UNIVERSITY OF KWAZULU-NATAL

Ecology, identification and genetic diversity of intermediate hosts of *Fasciola* species in South Africa.

By

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As the candidate's supervisor I have approved this thesis for submission.

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Abstract

Lymnaeid snails are known to transmit *Fasciola* spp. Moreover, the five lymnaeid snail species known to transmit *Fasciola* spp. have been reported in South Africa. However, there is paucity of information on the epidemiological role, ecology and distribution of these snails in South Africa. This study systematically assessed the prevalence of *Fasciola* spp. infections in lymnaeids and the geographical distribution of *P. columella* and its implications in the transmission of fasciolosis. Furthermore, the study aimed to determine the distribution, ecology, and identity of lymnaeids in South Africa and their potential species of infection. The susceptibility of lymnaeid snails to *Fasciola* spp. results showed that the infection rate was higher in experimental infections 50% (95% CI: 42-58%) compared to natural infections of field-collected snails 6% (95% CI: 0-22%). A systematic review showed that the wide geographical distribution of *P. columella* was related to its ability to adapt to and inhabit a vast array of freshwater bodies including thermal lakes and ditches with acidic soils. Freshwater snail survey was conducted in selected localities from six provinces of South Africa. One thousand and fifty-nine lymnaeid snails were collected, and only *Pseudosuccinea columella* and *Radix natalensis* were identified based on the 16S and COI genes, and the former species was shown to have a wider geographical distribution than *R. natalensis* which was restricted to Limpopo and Mpumalanga provinces. Furthermore, molecular analysis showed that *P. columella* populations lacked genetic variability, whilst *R. natalensis* formed two distinct sub-clades, showing genetic variations between Mpumalanga and Limpopo provinces. This was further supported by a moderate haplotype diversity of (16S: 0.618 and COI: 0.467) and a low nucleotide diversity (16S: 0.00685 and COI: 0.014) were recorded and a positive but non-significant Tajima's D (16S: 0.64672 and 1.4948; $p > 0.10$), which indicated allele deficiency, which may be due to recent population bottleneck. *Radix natalensis* was more abundant (n = 649) and cohabitated with *P. columella* in 2/3 (66.7 %) of the habitats and both species showed preference to perennial habitats. The 28S gene detected *Orientocreadium* spp. and *Trichobilharzia ocellata* DNA in *R. natalensis* and *P. columella*. No *Fasciola* DNA was detected in these Lymnaeidae snails. Results highlighted and emphasised the need for designing species specific *Fasciola* spp. primers that will detect and differentiate between species especially in the IHs. This will be crucial for detection of natural infections of *Fasciola* spp. and identifying habitats with circulating infections in surveys to better understand the geographical extension of fasciolosis and areas with potential high risk of infections to both animals and human.

Keywords: *Pseudosuccinea columella*, *Radix natalensis*, *Fasciola gigantica*, *Fasciola hepatica*, intermediate hosts, ecology, abundance, identification, genetic diversity, South Africa.

ABBREVIATIONS

IHS - Intermediate hosts

Spp. – Species

NCBI – National Center for Biotechnology Information

BLAST – Basic Local Alignment Search Tool

Anova – Analysis of variance

PCR – Polymerase chain reaction

DNA – Deoxynucleic acid

rDNA – ribosomal DNA

rRNA – ribosomal ribonucleic acid

COI – Mitochondrial Cytochrome c oxidase subunit I

Bp – base pairs

Hap – Haplotype

DECLARATION – PLAGIARISM

I, **Philile Ignecious Ngcamphalala** declare that:

1. The research reported in this thesis is my original research, except where otherwise indicated.
2. This thesis has not been submitted for any degree or examination at any other university.
3. This thesis does not contain other persons' data, pictures, graphs, or other information, unless specifically acknowledged as being sourced from other persons.
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DECLARATION 2: PUBLICATIONS

Details of publications:

