6 hours after collection. The activity of liver enzymes enzymes γ-glutamyl transferase (GGT), glutamate dehydrogenase (GLDH) was conducted spectrophotometrically to assess liver damage using commercial kits (Chemical analyser) and the levels were expressed as international units per liter.

3.3.10. Necropsy and histopathological examination

Postmortem examinations were carried out at the end of the experiment, 17 weeks post vaccination. Sheep were euthanized to examine the parenchyma of the livers, bile ducts and gall bladders for the presence of *F. hepatica* parasites using standard methods [12, 63]. Sheep were stunned before slaughter with nonpenetrative method with controlled obtuse blow to the head (manual blow).

The animal welfare body that comprises of veterinarians at Aklilu Lemma institute of pathology of Addis Abba University and the Science and Technology commission as well as College of Veterinary Medicine and Agriulture of Addis Abba University were approved the experiment.In the laboratory the livers and gall bladders were subjected to thorough investigation for the collection of parasites as well as for pathological studies. The gross pathological changes were recorded carefully. The bile ducts were opened first for chronic fasciolosis. For generalized liver fluke infection (fasciolosis) incision was given in different parts of the liver to examine the presence of fluke in the parenchyma. The liver was cut into slices of 4-5 mm thickness using a sharp knife and pressed to squeeze out flukes from its tissue and smaller bile ducts. Normal saline was used for quick removal of flukes from the liver tissue. Tissue samples were randomly

collected from the left and right hepatic lobes, gallbladder and hepatic lymph nodes, fixed in 10% buffered formalin and embedded in paraffin wax. The fixed specimens were trimmed, washed and dehydrated in ascending grades of alcohol, cleared in xylene and finally embedded in paraffin. The embedded samples were sectioned at 3-5 micrometre thickness using a rotary microtome. All the sections were stained routinely with Haematoxilin and Eosin for detailed histopathological examinations. The slides were then carefully observed under microscope (Olympus) for accurate interpretation of results (Scott and and Stockham, 2008).

The lesions were scored as per the generic grading criteria that can be applied to most, or even all, histopathologic findings as described previously (Frandsen and McCullough, 1980; Katherine et al., 2013; Kenneth et al., 2018). Briefly, histopathologic grades were assigned as level 1 (minimal), 2 (mild), 3 (moderate), 4 (marked), or 5 (severe) based on an increasing extent and/or complexity of change. Gross morphology was assessed to evaluate the damage to hepatic surface (Girard, 2002; Dijk et al., 2007; Zafra et al., 2010). Microscopic liver morphology was examined to evaluate, the area of portal spaces and hepatic damage (chronic tracts, cholangitis with eosinophils, lymphocytes and plasma cell infiltration, fibrosis and granulomas).

3.3.11. Body weight measurements

The bodyweight of each animal was measured using weighing balance in a weekly interval. The changes in body weight gain and loss recorded in different groups were compared to determine the effect of vaccinations.

3.3.12. Enzyme analysis

*3.3.12.1. Gamma-Glutamyl Transpeptidase (*GGT*) Test (γ-glutamyl transpeptidase)*

The gamma-glutamyl transpeptidase (GGT) test measures the amount of the enzyme GGT in the blood. In the liver, it is found primarily on biliary epithelial cells, but small amounts on canalicular and sinusoidal surfaces of hepatocytes. GGT functions in the body as a transport molecule, helping to move other molecules around the body. It is important in glutathione metabolism, amino acid absorption and protection against oxidant injury. It plays a significant role in helping the liver metabolize drugs and other toxins. GGT blood levels are usually high when the liver is damaged. This test is often done with other tests that measure liver enzymes if there's a possibility of liver damage. Gamma glutamyltransferase (GGT) is one of a broad group of enzymes that catalyze the transfer of amino acids from one peptide to another amino acid or peptide. This enzyme is sometimes referred to as a "transpeptidase" but is more appropriately included in the amino acid transferase group. Specifically it catalyzes the transfer of a gamma glutamyl group to another acceptor. The reference range with most commonly used methods is 0 to 50 IU/L in males and 0 to 30 IU/L in females. Higher activity in males is probably caused by high enzyme concentration in prostatic tissue. Although GGT is found in many tissues, the main source of serum activity is the liver (primarily biliary epithelium), thus GGT is used mainly as a sensitive indicator of cholestasis, but it also reflects biliary hyperplasia (Carakostas et al., 1986; Merrick et al., 2006; Evans, 2009; Beek et al., 2014; Draus; 2017).

3.3.12.2. Glutamate Dehydrogenase (GLDH, GDH*)*

Glutamate dehydrogenase (GLDH) is a mitochondrial enzyme that is involved in the metabolism of glutamate to 2-oxoglutarate. The GLDH enzyme is found primarily in liver, kidney, and cardiac muscle, with lower levels in brain, skeletal muscle, and leukocytes. The majority of GLDH in the serum originates from hepatocytes in healthy as well as diseased animals. The distribution of GLDH in the liver is mainly centrilobular, whereas ALT is periportal or centrilobular in rats, and may indicate the site of hepatocyte damage (Carakostas et al., 1986; Merrick et al., 2006). Levels of GLDH are normally low in most species, and increased serum GLDH is indicative of damaged or necrotic hepatocytes due to liver disease (Evans, 2009; Beek et al., 2014; Draus; 2017).

3.4. Statistical analysis

3.4.1. Coprology and abattoir survey

The data was entered into Excel spreadsheet for data management. The descriptive statistics was used to describe the overall prevalence of fasciolosis and the associated risk factors (age, sex, body condition, species and PA). A univariable logistic regression followed by multivariable logistic regression model was used to investigate the relation and statistical significance between positivity for coprology and liver examination and the factors considered. Data analysis was undertaken for the prevalence with species, breed, sex, body condition and age as independent variables. Fluke infection and egg detection with positive samples was considered as dependent variable. The results were analyzed for statistical significance by using STATA Version 11 (STATA, Stata corp. LP, 4905, Lakeway drive, College Station, Texas, USA). A P value of less than 0.05 was considered as significant

3.4.2. Analysis of experimenta animal and snail data

The data collected from each group (number and size of parasites, enzyme parameters, irradiation dose and the the immune response) were compared by two-way analysis of variance (ANOVA), followed by Tukey post hoc test for differences between the means of control and experimental samples at each time interval. The correlation between variables was tested to see how strongly pairs of variables related. p values lower than 0.05 were consi dered significant. STATA Tukey's HSD (Tukey's honest significance difference/test) was designed for a situation with equal sample sizes per group. p values lower than 0.05 were consi dered significant. STATA Version 11 (STATA, Stata corp. LP, 4905, Lakeway drive, College Station, Texas, USA). A comparison test of experimental frequencies and one- or two-way analysis of variance were used to establish levels of significance for the experimental snail infection.

4. RESULTS

4.1. Coprology

4.1.1. Prevalence

Among the 170 herds (80 sheep and 90 cattle) at the animal level, the overall prevalence of fasciolosis on the basis of coprologyy was 53.8% (605/1125) with 95% CI (0.4, 0.7). The highest prevalence was for sheep (60.1%) followed by cattle (49.2%). Among others, Birbrisa (77.7%) PA was shown the highest overall prevalence and Angolela (31.5%) the lowest. Among the risk factors considered the overall prevalence was highest for cross breed (62%) and female animals (56.6%) as well as those in young groups (59.8%) and animals with poor body condition (77.7%) (Table 4.1).

The prevalence of eggs of fasciola from bovine species was highest in Birbirsa PA (76%) and the least was in Angolela (31.7%). This similar trend was also recorded for sheep. In addition the other risk factors were noted to exhibit similar pattern as the case for the overall prevalence of fasciola eggs discharged from both sheep and cattle species (Table 4.2 and Table 4.3).

Table 4.1. Overall coprological prevalence (OR and 95% CI) of fasciolosis in ovine and bovine species with regards to the different risk factors assessed (species, PA, Age, breed, sex body condition).

Risk factors		Number examined	Number positive	Prevalence (%)	OR (95% CI)	P value
Species	Overall	1125	605	53.8	.095 [0.4, 0.7]	0.49
PA	*Debre Berhan	199	130	65.2	-	-
	Angolela	222	70	31.5	0.31 [0.19, 0.49]	0.000
	Birbirsa	211	164	77.7	1.72 [1.0 3.0]	0.04
	Kormergefya	161	76	47.2	0.83 [0.48 1.40]	.479
	Weiniye	179	95	53.3	0.67 [0.42 1.1]	0.108
	Goshbado	153	70	45.8	0.51 [0.38 0.84]	0.08
Age	Young	584	347	59.8	1.6 [1.3 20]	0.000
	Adult	541	258	47.6		
Breed	Cross	610	378	62	0.41 [.27 .59]	0.000
	Local	515	227	44.1		
Sex	Male	466	231	52	1.1 [.78 .15]	0.65
	Female	659	374	56.6		
Body C	Good	545	228	41.8	0.44 [0.35 0.56]	0.000
	Poor	580	377	58.2		

*Debre Berhan is a reference PA (Peasant Association), OR, odds ratio; CI, confidence interval; P value, Probability value; P value <0.05 considered significant

Table 4.2. Over all prevalence (OR and 95% CI) of Bovine fasciolosis with regards to the different risk factors assessed (species, PA, Age, breed, sex body condition). P value <0.05 considered significant

Risk factors (Virable)		Number examined	Number positive	Prevalence (%)	OR (95% CI)	P value
Bovine		677	333	49.2	-	-
PA	Debre Berhan	111	71	64.0	-	-
	Angolela	119	30	25.2	0.32 [.19 .36]	0.000
	Birbirsa	115	80	69.6	1.7 [.99 3.0]	0.05
	Kormergefya	94	45	47.9	0.39 [.23 .68]	0.001
	Weiniye	93	52	55.9	0.39 [.22 .67]	0.000
	Goshbado	145	55	37.9	0.25 [.15 .45]	0.000
Age	Young	324	175	54.0	1.4 [1.1 1.9]	0.016
	Adult	353	158	44.8		
Breed	Cross	346	183	52.8	0.34 [.11 1.0]	0.05
	Local	331	150	45.3		
Sex	Male	290	134	46.2	0.46 [0.15 1.6]	0.181
	Female	387	199	51.2		
Body Condition	Good	129	129	38.4	0.21 [.12 .38]	0.00
	Poor	205	205	60.6		

*Debre Berhan is a reference PA (Peasant Association), OR, Odds ratio; CI, confidence interval; P value, Probability value

Table 4.3 The prevalence of Ovine fasciolosis (OR and 95% CI) with regards to the different risk factors assessed (species, PA, Age, breed, sex body condition). P value <0.05 considered significant

Risk factors		Number examined	Number positive	Prevalence (%)	OR (95% CI)	p
Ovine		448	272	60.1	-	-
PA	Debre Berhan	83	57	68.7	0.16 [.08 .36]	.000
	Angolela	95	24	25.3	0.16 [.08 0.36]	.000
	Birbirsa	90	72	80	3.2 [1.4 7.4]	0.01
	Kormergefya	62	35	56.5	0.77 [.33 1.8]	0.55
	Weiniye	63	48	76.2	2.1 [.94 .84]	0.68
	Goshbado	55	39	70.9	1.6 [.68 3.7]	0.29
Age	Young	184	92	50.0	1.93 [1.1 3.3]	0.014
	Adult	264	180	68.2		
Breed	Cross	184	77	41.8	0.30 [.19 0.4]	0.000
	Local	264	195	73.6		
Sex	Male	176	97	55.1	2.2 [.14 .35]	0.001
	Female	272	195	71.9		
Body Condition	Good	205	100	48.8	0.38 [.22 .6]	0.001
	Poor	243	172	71		

*Debre Berhan is a reference PA (Peasant Association), OR, Odds ratio; CI, confidence interval; P value, Probability value

In cattle the egg output for fasciola species ranged from 14 to 890 with mean EPG of 57.1 ± 34.3 (mean ± SD) while in sheep the EPG ranged from 18 to 1300 with mean EPG of 63.5 ± 88.3 and the difference was not significant (P<0.05). The highest overall mean EPG (74 ± 87) was reported in Birbirsa PA and the least was in Angolela (42.4 ± 33.8) (Table 4.4).

Table 4.4. The prevalence (%) for faecal positive animals and the mean faecal egg counts of fasciola species with respect to the different risk factors assessed (species, PA, Age, breed, sex body condition). P value <0.05 considered significant

Risk factors		Number examined	Prevalence (%)	Mean EPG ± SD	P value	Mean Herd EPG ± SD	Mean Egg Shedding Index ± SD	P-value (EP2G*P)
Species	Bovine	677	49.2	57.1 ± 34.8		86.2 ± 89 .4		0.000
	Ovine	448	60.1	63.4 ± 88.3		98.8 ± 46.5	59.4 ± 27.9	
PA	Debre Berhan	199	65.2	59.7± 37.2		88.8 ± 53.4	57.9 34.8	Reference
	Angolela	222	31.5	42.4 ± 33.8	0.061	71.2 ± 37	22.4 11.7	0.000
	Birbirsa	211	77.7	73.7 ± 87.9	0.310	100 ± 87.7	77.7 ± 68.1	0.000

	Kormargefya	161	47.2	59.7 ± 57.8	1.000	89.2 ± 70.4	42.1 ± 33.5	±	0.000
	Weiniye	179	53.3	65.6 ± 75	1.000	85.6 ± 74.9	45.5 ± 39.6	±	0.023
	Goshbado	153	45.8	58.2 ± 58.5	1.000	84.8 ± 62.3	38.8 ± 84.1	±	0.025
Age	Young	584	59.8	64.5± 66.6	0.049	98.5 80.8	58.9 ± 39.8	±	0.000
	Adult	541	47.6	56.2± 58.3		79.4 ± 60.6	37.8 ± 29.6	±	
Breed	Cross	610	62	61.8± 71	0.024	97.8 ± 51	60.4± 60.6		0.000
	Local	515	44.1	57.1± 49.2		73.3 ± 62.3	32.3 ± 27.7	±	
Sex	Male	466	52	56.2± 58.3	0.027	85.3 ± 57.8	44.4 ± 23.4	±	0.043
	Female	659	56.8	64.5± 66.6		88.9 ± 44.9	50.5 ± 25.8	±	
Body condition	Good	545	51.8	59.2± 59.7	100	78.6 ± 80	51.7 ± 41.5	±	0.830
	Poor	580	65	60.2± 64.1		74 ± 60	48.1 ± 39	±	

*Debre Berhan is a reference PA (Peasant Association), OR, Odds ratio; CI, confidence interval; P value, Probability value.

4.1.2. The herd egg output

Across herds, the proportion with at least one infected animal, varied between 63 and 75 %. This proportion varied for sheep (63-70) and cattle (65-75) herds. However, there was a significant sampling effect at the herd-level; all herds where at least 605 animals sampled over the study period exhibited evidence of fluke infection (53.8%). There was significant variation in terms of within-herd infection prevalence (<0.05) (Table 4.4).

The egg shedding index estimated for the whole dry period (November to April) was 50.9 ± 29.3. According to this indices, the value of herd egg output estimated for bovine and ovine species was 42.41 ±30.75 and 59.4 ± 27.9, respectively and the variation was statistically significant ($p<0.05$). The overall mean egg shedding indices for young (58.9 ± 39.8) and adult (37.8 ± 29.6) as well as cross (60.4 ± 60.6) and local (32.3 ± 27.7) breeds were statistically significant ($P<0.05$). On the other hand, the herd egg output values obtained during this long dry seasons for male (44.4 ± 23.4) and female (50.5 ± 25.8) animals were not statistically significant ($P> 0.05$) as had been the case reported for good (51.7 ± 41.5) and poor (48.1 ± 39) body condition ($P>0.05$). The highest egg shedding index was recorded at Birbirsa PA (77.7 ± 68.1) and the least was at Angolela (22.4 ± 11.7).

4.2. Abattoir survey

Out of a total of 1312 sheep (509) and cattle (803) slaughtered, 1061 (81%) were found positive for fasciola species. However, the prevalence in sheep (84%) was significantly ($p<0.05$) higher than that of cattle (77.8%). The disease is 0. 25 times more likely occurs in sheep than in cattle. *F. hepatica* was a dominant (87.9%) species identified followed by *F. gigantica* (6.3 %) with some (5.8%) mixed infections. Age and sex appears to have no effect on the overall prevalence of fasciolosis. However, the prevalence was relatively higher in young (81.6%, 714/814) and male (81.1%, 618/762) groups than the adult (77.7%, 338/438) and female (78.7%, 433/550) animals. The effect of breed and body condition differences on the overall occurrence of fasciolosis was statistically significant ($P<0.05$) (Table 4.5).

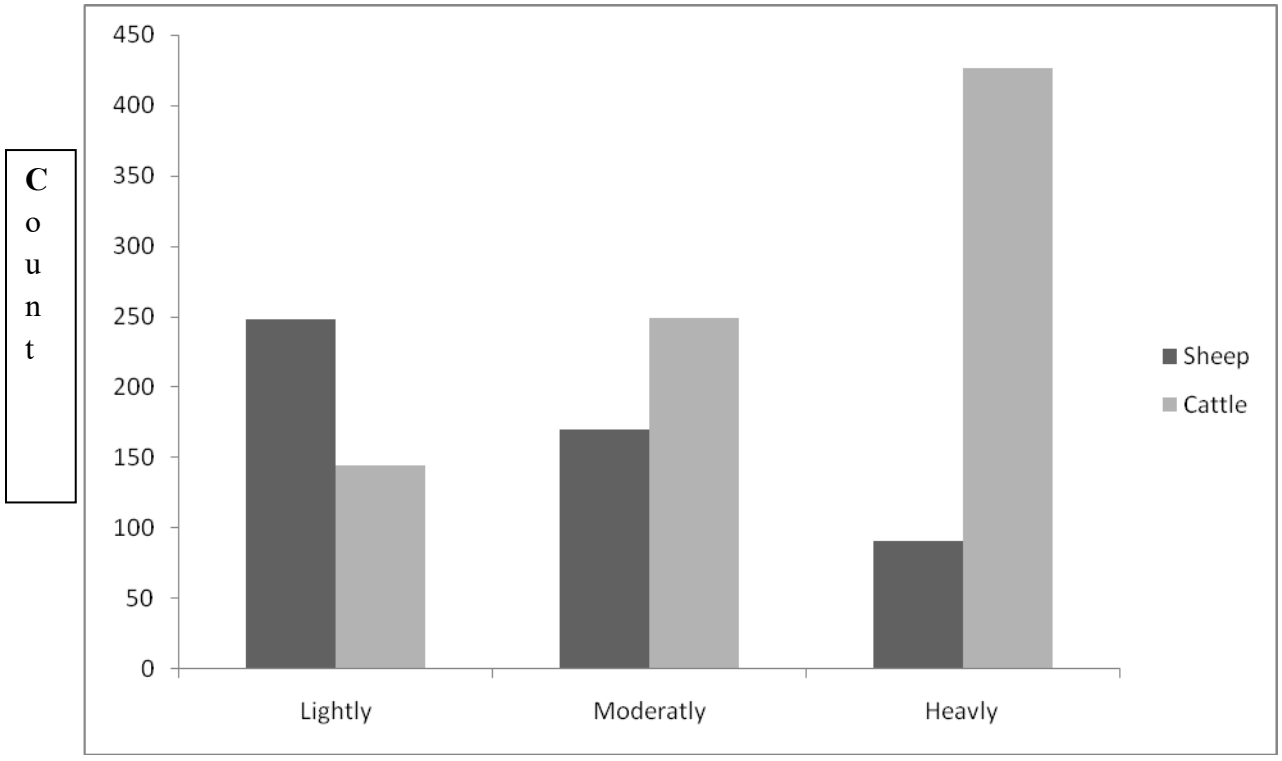
Table 4.5. The overall abattoir prevalence of fasciolosis in sheep and cattle fasciolosis (OR and 95% CI) with regards to the different risk factors assessed (species, Age, breed, sex body condition) and the associated risk factors.

Risk factors		Number examined	Number positive	Prevalence (%)	OR(95%CI)	SE	P value
Species	Bovine	803	623	77.7	.25[.15 .39]	0.6	0.00
	Ovine	509	428	84			
Age	Young	874	428	81.6	0.99 [.67 .48]	0.99	0.04
	Adult	438	623	77.7			
Breed	Cross	247	714	85	0.49 [0.62 1.3]	0.49	0.63
	Local	1065	338	79.2			
Sex	Male	762	210	81.1	0.91 [0.00 1.2]	0.18	0.00
	Female	550	1040	78.7			
Body condition	Good	360	618	95	13.7 [6.7 0.1]	7.12	0.00
	Poor	952	433	74.2			
F. species	*F. hepatica*	1050	923	87.8	11.3 [8.5 14.9]	1.6	0.00
	F. gigantica	1051	67	6.3			
	Mixed	1051	61	5.8			

In sheep, the probability of the occurrence of the disease in Awassi-Menz cross bred animals was 49% compared to the local Menz breed. Similarly, 20% of the Holstein-Friesian crosses are more prone to acquire the disease than the local Zebu cattle. In general, poor conditioned animals have high risk of acquiring fasciolosis with the respect to animals in good body condition.

In sheep, most of the livers (248/509) examined were lightly affected (47.8%) and about a third of those (170/509) were affected severely (33.3%). However, the severity of the infection was more pronounced in cattle (53%, 426/803) while the remaining 31% (249/803) and 18% (145/803) of the infected livers were affected moderately and lightly, respectively (Figure 4.1).

Figure 4.1. The level of severity in fasciola infected liver of sheep and cattle in Debreberhan abattoir



The liver weight of 1061 (sheep, 438; cattle, 623) fluke infected livers were measured. The mean (mean ± SE) live weight of fluke infected liver of local Menz breed sheep was 302.7 ± 29gm and this was lower than Aawassi Menz cross bred sheep (386 ± 48 gm). On the other hand, the corresponding mean live weight of a liver of local zebu cattle and Holiestine-Frezian crosses were 504 ± 34 and 583 ± 29gms, respectively (Table 6).

The overall mean number of flukes recovered from sheep and cattle were 67.5± 5.6 and this was ranged from 16 to 175. However, the mean number (77.5± 0.7) recovered from the cattle was relatively high in comparison to the lower count (59.6 ± 0.6) obtained from sheep. The live

weight of fluke infected liver and fluke count of both sheep (r= 0.65) and cattle (r=0.73) have been positively correlated.

Table 4.6. The mean live weight measurement of the fluke infected liver and the corresponding mean fluke burden

Risk factors		Live weight (gm) (M ± SE)	Range (M ± SE)	Fluke burden (M ± SE)	Range (M ± SE)
Species	Bovine (N=623)	539 ± 41	483-610 ± 30	77.5 ± 0.6	24 -174 ± 20
	Ovine (N=438)	344.4 ± 38.5	254- 392 ±28	59.6 ± 0.6	16-154 ± 15
Breed	Awassi-Menz cross	386 ± 48	296-423 ± 34	63.2 ± 0.3	36-154 ±12
	Menz sheep	302.7 ± 29	245-338 ±21	56 ± 0.3	16-145 ±13
	Holeistin-Fresien	583 ± 29	522-647 ±22	81 ± 0.7	32-175 ±20
	Zebu (Bos indicus)	504 ± 34	470-563 ±31	74 ± 0.6	24-162 ± 16

Prevalence = Number positive/Total number; OR, Odds ratio; CI, confidence interval; P value, Probability value

From a total 17800 intact flukes used for morphometric analysis in cattle, 13172 (91.5%) were matured and 4628 (8.5%) immature. Similarly in sheep, from a total of 8560 intact flukes 6755 (78.9%) were matured and 1805 (19.1%) were immature. Accordingly, the mean length (mm) and width (mm) of matured *F. hepatica* in cattle was 27.8 ± 0.23 (21-30± 4.5) and 5.92 ± 0.05,

respectively. The corresponding length and width *F. hepatica* in sheep was 23.4 ± 0.6 (20-25± 2.4) and 4.12 ± 0.54. In sheep, the mean length and width of matured *F. gigantica was* 25.1± 2.38 (22-28± 3.4) and 8.3± 3.2, respectively. In cattle both the length and width of *F. gigantica* are relatively larger than sheep. Hence, the mean length and width were 25.1± 2.3 and 8.3± 3.2 in sheep and 31± 3.2 (22-28± 3.4) and 9.5± 2.7 in cattle, respectively (Table 7).

Table 4.7. The morphometric analysis of the length and width of *F. hepatica* and *F. gigantica* from sheep and cattle analysis (Milimeter**,** mm)

Species	Fluke status		*Fasciola hepatica*			*Fasciola gigantica*		
			Length	Width	Range	Length	Width	Range
	Mature	Immature						
Bovine (N=17800)	13172	4628	27.8 ± 0.23	5.92 ± 0.05	21-30 ± 0.45	31 ±3.2	9.5 ± 2.7	25-35 ± 2.5
Ovine (N=8560)	6755	1805	23.4 ± 0.6	4.1 ± 0.54	20-25	25.1 ± 2.3	8.3 ± 3.2	22-28 ± 3.4

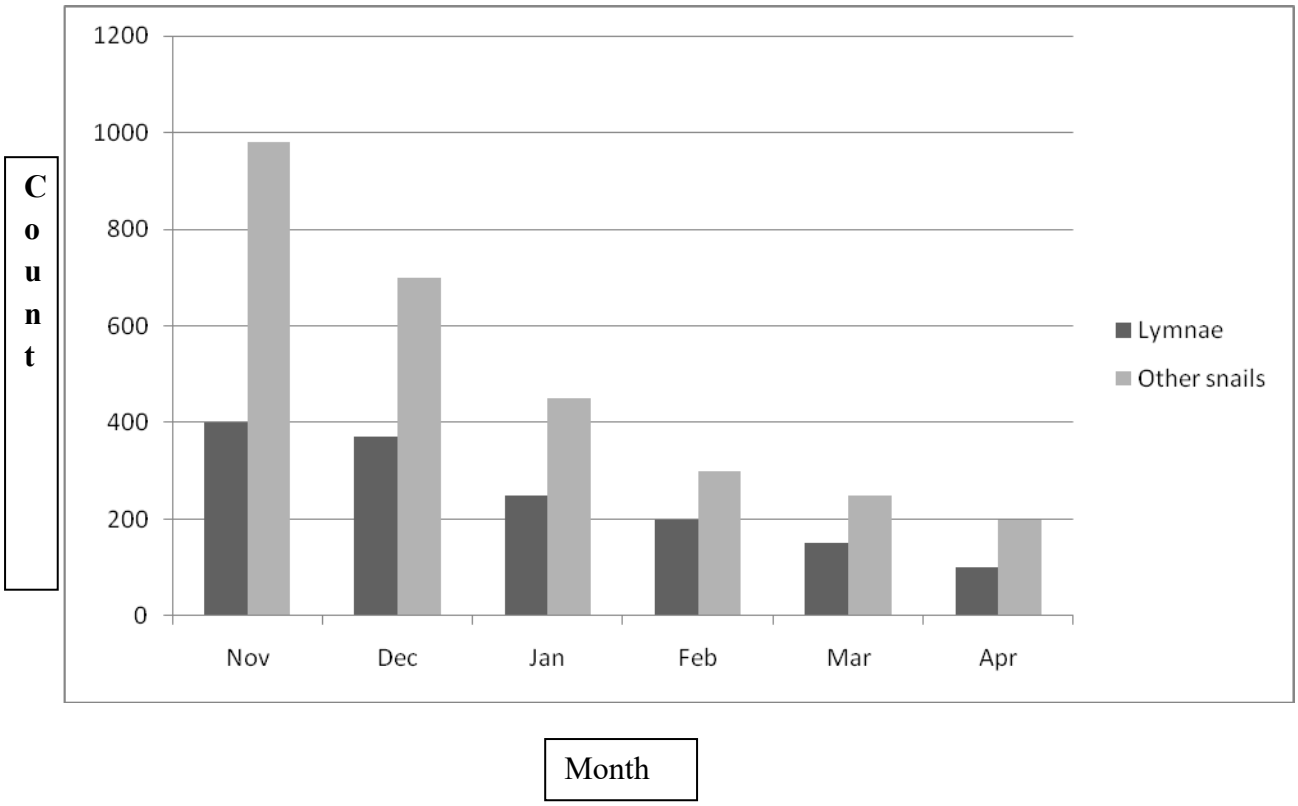
N= number of flukes recovered

4.3. Snail survey

A total of 4350 snails belonging to different genera (*Lymnaea*, *Biompholaria*, *Bulinus*, *Physa, Bivalvia* and *Ancylus* species) were collected during the long dry season of the snail survey in those villages around Deber Berhan town. The snail species identified were clearly disclosed the existence of *Lymnaea (Galba) truncatula, L. (Radix) natalensis, Biomphalaria pfeifferi, Bulunus truncates, Bulinus forscale,* Physa, Bivalvia and Ancylus species. Among the genera collected, *Lymnaea* species was the dominant and most abundant (1470) snail species encountered. The majority (905) were actually *L. truncatula*. However, *L. natalensis* (465) was encountered in those areas at the dearth of 1800masl and around Angolela river with higher elevation above 2600masl. The study clearly demonstrated the presence of the two recognized snails serving as intermediate hosts of fasciola species *L. truncatula* and *L. natalensis* snails were also collected from Rift valley area as well.