1. **Ngcamphalala Phililе Ignecious**, Malatji Mokgadi Pulane, Mukaratirwa Samson. 2022. Geography and ecology of invasive *Pseudosuccinea columella* (Gastropoda: Lymnaeidae) and implications in the transmission of *Fasciola* species (Digenea: Fasciolidae) – a review. *Journal of Helminthology* **96**: 1-18. https://doi.org/10.1017/S0022149X21000717.
2. **Ngcamphalala Phililе Ignecious**, Nyagura Ignore, Malatji Mokgadi Pulane, Mukaratirwa Samson. Susceptibility of lymnaeid snails to *Fasciola hepatica* and *Fasciola gigantica* (Digenea: Fasciolidae): A systematic review and meta-analysis. *PeerJ* **13:** e18976. https://doi.org/10.7717/peerj.18976.
3. **Ngcamphalala Phililе Ignecious**, Malatji Mokgadi Pulane, Nyagura Ignore, Nukeri Sophy, Sithole Msawenkosi, Ndlovu Innocent Siyanda, Tembe Danisile, Chaisi Mamohale, Mukaratirwa Samson. Geographical distribution, ecology and molecular identification of intermediate hosts of *Fasciola* species from selected localities in South Africa – Submitted, American Journal of Parasitology
4. **Ngcamphalala Phililе Ignecious**, Malatji Mokgadi Pulane, Nukeri Sophy, Chaisi Mamohale, Mukaratirwa Samson. Low genetic diversity of *Radix natalensis* and *Pseudosuccinea columella* from selected habitats from five provinces of South Africa – Submitted, PeerJ - Diversity & Conservation

From all the above publications, my role included collection of snails, conducting laboratory experimental work, data analysis and writing of draft manuscripts for publications along with my supervisors. Where other authors carried out aspects of the experimental, I either interpreted or consulted with them to understand the data. Co-authors contribution was also that of an editorial nature, checking on the scientific content in their field, and my correct interpretation of the data in their field. Based on their expertise, they may have added minor parts to the manuscripts.

Signed: …

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Table of contents

List of tables

List of figures

Chapter 1

General introduction

1.1. Introduction

Fasciola hepatica Linnaeus (1758) and *Fasciola gigantica* Cobbold (1856) are the two main species responsible for fascioliasis in livestock and humans (Robinson and Dalton, 2009; Devkota et al., 2011; Bista et al., 2018; Malatji and Mukaratirwa, 2019) in tropical and temperate regions of the world (Robinson and Dalton, 2009). These fasciolids affects a wide variety of mammalian hosts globally, predominately utilise domestic and wild ruminants as their definitive hosts (Robinson and Dalton, 2009; Mucheka et al., 2015; Bista et al., 2018; Malatji et al., 2019a) and occasionally humans as accidental hosts (Magalhaes et al., 2004; Admassu et al., 2015).

According to Admassu et al. (2015), liver fluke infections are highly prevalent and widely distributed in areas where there is a niche for lymnaeid snails and domesticated and wild ruminants. Freshwater snails from the family Lymnaeidae act as their intermediate hosts and facilitate the spread and possible introduction of *Fasciola* species to new areas (Mas-Coma et al., 2005; Admassu et al., 2015). *Fasciola hepatica* has been reported to utilise *Galba/Fossaria* species including *Galba truncatula* (Müller, 1774) and *Pseudosuccinea columella* (Say, 1817) as its IH (Mas-Coma et al., 2005; Malatji et al., 2019a) with *G. truncatula* reported as the main IH in some parts of Africa (Mucheka et al., 2015). *Fasciola gigantica* uses *Radix* species, *Radix auricularia* (Linnaeus, 1758) and *Radix natalensis* Krauss (1848) as IHs in Africa and *R. natalensis* is the main IH for *F. gigantica* in South Africa (Brown, 1994; Malatji and Mukaratirwa, 2019).

Both *F. gigantica* and *F. hepatica* have been reported to occur in South Africa (Mucheka et al., 2015; Chikowore et al., 2019; Malatji et al., 2019a) and the distribution pattern of these species seems to follow that of their presumed intermediate snail host (Mucheka et al., 2015; Malatji and Mukaratirwa, 2019). While several studies have recorded the presence of five lymnaeid snail species in South Africa, and these are *R. natalensis, R. auricularia, R. rubiginosa, G. truncatula* and *P. columella* (Appleton and Miranda, 2015; Malatji et al., 2019a; Malatji et al., 2019b), only *R. natalensis* and *G. truncatula* are considered predominant IHs of *F. gigantica* and *F. hepatica* in South Africa, respectively (Brown, 1994; Mucheka et al., 2015). However, the invasive snail *Pseudosuccinea columella* has been assumed to act as an IH of both *Fasciola* species, assumption led by the observed increment in the prevalence of both *Fasciola* species coinciding with its introduction in South Africa (De Kock et al., 1989). It was until recently that this species has been reported to be naturally infected with *F. gigantica* in KwaZulu-Natal and Eastern Cape provinces (Malatji and Mukaratirwa, 2019), suspected hybrid (*Fasciola* spp.) in KwaZulu-Natal province (Malatji and Mukaratirwa, 2019), and *F. hepatica* in Gauteng (Molaba et al.,

2023). Thus, this study aimed to survey and determine the geographical distribution and ecology of lymnaeid snails and to determine the identity and genetic diversity of lymnaeids and the potential species of infection in selected provinces of South Africa.

1.2. Problem statement

According to Beesley et al. (2017), better knowledge of the snail species acting as the IH, snail habitats and the prevalence of *F. hepatica* inside the snail host are the requirements needed to fully understand the epidemiology of *Fasciola* species. The distribution of freshwater molluscs is largely influenced by the availability of suitable water habitats (deKock et al., 2002). The habitat the snails inhabit may be permanent or temporary (Admassu et al., 2015), thus availability and abundance of freshwater snails depends on the prevailing climatic conditions (Kleiman et al., 2007; Admassu et al., 2015). According to Pfukenyi et al. (2005), variations in local rainfall, water temperature, type of vegetation available, seasonal water flow and the absence or presence of other mollusc species are the primary determinants of the differences observed in the abundance of freshwater snails between habitats.

The geographical distribution of lymnaeid species serve as biomarkers for the occurrence and distribution of *F. hepatica* and *F. gigantica* (Dar et al., 2016) and can thus be used to validate the mathematical modelling based on remote sensing and geographical information system (RS-GIS) tools for the control of fascioliasis (Fuentes et al., 1999, 2001). Therefore, there is a need for a reliable identification system and phylogenetic framework for Lymnaeidae snails to assist in defining areas of epidemiological risk (Correa et al., 2010; Standley et al., 2013) especially in southern Africa where fascioliasis is highly endemic (Malatji et al., 2019a). The present knowledge on the taxonomy of the Lymnaeidae and *Lymnaea-Fasciola* interrelationship is still scanty despite the interest in this family (Mas-Coma et al., 2005). Furthermore, the taxonomy of this family is still controversial (Standley et al., 2013), with interspecific uniformity in anatomical characters and great diversity in shell morphology, resulting in difficulties in specimen classification especially at a species level (Bargues et al., 2003, 2011; Standley et al., 2013). Moreover, the shell characters between and within species are extremely plastic and do not always accurately reflect the evolutionary relationship of the family as they appear to be linked to environmental conditions or other external forces (Mas-Coma et al., 2005; Bargues et al., 2011).

Despite the number of surveys previously conducted on freshwater snails, there is a paucity of genetic information on molecular phylogenies of both freshwater snails and their trematodes, subsequently resulting in limited knowledge required for critical examination of the extent of shared evolutionary history between freshwater snails and trematodes (Lockyer et al., 2004). Additionally, there is a need for the collection of molecular data on African snails to identify species diversity, delineation and

distribution across the different geographic regions and continents (Molaba et al., 2023). This will improve the availability of comparable sequences in GenBank database as some South African snail samples had previously shown a low percentage identity when compared to sequences available in the database. This will be achieved using DNA based PCR targeting the 16S ribosomal DNA and mitochondrial COI genes followed by sequencing and phylogenetic techniques.

1.3. Aims and objectives

The aims of this study were to:

a. To systematically assess the geographical distribution of *P. columella* and its implications in the transmission of *F. hepatica* and *F. gigantica*, and the susceptibility and prevalence of natural infections of *F. hepatica* and *F. gigantica* in lymnaeid snails.

b. To determine biogeography and genetic diversity of lymnaeid freshwater molluscs and the potential species of infection in the five provinces of South Africa.

The objectives of this study were to:

a. To identify and determine the genetic diversity of lymnaeid snails from five provinces of South Africa.

b. To determine the geographical distribution, ecology and infection status of *P. columella* and *R. natalensis* from selected habitats of six provinces of South Africa.