The pH of the different water bodies ranged from 6.9 to 8.35. The abundance of Lymnaea and other snails was highest during the cooler months with the highest record during the month of October and progressively decrease across the dry period with the lowest record during the month of April (Figure 2). The cercaria shedding pattern of the Lymnaied snails had shown that, out of those 1470 snails, 82 (5.6%) were positive for *Fasciola* cercariae and 45 (3.0%) were releasing cercariae of other trematodes.

Figure 4.2. Monthly prevalence of Lymnaea and other snail vectors in the study area



4.4. Postmortem examinations

4.4.1. Prevalence of fasciolosis in different abattoirs

From a total of 5427 ruminant livers examined in five abattoirs, 2530 (46.6% ± 0.059) were indicated the presence of liver flukes. The total number of livers observed in export abattoirs (ELFORA and HELIMIX) were 2330 (48.4%) while the remaining 3097 were (51.6%) from municipal abattoirs (Addis Ababa, Debre Berhan and Bahrdar). The overall prevalence of fasciolosis observed among the abattoirs had shown highly significance difference ($p<0.05$). Hence, the overall prevalence of fasciolosis observed in ruminants slaughtered in export abattoirs was 34.6% (877/2530) whereas it was significantly higher in ruminants slaughtered at municipal abattoirs 65.2% (1653/2530) as the whole. Comparison of the prevalence of fasciola positive ruminants in each of the four different abattoirs with respect to the prevalence observed at HELIMIX (44.9% ± 0.087) indicated significantly higher prevalence at Debre Berhan (80.1% ± 0.95) than the rest of the abattoirs (Table 4.8).

Table 4.8. The prevalence of fasciolosis in different abattoirs, origin, altitude of slaughtered ruminants

Risk factor		SE	Number Examined	Number Positive	Overall Prev. (%)	Prev. (%)	Sig	Exp (B)	95% CI	
									LB	UB
Abattoir	AA	.059	987	322	12.7	32.6	.000	1.681	1.411	2.004
	BD	.090	797	278	11	35	.000	1.520	1.263	1.829
	DB	.095	1313	1053	41.6	80.1	.000	.201	.168	.240
	ELFORA	.091	1149	349	13.3	30.3	.000	1.882	1.587	2.231
	Helimix	.087	1181	530	20.9	44.9	.	.	.	.
Origin	Afar	.	338	84	3.3	24.9	.119	1.389	.919	2.100
	Arsi	.211	1151	462	18.4	40.1	.035	.685	.482	.974
	Bale	.180	239	91	3.6	38.1	.176	.747	.490	1.140
	Borana	.215	524	118	4.7	22.5	.021	1.581	1.071	2.334
	Gojjam	.199	559	199	7.8	35.6	.333	.831	.572	1.208
	Gondar	.208	326	122	4.8	37.4	.197	.768	.515	1.146
	Hararghe	.223	387	203	8	52.4	.000	.416	.283	.613
	North Sh.	.197	1352	1073	42.4	79.4	.000	.119	.084	.171
	Somali	.188	287	86	3.4	30	.738	1.074	.708	1.629
	Wolaita	.213	102	41	1.6	40.2	.149	.684	.408	1.146
	Wollo*	.263	162	51	2	31.9	.000	.	.	.
Altitude	Highland	.052	2331	1358	53.7	58.3	.000	.497	.438	.562
	Lowland	.070	1276	427	16.8	33.4	.000	1.378	1.187	1.600
	Midland*	.081	1820	745	29.5	40.9	.	.	.	.

SE= Standard Error, Prev. = Prevalence, Exp (B)= Exponentiation of the coefficient, LB= Lower Bound, UB= Upper Bound, CI= Confidence Interval

4.4.2. Prevalence of fasciolosis in different geographical origin

The difference in the overall prevalence of fasciolosis based on their geographical origin was significant ($P<0.05$). The overall prevalence of liver fluke infection was higher in ruminants

from North Shoa (42.4% ± 0.2) followed by Arsi (18.4 ± 0.21%), Hararghe (8% ± 0.2), and Gojjam (7.8 ± 0.2%) respectively. However, the overall abattoir prevalence of fasciolosis in ruminants from the rest of the origins (<5%) considered in this study were much lower (Table 1).

The overall number of slaughtered ruminants brought to the abattoirs had shown that the majority had their origin from North Shoa (1352 / 24.9%) followed by Arsi (1150/ 21.2%) and Borana (524/ 9.7%), respectively. However, the greater part of ruminants brought to the abattoirs from North Shoa (79.4%) and Hararghe (52.4%) were found positive for liver fluke infection, whereas significant number of ruminants from Gondar (41.8%), Wolaita (40.2%) and Arsi (40.1%) were found to harbor fasciola species (Table 4.9).

4.4.3. Prevalence of fasciolosis in different altitude ranges

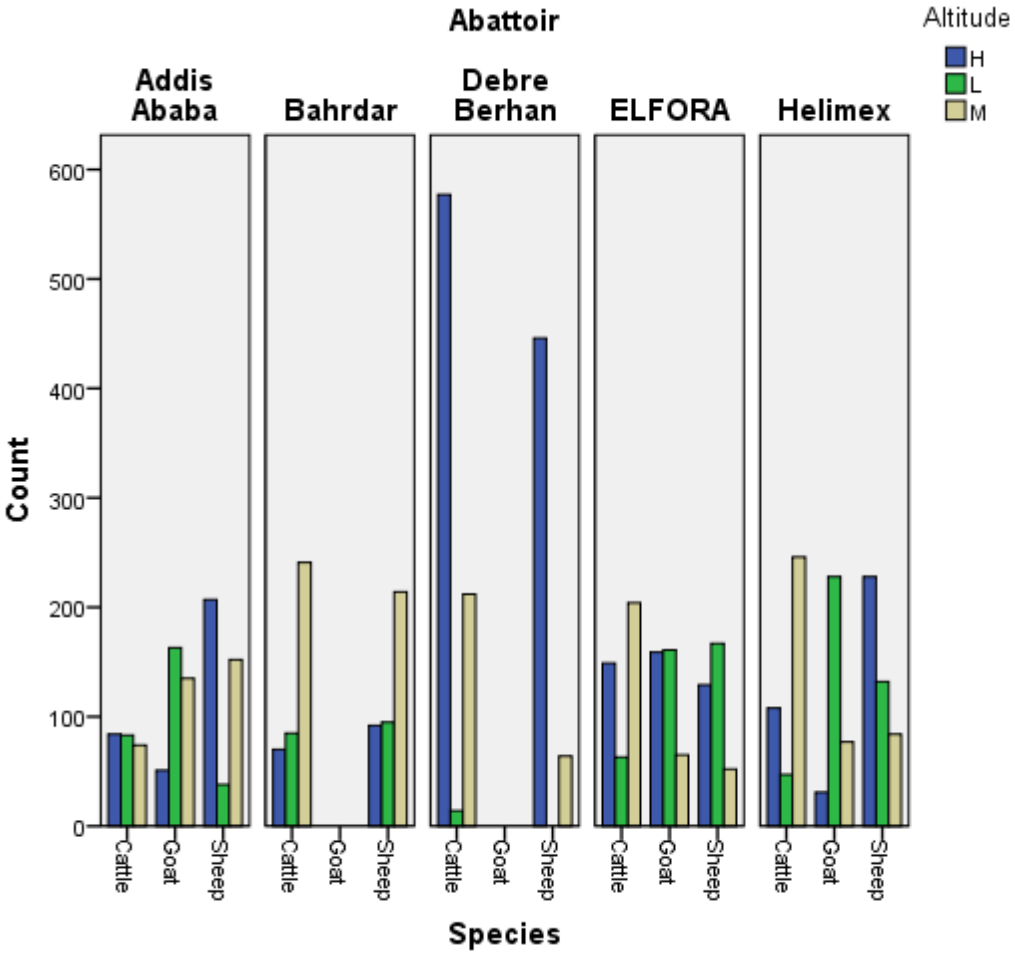
There have been significant differences ($P<0.05$) on the overall prevalence of fasciolosis among the slaughtered ruminants from various agroecological areas (altitude difference). Accordingly, it had significantly higher prevalence among ruminants from highlands (53.7% ± 0.52) followed by midlands (29.5% ± 0.81) and lowlands (16.8% ± 0.07), respectively (Table 1).

The majority of the ruminants slaughtered at abattoirs were from the highlands (43% / 2331) and the midlands (33.5% / 1820) followed by the lowlands (24% / 1276). Altogether, midlands and highlands had contributed more than three fourth (76.5% / 4151) of the total slaughtered ruminants in the abattoirs. Furthermore, the numbers of small ruminants (984 /) from the lowlands were lower in contrary to the relatively higher number of small ruminants supplied to the abattoirs from the highlands (57.6% / 1343) and midlands (46.3%/843). However, substantial

number of cattle (42.4% / 988) and sheep (24.1% / 564) had their origin from the highlands and midlands, respectively. On the other hand, more than half (51.6% / 552/1070) of goats slaughtered in the abattoirs were from the lowlands (Figure 4.3).

The present study indicated that the numbers of small ruminants supplied to the two export abattoirs from the different agroecological zones were lower than those supplied to the sum of the three municipal abattoirs and the different was not significant ($P>0.05$). However, those supplied to Debreberhan abattoir (only sheep) from the highlands was substantial (38.8% / 510). Similarly large numbers of ruminants supplied to Bahrdar (50% / 401) and Addis Ababa (37% / 361) municipal abattoirs were from the midlands. Meanwhile, sheep and cattle (68% / 3686) slaughtered in municipal abattoirs were mainly from the highlands and midlands as a whole. The supply of goats to the export abattoir is mainly from the lowlands. The present study revealed that goats were never encountered at Bahrdar and Debreberhan municipal abattoirs (Figure 4.3).

Figure 4.3. The supply of ruminants to the export and municipal abattoirs from different agroecological zones



4.4.4. The prevalence of fasciolosis in different species and age

From the overall prevalence of fasciolosis (46.6% ± 0.06) reported in ruminants, the overall prevalence encountered in sheep (23.8% ± 0.109) was significantly lower than that of cattle (45.3% ± .059), but it was significantly higher than the overall prevalence observed in goats (5.4% ± 0.72). Nonetheless, out of a total of 2257 cattle, 1189 sheep and 1070 goat livers examined, the prevalence was 50.8%, 58% and 12.2%, respectively (Table 4.9).

Table 4.9. The abattoir prevalence of ruminant fasciolosis OR (95% CI) with respect to different factors assessed (Sex, Age, Breed, Body condition)

Risk factor		SE	Number Examined	Number Positive	Overall Prev. (%)	Prev. (%)	P value	OR (95%) CI
Species	Cattle	.061	2257	1147	45.3	50.8	.000	1.4 [1.3 1.6]
	Goats	.102	1070	137	5.4	12.8	.000	9.9 [8.1 12.1]
	Sheep	.	2100	1246	23.8	59.3	.	.
Sex	Female	.061	1514	744	29.4	49.1	.021	0.87 [0.77 0.98]
	Male	.	3913	1786	70.6	45.6	.	.
Age	Adult	.055	2792	1320	52.3	47.3	.269	1.1 [0.95 1.2]
	Young	.	2234	1083	42.8	48.5	.	.
Breed	Cross	.066	1198	685	27.1	57.2	.000	0.58 [0.51 0.66]
	Local	.	4229	1845	72.9	43.6	.	.
*BC	Good	.061	1951	801	43.2	41	.002	1.2 [1.1 1.4]
	Poor	.040	2565	1173	56.8	59.4	.000	.
Fasciola	FH		1974	1399	70.9			
	FG		1974	424	21.5			
	Mixed		1974	135	6.8			
	Immature		1974	192	9.7			

SE= Standard Error, Prev. = Prevalence, P value = Probablity Value, CI= Confidence Interval, OR= Odds Ratio *BC= Body condition

The total numbers of adult ruminants (52.4% / 2792) slaughtered were relatively higher than the young animals (42.6% / 2234). However, the prevalence of fasciolosis was relatively lower in adult (47.3% ± 0.57) animals than the young (48.5% ± 0.42) ruminants and the difference was not significant (Table 2). In both export (42.9% / 2330) and municipal (57.1% / 3097) abattoirs the slaughtered ruminates were predominantly adults. In all abattoirs, irrespective of the origins the large numbers of the slaughtered ruminants were adults. Nonetheless, most of those that had

their origin from North Shoa (79.4%), Gondar (52.4%) and Hararghe (37.4%) were young ruminants (Figure 4.3).

The species analysis had shown that most of the total slaughtered cattle (53.3% / 1024), goats (55.6% / 595) and sheep (59.2% / 1243) were adult. Similarly, irrespective of the agroecological zones (highland (54.7%), midland (54.5%) and lowland (56.3%)) most of the slaughtered ruminants were adults.

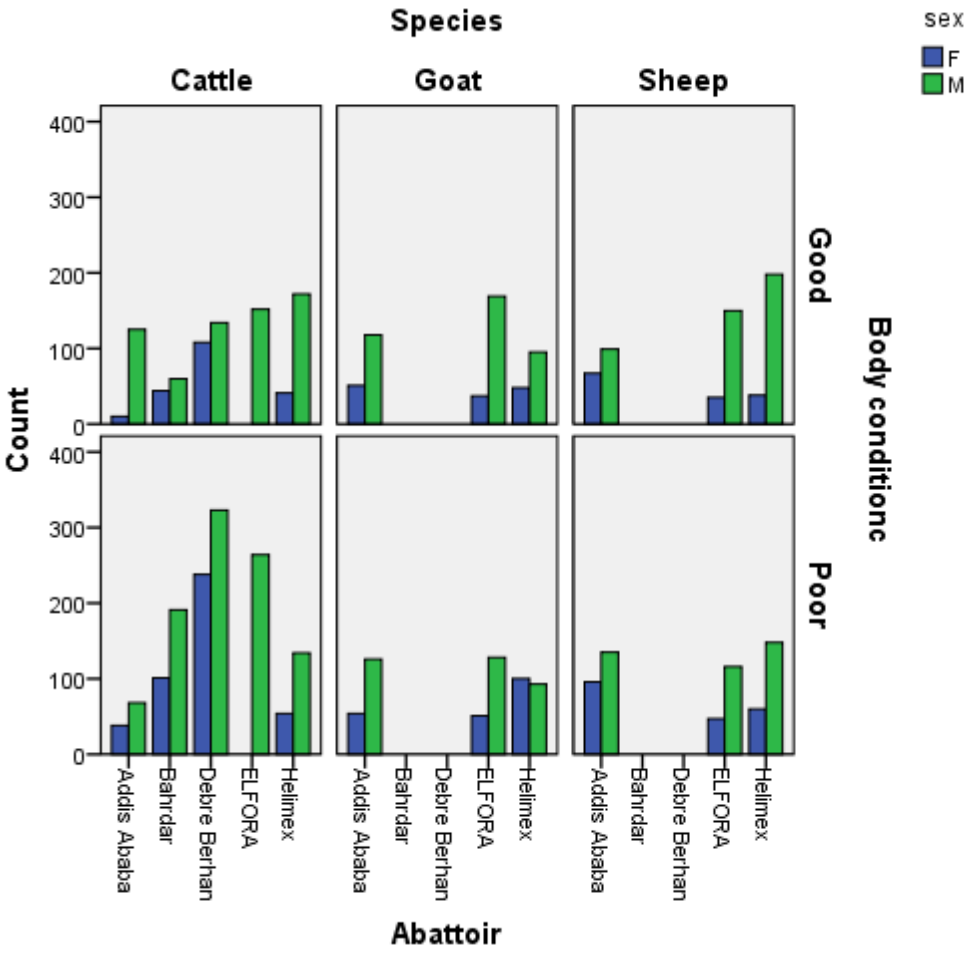
4.4.5. The prevalence of fasciolosis in different sex and breed

The slaughtered ruminants were predominantly male (71% / 3913). On the other hand, out of the total slaughtered ruminants, female animals account for about one third (29% / 1514) only. The difference in the overall prevalence between the sex groups was significant. The overall prevalence in male (71%) was significantly higher than the female (18.1%) ruminants, however, out of 1979 fluke positive animals, 52.4% were male and 22.8% were females (Table 2). In addition, out of 3913 male and 1514 female animals slaughtered, the prevalence was 45.6% and 49.1% respectively. Hence, the female appears to show higher prevalence despite the overall lower number of female animals slaughtered (Table 4.9).

Relatively large numbers of male animals were slaughtered in export abattoirs than the municipal abattoirs. Accordingly, 979 and 840 male animals were examined for fluke infection in ELFORA and HELIMIX export abattoirs, respectively. Meanwhile, the proportion of male ruminants were still significant than the female in all the remaining municipal abattoirs including those in Addis Ababa (671/987), Debreberhan (457/803) and Bahrdar (251/396) abattoirs. The number of male animals was also significant than the female animals with respect to the origin, species and

agroecological zones (altitude differences) and body conditions considered (Table 4.9 and Figure 4.4).

Figure 4.4.The sex of slaughtered ruminants with respect to the species, the abattoirs and body condition



The local breed of ruminants represented more than three fourth (78% /4229) of those entirely slaughtered at the abattoirs. Hence, the overall prevalence of fasciolosis was significantly higher in local breed (72.9% ± 0.66) of ruminants than the cross breed (27.1% ± 0.66) animals. However, out of 1198 cross bred and 4229 local bred ruminants slaughtered, 685 (57.2%) and 1845 (43.6%) were found to harbor liver flukes, respectively. Despite the lower number of cross

breed ruminants slaughtered, this result indicated the significantly higher prevalence of fasciolosis along with cross breed (57.2%) ruminants than the locals (43.6%) (Table 4.9). The trend in the number of ruminant slaughtered (cross and local breeds) was similar irrespective of the differences in origin, agro ecology (altitude), species and abattoirs considered.

4.4.6. The prevalence in different body condition

More than half of the slaughtered ruminants (56.8%) in poor body condition were positive for the liver fluke infection compared to the lower overall prevalence (43.3%) observed by ruminants in good body condition status. From a total of fasciola positive ruminants, the prevalence of liver flukes in poor body condition animals were 59.4% (Table 4.9).

However, the prevalence observed in ruminants with good body condition status was only 41%. 53.4% (1370/2565) of the ruminants slaughtered at municipal abattoirs were predominantly not in good body condition. Altogether, the prevalence of poor conditioned ruminants slaughtered at export abattoirs were 46.6%. On the other hand the prevalence of fasciolosis in ruminants with good body condition at export and municipal abattoirs was 58.2% and 41.8%, respectively.
Significant numbers of slaughtered cattle (62.5% / 1411) were in poor condition compared to the relatively lower number in goats (52%/ 552) and sheep (51%/ 602). Meanwhile, the difference in fasciola prevalence among the body condition status (good and poor) of cattle, sheep and goats was significant.

Similar trends have been observed for the prevalence of fasciolosis in body conditioned status of ruminants with their respective origins and agroecological zones. Hence, the poor body conditions and the higher prevalence of fasciolosis were positively related irrespective of their geographic origin (r=0.58) or altitudinal (r= 0.69). Young, male and local ruminants represented significantly higher number of poor body conditioned animals in both export and municipal abattoirs than ruminants with good condition.

4.4.7. The different prevalence of fasciola species

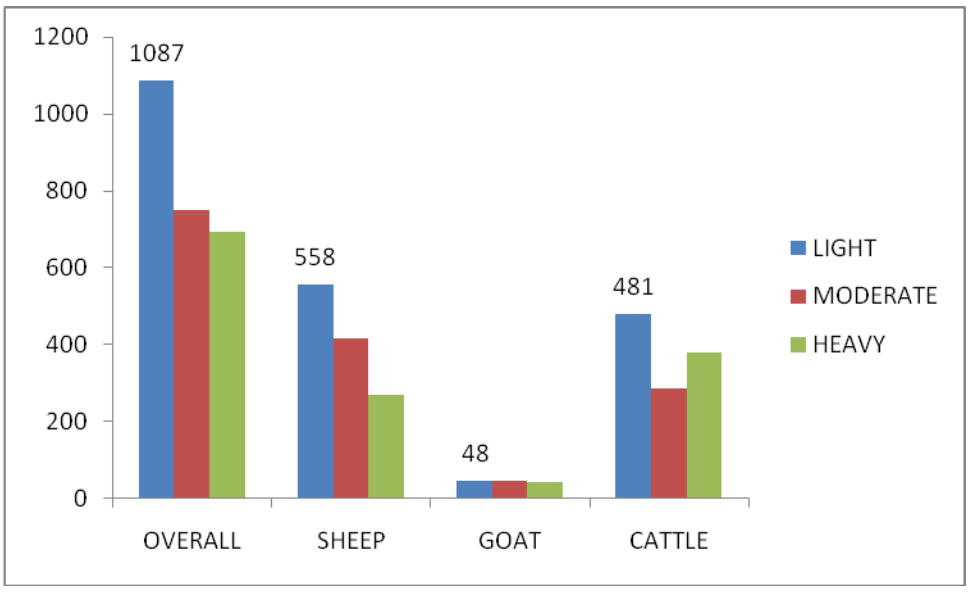
In general, *Fasciola hepatica* (70.9%) was a dominant fasciola species identified followed by *Fasciola gigantica* (21.5%), mixed infections (6.8%) and immature flukes (9.7%). *F. hepatica* and *F. gigantica* had been identified from all the slaughtered ruminants (cattle, sheep and goats) and in both the export and municipal abattoirs. However, *F. hepatica* was the only species identified from ruminants that had their origin from the highlands. Both *F. hepatica* and *F. gigantica* were identified from the midland ruminants. *F. gigantica* was the only diagnosed species from ruminants originated from the lowlands. Mixed infections were observed in ruminants from midlands and all the abattoirs included in the present study. The result indicated that, immature flukes were found in ruminants slaughtered in all of the abattoirs irrespective of the origin of the ruminants and altitudinal variations and other factors considered.

4.4.8. Severity of the liver lesions

The lesions of fluke infections were clearly observed in the livers of all the three species of ruminants slaughtered in the export and municipal abattoirs, irrespective of their geographical

origin and agroecological variations. The overall lesion observation had shown that most of the fluke infected livers examined were affected lightly (20%/ 1087). The numbers of moderately (13.8%/ 752) affected livers were slightly higher than those heavily infected livers (12.8%/ 692). However, the difference in the number of ruminants with light liver lesion was significant (P>0.05) than that of either the moderately or heavily infected ruminants. But, the difference in overall count of moderately and heavily affected livers was not significant (Figure 4.5).

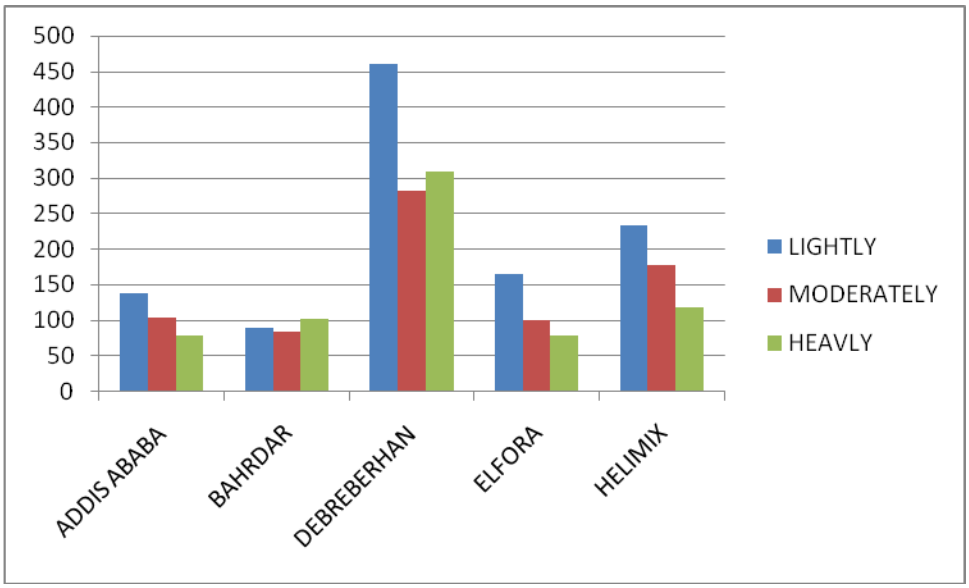
Figure 4.5. The severity of liver lesions in ruminant species (sheep cattler goats)



22.1% (558/2530) and 19% (481/2530) of lightely affected livers in sheep and cattle were outnumbered either the livers with moderate (417 and 287/2530) liver fluke infection or the heavely (271 and 379/2530) infected ones, respectively. In cattle alone significant number livers were heavely (15%) infected compared to the lower count of moderately (11.3%) infected livers. The severity of the diffrences in the number of fluke infected liver of goats were not significant in either light, moderate or the heavly infected groups (Figure).

Irrespective of the lesion types, fluke induced lesions were abundent in Debereberhan municipal abattoir followed by Helimix export abattoir. In all the abattoirs the predominant lesions encounterd were light type except the slightly higher count of heavy lesions observed at Bahrdar abattoir (Figure 4.6).

Figure 4.6.The severity of the lesions of fluke infected livers in different abattoirs



Irrespective of the orgin of the ruminants, fluke infected livers were predominantly lightly affected and the subsequently observed lesions were moderate type. However, observation of fluke infected ruminant livers from North Shoa had shown that most of the livers were lightly affected followed by heavly infected livers instead of the moderate types observed elsewhere.

The light type of lesion was predominant on ruminants from the highlands and followed by those from the midlands and lowlands, respectively. Similarly moderate lesion type outnumber in

highlands and midlands respectively. However, the heavily infected livers were more pronounced in ruminants from the lowlands with successive light type lesions. However, ruminants with heavy infection of fluke infeceted livers were niticeably the lowest in the midlands.

The predominant lesion types in both the local and cross breed ruminants were light type and successively encountered lesion was the moderate type. The difference in the number of lesions types between cross and local breed animals was not significant, although the light (283) types slightly outnumber the moderate (200) and the heavily (200) infected ones. However, in local breed animals the light type (804) lesions significantly higher than the lesions categorized as moderate (551) and heavily (442) infected ones (Figure 4.6).

The liver lesions were more pronounced in male and adult ruminants than the female and the young ones. The observation of fluke infected livers indicated the predominance of light type lesions. However, in male and young ruminants the moderate type of lesions was abundant. This is not the case in female and adult ruminants where the high count of the light types of lesion was succeeded by the relatively high number of heavily infected livers.

The overall mean number of flukes recovered from sheep and cattle were 65.5± 5.6 and this was ranged from 16 to 175. However, the mean number (74.5± 0.7) recovered from the cattle was relatively high in comparison to the lower count obtained from sheep (57.6 ± 0.6) and goats (35.6 ± 0.8).

4.4.9. Economic losses incurred at abattoirs

The retail price of the liver of cattle at export abattoirs was 30 ETB / 1.43 USD while that of small ruminants was 12 ETB / 0.57 USD only. However, this had shown variation among municipal abattoirs (25-48 ETB for liver of beef cattle and 12 to 22 ETB for shoats). The mean annual financial loss recorded altogether in export and municipal abattoirs was 7,049,638 ETB / 335, 697.1 USD. However, the overall mean financial loss observed in three municipal abattoirs (5,260,596 ETB / 250,504.6 USD) was significantly higher than the combined loss incurred in two export abattoirs (1,789,043ETB / 85,192.5 USD).

Comparison of the loss incurred in two export abattoirs for beef cattle had shown slightly lower loss at HELIMEX (474,682.5ETB/22,603.9USD) than the ELFORA (499,320/ 23,777) export abattoir. Similar pattern of rejection of fluke infected liver for human consumption and the associated loss of the wholesale price was observed for sheep at HELMIX (257,040 ETB / 12,240 USD) and ELFORA export abattoirs (294,000 ETB/ 14,000 USD) (Table1). The mean loss incurred in meat goats was relatively lower at HELIMIX (112,800 ETB/ 5,371.5 USD) than at ELFORA (151,200ETB/ 7,200 USD) export abattoir. The order of substantial economic loss due to liver condemnation was observed for beef cattle followed by sheep and goats, respectively (Table 4.10).