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Chapter 2

Geography and ecology of invasive *Pseudosuccinea columella* (Gastropoda: Lymnaeidae) and implications in the transmission of *Fasciola* species (Digenea: Fasciolidae) – a review

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2.1. Abstract

Pseudosuccinea columella is considered invasive and has become an important intermediate host of both *Fasciola* species in many regions of the world. This systematic review assessed the geographical distribution of *P. columella*, and its implications in the transmission of *Fasciola hepatica* and *Fasciola gigantica*, globally. A literature search was conducted on Google Scholar, JSTOR and PubMed databases using Boolean operators in combination with predetermined search terms for thematic analysis. Results show that *P. columella* has been documented in 22 countries from Europe (3), Africa (8), Oceania (2), North America (3) and South America (6). Furthermore, this snail species has shown to adapt to and inhabit a vast array of freshwater bodies including thermal lakes and ditches with acidic soils. Studies showed that *P. columella* transmits *F. hepatica*, with natural and experimental infections documented in sub-Saharan Africa, Europe, South America and North America. Experimental infection studies in Cuba showed the presence of *P. columella* populations resistant to *F. hepatica* infection. Furthermore, some populations of this invasive snail collected from *F. hepatica* endemic locations in Brazil, Venezuela, Australia, South Africa, Colombia and Argentina were found without *Fasciola* infection. As a result, the role played by this snail in the transmission of *Fasciola* spp. in these endemic areas is still uncertain. Therefore, further studies to detect natural infections are needed in regions/countries where the snail is deemed invasive to better understand the veterinary and public health importance of this snail species in *Fasciola* endemic areas and determine the global dispersion of resistant populations of *P. columella*.

Keywords: *Pseudosuccinea columella*, *Fasciola hepatica*, *Fasciola gigantica*, distribution, susceptibility, natural infections, experimental infection, invasive.

2.2. Introduction

Fasciolosis is a parasitic zoonotic disease caused by digenetic liver flukes *Fasciola hepatica* (Linnaeus, 1758) and *Fasciola gigantica* (Cobbold, 1856) (Valero et al., 2001; Mas-Coma, 2004; Bargues et al., 2007; Dung et al., 2013; Sabourin et al., 2018; Alemu, 2019). The disease affects a wide range of domesticated and wild ruminants (Correa et al., 2010; Beesley et al., 2017; Sabourin et al., 2018; Alemu, 2019) and occasionally humans as accidental hosts (Magalhães et al., 2004; Correa et al., 2010; Beesley et al., 2017; Alemu, 2019). This parasitic infection has been well recognized and documented

for its veterinary importance throughout the world (Mas-Coma, 2004; Bargues et al., 2007). The occurrences of human infections have been reported to be on the rise recently, documented in five continents except Antarctica (Mas-Coma, 2004; Alemu, 2019).

Previous research indicated that the epidemiology of fasciolosis is highly linked to the ecological characteristics of the snail vector involved in the transmission (Mas-Coma, 2004; Bargues et al., 2011), and the susceptibility of these snail intermediate hosts (IHs) to these *Fasciola* species may differ (Alemu, 2009) depending on variations in the immunological responses of the IHs (Beesley et al., 2017). Due to the wide range and distribution of IHs, *F. hepatica* has been documented as the most common and widely distributed liver fluke, particularly in temperate zones of Australia, Europe and the Americas (Dung et al., 2013; Admassu et al., 2015; Sabourin et al., 2018; Alemu, 2019). The transmission of this *Fasciola* species is globally linked to Lymnaeidae species, including *Lymnaea tomentosa* (Pfeiffer, 1855), *Lymnaea bulimoides* (Pilsbry and Ferriss, 1906), *Lymnaea viator* (d'Orbigny, 1835), *Pseudosuccinea columella* (Say, 1817), *Lymnaea humilis* (Say, 1822), *Lymnaea diaphena* (Evans & Shumard, 1856) (Vorster and Mapham, 2008; Alemu, 2019; Leka, 2019), *Lymnaea cubensis* (Pfeiffer, 1839) (Bargues et al., 2007; Pointier et al., 2009) and *Lymnaea neotropica* (Bargues, Artigas, Mera y Sierra, Pointier and Mas-Coma, 2007) (Bargues et al., 2007; Sanabria et al., 2012; Bargues et al., 2017). However, the main snail IH of *F. hepatica* in most regions of the world, particularly in Africa, South America, Europe and in some parts of Asia (Alemu, 2019), is *Galba truncatula* (Müller, 1774) (Caron et al., 2008; Alemu, 2019).

Fasciola gigantica is limited to the tropical and/or subtropical regions of Africa, Asia and the Far East (Correa et al., 2010; Mochankana and Robertson., 2018; Alemu, 2019). According to Mas-Coma (2005), limitations in the geographical distribution of this species is due to the slow spread of species from the genus *Radix* that have been implicated in the transmission of *F. gigantica*. These are species belonging to the *Radix auricularia* (Linnaeus, 1758) superspecies complex by Hubendick (1951), which comprises *Radix rubiginosa* (Minchelin, 1831) in Asia and *Radix natalensis* (Krauss, 1848) in Africa (Brown, 1994). Alemu (2019) named five Lymnaea (*Radix*) species involved in the transmission of *F. gigantica*, however, the author also reported *R. auricularia* and *R. natalensis* as the most important IHs of this *Fasciola* species.

The occurrence of overlapping distribution of both *Fasciola* spp. has been reported in some tropical regions of Asian and African countries (Mas-Coma et al., 2005; Dung et al., 2013; Malatji and Mukaratirwa, 2019) where co-infections in the definitive hosts have been documented (Chen et al., 2013; Sabourin et al., 2018). According to Mas-Coma et al. (2009), these overlaps occur in areas with climatic conditions that favour the existence of the intermediate hosts of both *F. hepatica* and *F. gigantica*. The overlap may also be caused by the presence of *P. columella*, the invasive snail originating

from Central America, the Caribbean and the southern parts of North America (Mas-Coma et al., 2005). This species succeeded in being one of the most widely distributed freshwater snails in some countries (Prepelitchi et al., 2011), where it plays an important role in the transmission of fasciolosis (Zarco et al., 2011). According to Mas-Coma (2005), this invasive snail contributes to the secondary transmission of *F. hepatica* and has been shown to transmit *F. gigantica* in South Africa (Malatji and Mukaratirwa, 2019) and Egypt (Grabner et al., 2014). In countries such as South Africa, *P. columella* is presumed to transmit both *Fasciola* species, due to the observed increased prevalence in infection rate of both trematodes, which coincided with the introduction of *P. columella* in the country (Malatji and Mukaratirwa, 2019). Therefore, this article reviewed the geographical distribution of *P. columella* and its implications in the transmission of *F. hepatica* and *F. gigantica* worldwide. The knowledge on the global distribution and role played by *P. columella* in the epidemiology and transmission of *Fasciola* spp. is crucial in predicting the potential veterinary and public health risk and burden of fasciolosis.

2.3. Methodology

2.3.1. Searching strategy

A systematic search of literature was conducted on the electronic databases Google Scholar, JSTOR and PubMed. A literature search was limited to peer-reviewed articles, written in English, and conducted and published between 1990 and 2020 (30 years). The search was performed using the following search terms and Boolean operators (OR, AND): *Pseudosuccinea columella* AND *Fasciol*a spp., *Fasciola hepatica* OR *Fasciola gigantica* AND *P. columella*, *P. columella* AND *Fasciola* infection. Additional articles were identified by screening through the reference lists of selected articles (snowballing). Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines were followed during the conduction and reporting of the systematic review.

2.3.2. Inclusion and exclusion criteria

Articles were included if they were published in peer-reviewed journals and explicitly reported on (1) the distribution and ecological preferences of *P. columella*, and (2) infections (natural and/or experimental) of *P. columella* with either *F. hepatica* or *F. gigantica* or both, globally.

The review excluded studies reporting on infections of *Fasciola* spp. in gastropods other than *P. columella*, those focusing on the distribution of *Fasciola* spp. with no link to *P. columella* and studies that did not identify *P. columella* up to species level.