Table 4.10. The annual economic loss due to ruminants liver condemnation in two export abattoirs

Species	Abattoir name	Location (town)	Annual slaughter capacity	Prevalence (%)	Retail price (ETB/USD)	Estimated loss (ETB/USD)
Bovine	ELFORA (export)	Debre Zeit	54,750	28.9	30	474,682.5/22,603.9
Bovine	Helimix (export)	Debre Zeit	54,750	30.40	30	499,320/23,777
Ovine	ELFORA (export)	Debre Zeit	100,000	24.5	12	294,000/14,000
Caprine	ELFORA (export)	Debre Zeit	100,000	12.6%	12	151,200/7200
Ovine	Helimix (export)	Debre-Zeit	100,000	21.42	12	257,040/12,240
Caprine	Helimix (export)	Debre-Zeit	100,000	9.4	12	112,800/5371.5
Total						1,789,043 / 1,681,614

Comparison of the financial loss observed for slaughtered animals at three municipal abattoirs had shown that, it was significantly higher at Addis Ababa (4,843,837ETB) municipal abattoir followed by Debreberhan (375,153.4ETB) and Bahrdar (41,605.2 ETB). The loss for bovine fasciolosis was significantly higher in both Addis Ababa and Debre Berhan abattoirs than the loss incurred in small ruminants (Table 4.11).

Table 4.11. The annual economic loss due to ruminants liver condemnation in three municipal abattoirs

Species	Abattoir name	Location (town)	Annual slaughter capacity	Prevalence (%)	Retail price (ETB/USD)	Total price (ETB/USD)
Bovine	DB (municipal)	DB	7392	77.8	48	288223.5/13724.9
Bovine	Bahrdar (municipal)	BD	4368	38.1	25	41605.2/1981.2
Bovine	AA (municipal)	AA	177,781	48.8	35	4311189/205294.7
Ovine	DB (municipal)	DB	4,704	84	22	86929.92/4139.5
Ovine	AA (municipal)	AA	117,780	30.3	12	428248.1/20392.8
Caprine	AA (municipal)	AA	60,000	14.5	12	104400/4971.4
Total						805391.3/38352

*DB = Debre Berhan, AA= Addis Ababa, BD= Bahrdar

4.5. Snail rearing and exposure to the infective miracidia

4.5.1. Snail collection exposure with miracidia

The snails collected for exposure with miracidia and subsequent harvest of the cercariae shed were identified in the laboratory as *G. truncatula* snails. The morphological measurements on the snail vectors of *F. hepatica* and *F. gigantica* were indicated in the table 4.12. The shells of *G. truncatula* were between 4.8 and 6.0 mm long at the start of the experiment.

4.5.1. Laboratory rearing of the snails

The present method used for the *G. truncatula* snail rearing, maintenance of the aquaria and diet are extremely suitable in the laboratory..

Table 4.12. The morphological measurements of the snail intermediate hosts *G. truncatula* and *R. natalensis* collected from rivers

Area	Snails	Source	Stage of the snail	Length of the shell (mm)	Breadth the shell (mm)	No. whorls	Aperture size (mm)	
							Length	Breadth
Angolela	*L. truncatula*	River	Adult	7.0±1.3	2.5±.1.5		1.3±0.2	0.8±0.9
Guta	*R. natalensis*	River	Adult	13.3±3.04	5.56±1.3	3.3±1.3	7.72±0.2	5.5±0.1
Wondo-Genet		Ponds & canals	Adult	12.3±2.3	4.50±1.3	2.9±1.3	6.72±1.2	4.4±0.1
Tikur Wuha		River	Adult	10.8±1.04	4.45±1.3	2.8±1.3	5.62±1.2	5.4±1.0

The size of freshwater pulmonate snails had shown variations from different rivers, ponds and canals. The *G. truncatula* collected from rivers had shell lengths ranging from 6.8 ± 1.3 to 7.2 ± 1.3 mm with largest being from Angolela and the smallest from Wondogenet. However, in *R. natalensis* this was ranges only from 10.8±1.04 in Tikur whuha to 13.3±3.04 in Guta river (Table 4.12 above). Shell length at collection and death, metacercarial production, length of days, prepatent and patent periods are indicated in the table 4.13 below

Table 4.13. Snail species collected from rivers, ponds and canals

Snail species	*L. truncatula*				*L. natalesis*			
Rivers	Angolela	Guta	Wondogenet	Tikurwuha	Angolela	Guta	Wondogenet	Tikurwuha
Number of snails at the beginning	100	10	50	40	-	80	60	70
Day 30 (%)	75 (75.0)	6 (60.0)	27 (54.0)	22 (55.0)	-	65 (81.3)	45 (75%)	52 (74.3)
Number of CS snails	45	4	23	16	-	33	24	26
Prevalence (%)	45	40	46	40	-	41.3	40	37.1
Shell height at collection	7.0±1.3	7.1±1.3	6.7±1.8	6.9±1.3		13.3±2.1	14.4±3.4	12.5±4.1
Shell height at death	8.0±1.3	8.2.1±1.3	8.7±1.8	8.9±1.3		14.3±2.1	15.4±3.4	13.5±4.1
Patent period	27.4 ± 6.8	26.7 ± 8.2	24.7 ± 7.2	22.7 ± 7.2	-	30.7 ± 8.2	28.7 ± 7.2	29.7 ± 7.2
Total metacercarae (Mean value ± SD)	13,500	11,125	9,450	7,800	-	14,500	12,360	13,100
Per SC snail	300	278.1	205	195		351.1	309	353.1

CS = Cercaria shedding snails, SD = Standard deviation

Compared to *G. truncatula* (55.4%, $p < 0.05$) collected from rivers, ponds and canals and subjected to follow up on their survival (Table 5.3.2) under laboratory conditions, the survival of *R. natalensis* on day 30 was significantly higher (77.1%, $p < 0.05$), while the prevalence of *F.*

hepatica infection was significantly lower ($p < 0.05$) and the shell height of cercaria shedding snails significantly greater ($H = 14.3 \pm 2.1$, $p < 0.05$). The prepatent period was significantly longer ($p < 0.5$) in *R. natalensis* than the *G. truncatula*. In contrast, slight but insignificant difference between the lengths of patent periods was noted. The mean numbers of metacercariae were significantly higher ($p < 0.05$) in *R. natalensis* than in *G. truncatula*. There was considerable difference on metacercarial production of individual snails in both groups (Table 4.14). The lowest and highest metacercarial production by individual *G. truncatula* snail ranges from 19 to 3950 larvae while that of *R. natalesis* was 25- 3280. The highest totals of metacercariae were recorded for *G. truncatula*, with 14 snails (out of 100 from river Angolela) and 10 (out of 45 in Wondogenet) shedding more than 500 larvae per individual.

Table 4.14. Metacercarial production of *G. truncatula* infected with *Fasciola hepatica* miracidia (one, three and five for groups A, B and C, respectively) and maintained at constant temperature 20^{o}C and kept on algal food (Blue green algae).

Experiment	Shell length at infection	Shell length at death	Days between Infection and first Cercarial shedding	Duration of shedding (days)	No. of cercariae shed
A	5.8±0.0	6.8±1.8	45±5.5	10.6±4.5	295.7±70.6
B	4.8±0.0	8.5±.1.2	38±6.5	18±5.5	1863.6±970.6
C	6.0±0.0	7.8±.1.5	25±0.4	23±1.3	721.8±390.6

In miracidial infections, for groups A, B and C the cercarial output ranged from 20-1083, 144-3879 and 28-2645 respectively. Snails infected with single miracidium were produced less cercaria than either those which infected with three (Group B) or five metacercaria (Group C)

(Table 4.14). The highest estimated number of cysts produced by each miracidium was about 3879.

The mean shell length at death of the snails was between 6.8±1.8 and 8.5±.1.2. Snails with larger shell size showed a lower infection rate, the groups presenting the highest (80%) and lowest (3%) proportions of positives being those between 6.8±1.8 mm to 7.8±.1.5 and 8.5±.1.2. mm or more, respectively. The shell lengths recorded at death concides with high cercaria shedding of snails in Group B. Snail of Group B produced more metacercaria than Groups A and C ($P < 0.05$). Cercariae were present in 78.1% of them at 30 days post-infection, and cercarial shedding was observed 65 days post infection. Duration of shedding days was longest in group C followed by groups B and A. It seems that the longevity of shedding days coincides with shell length at infection and it was concluded that there is a non-linear negative association between shell size and infection rate (Table 4.14).

In each groups of snail population considered separately, the shell height of CS snails in the two- and five-miracidia groups did not significantly differ from each other. However, this was significant in the one and five miracidia groups.

In groups A, B and C the survival of snails at day 30 post infection (78.1%) and the prevalences of *F. hepatica* infections (82.0%) did not show any significant variations ($p>0.05$). The mean number of cercariae was significantly higher ($p< 0.05$) in snails with relatively smaller shell length at infection group B. The patent period was significantly longer ($t = 6.41$, $p < 0.5$) in a group C with relatively larger shell size (at infection) than the other groups. There was

considerable difference on metacercarial production of individual snails in all experimental groups. The lowest and highest metacercarial production by individual *G. truncatula* snail was 20 and 3879 for groups A and B respectively. The highest totals of metacercariae were recorded for group B, with 2 snails (out of 20) shedding more than 500 larvae per individual (Table 4.15).

Table 4.15. The percentage of the survival at day 30 post exposures, the prevalence of infection in surviving snails and the total number of cercariae (mean ± SD).

Groups	Shell length at Infection	Shell length at death	Number of snails at exposure	Survival at day 30 p.e (%)	Prevalence of infection in surviving snails (%)	Total number of cercarae (mean ± SD)
A	5.8±0.0	6.8±1.8	10	80.5	81.0	100.3 ± 98.0
B	4.8±0.0	8.5±.1.2	10	78.3	83.0	185.1 ± 135.5
C	6.0±0.0	7.8±.1.5	10	75.5	82.0	152.3 ± 99.3

The values recorded in the three snail groups were also compared to determine whether the miracidial dose used for snail exposure had any significant effect on the characteristics of *F. hepatica* infection. In group B, the snail survival on day 30 post exposure was insignificantly higher (78.3%, $p > 0.05$) in the three-miracidia than in the five-miracidia group. In contrast, significant difference was recorded in snail survival between single miracidia (80.5%) and five-miracidia (75.5) groups. However, the prevalence of *F. hepatica* infection was insignificant in all infected groups (the single, thee and five-miracidia groups) of *G. truncatula* populations from river Angolela.

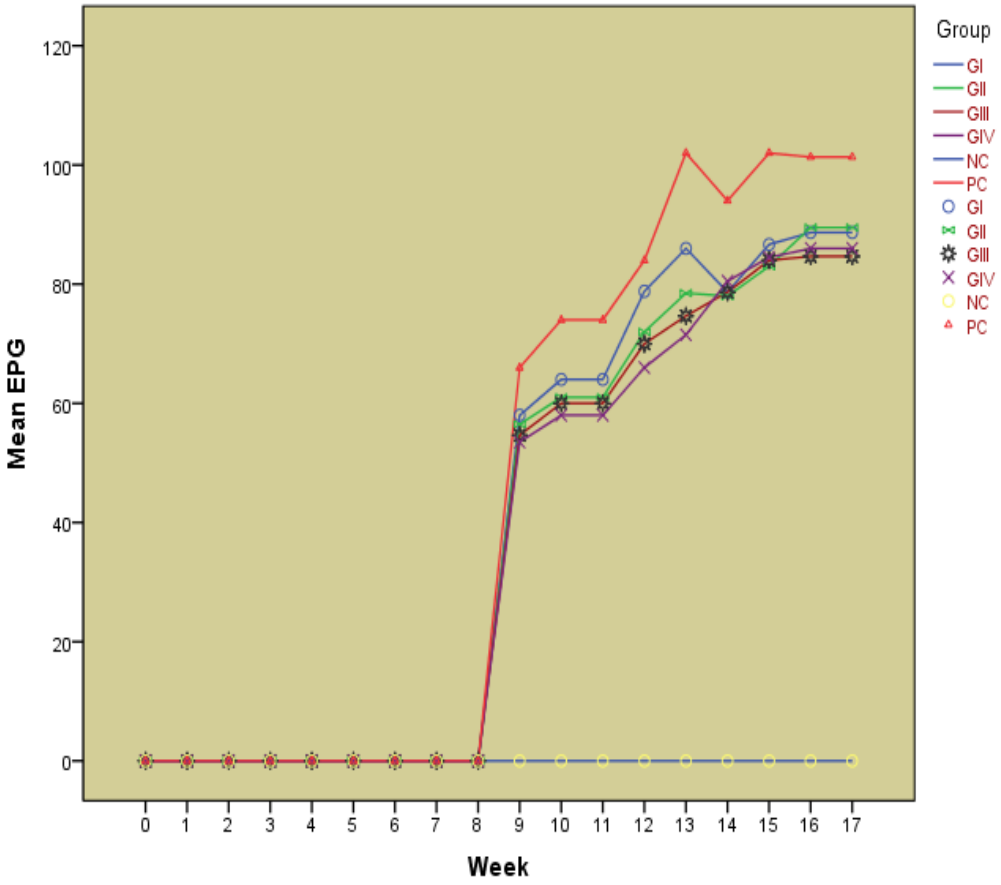
4.6. Experimental infection of sheep

4.6.1. The discharge of fluke eggs in the faeces and the EPG

All the vaccinated and infected control groups were found to shade eggs except the uninfected negative controls. Eggs were seen to shade starting from the eighth week post infection. These were first observed in infected control groups (PC) and those vaccinated with low dose of the parasite (GI dosed with 30 gray and GII sheep with 60 gray). The animals in high dose vaccinated groups (GIII received 120 gray and GIV dosed with 240 gray) began to shade eggs only after week 9 post infection. The uninfected control animals (NC) were never appeared to excrete eggs. The eggs discharged by all infected and vaccinated animals continued to be recovered until the end of the experiment 17 weeks post vaccination. Higher EPG output was observed in infected control (PC) followed by those animals vaccinated with low doses of the gamma irradiation (GI and GII).

The EPG of all groups steadily increased with time (Figure 4.6).

Figure 4.7. The eggs per gram of faeces in different experimental groups across time



The EPG of all groups steadily increased with time. The pattern of EPG raise was similar in all sheep that had received the irradiated infective metacercariae and those that were infected with the same number of non sensitized normal juvenile fluke. The clear differences in EPG output were noted between week 12 and 15 post infection. Infected control sheep had shown significantly ($P<0.05$) higher EPG level reaching peak 13 post infection compared to the group of sheep that were received different levels of gamma sensitized metacercariae.

The higher EPG of eggs (108) were recorded for positive control animals at the end of the experiment, 17 weeks post vaccination than the other groups. However, the EPG of higher dosed animals (GIII and IV) appeared lower than those vaccinated with lower doses (GI and II). In all cases slight variation of EPG was observed between those higher dosed group of animals in GIII and GIV. Similarly the EPG of GI animals was relatively higher than that of GII from the lower dosed animals (Figure). Generally with the exception of infected control animals, a relatively non significant EPG output was induced following vaccination with 500 metacercariae attenuated with 240Gy of γ-irradiation (GIV), or attenuated with 120Gy (GIII) of γ -irradiation compared to metacercariae γ-irradiated at 30Gy and 60Gys. These findings clearly disclosed the effect of degree of parasite attenuation (dose levels) on starting time of egg shedding and the level of EPG output in sheep irradiated with gamma cells provided, the levels of irradiation-attenuated infective stages were kept constant (500 metacercariae).

4.6.2. Fluke recovery and measurements

Out of the 500 flukes administered per os to each group, the mean (M± SE) number of flukes recovered vary significantly ($P<0.05$). The present finding indicated that, the higher the irradiation dose, the lowest was the recovery of flukes in necropsy examinations (Table 4.16). That means a strong dose response was evident in the number of parasites recovered in the groups dosed with incremental doses of γ-irradiation *F. hepatica* metacercariae. The recovered parasites were morphologically normal, patent, immature and adult liver flukes. The percentage of the flukes recovered (percentage reduction in fluke burden) unirradiated from the liver of the sheep were highest in infected control groups (22.5%; 112.3±4.3) followed by GI animals dosed with the lowest irradiation dose (18.6%; 93± 3.4). The percentage reduction in burden of flukes

in group II and III sheep was 13.4% (67.8 ± 2.8) and 11.2% (56.5± 7.18) respectively. Similarly, those animals in the highest irradiation dose (GIV) yield the lowest recovery (7.7%, 38.3± 3.3). Accordingly, the higher the irradiation dose, the lowest was the recovery of flukes in necropsy examinations.

A total of 350 (82%) recovered fluke lengths were measured from sheep in all infected (PC) and vaccinated groups. The overall number flukes counted from positive control group was significantly higher ($P<0.05$) than other vaccinated groups of the experimental animals.

Table 4.16. The number of metacercariae administered per os and the flukes recovered in different vaccination groups at necropsy.

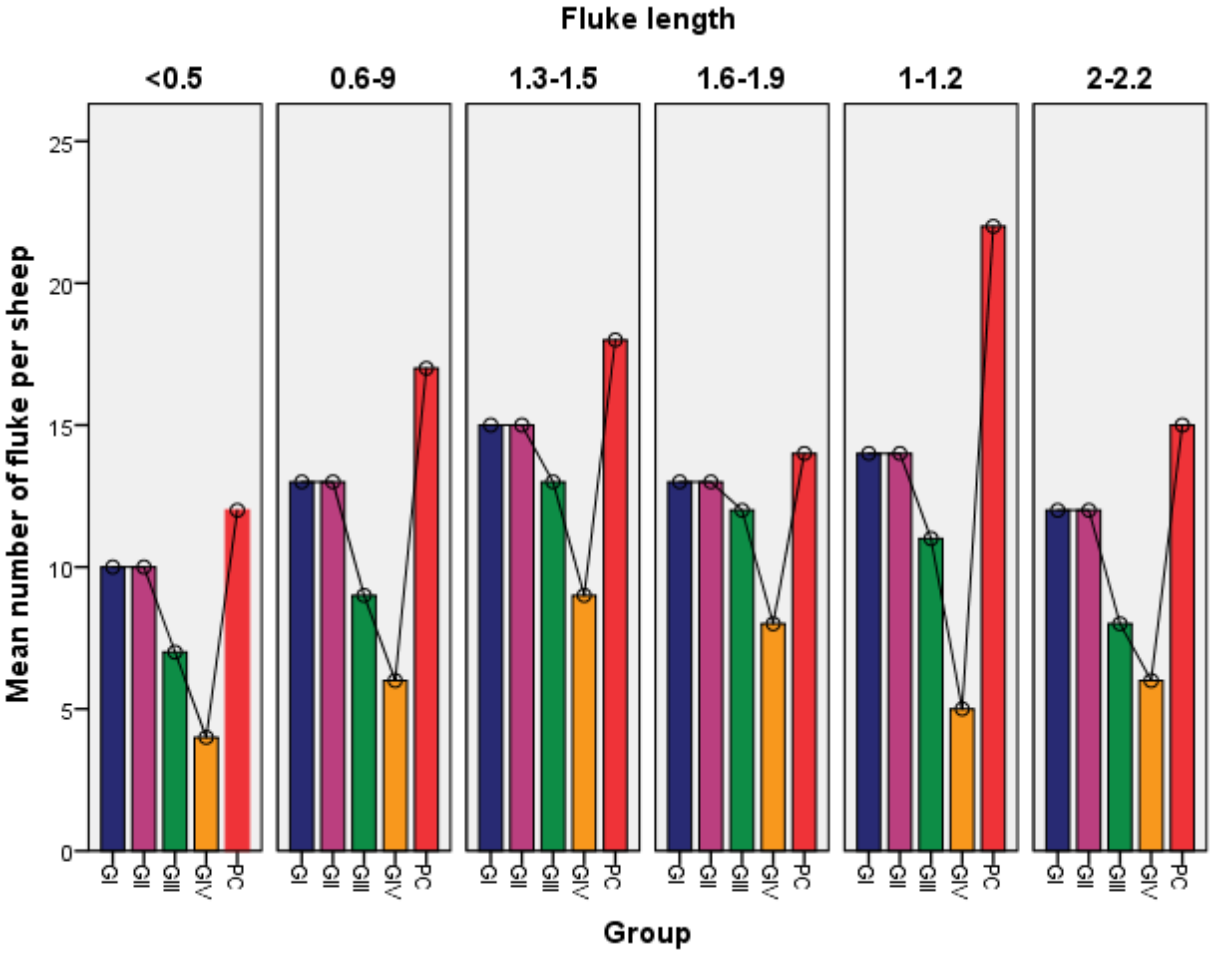
Group	Number of animals	Number of parasites (met)	Irradiation dose (gray)	Fluke recovery				
				Mean ±SE	%	Max	Min	Range
I	6	500	30	93± 3.4	18.6	103	80	23
II	6	500	60	67± 2.8	13.4	75	60	15
III	6	500	120	56.5± 7.18	11.2	75	30	45
IV	6	500	240	38.3± 3.3	7.7	50	30	20
Infected Control (PC)	6	500	-	112.3± 4.3	22.5	123	100	23
Non Vaccinated	6	-	-	-	-	-	-	-

Relatively large number of flukes were recovered and measured (98; 28%) from sheep in the positive control than the vaccinated groups. The number of flukes measured in groups GI, GII, GIII and GIV were, 77 (22%), 77 (22%), 60 (17.1%), and 38 (10.8%) respectively. The length of flukes recovered ranged from 0.30cm to 2.3cm. The mean length of *Fasciola hepatica* recovered in positive control group was 2.2cm. The corresponding length in GI, GII, GIII and GIV was 2.1, 1.8, 1.6, and 1.5cm respectively. However, in all the infected control and vaccinated groups the majority (Mean + SEM) of flukes recovered (69 $\pm$ 5.1) had lengths between 1.3cm and 1.5cm. Similarly significant number of flukes had length greater than 1.6cm and less than 1.9cm (66 $\pm$ 4.74). In all treatment groups, the mean number of those flukes with lengths less than 0.5cm (43 $\pm$ 8.3) and more than 2cm (57) was relatively small. The mean number of flukes measuring less than or equal to 1cm was significantly lower in the group infected with high irradiation dose in GIV (15 $\pm$ 1.3) and GIII (27 $\pm$ 3.1) respectively in that order. However, the reverse is true for animals received parasites exposed to lower irradiation dose in GI (37 $\pm$ 2.1) and GII (37 $\pm$ 3.4). The overall number flukes counted from positive control group was significantly higher ($P<0.05$) than other vaccinated groups of the experimental animals (Figure 4.10).

Similar trends were recorded on the width of flukes recovered corresponding to their lengths where the widths of the flukes had seen to rise with increasing length of liver flukes. The width of flukes recovered ranged from 0.30cm to 1.2cm. However, in all the infected control and vaccinated groups the majority (Mean + SEM) of flukes recovered (68$\pm$ 5.1) had widths between 0.7cm and 0.9cm. Similarly significant number of flukes had width greater than 0.5cm and less than 0.6cm (66 $\pm$ 4.74). In all treatment groups, the mean number of those flukes with lengths less than 0.3cm (43 $\pm$ 8.3) and more than 1.2cm (53) was relatively small. The mean number of

flukes measuring less than or equal to 0.5cm was significantly lower in the group infected with high irradiation dose in GIV (10 $\pm$ 2.3) and GIII (16 $\pm$ 2.1) respectively in that order. However, the reverse is true for animals received parasites exposed to lower irradiation dose in GI (23 $\pm$ 3.1) and GII (23 $\pm$ 3.4). The overall number of flukes counted from positive control group (29 $\pm$ 5.2) was significantly higher ($P<0.05$) than other vaccinated groups of the experimental animals (Figure 4.10). These findings are an indication for strong host immunological responses at higher gamma irradiated groups of animals (GIII and GIV) than those received parasites exposed to low irradiation and uninfected controls (PC).

Figure 4.8. The number of fluke recovered and the measurement of fluke length (cm) in different treatment groups.



Similar trends were recorded on the width of flukes recovered corresponding to their lengths where the widths of the flukes had seen to rise with increasing length of liver flukes. The width of flukes recovered ranged from 0.30cm to 1.2cm (Figure 4.9).

Figure 4.9. The number of fluke recovered and the measurement of fluke width (cm) in different treatment groups.

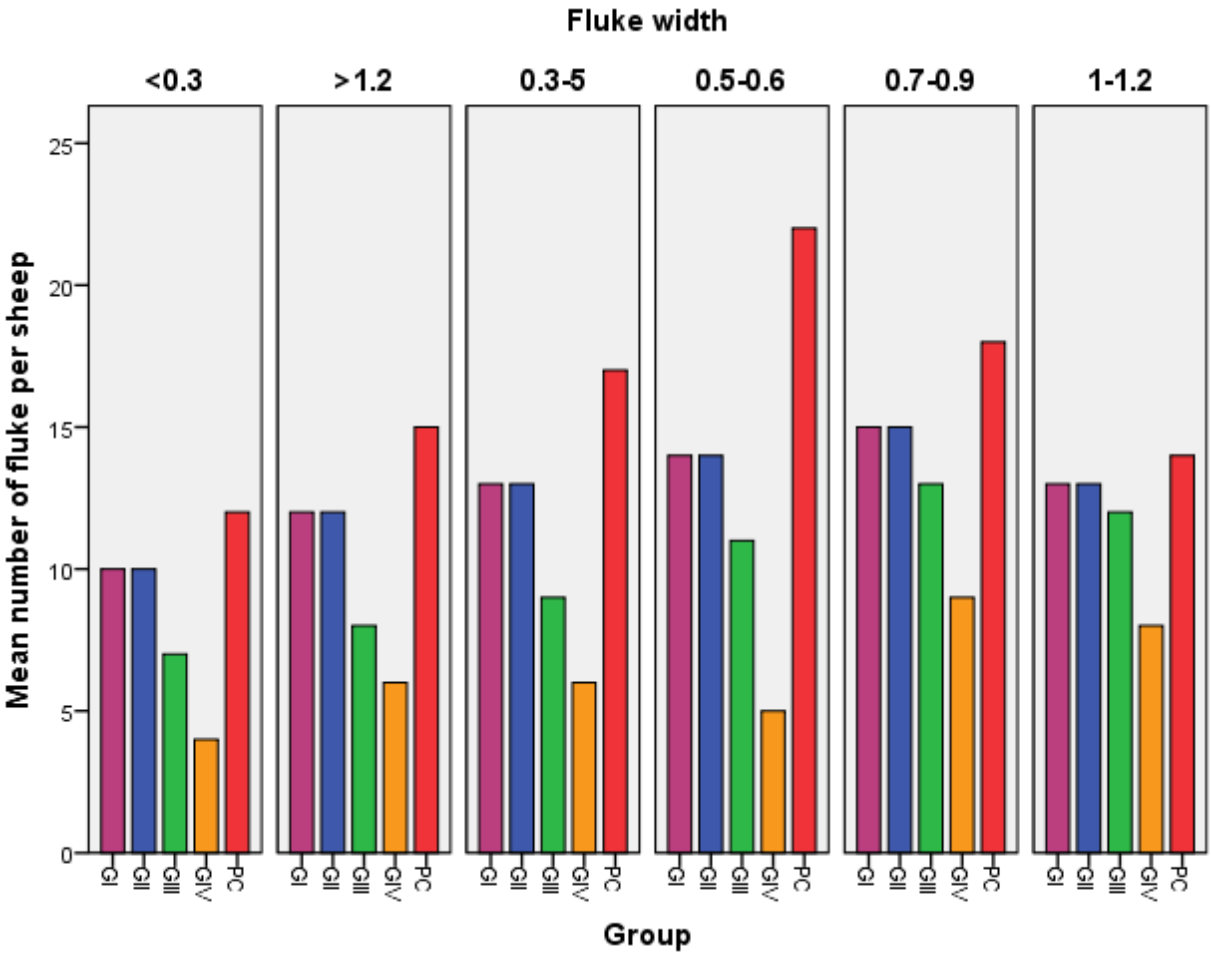


Figure 4.10. The number of flukes recovered from the different treatment groups at end of the experiment

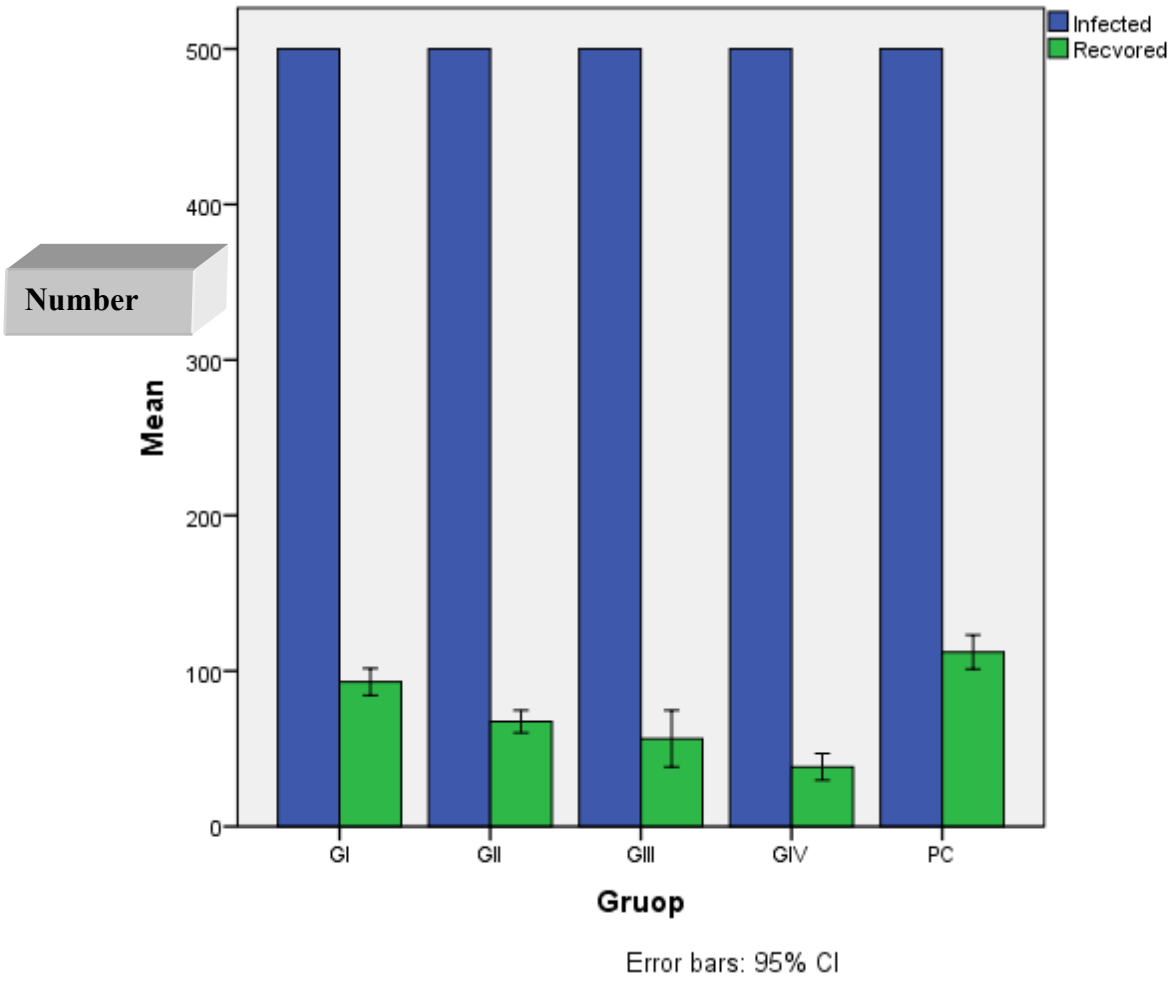


Table 4.17. The mean Fluke length, width and mean liver weight of infected and control groups

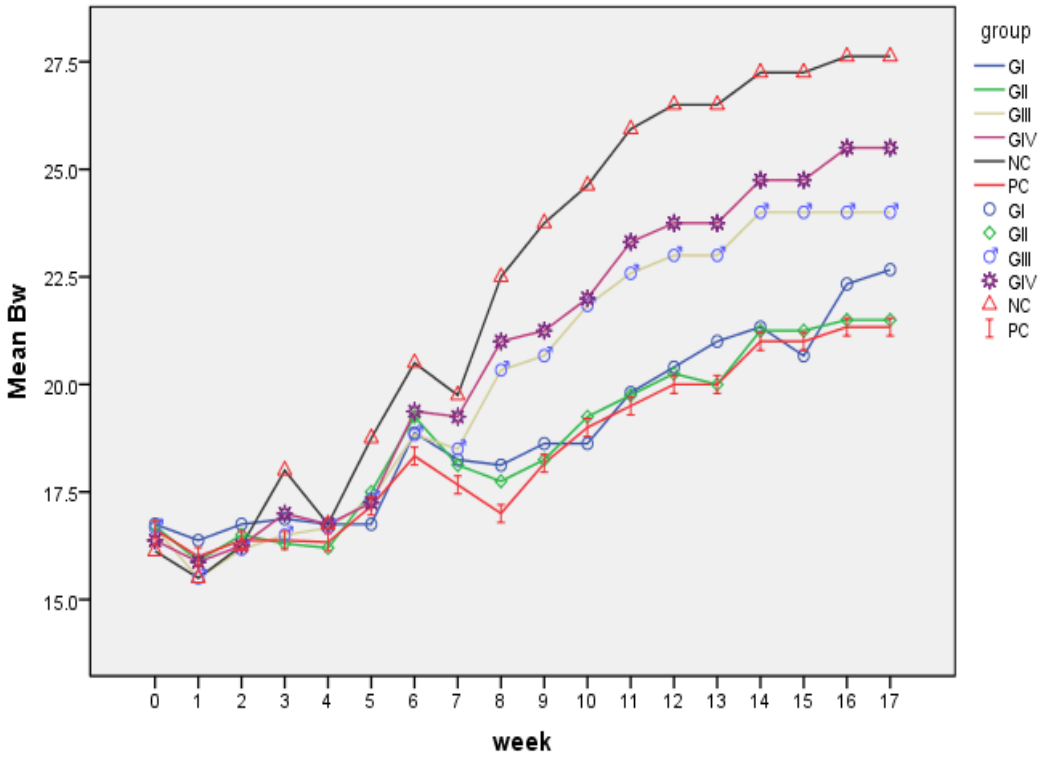
Group	Mean number of fluke ± SEM	Mean Length ± SEM (cm)	Mean width ± SEM (cm)	Mean liver weight ± SEM (gm)	Mean differences of liver weight (PC-other groups) ± SEM (gm)
PC	112.3± 4.3	2.3 ± 0.2	1.75 ± 0.1	396	Reference
GI	93± 3.4	2.1 ± 0.3	1.5 ± 0.2	378	18 ± 2.3
GII	67± 2.8	1.8 ± 0.1	1.3 ± 0.1	368	28 ± 3.4
GIII	56.5± 7.18	1.6 ± 0.1	1.2 ± 0.1	356	40 ± 2.3
GIV	38.3± 3.3	1.5 ± 0.15	1.1 ± 0.1	348	48± 3.3
NC	-	-	-	325	71 ± 2.5

4.6.3. The liver weight and the irradiation dose

The results clearly disclosed the difference in the mean change of the liver weight between the infected and control groups at necropsy (table 4.16 above). The highest mean live weight changes were observed between positive and negative control groups with a difference of 71± 2.5 g and this was significant (P<0.5%). Comparison between highly dosed groups at GIV with that of positive control had shown a mean difference of 48± 3.3 g. This was followed by the mean difference of 40± 2.3 g with that of group III animals. The relative comparative mean live weight changes recorded for GI and GII were 18 ± 2.3 and 28 ± 3.4gm. Generally the liver weight relatively tends to decline with progressive increase in irradiation dose and subsequent decrease in the recovery of the fluke and reduction in size of *F. hepatica* at necropsy examinations.

4.6.4. Body weight changes

Figure 4.11. Body weight changes of sheep vaccinated with different doses of *Fasciola hepatica* across time

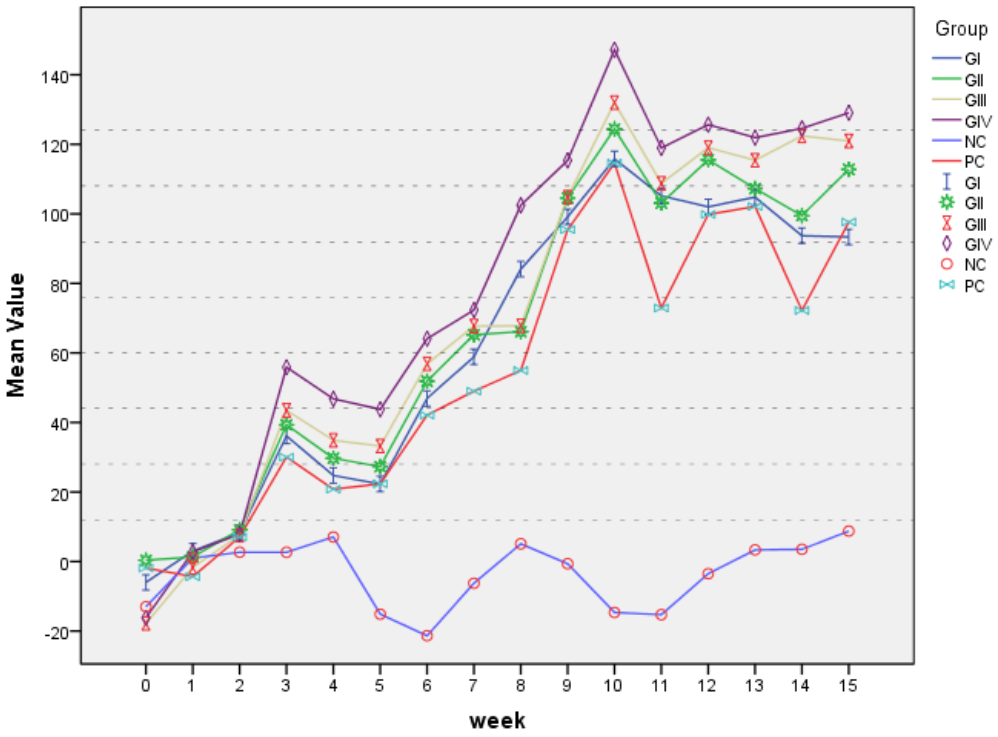


4.6.5. Serum Immunoglobulin response to irradiated F. hepatica metacercarae on sheep

The ELISA antibody index for the host immunological response of sheep infected with irradiated larvae of *F. hepatica* was presented in Figure. All experimental groups of sheep were responded to sensitization with γ-irradiated metacercariae by developing circulating antibodies to *F. hepatica* infections (Fig). The antibody titers measured by ELISA varied with the dose of γ-irradiation provided by stimulation with equal number of metacercariae to each of the infectious groups. The uninfected control animals maintained the same low values of IgG1 just below the cutoff point of 10% throughout the experimental period. The anti Fasciola IgG1 antibodies were

detected within 1 week post infection in all irradiated and control groups and it remained low until the second week post infection. However, beginning from week 2 post infections all the infected sheep that received different levels of γ-irradiation doses of *Fasciola hepatica*, including the infected controls shown an elevation in anti Fasciola IgG1 antibodies until week 10 post infections.

Figure 4.12. The opitical density values showing IgG1 response of sheep in vaccinated and control groups across time



The present findings indicated that the dynamics of serum anti Fasciola IgG1 response were comparable in all infected sheep. There was a steady increase, reaching a peak at week 10 post

infections, after which antibody levels decreased and became invariably stable until 15 weeks post infection. The peak opitical density values of the groups GIV, GIII, GII, GI and PC animals were recorded as 147, 132, 124, 116 and 115 percent respectively. At week 3 post infection the relatively sharp rise in IgG1 level was observed in all infected sheep. Apart from the GIV sheep that received 500 metacercariae γ-irradiated at 240 Gy and induced relatively sharp and higher anti Fasciola antibody production, the other groups showed nearly similar steady elevation in the levels of IgG1 titers. The anti Fasciola antibody titers of all infected groups seems to decline slightly between weeks 3 and 5 and continue then with a steady rise until week 10. After this an invariably slight decline of IgG1 level was shown in different groups between week 10 and week 11 post infection. The anti Fasciola IgG1 of sheep, relatively maintained constant levels for groups that had received the γ-irradiated metacercariae and dosed with 120 and 240 Gy between weeks 11 and 15 post infections.

Sheep vaccinated with 500Gy γ-irradiated metacercariae (GIV) produced the highest antibody titer compared to the non sensitized (irradiated) positive controls. Sheep from group GIV γ-irradiated with 240Gy and GIII with 120Gy showed relatively higher levels of IgG1 level compared to those groups that had received metacercariae dosed with low dose of γ-irradiation (30 and 60 Gy) respectively. However, the difference was not statistically significant. The same holds true for groups which had received metacercariae γ-irradiated with 60 and 30Gy where the later produced slightly lower levels of IgG1 but the different was not statistically significant. Furthermore, the IgG1 responses between animals that were kept at 240 γ-irradiated vaccine and the lower dosed groups (group I and II) were significant ($p<0.05$). The difference of IgG1 levels

in positive controls and in those sheep in groups I and II were also significant ($p<0.05$). Likewise the difference in IgG1 levels of GIII and Groups (I and II) were l significant ($p<0.05$).

4.6.6. Gross and histopathological examinations

4.6.6.1. Gross changes

The livers from uninfected controls (NC) showed no gross lesions on either the diaphragmatic or visceral surfaces. Gross hepatic lesions were similar in sheep of the positive control (PC) and those infected with the relatively lower doses of 30Gy and 60Gy of γ-irradiation at GI and GII. The lesions consisted of superficial scars, fibrous perihepatitis and variable numbers of white tortuous fluke migration tracts, mainly involving the left hepatic lobe, whereas gross changes in the right and quadrate lobes were variable and generally less severe (Table 1). Nevertheless, the extensive fibrinous perihepatitis observed in sheep of non sensitized control groups (PC) and infected sheep of group GI and GII was probably due to rupture of small superficial bile ducts. Sheep in groups II and I showed moderate to severe gross hepatic changes. Gall bladders and large superficial bile ducts were whitish in color and enlarged.

Table 4.18. The fluke burdens, macroscopic and microscopic liver morphometric analyses in sheep from different treatment groups

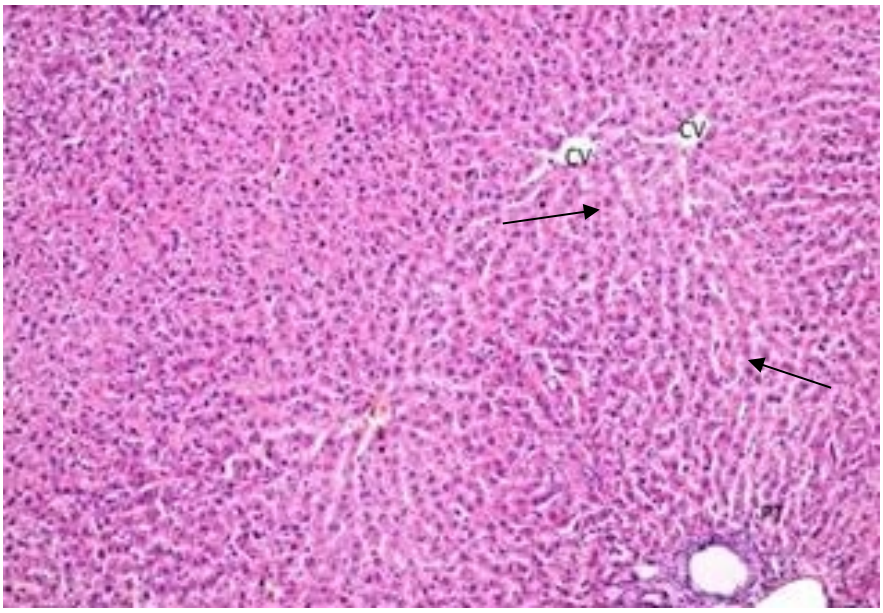
Group	Fluke burden	Gross damage (%)	Microscopic damage (%)	Bile duct (%)
GI	93± 3.4	50.3±14.3	39.2±24.3	5.4±9.3
GII	67± 2.8	44.8±15.3	34.8±20.8	4.4±7.1
GIII	56.5± 7.18	36.1±13.3	31.9±19.1	4.4±6.2
GIV	38.3± 3.3	32.1±14.3	28.9±19.1	4.4±6.2
PC	112.3± 4.3	55.3±18.3	48±24.3	6.2±8.3
NC	-	-	-	-

Mean ± standard deviation, % = percentage

4.6.6.2. Histopathology

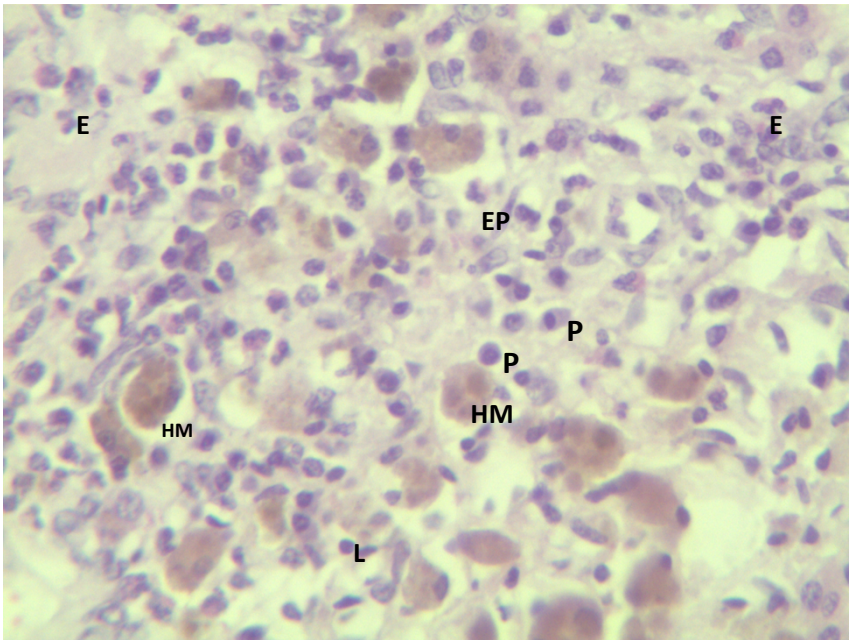
The livers of uninfected control sheep (NC) showed the typical lobular architecture with isolated eosinophils and lymphocytes in portal spaces. Fewer macrophages and plasma cells were seen in portal areas. Sheep of positive control group (PC) that had received normal 500 unirradiated F. hepatica metacercariae showed widespread portal fibrosis with extensive distortion of hepatic lobules, marked bile duct proliferation (hyperplasia of mucosal epithelium of the bile duct with globule leucocytes showing severe cholangitis), severe portal hepatitis with inflammatory infiltration of lymphocytes, hemosiderin laden macrophages and plasma cells and extensive infiltration with eosinophils and epitheloid cells resulting in loss of lobular architecture and moderate cirrhosis (Figure 4.13).

Figure 4.13. NC, sheep: The classical hepatic architecture. Hepatocytes are arranged in cords (arrows) radiating from the central vein (CV) and separated by blood sinusoids.



Sheep of non sensitized control group (PC) that had received normal 500 unirradiated *F. hepatica* metacercariae showed widespread portal fibrosis with extensive distortion of hepatic lobules, marked bile duct proliferation (hyperplasia of mucosal epithelium of the bile duct with globule leucocytes showing severe cholangitis), severe portal hepatitis with inflammatory infiltration of lymphocytes, hemosiderin laden macrophages and plasma cells and extensive infiltration with eosinophils and epitheloid cells resulting in loss of lobular architecture and moderate cirrhosis (Figure 4.14).

Figure 4.14. PC: Massive infiltration of plasma cells (P), Hemosiderin laden macrophages (HM), lymphocytes (L) Eosinophils (E), and Epithelial cells (EP)



Larger bile ducts often contained cell debris and fluke eggs, and were surrounded by numerous globule leucocytes. Fresh migrational tracks of all sizes were mainly composed of eosinophilic debris of disintegrated hepatocytes infiltrated by numerous eosinophils and some lymphocytes and macrophages. In smaller tracks tendency to haemorrhages was slight. In the larger tracks the macrophages contained a profusion of iron (hemosiderin filled cells) in consequence of pronounced haemorrhages. Often tissue elements surrounding the tracks were affected by a pronounced coagulative necrosis. Chronic tracts with numerous macrophages containing hemosiderin pigment were often seen. Granulomas with necrotic or mineralized centers

surrounded by macrophages, multinucleate giant cells, numerous eosinophils, and more peripherally by lymphocytes and plasma cells, were observed in all sheep. Fluke eggs were identified in some of these granulomas (Figure 4.15; Figure 4.16).

Figure 4.15. PC: Hyperplasia of mucosal epithelium of the bile duct with globule leucocytes: Cells containing globules= macrophages and lymphocytes (arrows); several mitoses (proliferation of bile ducts=BD), abundant and diffuse infiltration of inflammatory cells (macrophages and lymphocytes = I) associated with bile ducts (Cholangitis), extensive fibrosis of liver parenchyma (proliferation of fibrous connective tissues) with extensive distortion of hepatic lobules

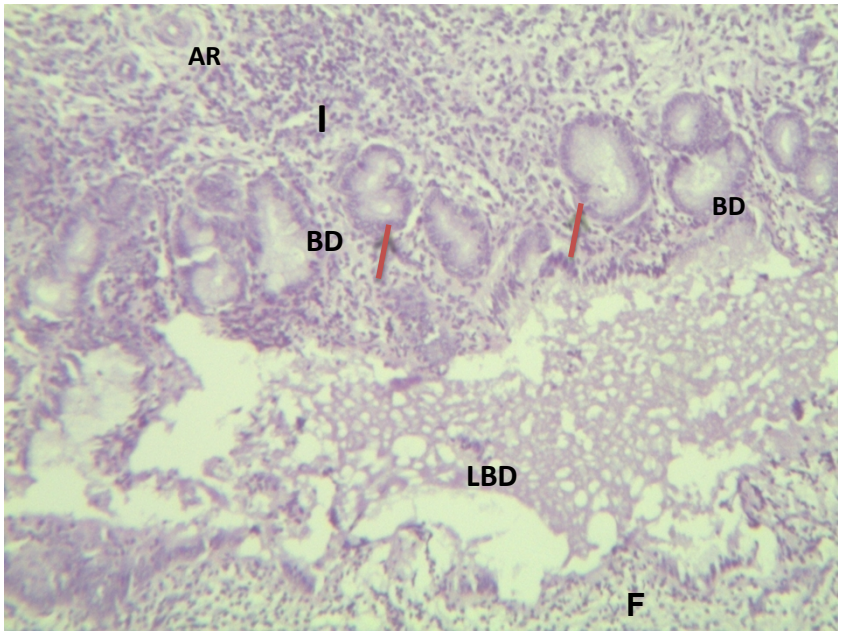
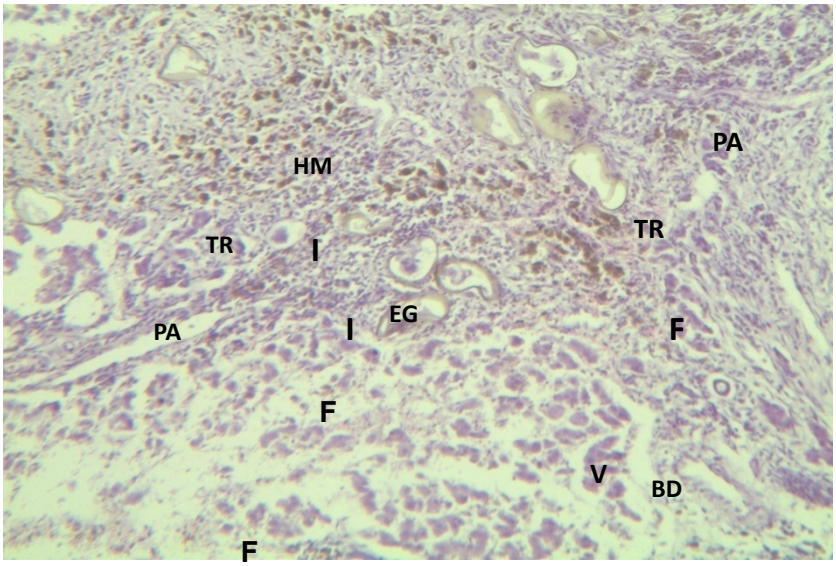
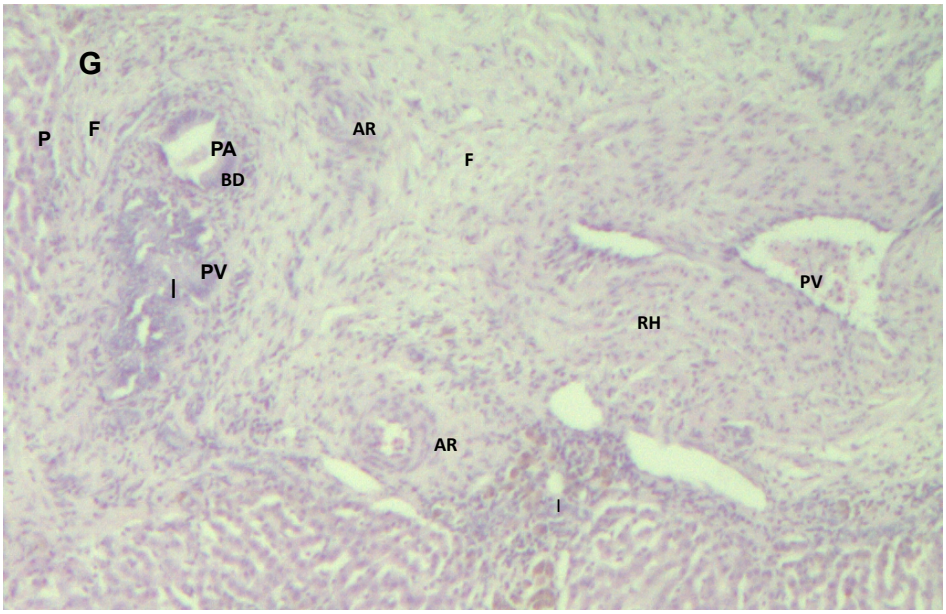


Figure 4.16. PC: Chronic inflammatory reaction is evident in portal areas (portal hepatitis): Massive hemosiderin laden macrophages, bile duct, mass of cellular debris and parasite eggs (EG), immature flukes in the parenchyma (PA) and fluke migratory tunnels (TR), fibrosis (F)



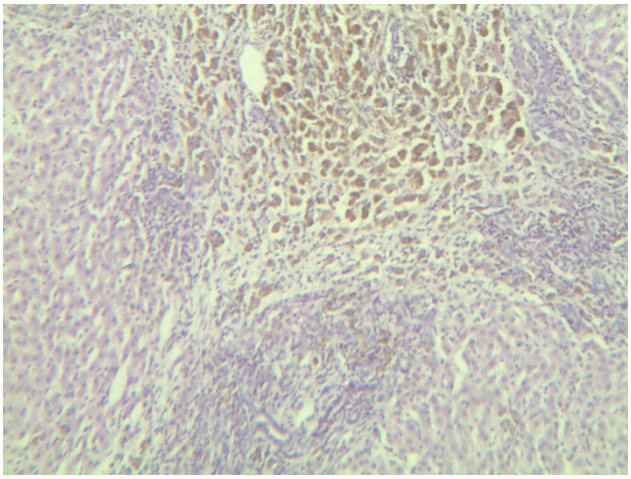
Comparatively more severe damage to the liver were observed in positive control sheep than that of sheep kept either in group GI (30Gy) and GII (60Gy) exposed to relatively lower doses of γ-irradiation or groups of sheep maintained at relatively higher doses at 120Gy and 240Gy in groups GIII and GIV (Figure 4.17; Figure 4.18)

Figure 4.17. Early Cirrhosis (GI): Extensive periportal fibrosis (F) and mononuclear infiltration (I) with regenerative response (RH) of hepatosites and arterial dilatation (AR) due to portal hypertension; Granuloma (G) with parasites (PA) within the bile ducts (BD)associated with globule leucocytes (cholangitis) and inflammatory infiltration of lymphocytes and macrophages (I) in the portal vein (portal hepatitis)



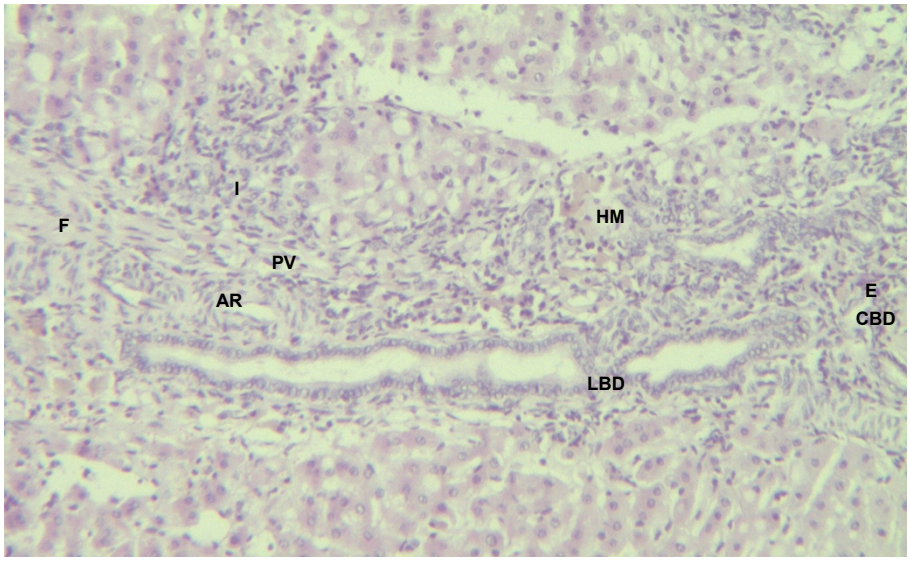
Sheep of groups GI and GII showed hepatic changes which were comparatively less severe than those of group uninfected control (PC).