2.4. Results

A literature search from the three databases yielded a total of 827 studies (Figure 2.1). An additional 26 articles were obtained through bibliographic searches from relevant articles. Thirty articles were

removed because they were duplicated. A total of 719 were excluded after screening their titles and abstracts. The full texts of 89 articles were downloaded and screened for eligibility, and 33 studies were deemed ineligible since they did not explicitly report on the distribution of *P. columella* and the role it plays in the transmission of *F. hepatica* and *F. gigantica* globally, but reported on *Fasciola* species infections exclusively on definitive hosts and other Lymnaeidae snail species. A total of 56 studied were retained and used in this systematic review.

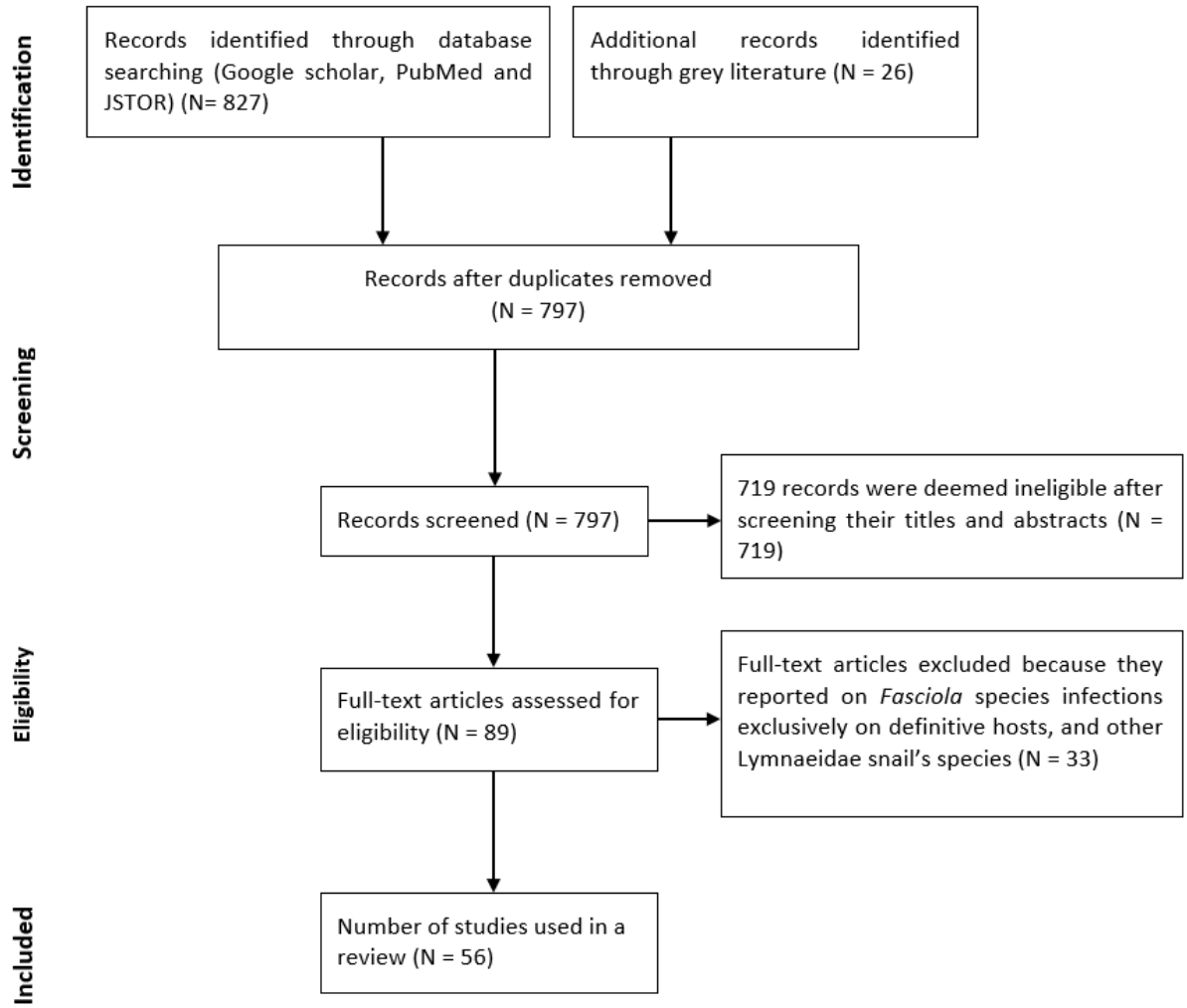

Figure 2.1 Prisma diagram.