Figure 4.18. Portal hepatitis with excessive infiltration of hemosiderin laden macrophages at periportal spaces with moderate infiltration lymphocytes and portal fibrosis (GI)



Extensive portal fibrosis, bile duct proliferation and infiltration of eosinophils were very severe, causing loss of lobular architecture in large areas of the hepatic parenchyma. These changes were more severe in group GI than in either group GII or sheep maintained at relatively higher doses at groups GIII and GIV. Mononuclear cell infiltration with lymphocytes and plasma cells were very severe, especially in group GI (Figure 4.18.).

Figure 4.19. Diffuse massive inflammatory infiltration (I) of portal spaces: (portal hepatitis; infiltration of lymphocytes, macrophages, plasma cells) with mild hemosiderin laden macrophages (HM) hyperplastic and enlarged bile ducts (LBD) with globule leukocytes, arterial dialatation (AR) moderate portal fibrosis (PF), cellular (Eosinophils and lymphocytes) reaction and parasite debris in bile ducts (CBD) and portal vein (PV): GII



Lymphoid follicles with germinal centers (regenerative response of hepatocytes) were observed in the larger portal tracts indicating early stage of liver cirrhosis. Granulomas composed of an eosinophilic necrotic centre, surrounded by multinucleate giant cells and macrophages and more peripherally by lymphocytes and plasma cells were found in sheep of group GI and group GII similar those seen in non sensitized positive control group. Diffuse infiltration of eosinophils was often observed in areas of hepatic parenchyma and portal spaces, particularly next to granulomas, or associated with parasite eggs that had reached the hepatic parenchyma.

Thickening of arterial wall was evident in portal areas due to portal hypertension. The number of fluke eggs within bile ducts was higher than in group GII. Numerous eggs were observed in the hepatic parenchyma causing a heavy infiltration of eosinophils and granulomas similar to those described in non sensitized positive control sheep. Some fluke eggs were also found within portal veins and centrilobular veins, causing thrombi. Chronic tracts were numerous in both groups GI and GII sheep as evidenced similarly in infected control group (Figure 4.20; Figure 4.21; Figure 4.22).

Figure 4.20. Chronic inflammatory reactions in portal areas: Portal hepatitis with moderate infiltration (I) of mononuclear cells (lymphocytes) and hemosiderin laden macrophages (HM), bile duct (BD), mild cholangitis and portal fibrosis (PF) (GIII)

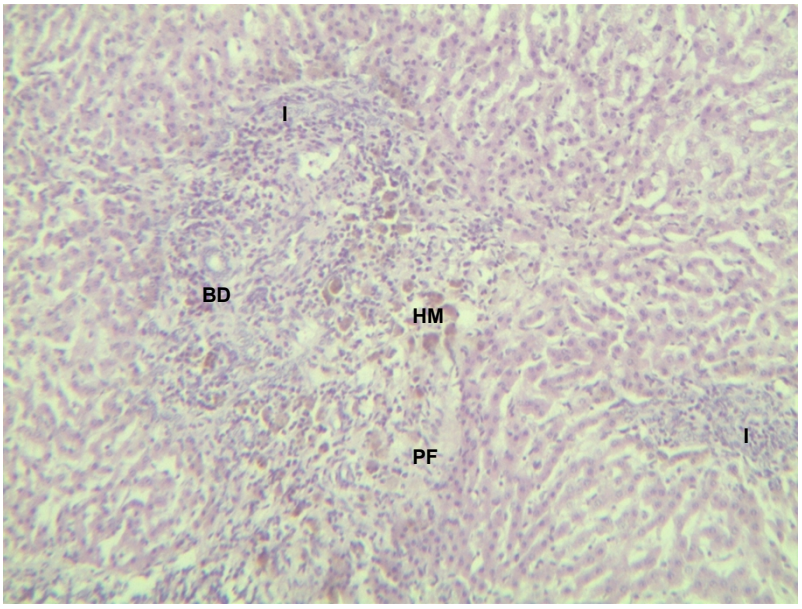


Figure 4.21 Granuloma (G) with massive infiltration of inflammatory cells in portal areas: Portal hepatitis having diffuse infiltration of mononuclear cells (I), polymorphonuclear cells (PMC), bile duct (BD) hyperplasia with associated globule leukocytes and immature parasites (Pa) inside the bile duct lumen, moderate arterial dilatation (Ar) , hemosiderin filled cells in hepatic parenchyma, degenrating hepatosites (DH), fat laden cells (FC) ; (GIII)

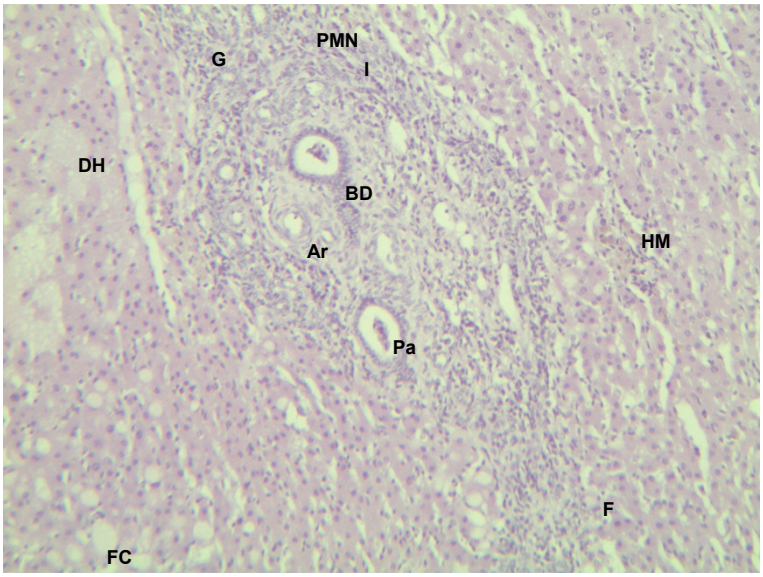
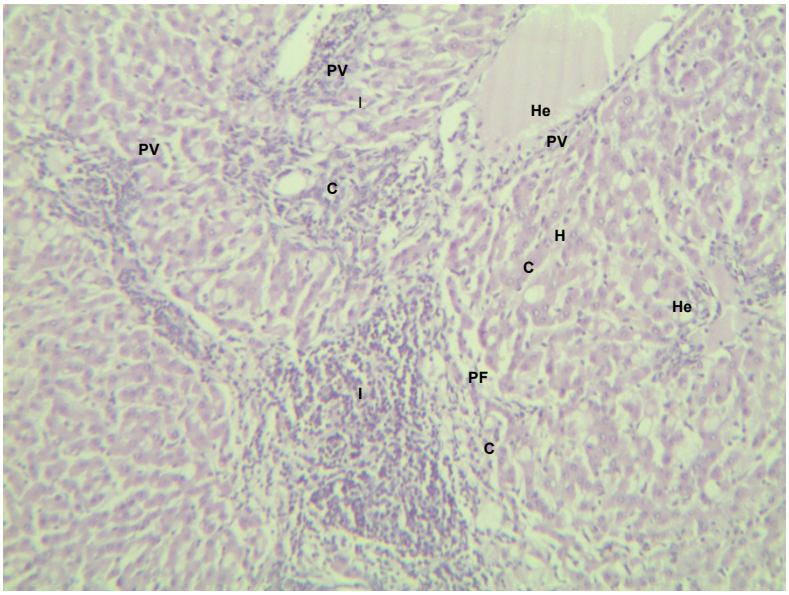


Figure 4.22. Chronic inflammatory reactions in portal areas (portal hepatitis) with moderate diffuse infiltration of mononuclear cells (I), hemorrhagic vein (He), portal fibrosis (PF) and normal hepatic lobule (H) (GIII)



The hepatic changes in group III sheep was marked by chronic inflammatory reactions in portal areas with moderate diffuse infiltration of mononuclear cells, polymorphonuclear cells (eosinophils), moderate hyperplasia of the bile duct with associated globule leukocytes and immature parasites inside the bile duct lumen, moderate arterial dilatation, hemosiderin filled cells in hepatic parenchyma, degenerating hepatocytes, fat laden cells (fatty degeneration) hemorrhagic vein, portal fibrosis and granulomas with moderate infiltration of mononuclear and polymorphonuclear cells. Comparatively similar but less marked lesions and inflammatory cells were seen in group GIV sheep that had received the highest dose of 240Gy irradiated metacercariae of *F. hepatica*. Multifocal mild inflammatory infiltration in the portal spaces with

lymphocytes and macrophages; hemosiderin laden macrophages and other inflammatory cells within the portal vein, fluke migratory tracks and wide area of normal architecture of hepatic parenchyma (4.22; Figure 4.23; Figure 4.24).

Figure 4.23. GIV Less remarkable inflammatory (I) reactions in the portal areas with dilatation of artery and bile duct associated with globule leukocytes (mild cholangitis) and parasite debris (Pa) and cells within the bile duct (BD), normal hepatic parenchyma with lobular pattern (H), hemorrhage inside the hepatic vein (He), mild hemosiderin laden macrophages (HM)

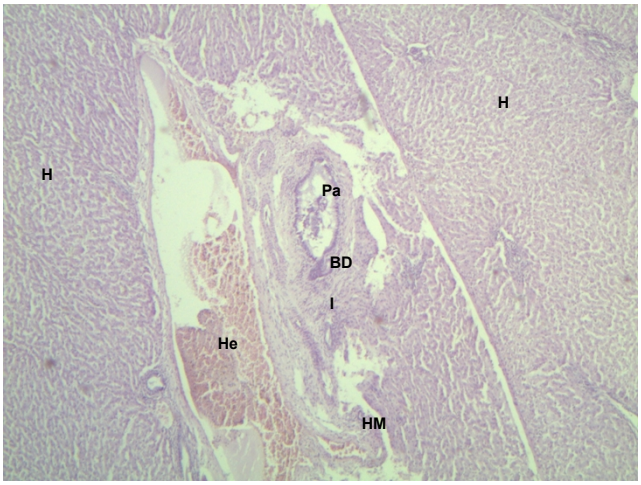


Figure 4.24. Multifocal mild inflammatory infiltration in the portal spaces with lymphocytes and macrophages: Hemosiderin laden macrophages (HM) and other inflammatory cells within the portal vein, fluke migratory tracks (Tr) and normal architecture of hepatic parenchyma (HP) (GIV)

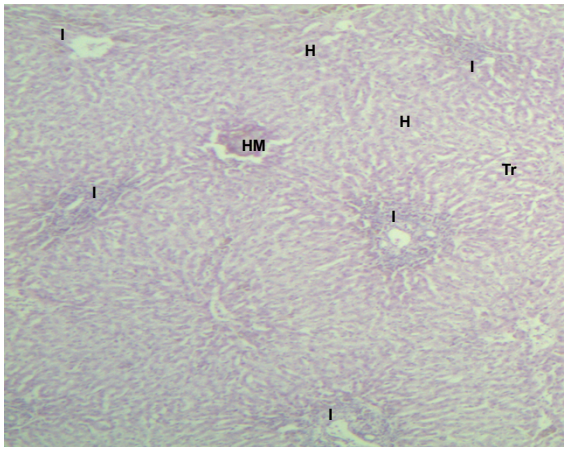


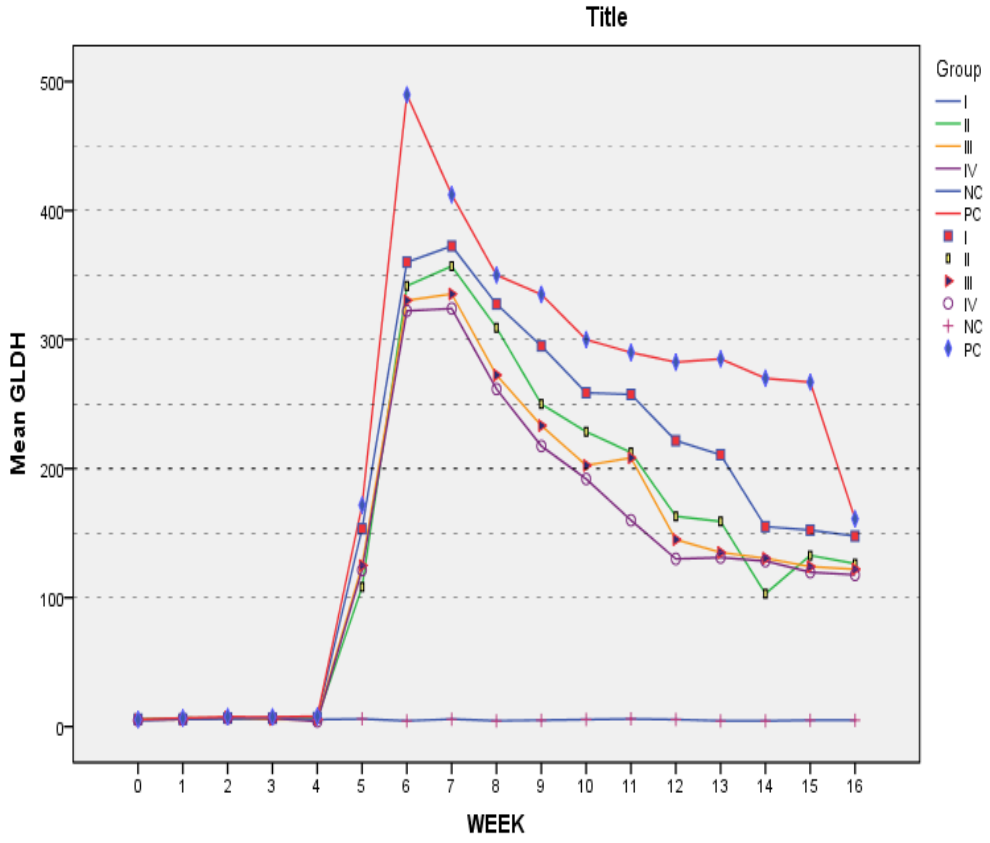
Table 5.19. Summary of microscopic lesions and the infiltrating cells of the liver and their severity in different dose groups that received unirradiated and irradiated *F. hepatica* metacercariae.

Lesions and infiltrating cells	Grading of lesions* and cell counts^{+} in the stated lesions					
	PC	GI	GII	GIII	GIV	NC
Lesions						
Bile duct Hyperplasia	+++	+++	++	++	+	
Cholangitis	+++	+++	++	+	+	
Portal hepatitis	+++	+++	++	++	±	
Perihepatitis	+++	++	++	+	+	
Migratory tunnels	+++	+++	++	+	±	
Eggs (Parenchyma)	+++	+++	+	-	-	
Eggs (intravascular)	++	++	+	-	_	
Arterial dilatation	+++	++	+	-	-	
Hemorrhagic vein	+++	++	+		+	
Hypertrophy of arteries	+++	++	++	+	±	
Bile duct dilatation	+++	++	++	+	±	
Hemosiderin	+++	+++	++	++	++	
Granuloma	++	++	++	±	-	
Fibrosis	+++	+++	++	+	±	
Lymphoid follicle (LF)	++	++	+	+	±	
Cirrhosis	++	++	+	±	±	
Cells						
Eosinophilis	+++	+++	++	+	+	
Lymphocytes	+++	+++	++	+++	+++	
Macrophages	+++	++	++	++	++	
Hemosiderin Laden Macrophages	+++	+++	+++	+++	+++	
Epitheloid cells	+++	++	++	++	++	
Fibroblasts	+++	++	++	++	++	
Fat laden cells (fatty cells)	+++	+++	++	+	+	

4.7. Dynamics of hepatic enzymes

The Dynamics of hepatic enzymes during experimental period on sheep γ-irradiated by metacercariae of *F. hepatica* was shown in Figure 1 and 2 below. Animals exposed were responded well to the infection as shown by evolution of hepatic and gall bladder enzymes. The enzyme level varies with the dose of γ-irradiation and non sensitized groups of animals. Generally irrespective of immunization, all the infected animals exhibited increasing trends in the GLDH and GGT levels from week 2 onward post infection. In all cases the uninfected control sheep (NC) maintained the low profile of enzymes that were well within the normal ranges of each enzyme (GLDH= 1-12; GGT= 34-100 IU) in sheep.

Figure 4.25 The weekly dynamics of GLDH during the experimental period in sheep



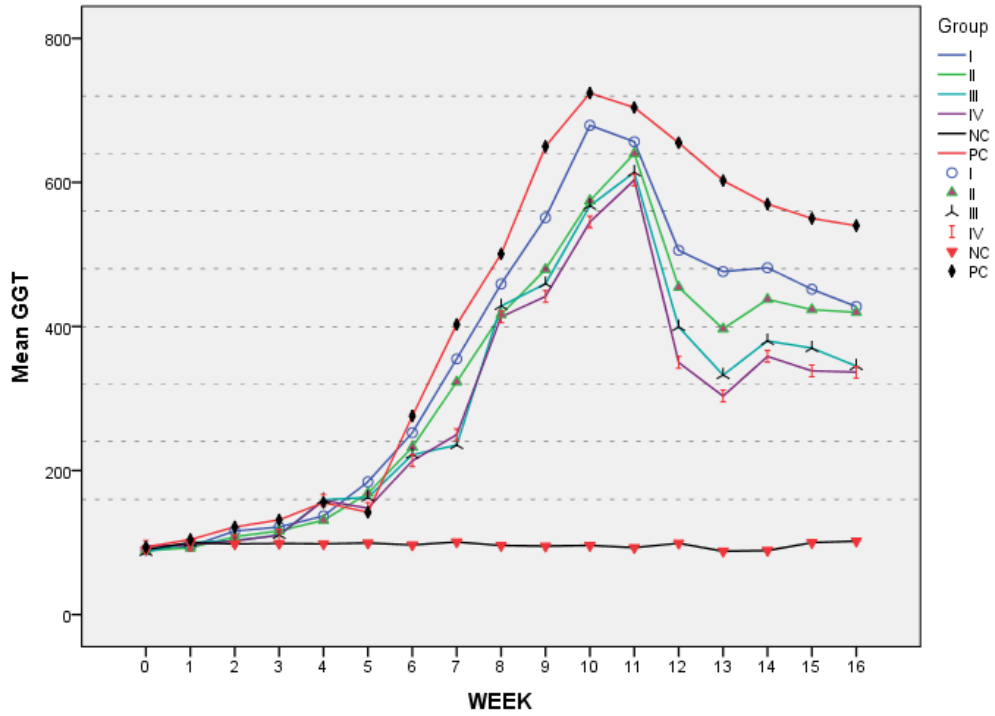
The mean (mean ±SE) GLDH level of positive control sheep (489.67±16 IU) was significantly ($p < 0.05$) higher compared to the other vaccinated groups at GI (360±9 IU), GII (356.6±8 IU), GIII (335.3±8 IU) and GIV (324.4±12). The comparative progressive decline in the mean levels of serum GLDH of the infected control sheep observed between weeks 6 and 16 was in line the decline observed in all other treatments.

All vaccinated groups had maintained the serum GLDH level greater than 100 IU at week 16 post infection. However, sheep that were dosed with 30 Gy (minimum γ-irradiation dose used in

the present experiment) had shown significantly higher ($p<0.05$) mean serum GLDH level between week 5 and 16 compared to the relatively lower levels of other treatment groups during the same period.

In group of sheep dosed with 60, 120 and 240 Gy the mean serum GLDH level lies in a range of 100 and 150 IU after week 6 post infections. However, these values were relatively stable between weeks 12 and 16 post infection, regardless of statistically insignificant ($P>0.05$) difference between the groups. On the other hand animals vaccinated with low dose of 30 Gy kept the stable serum GLDH level in the range of 150 - 200 IU between weeks 14 and 16 post infection. The serum GLDH profile of non sensitized positive control sheep reached a minimum level of 200 IU only at week 16 following a sharp decline from 267± at week 15 to 200 at week 16 post infection. The more pronounced changes in levels of enzyme at positive control and lower dosed groups were indicated the relative higher level of damage to the liver.

Figure 4.26. Weekly dynamics of GGT during the experimental period in sheep



5. DISCUSSION

In Ethiopia, the prevalence of fasciolosis in sheep and cattle had been reported in different altitudinal ranges where the levels of climatic (temperature, rain fall) and ecological factors affect the prevalence, epidemiology and species of the parasite (Graber and Daynes, 1974; Yilma and Malone, 1998, Yilma and Mesfin, 2000; Biffa et al., 2006). Accordingly, the dearth of the *Fasciola hepatica* and *Fasciola gigantica,* determined by different authors (Yilma and Malone, 1998; Graber and Daynes, 1974), was below 1200 masl and above 1700 masl respectively.

The present study had clearly disclosed the occurrence of higher prevalence of fasciolosis in sheep and cattle from highland areas. The overall coprologic, herd and abattoir prevalence of fasciolosis in cattle and sheep were, 53.8%, 50.9 ± 60.8%, 81%, in that order. The subsequent findings in sheep were, 60.1%, 59.4 ± 27.9% and 84%, respectively. Unlike in sheep, significantly lower prevalence were reported in cattle for coprology, herd and abattoir survey with a prevalence of 49.2%, 42.41 ±30.8%, 77.8%, respectively. This result is higher than the coprologic findings of both individual animal (33.4 %) and herd (41.2%) level prevalence reports from North Gondar and lower than the abattoir reports (90.7%) of the same authors in cattle during the long dry season (Yilma and Mesfin, 2000). Contrary to the present finding (49.2%), higher prevalence (64.6%) was reported from the different PA's of the same districts around Debre Berhan town; however, the prevalence in sheep (54.2%) was relatively lower than the present (65.2%) finding (Abegaz, 2006). Birbrisa (77.7%) PA was showed the highest overall fluke egg prevalence and Angolela (31.5%) the lowest. The difference in climatic and ecological factors (altitude) may have positively contributed to the observed differences in prevalence.

Treatment provision (fasciolicidal) and vector control (endod) are also other factors that can affect the prevalence of fasciolosis.

The abattoir prevalence of fasciolosis in cattle (77.8%) was nearly in agreement with the 83% report of other workers in the same site (Dagne, 1994; Abegaz, 2006). Comparable reports were observed in some parts of the country with similar ecological and climatic conditions in cattle and sheep. Accordingly, the report from the predominantly (75%) highland neighbor district of Menz Gera Midir of North Shoa zone indicated a prevalence of 40.5% in sheep (Gebreyohannes et al., 2013). However, other authors (Dagnachew et al., 2011; Kedir et al., 2012) revealed, significantly lower prevalence of 15.5% and 14.6% of fasciolosis in sheep from highland district of Dabat in North Gondar and Mid land areas of Jimma, respectively. The high abattoir prevalence of fasciolosis than the coprologial (low sensitivity) findings may be due to the high sensitivity of postmortem examination, which is considered gold standard.

Similar lower prevalence of fasciolosis was reported in other parts of the country. The report on cattle by Abuna et al. (2009) had shown relatively lower prevalence of fasciolosis in abattoir (14%) and by coprological survey (4.9%) in Wolaita soddo. The overall high prevalence (44.7%) of fasciolosis in working donkeys had been reported during coprological examination in different agroecological zones of the country (Getachew et al., 2009; Getachew et al., 2010). Accordingly, comparably high prevalence was reported in donkeys of the highland district (Bereh, 72.6%) and this was statistically significantly higher than the mid (Akaki, 43.9%; Ada, 52.6%) and lowland (Boset, 21.5%) districts.

The predisposing factors like the PA's, species, age, breed and body condition observed were statistically significant variables on both individual animals and herd level by coprological survey. However, the abattoir study indicated that age and sex appears to have no effect on the overall prevalence of fasciolosis. On the other hand the effect of breed and body condition differences on the overall occurrence of fasciolosis in abattoir was significant. In sheep, the probability of the occurrence of the disease in Awassi-Menz cross bred animals was 15% compared to the local Menz breed. Similarly, 20% of the Holstein Friesian crosses are more prone to acquire the disease than the local Zebu cattle. In general, poor conditioned animals have 35% risk of acquiring fasciolosis with the respect to animals in good body condition. Furthermore the abattoir survey indicated that the disease is 0. 25 times more likely occurs in sheep than in cattle.

The presence of conducive environments (rainfall, temperature and high altitude) for the existence of the intermediate host (*Lymnaea truncatula*) have been known as the main recognized factors for *F. hepatica* transmission in Ethiopian highlands (Graber and Daynes, 1974; Yilma and Malone, 1998, Yilma and Mesfin, 2000; Biffa et al., 2006). However, most of the slaughtered animals at the abattoir in the present study were supplied from highland and middle altitude zones where the vector (*Lymnaea truncatula*) is prevalent. It is not uncommon to find favorable areas where the existence of *F. gigantica* in those areas provided suitable habitats for the vector (*L. natalensis*) like irrigation canals, flood prone areas and drainage ditches (Bekele et al., 1992). Agroecological areas that are suitable for the existence of both vector species and keeping together animals from different herds in the same grazing area may contribute for mixed infections in highlands. A number of reports suggested (Fekadu, 1987; Roman, 1987; Rahmeto 1992, Dinka et al., 2009) in different parts of the country were in

agreement to the present study for the existence of mixed infections in the livers of infected animals.

The limitations of the gold standard method (post mortem examination) for fasciolosis in terms of labour, time and cost relatively makes it difficult approach compared to the egg-shedding index, as an estimate of herd-infection prevalence and individual coproscopic examination. This is mainly depends on the selection pressure for animals to be slaughtered at post mortem examinations (Malone et al., 1992). Since, Selection pressures are external agents which affect an organism's ability to survive in a given environment. Selection pressures can be negative (decreases the prevalence fasciolosis) or positive (increases the prevalence). However, from the findings of the present study the approach with egg-shedding index was relatively stronger (labour and cost) than the results obtained by individual coproscopic examination. Similar findings were reported by other authors for the superiority of the egg shedding index in terms of labour and cost (Yilma and Mesfin, 2000; Malone et al., 1992).

The number of flukes recovered and the fibrosis of the liver were used to classify the severity of fluke infections as light, medium and heavy. Accordingly, the worm burden in lightly affected sheep liver had relatively higher fluke counts compared to the heavily affected liver. However, in cattle, fluke counts in moderately affected livers exceeded that obtained from heavily affected ones. The less worm burden in heavily affected livers of sheep and cattle was associated with the severe fibrosis that impedes the passage of immature flukes and acquired resistance that resulted in the expulsion of flukes from the bile ducts (Dwinger et. al., 1982).

The present result on the mean fluke burden in sheep and cattle was 59.6 ± 0.6 and 77.5 ± 0.7, respectively. This finding relatively exceeds the mean fluke count of 66.23 reported in Gondar (Yilma and Mesfin, 2000). Similar high mean fluke burden of 73.3 and 67 was reported in the Gondar and Bahrdar by other authors (Roman, 1987; and Fekadu, 1987). The reports from other parts of the country in Nekemte area had also shown a mean fluke count of 85.5 in cattle (Abebe, 1988). Adem (1994) and Abduljebar (1994) were reported similar high mean fluke burden of 74 and 68 in Zeway and Sinana districts from cattle, respectively.

According to Soulsby (1982) the presence of more than 50 flukes per liver indicates high pathogenecity. Other authors (Marcos et al., 2007) were also concluded that as the number of *F. hepatica* adult forms increases, the likelihood of developing liver fibrosis will also increase in cattle. Therefore, the present finding clearly disclosed the high pathogenecity of fluke infections in both sheep and cattle of the study area, and thereby in agreement with aforementioned authors. The mean (mean ± SE) live weight of fluke infected liver of local Menz breed sheep was 302.7 $\pm$ 29g and this was lower than Aawassi Menz cross bred sheep (386 $\pm$ 48 g). On the other hand, the corresponding mean live weight of a liver of local zebu cattle and Holiestine Frezian crosses were 504 $\pm$ 34 and 583 $\pm$ 29g, respectively. The live weight of infected liver and fluke count of both sheep (r= 0.65) and cattle (r=0.75) have been positively correlated. That means, the larger the weight of the infected liver, the higher the count of the liver fluke.

The most remarkable features of the morphometric analysis of fasciola species (*F. hepatica* and *F. gigantica)* are variations in their length and width of both mature and immature flukes. The variation was actually associated with differences in the development stages of the parasites within the species and the clear morphologic difference existing between the two fasciola species

identified. Accordingly, the mean length (mm) and width (mm) of matured *F. hepatica* in cattle was 27.8 ± 0.23 and 4.92 ± 0.05, respectively. The corresponding mean length and width of *F. hepatica* in sheep was 23.4 ± 0.6 and 3.12 ± 0.54. This finding is relatively larger than those reported in cattle by Yilma and Mesfin (2000) in Gondar. However, the length of adult *F. gigantica* was relatively larger and narrower than *F. hepatica*. Accordingly, both adult *F. hepatica* and *F. gigantica* are leaf-shaped with tapered anterior and posterior ends with differences in their measure of mean body length and width. Moreover, morphological intermediate forms of fasciola have been reported elsewhere (Mas-Coma et al, 2005).

The malacological survey clearly disclosed the existence of different genera of snails in the study areas. The vectors of *Fasciola* species (Lymneaa species) were the dominant snails collected during the survey. The presence of both Lymnaea species (*L. truncatula* and *L. natalensis*) demonstrated the potential of the intermediate hosts to transmit both *F. hepatica* and *F. gigantica* in the study areas (in all agro climatic zones despite the altitudinal difference). The existence and the report of *L. natalensis* at higher elevations were not uncommon. The finding of this snail above 2600 masl in river angolela was also reported by other authors. Abegaz (2006) indicated the presence of the intermediate host in the same river and Rahmeto (1992) reported the existence of both species at 2070 elevations in West Shoa. From other snails collected, Bulinus species were dominant. The effect of the soil, temperature and rainfall on the occurrence, abundance and distribution of the snail intermediate hosts has been demonstrated by Yilma and Malone (1998).

The presence study plainly disclosed the high prevalence of fasciolosis both at herd and individual animal level coprologically and at abattoir survey. *F. hepatica* was the dominant fluke species prevailing in the study area with the presence of some *F. gigantica* and mixed infections. The morphometric analysis clearly distinguished *F. hepatica* from *F. gigantica* where both adult matured *F. hepatica* and *F. gigantica* are leaf-shaped with tapered anterior and posterior ends with differences in their measure of mean body length and width.

At present there are seven large scale meat processing abattoirs that have been established in Ethiopia in response to the emerging meat export opportunities to the Middle East and North African Countries. There are also several meat export abattoirs under construction and more are planned to be established in the near future in different regions of the country (Negassa and Jabbar, 2007; EMPEA, 2010). However, the large majority are municipal abattoirs and slaughterer slabs. The export abattoirs are competing for the domestic supply of live cattle and shoats with the demand for live animals for domestic consumption, and for formal and informal trade (Negassa and Jabbar, 2007; EMPEA, 2010).

Fasciolosis has direct and indirect impact on Ethiopian economy affecting animal production and productivity (Njau, et al., 1988; Njau et al., 1989; Njau et al., 1991; Yilma & Mesfin, 2000; Taylor, et al., 2007). The economic loss is more significant when animals show prevalence above 25% with evident clinical signs. It is estimated that more than 300 million cattle and 250 million sheep in the world that are grazing in areas where infective forms of the parasite present, represent annual losses of more than, USD 3billion (Taylor, et al., 2007; Mas-Coma, 2005).

Moderate to heavy fasciola infections (over 40 flukes) result in production losses in the major cow calf operations in the United States.

From a total of 5427 ruminant livers examined in five abattoirs, 2530 (46.6% ± 0.059) were indicated the presence of liver flukes. The overall prevalence of fasciolosis observed among the abattoirs had shown highly significance difference. Hence, the overall prevalence of fasciolosis observed in ruminants slaughtered in export abattoirs was 34.6%, whereas this was significantly lower in ruminants slaughtered at municipal abattoirs 65.2% as the whole. Comparison of the prevalence of fasciola positive ruminants in each of the four different abattoirs with respect to the prevalence observed at HELIMIX (44.9% ± 0.087) indicated significantly higher prevalence at Debreberhan (80.1% ± 0.95) than the rest of the abattoirs.

There have been significant differences on the overall prevalence of fasciolosis among the slaughtered ruminants from various agroecological areas with altitude differences. Accordingly, it had significantly shown higher prevalence among ruminants from highlands followed by midlands and lowlands, respectively. Altogether, midlands and highlands had contributed more than three fourth of the total slaughtered ruminants in the abattoirs. The higher fasciola prevalence in municipal abattoirs (Debre Berhan) may be related to the supply of slaughter animals from the highlands and midlands with abundant of snail intermediate host in contrary to the relatively large number of slaughter animals supplied to the export abattoirs from the lowlands.

From the overall prevalence of fasciolosis reported in ruminants, the overall prevalence encountered in sheep was significantly lower than that of cattle, but it was significantly higher than the overall prevalence observed in goats. Nonetheless, out of a total of 2257 cattle, 1189 sheep and 1070 goat livers examined, the prevalence was 50.8%, 58% and 12.2%, respectively. The slaughtered ruminants were predominantly male (71%). The difference in the overall prevalence between the sex groups was significant. The female appears to show higher prevalence despite the overall lower number of female animals slaughtered.

Despite the lower number of cross breed ruminants slaughtered, this result indicated the significantly higher prevalence of fasciolosis along with cross breed (57.2%) ruminants than the locals (43.6%). Altogether, the prevalence of poor conditioned ruminants slaughtered at export abattoirs were 46.6%. On the other hand the prevalence of fasciolosis in ruminants with good body condition at export and municipal abattoirs was 58.2% and 41.8%, respectively.

The present study in Debreberhan abattoir indicated that the dominant fluke species of the study area is *Fasciola hepatica (*87.8%). The proportions of cattle and sheep infected with *F. gigantica* alone (6.3%) or mixed infection with both species (5.8%) was relatively small. In general, *Fasciola hepatica* (70.9%) was a dominant fasciola species identified followed by *Fasciola gigantica* (21.5%), mixed infections (6.8%) and immature flukes (9.7%). *F. hepatica* and *F. gigantica* had been identified from all the slaughtered ruminants (cattle, sheep and goats) and in both the export and municipal abattoirs.

The lesions of fluke infections were clearly observed in the livers of all the three species of ruminants slaughtered in the export and municipal abattoirs, irrespective of their geographical origin and agroecological variations. The overall lesion observation had shown that most of the fluke infected livers examined were affected lightly (20%). Irrespective of the lesion types, fluke induced lesions were abundent in Debereberhan municipal abattoir followed by Helimix export abattoir. In all the abattoirs the predominant lesions encounterd were light type except the slightly higher count of heavy lesions observed at Bahrdar abattoir. The predominant lesion types in both the local and cross breed ruminants were light type and successively encountered lesion was the moderate type.

The overall mean number of flukes recovered from sheep and cattle were 65.5± 5.6 and this was ranged from 16 to 175. However, the mean number (74.5± 0.7) recovered from the cattle was relatively high in comparison to the lower count obtained from sheep (57.6 ± 0.6) and goats (35.6 ± 0.8).

In Ethiopia, various authors reported the wide distribution and occurrence of fasciolosis with significance economic loss associated with the rejection of fluke infected liver at abattoirs. The overall financial loss observed in the present study (7,049,638 ETB/335,697.1USD) was significantly higher than the work of other authors elsewhere in the country. Rahmeto et al., (2010) reported a loss of 8312 USD at Hawassa abattoir whereas, Tolossa et al. (2007) and Abuna et al. (2010) reported a financial loss of 6300 USD and 4000 USD at Jimma and Wolaita Soddo municipal abattoirs, respectively.

The relatively higher fluke prevalence at ELFORA (28.9%) and HELMIX (30.4%) export abattoirs coupled with higher annual slaughter rate (54,700 cattle / annum) were responsible for higher overall financial loss incurred due to condemnation of fluke infected bovine liver. Similarly the prevalence and annual number of animals slaughtered had significant effect on the higher overall financial loss incurred in small ruminants (HELIMIX, sheep (24.5%); ELFORA, sheep (21.42%); HELIMIX, goats (9.4%) and ELFORA, goats (12.6%) with annual slaughter capacity of 100, 000 each) (EMPEA, 2010; for slaughter capacity).

The Addis Ababa municipal abattoir has also high production capacity (177,781) compared to the other municipal abattoirs with much lower number of slaughtered animals per annum (Hawassa, 20,000; Jimma, 14,000; Woliata Soddo, 5678, Bahrdar 4368, Debreberhan, 7728 and Gondar, 7392) (EMPEA, 2010). Comparison of the loss that incurred for beef cattle at Debreberhan abattoir (288, 223 ETB /13,724 USD) was significantly higher than the report of other workers (Abebe, et. al., 2010; Abuna et al., 2009; Tolossa et al, 2007) (AACCSA, 2005). However, at Bahrdar (41,605.2ETB / 1981.2 USD) it was much lower compared to the previous reports of the same authors.

Despite the low annual slaughter rate, the retail price of the liver of beef cattle at Debreberhan (48 ETB) was relatively higher than the price at Addis Ababa (35 ETB). Furthermore, the prevalence of bovine (77.7%) and ovine (81%) fasciolosis was significantly higher at Debreberhan than that of Addis Ababa (48.8%, 30.3% and 14.5 for cattle, sheep and goats respectively).

In general, the high fasciola prevalence reports in the abattoirs of the present study was responsible for clear observed differences in the economic loss incurred as a result of the rejection of fluke infected liver. The retail price of the liver of cattle at export abattoirs was only 30 ETB / 1.43USD while that of small ruminants was 12/0.57USD. However, these had shown variation among municipal abattoirs (25-48 ETB) for liver of beef cattle and 12 to 22 ETB for shoats. The variation of the retail price of the liver had significant effect on the observed financial loss due to fluke infected liver. The retail price differs with the demand of the liver in different towns, as it is clear that the price is affected by the demand and supply for uninfected healthy liver to the market. Thus, there was relatively high price tag for liver of cattle at Addis Ababa (35 ETB) and Deberberhan (48 ETB) towns compared to the lower prices observed at Bahrdar (25 ETB) and Debre Zeit (30 ETB). The present findings plainly indicated that, the retail price of the liver of sheep (22 ETB) was relatively higher at Debreberhan town than the other towns (12 ETB).

The combined mean financial loss recorded in both export and municipal abattoirs was 7,049638 ETB /335, 697.1 USD. However, the overall mean financial loss observed in three municipal abattoirs altogether (5,260,596 ETB/ 250504.6 USD) was significantly higher than the sum of combined loss incurred in two export abattoirs (1,789,043 ETB/ 85,192.5USD). Comparison of the financial loss observed for slaughtered animals at three municipal abattoirs had shown significantly higher loss at Addis Ababa (4,843,837ETB) municipal abattoir followed by Debreberhan (375,153.4ETB) and Bahrdar (41,605.2 ETB).

The financial loss observed due to fluke infected liver of bovine was significantly higher in Addis Ababa (4,311,189ETB/205,294 USD) abattoirs than on the whole loss incurred in small ruminants at municipal abattoirs (549, 978 ETB/ 26,189.43USD). However, the loss in small ruminants was almost twice the loss observed in rejection of fluke infected liver at Debreberhan (288, 223.5 ETB/13724.9) for cattle alone.

The overall mean fluke count (M ± SE) indicated that moderately affected sheep and goats liver had relatively higher fluke counts compared to the severely affected ones. However, in cattle, fluke counts in heavily affected livers exceeded that obtained from moderately affected ones. The worm burden in lightly affected sheep liver had relatively higher fluke counts compared to the severely affected liver. The less worm burden in severely affected livers of sheep may be associated with the severe fibrosis that impedes the passage of immature flukes and acquired resistance that resulted in the expulsion of flukes from the bile ducts (Dwinger et al., 1982).

In present study, the collections of freshwater pulmonate snails (G. *truncatula* and R. *natalesis*) were made possible from rivers, ponds and canals and their sizes vary. The morphological measurements on the snail vectors of *F. hepatica* and *F. gigantica* indicated that they were in the normal range. The measurements do not show much variation in their shell length and width for *Radix (Fossaria)* species collected from the rivers and ponds in the central Rift Valley. For *G. truncatula*, the shell lengths at collection were the largest from Angolela river and the smallest were from Wondogenet. However, in *R. natalensis* the largest were collected from Tikur Whuha and the smallest in Guta River. The findings indicated no *Radix* species from Angolela river at

the time of collection. The presence of both *Radix* and *Galba* species from fresh water bodies in Ethiopia has been reported since (Graber and Daynes, 1974).

The snails collected for exposure to the miracidia and subsequent harvest of the cercaria shed were identified in the laboratory as *G. truncatula* snails. The snails were reared in the laboratory and the subsequent generations of *G. truncatula* snails were finally used for artificial infection with *Fasciola hepatica* miracidia. The eggs of *Fasciola hepatica* collected from the Debre Berhan Municipal abattoir were successfully used to produce the required miracidia. The cercaria shed from the infected snails were made to encyst to give rise for the metacercariae needed for the present experimental trail.

The shells of *G. truncatula* were between 4.8 and 6.0 mm long at the start of the experiment. Other works had shown that at these shell sizes the snails had reached sexual maturity (Boray, 1969; Kendal, 1953; Harris 1974). The snails were kept on blue green algae as food source and this was successfully used to rear the snails to produce the metacercaria. The greater production of *F. hepatica* cercariae in snails which fed on Algae or the Boray diet had already been reported by different writers (Boray, 1969; Kendall, 1963; or Lee et al., 1995) when these authors used the same sources of food for the rearing of *G. truncatula* or *G. viridis* under laboratory conditions.

Shell length at death of the snails was between 6.8±1.8 and 8.5±.1.2. Snails with larger shell size showed a lower infection rate, the groups presenting the highest (76%) and lowest (3%) proportions of positives being those of 5-6 mm and 13 mm or more, respectively. Cercariae were

present in 18% of them at 25 days post-infection, and cercarial shedding was observed 65 days postinfection. It was concluded that there is a non-linear negative association between shell size and infection rate.

The present study plainly disclosed that metacercarial production of artificially infected *Lymnae truncatula* snails vary considerably. The 20^{o}C temperature for oviposition and rearing of these snails under laboratory appears to be optimum. This variation in the total output of cercariae of individual snails was also reported by other authors elsewhere (Krull, 1941; Roberts, 1950; Bitakaramire, 1968; Hodasi, 1972 and Lee et al., 1995). The highest estimated number of cysts produced by each miracidium was about 3879. Shell length at death of the snails was between 6.8±1.8 and 8.5±.1.2. The shell lengths recorded at death concides with high cercaria shedding of sanils in Group B when they were kept at temperatures above 20°C during infection. This finding was agreed with thee reports of Lee et (1995) which disclosed that *Lymnaea viridis* produced high metacercaria when kept at tempreture range of 20-24°C and when the shell length at death was relatively larger than other groups. Snails with larger shell size showed a higher infection rate, the groups presenting the highest (80%) and lowest (3%) proportions of positives being those of 5-6 mm and 13 mm or more, respectively. Cercariae were present in 18% of them at 25 days post-infection, and cercarial shedding was observed 65 days postinfection. It was concluded that there is a linear positive association between shell size and infection rate.

The only record of oviposition by either *L. columella* or *L. tomentosa* at low temperatures is Boray's (1964b) observation that *L. tomentosa* will lay eggs at 15°C. *L. truncatula*, whose temperature tolerance zone is below the upper limits of these 2 species, is said to begin

oviposition at 10-11°C (Kendall, 1953). The maximum and optimum temperatures for oviposition were not determined in these experiments. *L. tomentosa* has been recorded as laying eggs at 30°C (Lynch, 1963), and optimum temperatures are said to be about 26°C (Boray 1964a); for *L. truncatula* 18-21°C appears to be the optimum range and 25°C the upper tolerance limit (Kendall 1953).

The present study revealed that a number of *Galba truncatula* snails initiated cercarial shedding 25 days after they were exposed to *F. hepatica* miracidia. It is known that optimum temperature and moisture condition favour the rapid growth *Fasciola* in the snail interediate host. The time period between infection and first cercarial shedding of the snails in this study was shorter than the results of most other studies in which cercariae were shed varies from 27th to 73rd day (Lee et al., 1995 Roberts, 1950; Hodasi, 1972; Bitakaramire, 1968; Krull, 1941) after infection. The findings indicated invariable minimum development times for *F. hepatica* in different intermediate hosts. Together with the susceptibilities of *G. truncatula* to infection in various stages of growth, the result suggests a well-adjusted relationship between the parasite and its snail. The present method used for the *G. truncatula* snail rearing, maintenance of the aquaria and diet are extremely suitable in the laboratory.

The values reported for the characteristics of *F. hepatica* infection in *G. truncatula* and *R. natalensis* agreed with those reported by other authors (Rondelaud et al., 2002; Vignoles et al., 2015; Sanabria et al., 2015). Although the prevalence of *F. hepatica* natural infection in *G. truncatula* was higher than the infection of Fasciola species reported in *R. natalensis*, in present study the survival of *F. hepatica* infected *G. truncatula* that was brought to laboratory on day 30

was significantly lower than *R. natalensis* and the relative change in mean height of cercaria shedding snails at their death was greater (13.2–15.4 mm compared to 6.7–.8.9 mm for *G. truncatula*. Furthermore, the total metacercarial production in *R. natalensis* was significantly higher than that of *G. truncatula*.

In experimental production of metacercarae of *F. hepatica* with miracidial infections of *G. truncatula* snails were kept under optimal temperature of 20^0C. The cercarial outputs of individual snails vary significantly. The current experiment was based on suitable temperature range recommended for rearing of *F. hepatica* under laboratory condition by various authors (Kim et al., 1978; Jang et al., 1987; Rondelaud et al., 2002; Vignoles et al., 2015). Snails infected with single miracidium were produced less cercaria than either that infected with three or five metacercaria. The higher cercarial output with shorter survival of *G. truncatula* infected with increasing number of infective miracidium has been reported by other authors (Rondelaud et al., 2002; Sanabria et al., 2015; Vignoles et al., 2015).

In miracidia infected groups of *G. truncatula*, the prevalence did not show any significant variation when the number of miracidia per snail went from one to five, whereas snail survival on day 30 post exposure was significantly lower in the five-miracidia than in the two-miracidia groups. Thus it antagonizes the positive relationship between the number of miracidia used for each snail and that of free rediae developing within the snail body (Rondelaud et al., 2002). In contrast, Vignoles et al. (2015) reported a significant increase in prevalence when the number of miracidia per snail went from two to five, whereas snail survival on day 30 post exposure did not differ significantly in the four groups of infected with quinquemiracidia of *P. columella*.

However, the number of metacercariae in the other groups (originated from other area) did not significantly differ from each other when the miracidial dose increased from two to five (Sanabria et al., 2015). This result was in contrary to the concept that the competition occurring between free rediae during their development (Combes 1995; Rondelaud et al., 2002) induced a delay in the differentiation of intraredial cercariae and their exit from the snail so that the numbers of shed cercariae were within the same scale of values in the two- and five-miracidia infections. In each groups of snail population considered separately, the shell height of cercaria shading snails in the two- and five-miracidia groups did not significantly differ from each other. However, this was significant in the one and five miracidia groups.

Boray, (1964a) emphasized the importance of trematode infections of snails. The larval stages of trematodes live as true parasites within the snail's body and may cause severe pathological changes. Infection with *F. hepatica* causes severe damage to the snails and mortality may occur from acute (infection phase), subacute (developmental phase) and chronic (accumulation phase) infections. The death of many snails may be one of the controlling factors of the intensity of fasciolosis in livestock. However under natural conditions the death of the snail host often occurs when the life cycle of *F. hepatica* has been completed and the result of pathogenic effect of *F. hepatica* in snails may be favorable for the completion of the life cycle by forcing the snails to leave deeper water.

The greater survival, increased size of cercaria shedding snails at death and higher metacercarial production by *Psedosuccinate Columella* compared to *G. truncatulla* has been reported by other authors (Vignoles et al., 2015). However, given the significant presence of sheep and cattle in

highlands and the predominant presence of *G. truncatula* well adapted to its natural habitat as the intermediate host of *F. hepatica* (Yilma and Malone, 1998), it may necessitate the propagation this snail for metacercarial production under experimental conditions for sheep and cattle in Ethiopia.

The pathogenesis of fasciolosis is associated with liver damage that is inflicted by migrating and feeding immature flukes as well as host inflammatory immune responses to parasite-secreted molecules and tissue damage (Urquhart, 1996; Taylor, 2007; Molina-Hernándeza). One of the advantages of live, attenuated vaccines, and a reason to re-investigate the potential of radiation attenuation, is their potent immunogenicity since the organisms are still able to replicate and behave initially in a similar manner to a natural infection, thereby stimulating the immune system to secrete the immunoregulatory products and induce the cellular activation that would normally occur (Viljoen and Luckins, 2012). Both humoral and cell mediated immune responses appear to be important for resistance to *F. hepatica,* although cellular response seem to be more relevant. Some mechanisms of immunemodulation in *F. hepatica* infection have also been described in different hosts, affecting either antibody activity (Chapman and Mitchell, 1982) or lymphocyte response (Zimmerman, et al., 1983).

There have been inconclusive and no persistent evidence on scientific community for the effective natural immune response of sheep and cattle against Fasciola infections so far. An effective immune response against Fasciola infection varies between the ruminant hosts and between liver fluke species. Some authors reported the failure to develop natural immune response in sheep. By contrast, cattle exhibit a high level of natural resistance to the acute effects

of primary and secondary infections, even in large doses (Dalton et al., 2013). According to some authors (Haroun and Hillyer, 1986; Jayaraj et al., 2009) in rats and cattle a development of resistance to challenge infection has been established. In other hosts (sheep, rabbits, mice) there is no evidence of acquired resistance to primary or secondary infection (Dalton et al., 2013; Haroun and Hillyer, 1986; Jayaraj et al., 2009). However, several irradiation-attenuated parasite species have been used experimentally to induce protection in various host species (Chapman and Mitchell, 1982; Haroun, and Hillyer, 1986; Taylor et al., 2007; Jayaraj, et al., 2009; Viljoen and Luckins, 2012; Molina-Hernándeza, et al., 2015), including cattle against infection with the liver fluke, *F. hepatica,* and sheep and cattle against infection with *F. gigantica* (Haroun, and Hillyer, 1986; Molina-Hernándeza, et al., 2015). A variety of studies have demonstrated that acquired immunity can be demonstrated in ruminants following *F. hepatica* or *F. gigantica* infections in animals, suggesting that development of vaccine is potentially feasible (McManus, and Dalton, 2006). Studies on biochemical difference between *F. hepatica and F. gigantica* (Spithill et al., 1997; Piedrafita et al., 2010) also suggested that a vaccine which can harness mechanisms of acquired immunity for *F. gigantica* in sheep may be feasible. The different studies provided evidence that acquired immunity could be induced following fasciola infection in animals. Based on the recovery of flukes after primary challenge, sheep do not develop acquired immunity to *F. hepatica* (Boyce et al., 1987; Chauvin et al., 1995; Spithill et al., 1999; Pleasance et al., 2011).

The local cellular response in the liver, the definitive site of *Fasciola hepatica* infection, has been characterized histologically during primary infection in sheep fasciolosis (Chauvin et a., 1995). Sudanese desert sheep can be protected by vaccination with irradiated metacercariae of *F.*

gigantica (A'Gadira, et al., 1987) and Indonesian thin-tail (ITT) sheep express high resistance to *F. gigantica* infection (Wiedosari and Copeman, 1990; Piedrafita et al., 2004; Pleasance et al., 2011; Roberts, 1997a, b). In ITT sheep, resistance to *F. gigantica* is expressed within a few weeks of infection and higher resistance is observed after exposure (Pleasance et al., 2011; Roberts, 1997a). This resistance appears to be immunologically based, as it is suppressed by dexamethasone treatment (Spithill et al., 1999a).

The present results revealed that *F. hepatica* infections in sheep induced an immune response, both humoral and cell mediated. A humoral immune response was developed in infections as early production of specific anti *F. hepatica* IgG1 week by 2 post infection. The dynamics of serum anti Fasciola IgG1 response was comparable in all infected sheep. There was a steady increase, reaching a peak at week 10 post infections, after which antibody levels decreased and became invariably stable until 15 weeks post infection. Apart from the GIV sheep that received 500 metacercariae γ-irradiated at 240 Gy and induced relatively sharp and higher anti fasciola antibody production, other groups had shown nearly similar steady elevation in the level of IgG1 titers. Sheep vaccinated with 500Gy γ-irradiated metacercariae (GIV) produced the highest antibody titre compared to the positive control group. This response was 2.5 times greater than the anti Fasciola antibody titre observed in the positive control. Sheep from group GIV (γ-irradiated with 240Gy) and GIII (with 120Gy) showed relatively higher production of IgG1 compared to those groups that had received metacercariae dosed with lower doses of γ-irradiation (30 and 60 Gy, respectively). However, comparative relative difference was noted on immune response of the host infected with metacercariae γ-irradiated with 240 and 120 Gy,

where the former shown slightly higher elevation of anti Fasciola IgG1 titer and the difference was not statistically significant.

In line with these results, acquired resistance of goats to *F. gigantica* has been reported both after primary infections ceased with anthelmintics (Haroun et al., 1989) and after the administration of irradiated metacercaria (El Sanhouri et al., 1987). However, contrary to the present result no resistance to *F. hepatica* has been described in sheep (Chauvin, et al., 1995) while high resistance to *F. gigantica* has been proved (Roberts et al., 1997b). The effective response of sheep infected with *Fasciola hepatica* in the present study may indicate the possibility that the factors assessed (significant reduction in egg output, worm count, minimal damage to the liver, high immune response (IgG1 level and cells) can be useful indicators to say that sheep can develop protective immunity to *F. hepatica*. On the other hand studies conducted on goat indicated that secondary infection did not induce any modification in IgG response and only minor differences could be seen between primary and secondary infected animals. That means, it seems that serum IgG reached a level where further exposure to the antigen failed to elicit an increased response (Robersts et al., 1997a; Piedrafita et al., 2007; Piedrafita et al., 2010).

Indeed, previous workers performed a challenge infection after vaccinating with irradiated parasites have shown that the immune response to reinfection has failed to elicit an increased response to IgG in sheep (Chauvin et al., 1995) and cattle (Clery et al., 1996). Accordingly, sheep and cattle did not develop resistance against secondary infection Chauvin et al., 1995; Clery et al., 1996; Martinez Moreno et al., 1997).

Within seven days of infection in mice, a systemic antigen-specific Th2 response is firmly established and is characterized by the secretion of IL-4, IL-5 and IL-13 from splenocytes (Cervi et al., 1996; Walsh et al., 1998). As the infection develops (3 weeks) regulatory macrophages (TGF- and IL-10 producing) and dendritic cells (IL-10 producing) are recruited to the peritoneum and DC maturation is inhibited (Walsh et al., 1998). Chronic disease in sheep and cattle is also typified by Th2 responses and suppressed Th1 responses. Serologically, this polarity of immune response is strikingly displayed in the isotype of circulating antibodies; both fluke-infected sheep and cattle secrete high titres of IgG1 antibodies and virtually no IgG2 (Hoyle et al., 2003; Mulcahy et al., 1999). A study (Pleasance 2011a) found that the responses of Indonesian Thin Tailed (ITT) sheep, that exhibit resistance to *F. gigantica* but susceptibility to *F. hepatica*, were predominantly Th2 biased and that susceptibility to the latter parasite was associated with an increase in the IgG1/IgG2 and IL-4/IFN ratios.

The present study plainly depicts vigorous cellular response against the parasite in the livers of sheep post infection. In spite of the fact that some fluke eggs, immature or mature flukes were observed in the hepatic parenchyma, this clearly shows some level of protective response, as there was no evidence of consistent killing of either immature or mature flukes in the hepatic parenchyma. The effector mechanism of protective immunity has not been definitely established, but reported data suggest it occurs at the early phase of the infection, in three different sites: the wall of the small intestine (Charbon et al., 1991), the peritoneal cavity (Burden et al., 1983) and the liver surface and parenchyma (Keegan and Trudgett 1992). This mechanism is manifested against immature parasites and, only in a lesser extent, against mature flukes. It is a nitric oxide-

mediated killing that requires the attachment of the effector cells (eosinophils, neutrophils and macrophages) to the tegument of the parasite (Spithill et al., 1997).

Infection with irradiated metacercariae reduced hepatic damage and local inflammatory infiltration in the liver. Sheep of from the positive control group (PC) that had received normal 500 unirradiated *F. hepatica* metacercariae showed widespread portal fibrosis with extensive distortion of hepatic lobules, marked bile duct proliferation (hyperplasia of mucosal epithelium of the bile duct with globule leucocytes showing severe cholangitis), severe portal hepatitis with inflammatory infiltration of lymphocytes, hemosiderin laden macrophages and plasma cells and extensive infiltration with eosinophils and epitheloid cells resulting in loss of lobular architecture and moderate cirrhosis.

The fact that an immune response is induced but no resistance is acquired against *F. hepatica* implies that the response is ineffective due to some defense mechanisms operating in this species. Martinez Moreno et al. (1997) have rarely observed the immune inflammatory cells in close association with the flukes, and consequently, they have recorded few images of destruction of the parasite. This may confirm the existence of some immune evasion mechanisms in goat fasciolosis. One of them may be the rapid migration of the flukes throughout the liver, as has been previously reported in goats (Martinez Moreno et al., 1997) and sheep (Meeusen et al., 1995) that makes it impossible for the leucocytic infiltration to form around the parasite (Chauvin et al., 1995). Another mechanism may be the depression of the local inflammatory and immune responses around the parasite, as was described in the sheep (Chauvin et al., 1995). The scarcity of $CD3^+$ T cells in the infiltrate surrounding acute tunnels suggests that *F. hepatica*

inhibits their migration through the liver parenchyma. This hypothesis is supported by the involvement of ESP from *F. hepatica* in the suppression of the PBL proliferative response of goats (Martinez Moreno et al., 1997), rats (Cervi et al., 1996) and sheep (Zimmerman et al., 1983).

The present study clearly depicts that the immune response was vigorous in all infected groups but this was more pronounced on sheep that have received high dose of irradiation (GIII and GIV). As evidenced by the pathological findings of fluke infected liver, the damage was much higher in sheep from positive control group and those dosed with low level of gamma irradiation (GI and GII). But whether this response was protective against primary infection has not been established yet. In all infected groups and PC sheep, the plasma cell (B lymphocytes) response is vigorous as the case for other inflammatory cells (eosinophil's and macrophages). In present study larger bile ducts often contained cell debris and fluke eggs, and were surrounded by numerous globule leucocytes. Fresh migrational tracks of all sizes were mainly composed of eosinophilic debris of disintegrated hepatocytes infiltrated by numerous eosinophils and some lymphocytes and macrophages. In smaller tracks tendency to haemorrhages was slight. In the larger tracks the macrophages contained a profusion of iron (hemosiderin filled cells) in consequence of pronounced haemorrhages. Often tissue elements surrounding the tracks were affected by a pronounced coagulative necrosis. Chronic tracts with numerous macrophages containing hemosiderin pigment were often seen. Granulomas with necrotic or mineralized centers surrounded by macrophages, multinucleate giant cells, numerous eosinophils, and more peripherally by lymphocytes and plasma cells, were observed in all sheep. Fluke eggs were identified in some of these granulomas.

A study (Campbell et al., 1978) demonstrated the cellular response to *Fasciola hepatica* metacercarae in challenge infection of both vaccinated and unvaccinated sheep had significantly increased the numbers of eosinophils and globule leucocytes in the parenchymal bile duct and the numbers of mast cells and globule leucocytes in the abdominal bile duct. In addition the numbers of eosinophils and globule leucocytes in the parenchymal bile duct were significantly correlated with the percentage of retarded flukes in both vaccinated groups.

The innate immune system plays an important role in the defense against *F. hepatica* infection and also in priming the adaptive immune response. Innate effector mechanisms elicited upon infection include rapid eosinophilia and macrophage activation. Eosinophilia is a pronounced, yet controversial, characteristic response to most helminth infections. Studies using murine hosts deficient in eosinophils fail to show overall differences in helminth parasite burdens (Bossaert et al., 2000; Swartz et al., 2006). In cattle infected with *F. hepatica*, elevated eosinophil counts were found 4 weeks post infection and persisted over a 16-week period (Bossaert et al., 2000), while the presence of biphasic eosinophilia has been demonstrated in sheep (Zhang et al., 2005), occurring at weeks 4 and again at 9–10 post-infection. Elevated eosinophil counts in the lamina propria of gut loops of immune rats have been correlated with protection in an ex vivo model (Van Milligen, 1999).

The liver fluke has proved to have several detrimental effects on its intermediate host, including castration or decrease in fecundity, increased mortality, destruction of the digestive gland, metabolic changes (reallocation of energy from reproduction to growth, inducing gigantism), increased sensitivity to environmental stress (Graczyk 1999; Gutierrez 2000).

To deter the detrimental effects of pathogens to the host, huge successes have been made in vaccine development against viruses and bacteria over the past several years. With the exception of the live attenuated Huskvac vaccine for lungworm, there are no commercially viable vaccines for animal helminth parasitic pathogens (Matthews, 2000). Recent advances in vaccination with recombinant helminth antigens have been successful against cestode infections of livestock and new vaccines are being tested against nematode parasites of animals (Hewitson et al., 2014). More recently, a new vaccine for *H. contortus*, Barbervax, consisting of native gut-derived antigen complex has been launched in Australia (www.barbervax.com.au).

Attenuated antiparasite vaccines enable the host to mount a protective immune response against the organism without the development of the pathological symptoms of infection. Despite the practical nature of this strategy, several irradiation-attenuated parasite species have been used experimentally to induce protection in various host species (Smith et al., 1993), including cattle against infection with the liver fluke, *Fasciola hepatica,* and sheep and cattle against infection with *F. gigantica* (Haroun and Hillyer, 1986).

Several authors previously depicted that plasma activities of liver enzymes are sensitive indicators of liver damage in sheep and cattle (Rowlands and Clampitt, 1979; Bulgin et al., 1984; Ferre et al., 1994; Ferre et al., 19995a; Ferre et al., 19996) and with this regard our findings were not different. In ruminant (bovine and ovine) fasciolosis young flukes may be found in the hepatic parenchyma for approximately 6-8 weeks, migrating through the tissue and growing. The flukes then enter the biliary system, mature and lay eggs which can be found in faeces many months after initial infection (Urquhart et al., 1996; Taylor et al., 2007).

In the present study the parasitological findings clearly disclosed that, sheep appear to develop immunity against *F. hepatica* infection when dosed with 120 and 240 gray while the size of the inocula is 500 metacercarae in single infection. This is evidenced by the significant reduction of worm egg outputs and the recovery of mature flukes in groups γ-irradiated with 120 and 240 gray compared to the positive control and groups kept at 30 and 60 gray of γ-irradiated metacercarae. Furthermore, the enzyme profile indicated minimum damage to the liver in groups with high doses (120 and 240 gray) of γ-irradiation with the inocula size of 500.

In this study, primary vaccination of sheep with 500 γ-irradiated (30 and 60 gray) metacercariae of *Fasciola hepatica* did not generate significant protection compared with 500 γ-irradiated (120 and 240 gray) metacercariae and the positive control as measured by the reduction of faecal egg output and recovery of flukes from liver and bile ducts. All the vaccinated and infected control groups were found to shade eggs except the uninfected negative controls. Higher EPG output were observed in infected control (PC) followed by those animals vaccinated with low doses of the gamma irradiation (GI and GII). However, a study (Campbell et al., 1978) stated that vaccination of sheep with either 100 or 1000 γ-irradiated (25 gray) metacercariae of *Fasciola hepatica*, on two occasions six weeks apart, did not generate significant protection against intraruminal challenge with *F. hepatica* six weeks after the second vaccinating dose as measured by recovery of flukes from liver and bile ducts, twenty weeks after challenge.

The present results were in agreement with most of the previous studies in that vaccination of sheep with irradiated metacercarae of *F. hepatica* yielded matured and immature parasites with varying level of reduction across treatment groups. In contrary to some authors (Haroun and

Hillyer, 1986; Rickard and Howell, 1982; Boyce et al., 1987) that stated sheep do not acquire resistance against *F. hepatica* as indicated by the yields of mature parasites from primary and secondary infections, the result of the present study revealed differences in response of sheep to yield matured parasites in vaccinated and control groups from primary infection. In coincident to this, the mean number of flukes recovered from infected groups vary significantly ($P<0.05$). That means a strong dose response was evident in the number of parasites recovered in the groups dosed with incremental doses of γ-irradiation with *F. hepatica* metacercariae. Accordingly, the yield of matured parasites from Ethiopian sheep ranged from 7.7% to 22.5%. In the studies with European fleece sheep, yields of *F. hepatica* ranged from 16 to 38% after primary infection and from13 to 31% after secondary infection (Boyce et al., 1987). A significant reduction in parasite numbers (from 17% in control animals to 3.4%) was reported in Sudanese desert sheep vaccinated with irradiated metacercariae of *F. gigantica* following a secondary challenge (A'Gadira et al., 1987).

Presently, the percentage of the flukes recovered (percentage reduction in fluke burden 81.5%) unirradiated from the liver of the sheep were highest in infected control groups (22.5%; 112.3±4.3) followed by GI animals dosed with the lowest irradiation dose (18.6%; 93± 3.4 recovered / 83.4% reduction). The percentage reduction in burden of flukes in group II and III sheep was 86.7% (13.4%; 67.8 ± 2.8 recovered) and 88.8% (11.2%; 56.5± 7.18 recovered) respectively. Similarly, those animals in the highest irradiation dose (GIV) yield the lowest recovery 92.3% (7.7%, 38.3± 3.3 recovered). Accordingly, the higher the irradiation dose, the lowest was the recovery of flukes in necropsy examinations. There was, however, a significant increase in the proportion of flukes retarded in the parenchyma of vaccinated groups with

significant effect observed in those vaccinated with 120 and 240 gray of γ-irradiated (30 and 60 gray) metacercariae. The percentage of retarded flukes was positively correlated (r = 0.72) with the degree of liver damage. According to the works of some authors [36], no significant reduction of fluke burdens were observed in any group, although a non-significant 20% reduction was observed in sheep vaccinated with 2000 metacercariae irradiated with 100 gray.

The present study used parasitological parameters such as recovered liver fluke length and width, total mass of recovered flukes and liver damage (enzyme profile) which are major factors often used to indicate the protection against the severity of parasite infection. This method of assessing has been used in evaluating vaccination against fasciolosis by different studies including a multivalent vaccine of recombinant stage-specific antigens (Valero et al., 2006; Valero et al., 2002; Jayaraj et al., 2009).

Cysteine proteases are common virulence mediaters of parasites, and are produced by all stages of the fluke lifecycle. They mediate biological functions including excystment, tissue invasion and immune evasion (Bulgin et al., 1984). Adult fluke cathepsin L and Newly Excysted Juvenile (NEJ) cathepsin B are the prominent proteolytic enzymes of their respective ES materials. *F. hepatica* cathepsin L5 (Creaney et al., 1995; Irving et al., 2003), cathepsin B (Smooker et al., 2000, Beckham et al., 2006; Kennedy et al., 2006; Law et al., 2006; Meemon et al., 2004) and *F. gigantica* cathepsin L1 (Grams et al., 2001) are promising targets for vaccines against *Fasciola* infection.

Similar to the current finding, other authors (Jayaraj et al., 2009) depicted that, in single and multivalent recombinant protein vaccinations of adult stage *F. hepatica* cathepsin L5, metacercarial stage *F. gigantica* cathepsin L1g and juvenile stage *F. hepatica* cathepsin B

against *F. hepatica* challenge infection, the rats vaccinated with recombinant proteins were shown to have significantly fewer and smaller flukes than the control rats. That means a maximum protection of 83% was seen in the group vaccinated with a combination of cathepsin B and cathepsin L5 (Jayaraj et al., 2009). Although there was variation on the level of protection, the percentage reduction of fluke population of gamma irradiated *F. hepatica* metacercarial infection in Ethiopian sheep ranged from 7.7 to 22.5%.

In agreement with the present study, vaccination with the multivalent cathepsin B/L5 leads to less liver damage, lower fluke numbers and the lowest mean wet weight compared to control rats. This result indicates liver fluke development was retarded in the vaccinated groups. It may be that a fluke vaccine does not need to induce sterile immunity, but reduce the pathology to a level that is tolerated by the animal. A reduction in fluke burdens will also reduce the numbers of eggs passed by infected animals, reducing pasture contamination. A study demonstrated (Jayaraj et al., 2009) that juvenile stage-specific recombinant proteins (B, L1g) are able to mediate immunity to liver fluke infection, but that protection is greatest when a juvenile stage protease (cathepsin B) is used in concert with an adult stage protease, L5. A work reported (Jayaraj et al., 2009), the protective immunity elicited by recombinant protein vaccination appears to evoke effector and memory responses against infection. Although such vaccination induces strong humoral responses (Jayaraj et al., 2009), the precise effecter mechanisms leading to fluke control have not been elucidated. That a cocktail of juvenile and adult stage *Fasciola* recombinant proteins induced the better protective immunity than individual protein alone indicates that stage specific, multivalent recombinant vaccines against parasites may feasible (Jayaraj et al., 2009).

The number and size of fluke's recovered had a significant effect on the observed weight changes of the liver in different groups. The results clearly disclosed the difference in the mean change of the liver weight between the infected and control groups at necropsy. The highest mean live weight changes were observed between positive and negative control groups with a difference of 71± 2.5 g and this was significant ($p<0.5\%$). The presence of flukes in positive control was defiantly responsible for the increase weight of the liver than the uninfected negative control sheep. Comparison between highly dosed groups at GIV with that of positive control had shown a mean difference of 48± 3.3 g. This was followed by the mean difference of 40± 2.3 g with that of group III animals. The relative comparative mean live weight changes recorded for GI and GII were 18 ± 2.3 and 28 ± 3.4gm. Generally the liver weight relatively tends to decline with progressive increase in irradiation dose and subsequent decrease in the recovery of the fluke and reduction in size of *F. hepatica* at necropsy examinations.

The experiment was only resumed after three week period of acclimatization and all animals began to gain weight after the second week post infection and this gain was continued till the end of the experimental period. Generally the mean weight gain of all groups tends to increase with time despite the differences in weight gain of the treatment groups used for the vaccine trails. Despite the nearly linear increase in the mean weight gain of positive controls and the groups received the lower irradiation doses of 30 and 60 gray, a significant body weight loss was observed between week 6 and 8 post infection followed by a steady increase of the mean weight of infected animals across time. Given the young growing age of sheep used in this experiment, the initial live weight gain has been expected. However, the effect of fasciola on body weight loss of treatment groups has been described between weeks 6 and 8. This might be due to

damage caused by the migrating flukes to the liver parenchyma. Rates of growth were significantly reduced by 14.7% and 14.1% in steers receiving a superimposed artificial infection rate of 1200 metacercariae and grazed at 3.54 beasts/hectare and 4.39 beasts/hectare respectively. Similarly group body weights were depressed 3% and 20% in steers receiving 600 metacercariae and grazed at 3.54 beasts/hectare and 4.39 beasts/hectare respectively (Chick, 2008).

Along with parasitological tests (faecal examination for fluke eggs and the EPG count), laboratory analysis of the hepatic enzymes has been useful indicator for damages caused by the liver flukes. In this regard, the first is the estimation of plasma levels of enzymes released by damaged liver cells. Two enzymes are usually measured. Glutamate dehydrogenase (GLDH) is released when parenchymal cells are damaged and levels become elevated within the first few weeks of infection. The other, gamma glutamiyl transpeptidase (GGT) indicates damage to the epithelial cells lining the bile ducts; elevation of this enzyme takes place mainly after the flukes reach the bile ducts and raised levels are maintained for a longer period (Urquhart et al., 1996; Hoffmann & Solter, 2008; Washington and Hoosier, 2012).

The results of the present study indicated that a peak in GGT activity was associated with the onset of patency. Tissue damage, which is shown by fluctuations in GLDH and GGT levels after adult flukes have become established in the bile ducts, is considered to be due to the feeding activity of adult flukes and the deposition of immune complexes in the liver parenchyma. In this sense the serum was successfully assayed for the presence of the enzymes glutamate dehydrogenase (GLDH) and gamma-glutamyl transferase (GGT), as indicators of liver and bile duct damage respectively.

In present study animals that have been exposed to infection of the metacercariae of *F. hepatica* were responded well to sensitization and infection as shown by evolution of hepatic (GLDH), bile duct and gall bladder (GGT) enzymes. The degree of visual hepatic damage and burden of *F. hepatica* were significantly positively related to levels of GGT and GLDH. The enzyme level varies with the dose of γ-irradiation and non sensitized groups of animals. In all cases the uninfected control sheep (NC) maintained the low profile of enzymes that were well within the normal ranges of each enzyme (GLDH= 1-12; GGT= 34-100 IU).

Glutamate dehydrogenase (GLDH) is a mitochondrial enzyme that catalyzes the removal of hydrogen from L-glutamate to form the corresponding ketimine acid that then undergoes spontaneous hydrolysis to 2-oxoglutarate. The liver has by far the highest concentration of GLDH activity (Boyd, 1962; Keller, 1981). Lesser amounts are found in the kidney and small intestine, where the GLDH activity is located in the proximal and distal tubular epithelial cells and in the mucosal epithelial cells, respectively. The GLDH activity of non hepatic tissues is relatively small compared to that found in liver, where GLDH is concentrated in the central areas of the lobule. In all species, increases in serum GLDH activity are considered liver specific (Washington and Hoosier, 2012; Pearson et al., 1995).

Serum GLDH activity is used most commonly in food animals. Because of its location within mitochondria, GLDH should be released only with irreversible cell injury. The sensitivity of GLDH activity varies depending on the nature of the disease. For example, in a study of calves with hepatic disease, GLDH activity increased in only 60% of the animals (Pearson et al., 1995).

Similarly, in cattle, the sensitivity of GLDH activity for the detection of hepatic lipidosis, hepatic abscessation, leptospirosis, and fascioliasis was only 28%, 53%, 71%, and 72%, respectively (West, 1991). The determination of GLDH activity is best done in conjunction with the determination of other hepatic enzymes and other indicators of hepatic injury or disease (Hoffmann & Solter, 2008; Washington and Hoosier, 2012).

The elevation of the enzymes and the damage inflicted on bile ducts has shown that they have positively correlated (r = 0.68). Irrespective of the infectious groups, similar trends in the elevation GGT profile (indicator of epithelial damage in the bile duct) was observed for all sheep infected with *F. hepatica* metacercariae until the end of the experimental period. All animals kept in the sensitized and infected groups had maintained the steady rise in the level of GGT until week 4 post infection, then after the GGT profile had shown marked elevation before reaching peak levels between weeks 9 and 11 post infection where the majority of flukes reach patency (epithelial damage to the bile ducts and gall bladder). Those it can be said that a peak in GGT activity was associated with the onset of patency. Then after the level of enzymes progressively decline before it had shown relatively stable enzyme levels between weeks 14-16 post infection. However, sheep infected with non irradiated normal *F. hepatica* metacercarae (PC) and Group I animals received the infective parasite that have been exposed to low levels γ-irradiation of 30Gy had shown significantly higher GGT levels compared to the animals in other infected groups that received 120Gy and 60Gy of irradiated *F. hepatica* metacercariae.

Although sheep received the 500 metacercariae and irradiated with 30Gy had relatively higher GGT profile compared to sheep that were treated with the same number of metacercariae and

kept at 60 and 120 Gy, the difference was not statistically significant (P>0.05). A statistically significance difference (P<0.05) in serum GGT levels was only observed between infected control sheep (PC) and the other sensitized groups (GI, GII, GIII and GIV) after week 8 post infection. However, between week 9 and 11 a statistically significant (P<0.05) rise in serum GGT profile was observed in GI (γ-irradiated with 30Gy) sheep compared to other sheep that had received a relatively higher doses of γ-irradiation (60 and 120 Gy). The difference in mean (M±SD) GGT profile of group GII and GIII and also GIII and GIV was not significant (P>0.05). Particularly the steady rise of GGT levels of groups GII, GIII and GIV were indistinguishable until the values had shown clear relative comparative difference after week 12 post infection.

The traditional method employed in the diagnosis of fasciolosis is the direct diagnosis method based on the detection and counting of eggs in faecal samples following the prepatent period of the disease. Juvenile flukes complete their migration in the liver and develop in bile ducts so that the parasite can leave its egg in the environment through faeces. Therefore, early diagnosis of the parasite through faecal examination is possible only 8 weeks after the infection (Moazeni and Ahmed, 2016).

Presently, despite the conventional coprological method employed to detect fasciola eggs and quantify it in the epidemiological study, the result had still revealed high coprological prevalence in both naturally infected sheep (60.1%) and cattle (49.2%). Similarly the recorded EPG counts in sheep and cattle were 63.4 ± 88.3 and 57.1 ± 34.8, respectively (Abebayehu et al., 2019a). The results actually did not include undetected mild infections and the effects of acquired host immunological responses against liver fluke infection in cattle and other factors affecting the

daily variations on egg shedding. Some of the findings of the higher values on detection of eggs may also be attributed to the false positive result due to the retention of eggs in the gall bladder.

Presently, alongside coprological examination, an antibody ELISA test was employed to detect the circulating IgG1 antibody response by adaptive B cells of all experimentally infected sheep. The result revealed that *F. hepatica* infections in sheep were induced an immune response, both humoral and cell mediated. A humoral immune response was developed in infections as early production of specific anti *F. hepatica* IgG1 week 2 post infections. The dynamics of serum anti Fasciola IgG1 response was comparable in all infected sheep. There was a steady increase, reaching a peak at week 10 post infections, after which antibody levels decreased and became invariably stable until 15 weeks post infection.

This finding is supported by evidence from studies using infected sheep where IgG1 was the dominant isotype (Raadsma et al., 2007). Studies have shown that antibody subclass involvement is dominated by IgG1 as serology conducted to date has shown poor IgG2a and IgM responses along with transient IgA in infected animals (Clery et al., 1996). Other workers have also found that IgG1 to be the main isotype produced (Movesijan and Jovanovic, 1975). Nansen (1970) and Clery et al. (1996) observed a markedly increased rate of synthesis of IgG1 compared with IgG2 during chronic fasciolosis in cattle. Furthermore IgGl appears to be the principal isotype involved in other helminth infections (Mansour et al., 1990). In accordance with any other typical Th2-mediated immune responses, studies have shown the generation of antibody responses to be reliant on IL-4 (O'Neill et al., 2000).

Immunodiagnostic detection of fasciolosis in animals using antibody detection has been developed and used for a number of years (Zimmerman et al., 1985; Swarup et al., 1987; Fagbemi and Obarisiagbon, 1990; Fagbemi, et al., 1995). This antibody detection is quite sensitive for detecting the infection in the late stage but not the early one (Santiago et al., 1986); and it has major limitation as it also detects antibodies from previous exposure as the antibodies may persist even though the parasites may have already been killed. Hence, antigen-detection assay is better as it can identify animals with prepatent or occult infection, which could not be detected by the usual parasitological test.

Serologic methods provide the advantage of detecting infection 4–5 weeks earlier than faecal examination, while fasciola eggs are detectable in faecal samples only 10–11 weeks post infection (Bürger, 1992). However, other authors (Guobadia and Fagbemi, 1997) demonstrated this is possible only after 13–14 weeks post infection when the mature parasites start to produce and release the eggs. Additionally, faecal examination has the disadvantage of having a low sensitivity of approximately 30% (Happich and Boray, 1969).

On the other hand, detection of circulating antigens or of coproantigens in faeces may provide a reliable indication of current infection with *F. hepatica* (Krailas et al., 1999, 2000; Langley and Hillyer, 1989; Zheng et al., 1990). This is unlike the situation with detection of serum antibodies against the parasite, which do not necessarily reflect current infection (Hillyer, 1999).

Presently, on the experimental infections of sheep with γ-irradiated metacercarial stage, the diagnosis of fasciolosis is performed by conventional method through the demonstration of the

fluke's eggs in the faeces. Sheep that had received unirradiated flukes (PC) and those vaccinated with low dose of the parasite (GI dosed with 30 gray and GII sheep with 60 gray) had only begun to shed eggs after 8 weeks post infection when the mature parasites start to produce and release the eggs. By this time, major damage to the host hepatic system has already occurred. The early detection of fasciola eggs by these three groups compared to the highly dosed Groups III (120) and IV (240) which only began to shed eggs after week 9 post infection were in agreement with study of Moazeni and Ahmed (2016) that demonstrated early diagnosis of the parasite through faecal examination is possible only 8 weeks after the infection unlike the results of other authors that described the detection after week 10 (Bürger, 1992) and week 13 (Guobadia and Fagbemi, 1997).

The mean EPG result of cattle (57.1 ± 34.8) was significantly ($p<0.05$) lower than that of sheep (63.4 ± 88.3). The present result on the mean fluke burden in sheep and cattle was 59.6 ± 0.6 and 77.5 ± 0.7, respectively. However, certain difficulties and inaccuracies are associated with the use of faecal egg counts. This is because egg counts are known to be influenced by many factors including varying fecundity of species of parasites, ingesta volume, age of worms and host resistance (Tarazona, 1986). Although some studies had shown that there is no precise correlation between egg output and fluke burdens (McCaughey and Hatch, 1964), the present study on experimental infections and other workers had argued that there is strong correlation (Conceição et al., 2001). That means higher EPG counts are result of higher worm number and fecundity. Bossaert et al. (2000) found no correlation between antibody titres and fluke burdens. However, other authors (Conceição et al., 2001) did register two significant different levels of IgG which had shown strong correlation. Otherwise the samples examined represent only a small

fraction of the animal's daily output, in which eggs may be aggregated, skewing their distribution. Second, animals with prepatent infections will give false negative results (De Leon et al., 1981; Flanagan et al., 2011a). Finally, liver fluke eggs retained in the gall bladder after successful anthelmintic treatment and removal of flukes from bile ducts may generate false positive results (Flanagan et al., 2011a, b; Sargison, 2012).

The test carried out by Düwel and Reisenleiter (1990) in over 800 individual faeces samples from artificially infected bulls with a varying number of metacercariae of *Fasciola hepatica*, showed that the excretion of fasciola eggs over a period of several days fluctuates considerably within one animal and within one infection group. The excretion of fasciola eggs over one day varies widely at different times in each animal and also in each infection group. The distribution of fasciola eggs in the faeces is always irregular within one day and also over several days in all animals.

A number of studies have indicated that, no correlation was established between the number of flukes from condemned and non-condemned livers and faecal fluke egg counts. This finding disagrees with that of Coyle (1958), Hammond (1970) and Duwel & Reisenleiter (1984). Coyle (1958) found that naturally infected cattle in Uganda had low egg counts and that there was no correlation between faecal egg counts and fluke burden. Boray (1969) and Duwel & Reisenleiter (1984) also reported finding neither uniformity nor consistency in low, medium and high fluke burdens after examining faecal egg counts. A positive correlation between egg counts and worm burden was reported by Bisset, Vlassoff, Douch, Jonas, West & Green (1996) in young animals especially those in their first grazing period. No correlation has been reported to exist between

the number of worms at necropsy and EPGs (Barth et al., 1981; Duwel & Reisenleiter, 1984; Ndao et al., 1995).

In contrary to the parasitological and pathological results on goats immunized with native FhGST (Buffoni et al. 2010), the results of the present study indicate that protection was developed in the sheep immunized with γ-irradiated *Fasciola hepatica* metacercarae following primary infection. Irradiated sheep invariably responded with significant reductions in faecal egg output and parasite load resulting minimal hepatic damage. According to Sexiton et al. (1990), GST of adult *F. hepatica* is a novel Ag that can significantly protect sheep against liver fluke infection. The results suggest that the immune response to GST is directed to the juvenile worm reducing the number of worms that can establish in the liver of the vaccinated animals. The findings of Buffoni et al. (2010) were in direct contradiction with that of Sexiton et al. (1990). The result indicated that there was no significant reduction of fluke burden (9.3%) or faecal egg counts; hepatic damage was also similar in both infected groups.Sheep vaccinated with several doses of FhGST in FCA showed a 57% reduction in fluke burden (Sexton et al., 1990), however it was not possible to consistently induce a protective response despite using comparable vaccine at ion protocols in secondary infection (Spithill et al., 1999). FhGST also induced significant (41–69%) levels of protection in cattle; protection was dependent on the choice of adjuvant (Morrison et al., 1996).

Compared to the protection percentage of vaccine trials using *Fasciola hepatica* procathepsin L3 protein expressed by baculovirus produced FheCL3 glycoprotein that resulted in 52% reduction of liver flukes and concentrated or more purified or N-glycosidase F treated FheCL3 protein that

resulted 33–44% reductions (Reszka et al., 2005), the results of the present findings were in fact encouraging (81.5-92.3% protection).

In contrary to some authors (Wiedosari and Copeman, 1990; Roberts et al., 1997a, b) that argued sheep do not acquire resistance against *F. hepatica* as indicated by the yields of mature parasites from primary and secondary infections, the result of the present study revealed differences in response of sheep to yield matured parasites in vaccinated and control groups from primary infection. In coincident to this, the mean number of flukes recovered from infected groups vary significantly ($p<0.05$). That means a strong dose response was evident in the number of parasites recovered in the groups dosed with incremental doses of γ-irradiation with *F. hepatica* metacercariae. Accordingly, the yield of matured parasites from Ethiopian sheep ranged from 7.7% to 22.5%. In the studies with European fleece sheep, yields of *F. hepatica* ranged from 16 to 38% after primary infection and from13 to 31% after secondary infection (Roberts et al., 1997b). A significant reduction in parasite numbers (from 17% in control animals to 3.4%) was reported in Sudanese desert sheep vaccinated with irradiated metacercariae of *F. gigantica* following a secondary challenge (Roberts et al., 1994b).

G. truncatula is the predominant intermediate host collected and identified. Compared to *G. truncatula* (55.4%, $p < 0.05$), the survival of *R. natalensis* on day 30 was significantly higher (77.1%, $p < 0.05$), while the prevalence of *F. hepatica* infection was significantly lower ($p < 0.05$) and the shell height of cercaria shedding snails significantly greater ($H = 14.3 \pm 2.1$, $p < 0.05$). The prepatent period was significantly longer ($p < 0.5$) in *R. natalensis* than the *G.*

truncatula. In contrast, slight but insignificant difference between the lengths of patent periods was noted.

Presently, the experimental infections of the *G. truncatula* snails with the miracidia of *F. hepatica* have revealed high mortality. Studies have indicated that in experimental infections the liver fluke induces higher mortality in snails originating from populations with low natural prevalences than in those originating from populations with high prevalences (Rondelaud, 1993; Rondelaud et al., 1997; Bargues et al., 1995). Although the prevalencees of *F. hepatica* in *G. truncatula* population have never been well documented from different localities in Ethiopia, the present reports from North Shoa had indicated high prevalence on naturally infected population (45%). This result was relatively equal for the prevalence recorded from Wondogenet (46%). However, it was slightly lower in other populations of snails collected from Guta river (40%) and Tikur Wuha (40%).

Only *F. hepatica* strain from the abattoir at North Shoa was used experimentally to infect the *G. truncatula* in our laboratory and resulted in high mortality. The earlier reports by other workers indicated that there would be co-adaptation (coevolution) between host and parasite in which parasites continuously evolve to evade strategies developed by the hosts against them and, in turn, hosts must adapt to the parasite changes when population of snails with high natural prevalence were used (reviewed by Hurtrez-Boussè, et al., 2001; Rondelaud, 1993; Rondelaud et al., 1997; Bargues et al., 1995). A better understanding of the relationships between the liver fluke and its definitive hosts is required to elaborate control programs. Moreover, Boray (1966) has experimentally shown that Australian strains of *F. hepatica* are more infective than European

strains for the local intermediate host (*L. tomentosa*), suggesting a local adaptation of the introduced parasite to its new host.

6. CONCLUSIONS AND RECOMMENDATIONS

- ✓ The present study (coprological and abattoir) disclosed high prevalence of the disease in both sheep and cattle with the occurrence of the intermediate snail hosts (*G. truncatula* and *R. natalensis*).
- ✓ The different in prevalence was noted between export and manucipal abattoirs.
- ✓ Agroecological diffrences, origin, body condition, sex and age appear to affect the prevalences in different settings.
- ✓ The disease incurred significant losses as a result of liver condemnation at abattoirs.
- ✓ *F. hepatica* was a dominant fasciola species identified followed by *F. gigantica*.
- ✓ This trial was the first attempt on experimental evaluation of sheep against *F. hepatica* with gamma irradiation of *F. hepatica* metacercariae in Ethiopia.
- ✓ Metacercarial production of artificially infected *Lymnae truncatula* snails varied considerably.
- ✓ A number of *Lymnaea truncatula* snails initiated cercarial shedding 25 days after they were exposed to *F. hepatica* miracidia.
- ✓ The present method used for the *L. truncatula* snail rearing, maintenance of the aquaria and diet are very suitable in the laboratory.
- ✓ In the present settings, that include faecal examinations for *F. hepatica* eggs and the EPG, recovery of flukes and measurement of the sizes, serum IgG1 level (ELISA test), cellular responses (lymphocytes, plasma cells, macrophages, eosinophils in histopathological examinations of the liver), enzyme analysis (GLDH and GGT) became useful indicators for primary vaccination trail of sheep in determining both the irradiation attenuating dose and the level of inocula of *F.hepatica* metacercarae.

- ✓ The present finding revealed that acquired immunity can be demonstrated in sheep following *Fasciola hepatica* primary infections, suggesting that development of vaccine is potentially feasible. In this result vaccination with the metacercarae of *F. hepatica* in sheep have induced an immune response, both humoral and cell mediated. The findings clearly disclosed that, sheep appear to develop immunity against *F. hepatica* infection when dosed with 120 and 240 gray of gamma irradiation while the size of the inocula is 500 metacercarae in single infection. This is evidenced by the significant reduction of worm egg outputs and the recovery of mature flukes in groups γ-irradiated with 120 and 240 gray compared to the positive control and groups kept at 30 and 60 gray of γ-irradiated metacercarae. Furthermore in the present setting, the enzyme profile, histopathology and immunological findings indicated minimum damage to the liver and high immune response (IgG1 and cellular) in groups with high doses (120 and 240 gray) γ-irradiation with the inocula size of 500 *F. hepatica* metacercara in sheep.
- ✓ At 120 and 240 doses of irradiation the damage to the liver was minimal with high levels of cellular and anti fasciola immunoglobulin G1 (IgG1) production.
- ✓ The number and size of fluke's recovered had a significant effect on the observed weight changes of the liver in different groups.
- ✓ The present study depicts vigorous cellular response against the parasite in the livers of sheep post infection. Irradiation of the metacercarae reduced hepatic damage as shown by the local inflammatory infiltration in the liver and lesions.
- ✓ The determination of GLDH activity was best done in conjunction with the determination GGT as indicators of hepatic injury. The degree of visual hepatic damage and burden of

F. hepatica were significantly positively related to levels of GGT and GLDH. The enzyme level varies with the dose of γ-irradiation and non sensitized groups of animals.

Based upon the above conlusions the follwing recommendations are theforwarded.

- ✓ The high prevalence of the disease in both sheep and cattle with the occurrence of the intermediate snail hosts with favorable climatic and agro-ecological conditions necessitates and urges the application of control measures focusing on curative and preventive measures that are integrated with management practices. Furthermore, the findings on the vector survey and the prevalence of the disease on host animals are dispensable for strategic application of control measures in study areas. Given the significant effect of the risk factors assessed on the prevalence of the disease, due attention has to be given in managing the risk of mortality and morbidity of animals associated with the difference in breed and other factors like age and body condition.
- ✓ It is clear that ruminant fasciolosis has impending effect on Ethiopian economy by incurring significant loss as a result of liver condemnation. The loss estimated by this study is only a piece of the iceberg reported from a few enterprises and municipal abattoirs. The actual losses are expected to be much higher and necessities further studies.
- ✓—Therefore the findings should be replicated with the same sheep breed and secondary infections to boost the immune response. It should be carried out with the recommended irradiation doses and number of infective metacercara. The differences in immune responses of sheep breeds in Ethiopia to *F. hepatica* infections should also be tested.

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8. ANNEXES

8.1. Data collection record sheets

8.1.1. Record sheets for cross sectional study

Region.....
Zone……..…..
Woreda……...
Kebele (PA)……..…..
Village----------
Altitude----------
Longitude----------

Owners name	Sampling site (PA)	Age	Sex	Species	Breed	Body condition	Faecal exam result	Agroecology

8.1.3. Snail record sheet

Region_____
Zone_____
Wereda_____
Kebel_____
Altitude_____

Date	Site/locality	No. of Snails collected	Species Identified	Infected lymnaea snails		Remark
				Number	%	

8.2. Post mortem examination procedure for liver fluke

For more precise assessment of the liver fluke burden of an animal the liver can be examined post-mortem for its content of immature and adult fluke.

Equipments:

- A sharp knife
- A cutting board
- A medium size tray
- A wash bottle
- Petri dishes
- A laboratory counter

Procedure:

A) Place the liver on a board and cut it into fine slices with a sharp knife.

B) After each cut is made apply pressure to the liver to squeeze out the flukes and wipe these off gently before making the next cut.

C) When the whole organ has sliced, place all of the material in a tray and cover with water.

D) Remove the pieces of liver, again squeezing each piece as it is removed from the water.

E) Pour water and flukes into a sieve and wash parasites until clean.

F) Pour into petri dishes and count the flukes present.

G) If very large numbers of immature flukes are present, count by a dilution technique.

8.3. Pathological Categorization of affected livers:

According to Ogunrinade, 1982 they were classified into 3 groups; lightly affected, moderately affected and severely affected.

- Lightly affected - if the quarter of the liver is affected or if one bile duct is prominently enlarged on the ventral surface of the liver
- Moderately affected - if half of the organ is affected or if two or three bile ducts are hyperplastic)

- Severely affected - if the entire organ is involved or if the liver is cirrhotic and triangular in outline for the right lobe is atrophied.

8.3.1. Lesions record sheet due to fasciola hepatica infection in different treatment groups

Sheep	Cellular infiltrate		Cirrhosis	Chronic tracts	SER (hypertrophy	Abscesses or granulomas
	CD3	l-IgG				
Group 1	+/-		Mild, moderate, severe, very severe	Low, moderate, abundant, highly abundant	Mild, moderate, severe, very severe	Low, moderate, abundant, highly abundant
1						
2						
3						
4						
5						
6						
Group2						
1						
2						
3						
4						
5						
6						
Group3						

8.4. Body condition score

A body condition score estimates condition of muscling and fat development. Scoring is based on feeling the level of muscling and fat deposition over and around the vertebrae in the loin region. In addition to the central spinal column, loin vertebrae have a vertical bone protrusion (spinous process) and a short horizontal protrusion on each side (transverse process). Both of these protrusions are felt and used to assess an individual body condition score.

Rank	**Condition score**	**Description**	**Grouping**
Condition Score 1	Very thin	Very thin Spine prominent and sharp	poor
Condition Score 2	Thin	Thin Spine prominent and Smooth	
Condition Score 3	Average	Average Spine smooth and rounded	Good
Condition Score 4	Fat	Fat Spine only detected as a line	
Condition Score 5	Very fat	Very fat Spine not detectable; fat dimple over spine	Very good

Adapted from Thompson and Meyer, 1986, 1994.

The systems used most widely are listed as follows based on a scale of 1 to 5. The five scores are:

Condition 1 (Emaciated) Spinous processes are sharp and prominent. Loin eye muscle is shallow with no fat cover. Transverse processes are sharp; one can pass fingers under ends. It is possible to feel between each process.

Condition 2 (Thin) Spinous processes are sharp and prominent. Loin eye muscle has little fat cover but is full. Transverse processes are smooth and slightly rounded. It is possible to pass fingers under the ends of the transverse processes with a little pressure.

Condition 3 (Average) Spinous processes are smooth and rounded and one can feel individual processes only with pressure. Transverse processes are smooth and well covered, and firm pressure is needed to feel over the ends. Loin eye muscle is full with some fat cover.

Condition 4 (Fat) Spinous processes can be detected only with pressure as a hard line. Transverse processes cannot be felt. Loin eye muscle is full with a thick fat cover.

Condition5 (Obese) Spinous processes cannot be detected. There is a depression between fat where spine would normally be felt. Transverse processes cannot be detected. Loin eye muscle is very full with a very thick fat cover.

8.5. Description of Body condition score (BCS) of African Zebu cattle

Score	Condition	Feature	Grouping
1	L-	Marked emaciation	Poor
2	L	Transverse processes projection prominently, Neutral spines apper sharply	
3	L+	Individual dorsal spines are pointed to the touch; hips, pins, tail-head and ribs are prominent. Transverse processes visible, usually individually.	
4	M-	Ribs, hips, and pins clearly visible. Muscle mass between hooks and pins slightly concave. Slightly more flesh above the transverse processes than in L+.	Good
5	M	Ribs usually visible, little fat cover, dorsal spines barely visible.	
6	M+	Animal smooth and well covered; dorsal spines cannot be seen,	

		But are easily felt.	
7	F-	Animal smooth and well covered, but fat deposits are not marked. Dorsal spines can be felt with firm pressure, but feel rounded father than sharp.	Very good
8	F	Fat cover in critical areas can be easily seen and felt; transverse Processes cannot be seen or felt.	
9	F+	Heavy deposits of fat clearly visible on tail-hooks and pins fully covered and cannot be felt even with firm pressure. head, brisket, and cod; dorsal spines, ribs,	

Adapted from Nicholson, M.J and Butterworth, M.H, 1986

8.5. DRG ELISA KIT Test procedure

The test blood sera will be diluted in the dilution buffer. The plate will be incubated and washed, then the conjugate, a peroxidase-labelled anti-ruminant IgG1 monoclonal antibody, will be added to the wells. The plate will then incubated a second time at 21°C+/- 3°C, washed again and the enzyme's substrate (hydrogen peroxide) and the chromogen tetramethylbenzidine (TMB) are added. This chromogen has the advantages of being more sensitive than the other peroxidase chromogens and not being carcinogenic. If specific *Fasciola hepatica* immunoglobulins are present in the test sera the conjugate remains bound to the microwell that contains the antigen and the enzyme catalyses the transformation of the colorless chromogen into a pigmented compound. The intensity of the resulting blue colour is proportionate to the titer of specific

antibody in the sample. The signal read off the negative control microwell is subtracted from that of the positive microwell sensitised by the antigen. The interpretation of the results is done by comparing the signals of the samples (serum) with those of the positive controls.

8.5.1. Interpreting the results

Each value recorded for the odd columns will be subtracted from the signal of the corresponding negative control well and the result will be written down. In performing this calculation, any negative values that may exist will be allowed. the same operations for the column corresponding to the positive control will be carried out. The test can be validated only if the positive serum yields a difference in optical density at 10 minutes that is greater than the value given in the QC data sheet: (validation: ...).

The signal read for each sample well will be divided by the corresponding positive control serum signal and this result will be multiplied by 100 to express it as a percentage.

$$\text{Val} = \frac{\text{Delta OD Sample} * 100}{\text{Delta OD positive}}$$

Using the first table in the quality control procedure, each serum's degree of positivity will be determined.

A reliable diagnosis can be made only if frank seroconversion can be documented using two coupled serum samples taken at 2- to 3-week intervals. The first sample must be taken during the acute phase of the infection. A frank seroconversion is considered to have occurred if the signal increases by two orders of magnitude (two plusses; for example, ++ -> ++++ or + -> +++). A sample must be considered positive if it yields a result that is greater than or equal to one plus sign (+).

8.6. Diagnostic reagent for quantitative in vitro determination of glutamate dehydrogenase (GLDH) in serum or plasma on photometric systems

Summary [1,2]

Glutamate dehydrogenase (GLDH) is a mitochondrial enzyme which is present in many tissues. The measurement of GLDH is used to evaluate the extent of parenchymal liver damage.

Method
Optimized UV test, according to recommendations of the DGKC
(German Society of Clinical Chemistry)

Principle
α-Ketoglutarate + NADH + NH4+ < GLDH > L-Glutamate + NAD+ + H2O

Reagents
Components and Concentrations
R1: Triethanolamine pH 8.0 75 mmol/L

α-Ketoglutarate Ammonium acetate	10 mmol/L 150 mmol/L
EDTA	3.75 mmol/L
ADP	1.5 mmol/L
LDH R2: NADH	≥ 2.3 kU/L 1.3 mmol

Storage Instructions and Reagent Stability

The reagents are stable up to the end of the indicated month of expiry, if stored at 2 – 8°C and contamination is avoided. Reagents must be protected from light. Do not freeze the reagents!

Reagent Preparation
The reagents are ready to use.
Materials required
NaCl solution 9 g/L
General laboratory equipment

Specimen
Serum, heparin or EDTA plasma

Stability [4]:	7 days 7 days	at at	20 – 25°C 4 – 8°C

4 weeks at –20°C
Discard contaminated specimens! Only freeze once!

Assay Procedure
Application sheets for automated systems are available on request.

Wavelength	340 nm, Hg 334 nm
Optical path	1 cm
Temperature	37°C
Measurement	Against air

Sample/Calibrator 150 µL
Reagent 1 1000 µL
Mix, incubate for approx. 3 min., then add:
Reagent 2 250 µL
Mix, read absorbance after 30 sec. and start stopwatch.
Read absorbance again after 1, 2 and 3 min.
Calculation
With factor
From absorbance readings calculate ΔA/min and multiply by the corresponding factor from the table below:
ΔA/min x factor = GLDH activity [U/L]
340 nm -1485
334 nm -1515
With calibrator

$$\text{GLDH [U/L]} = \frac{\Delta/A \text{ min Sample}}{\Delta/A} \times \text{Conc. Calibrator [U/L]}$$

Conversion factor

GLDH [U/L] x 0.0167 = GLDH [µkat/L]

Performance Characteristics Measuring range

The test has been developed to determine GLDH activities within a measuring range from 2 – 120 U/L. When values exceed this range samples should be diluted 1 + 5 with NaCl solution (9 g/L) and results multiplied by 6.

Specificity/Interferences
No interference was observed by ascorbic acid up to 30 mg/dL, bilirubin up to 60 mg/dL and hemoglobin up to 500 mg/dL. Lipemia interferes.

Sensitivity/Limit of Detection

The lower limit of detection is 2 U/L. Precision

Intra-assay precision n = 20	Mean [U/L]	SD [U/L]	CV [%]
Sample 1	5.77	0.51	8.78
Sample 2	18.3	0.39	2.11
Sample 3	32.0	0.78	2.43

Inter-assay precision n = 20	Mean [U/L]	SD [U/L]	CV [%]
Sample 1	6.18	0.43	6.98
Sample 2	16.1	0.49	3.02
Sample 3	33.2	0.80	2.40

8.7. Estimation Hay price for experimental animals

Average body weight (kg)	Total number of sheep	Weight of a bale	Total weight of sheep	Hay per sheep per day (4%of BW)	Total days	Total weight of bales
20	100	20 kg	2000	0.04	125*2=250	250*2000*.04= 20,000 kg
Total weight of bale = total days to feed * Total weight of sheep * Hay per sheep per day (4%) Number of bales= 20,000/20= 1000 bale 500 bale for the first experiment and 600 for the second experiment (½)= 300 bale						

500*50= 25,000*2=50,000

Estimation concentrates for experimental animals

Sheep should be supplemented about 250gm concentrate per day. From this 250 gm, 75% is wheat bran and 25% is Nug cake.

8.8. Certificate of ethical clearnce

9. APPENDICIES

Appendix I. Scope of future studies

- The significance presence of fasciolosis in ruminants and their vectors necessitates future study on its impact on animal production and its significance for the health of the inhabitants
- Molecular characterization of fasciola species (*F. hepatica, F. gigantica* and intermediate forms) from Ethiopia based on mitochondrial and nuclear ribosomal DNA sequences
- Assessment of the impact of fasciolosis control on food security: monitoring the health and productivity in selected parts of Ethiopia
- Predicting impacts of climate change on *Fasciola hepatica* risk in Ethiopia
- Establish the seasonal epidemiology of bovine fasciolosis in some districts of Ethiopia having different agro-climatic conditions and topography
- Determine the effects of treatment on production using most efficacious fasciolicide
- Estimate cost benefit ratio in terms of cost of treatment and loss of productivity of animals.

Appendex II Curriculum Vitae

1. Bio data

Name Abebayehu Tadesse Wazza

(DVM, MSc, PhD)

Associate Professor Phone: Office

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Faculty of Veterinary Medicine Fax: +251 462 205421
P.O.Box 05, Hawassa Ethiopia E-mail: abebayehutade20@gmail.com

Nationality: Ethiopian

Date of birth: October 29, 1974

Place of Birth: Bele, Wolyta Zone, Southern Ethiopia

Marital status: Single

2. Educational Background

2009- : PhD study in Veterinary Parasitology, Faculty of Veterinary Medicine, Addis Ababa University, Debre-Zeit Ethiopia

2004-2006: Msc in Tropical Veterinary Medicine, Faculty of Veterinary Medicine, Addis Ababa University, Debre-Zeit Ethiopia

1996-2001: DVM Degree, Faculty of Veterinary Medicine, Addis Ababa University, Debre-Zeit Ethiopia

1995-1996: Preveterinary Study at Faculty of Natural Sciences, Addis Ababa University

1991-1994: Secondary School Education at Jinka Junior Secondary School, Jinka, Ethiopia

1989-1990: Junior School at Soddo Ber Junior School (7-8 Grade), Woliata Ethiopia

1982-1989: Primary school, at Ligaba Beyene Primary School, Woliata Ethiopia

Certificates:

2008: Certificate on control of Parasites of goats

2007: Certificate on computer skills

1994: Ethiopian School Leaving Certificate

3. Dissertations:

Doctor of Philosophy Degree (PhD) Thesis:

- Epidemiology of ruminant Fasciolosis and Its Vectors in Selected Endemic areas of Ethiopia and Evaluation of the Immune Response of sheep against primary infection with *Fasciola hepatica* metacercarae

Masters Degree (Msc) Thesis:

- Concurrent Trypanosoma congolense and Haemonchus contortus experimental infections in Goats: interactions and Pathogenic effects

DVM Thesis:

- Cutaneaus Lesions of camels (Camalus Dromedari) Associated with Bacterial Pathogens in North Eastern Ethiopia.

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4. Academic, research and professional experience

Teaching Experience

2006 till now: Veterinary Parasitology, Veterinary Physiology, Fisheries and Fish diseases, Veterinary Toxicology, Veterinary Clinical Pathology, Veterinary Biochemistry, Introduction to Parasitology, Clinical experience (practical) courses at Faculty of Veterinary Medicine, Hawassa University

Research Experience:

2018- : Study on trematodes (Fasciola and Paramphistomum) on selected areas of Southern Ethiopia

2011-2017: Epidemiology of ruminant Fasciolosis and Its Vectors in Selected Endemic areas of Ethiopia and Evaluation of the Immune Response of sheep against primary infection with Fasciola hepatica Metacercarae

2007: Study on anthelmintics resistance nematodes at goat farm of Hawassa University

2008: Study on Internal Parasites of Chicken on Different Agroecologoical Zones of Southern Ethiopia

2008: Study on internal parasites of small ruminants in southern Ethiopia

Professional services

- 2006 till now: Consultancy service on health care and management of livestock owning communities in and around Hawassa
- 2009-2015: DVM and Masters student advising, internal examiner of Msc thesis, College of Veterinary Medicine and Agriculture, Addis Ababa University
- 2001-2004: Field Veterinarian and head of district veterinary service and agricultural office, southern Ethiopia, ministry of agriculture, Ethiopia
- 2000-2001: Externship Fellow, South Wello and Afar regional state, Ethiopia. Provision of routine clinical and diagnostic service

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5. Research Publications in Peer Reviwed Scientific Journals

- ✓ Abattoir and Coprological Prevalence of Fasciolosis and its Vectors: Infection Intensity and Species Diversity. Global Veterinaria 21 (2): 65-76, 2019
- ✓ Economic Loss of Ruminant Fasciolosis Due to Liver Condemnation in Two Municipal and Three Export Abattoirs of Ethiopia, European Journal of Biological Sciences 11 (3): 70-81, 2019
- ✓ Bovine trypanosomosis and its vectors in two districts of bench Maji zone, South Western Ethiopia, Trop Anim Health Prod 42:1757-1762, 2010
- ✓ Study on ectoparasitic defects of processed skins at Sheba tannery, Tigray, Northern Ethiopia, Trop Anim Health Prod 42:1719-1722, 2010
- ✓ Study on anthelmintics resistance nematodes at goat farm of Hawassa College of Agriculture. Ethiop. Vet. J., 2009, 13(2): 50-57.
- ✓ Prevalence of Trepanosomsosis in small ruminants of Guto Gidda district, East Wellega Zone, Western Ethiopia. Ethiop. Vet. J., 2010.,14(2): 67-77

- ✓ Study on the prevalence of ectoparasite infestation of ruminants in and around Kombolcha and damage to fresh goat pelts and wet blue (pickled) skin at Kombolcha Tannery, North-eastern Ethiopia. Ethiopian Veterinary Journal 15(2), 87-101.
- ✓ Comparative pathological study of liver infection in ruminants. Indian J. Vet. Pathol., 32:113-120
- ✓ Mechanically transmitted bovine trypanosomosis in Tselemty Woreda, Western Tigray, Northern Ethiopia, Agricultural Journal 6(1): 10-13.
- ✓ A cross-sectional study of equine trypanosomosis and its vectors in Wolayta Zone, Southern Ethiopia, Journal of Animal and Veterinary Advances 9 (15): 2061-2066.
- ✓ Prevalence of haemoparasites and associated risk factors in working donkeys in Adigudem and Kwiha districts of Tigray region, Northern Ethiopia. Journal of Animal and Veterinary Advances 9(17):2249-2255.
- ✓ Cutaneous Lesions of Camels (Camelus dromedary) Associated with Bacterial Pathogens in North Eastern Ethiopia. Journal of Camel Practice, 1(9): 34-41

6. Speaking engagements

- November 2012: PhD Proposal on "Epidemiology of ruminant Fasciolosis and Its Vectors in Selected Endemic areas of Ethiopia and Evaluation of the Immune Response of sheep against primary infection with *Fasciola hepatica* Metacercarae. Faculty of Veterinary Medicine, Debre-Zeit Ethiopia
- April 2010: PhD Proposal on "Population Genetics and Characterization of *Trypanosoma congolense* strains in selected endemic areas of Ethiopia" Faculty of Veterinary Medicine, Debre-Zeit Ethiopia
- August 2009: PhD Seminar paper on "Molecular tools for diagnosis of Trypanosome infection in animals" Faculty of Veterinary Medicine, Debre-Zeit Ethiopia
- April 2006: Msc thesis on "Concurrent Trypanosoma congolense and Haemonchus contortus experimental infections in Goats: interactions and Pathogenic effects" Faculty of Veterinary Medicine, Debre-Zeit Ethiopia

- February 2005: Masters seminar paper on "Review on concurrent trypanosome and helminth infection on domestic animals" Faculty of Veterinary Medicine, Debre-Zeit Ethiopia
- Aprill 2000: Senior students seminar paper on "Synthesis study on small ruminant Parasitism in Ethiopia" Faculty of Veterinary Medicine, Debre-Zeit Ethiopia

7. Mentoring

- Advisor-ship in the course senior students scientific paper writing and presentation
- Supervision of final year DVM students in research undertaking and thesis write up

8. Awards and research grants achieved

- Research and publication fund, Hawassa University, Ethiopia

9. Academic positions and appointments achived

Academic promotions Achieved:

2019: Associate Professor, department of parasitology and pathology, Faculty of Veterinary Medicine, Hawassa University, Ethiopia

2006: Assistant Professor, department of parasitology and pathology, Faculty of Veterinary Medicine, Hawassa University, Ethiopia

Appointments:

2008-2009: Chairman of the department of Parasitology and pathology

10. Professional membership and extracurricular activity

- Member of Ethiopian Veterinary Association
- Participated at various positions in several standing and ad hoc committees in the Faculty of Veterinary Medicine

11. References

Professor Hagos Ashenafi,

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