The distribution of studies which fulfilled the inclusion criteria on a geographical scale and scope varied across continents. Of the 56 articles reviewed, 19 were from the African continent (Table 2.1), 17 from South America (Table 2.2), 11 from North America and the Caribbeans (Table 2.3), eight from Europe (Table 2.4) and Oceania had one article documented in Australia by Molloy and Anderson (2006). Thirty-nine of these articles were field studies and 17 were experimental studies. Africa had the highest

number of field-based studies, followed by South America, with 16 and 14 articles, respectively. Twenty-three studies assessed *Fasciola* infections in *P. columella*, while 33 articles reported solely on the distribution of *P. columella*.

Table 2.1 Summary of studies included in the distribution of *Pseudosuccinea columella* and its role in the transmission of *Fasciola* spp. in Africa for a period of 30 years (1990 – 2020).

Country	Author	Objective(s)	Type of study	Sample size (N)	Diagnostic method	Outcome(s)
South Africa	Malatji and Mukaratirwa, 2019	To confirm whether *P. columella* was transmitting *F. gigantica* and/or *F. hepatica* in selected locations of KwaZulu-Natal and Eastern Cape provinces of South Africa.	Field	100	Molecular	- *P. columella* from Eastern Cape and KwaZulu-Natal provinces were naturally infected with *F. gigantica.* - No *F. hepatica* natural infections were recorded.
	Malatji et al., 2019	To identify populations of Lymnaeidae snails collected from selected areas of Botswana and South Africa.	Field	100	Molecular	- *P. columella* was reported in both Mpumalanga and KwaZulu-Natal provinces of South Africa.
	Wolmarans and de Kock, 2006	To determine the current status of the mollusc species diversity and to compare findings with a survey conducted in 2001 in Kruger National Park (KNP), South Africa.	Field	-	Morphology	- *P. columella* was found in 3 of the sampled 42 habitats.
	de Kock and Wolmarans, 2008	To evaluate the progress of four alien species colonising water bodies in the KNP-South Africa.	Field	-	-	- 10 new localities in the KNP were added to the distribution of *P. columella* in the KNP.
	de Kock and Wolmarans, 1998	To evaluate the effects of droughts which struck between 1966 and 1995 on the population of freshwater molluscs in the KNP.	Field	-	Morphology	- The droughts led to some molluscs species disappearance from the KNP. - *P. columella* screened for *Fasciola* spp. was negative for any infections.
	de Kock et al., 2002	To establish the effects of high rainfall experienced between 1995 and 2000 on the population of freshwater molluscs in the KNP.	Field	-	Morphology	- Of the 43 sampled localities, *P. columella* was found in 5 of them.
	Perissinotto et al., 2014	To provide a comprehensive list of the diversity of gastropod molluscs in the St Lucia estuarine lake.	Field	-	Morphology	- A total of 37 gastropod species have been reported from Lake St Lucia, iSimangaliso Wetland Park.

						- *P. columella* was reported to colonize areas that have low salinity.
	Kemp et al., 2016	To compare mollusc diversity between the Marico and crocodile rivers in South Africa.	Field	5	Morphology	- There was a high mollusc diversity in the Marico River with juvenile specimens recorded throughout the study period when compared to the diversity from the Crocodile River. - *P. columella* was found in low abundance in both rivers.
Cameroon	Tchakonté et al., 2014	To investigate and study the diversity, ecology and dynamic of freshwater snails related to environmental factors in Cameroon.	Field	13	Morphology	- Ten species of freshwater snails were identified and recorded at nine of the ten urban stations. - *P. columella* was among the very rare species recorded with an occurrence frequency of less than 25%.
Zimbabwe	Carolus et al., 2019	To illustrate how the construction of an artificial lake may lead to a cascade of biological invasions leading to parasite spillback.	Field	-	Molecular	- Lake Kariba only had *P. columella* and *Radix* sp. Snails. - There was a high recorded prevalence of unidentified *Fasciola* sp. (that were neither *F. gigantica* nor *F. hepatica*) in *P. columella.*
Namibia	Curtis, 1991	To discuss the identification and conservation of Namibian freshwater invertebrate phyla.	Field	-	-	-In Namibia, *P. columella* occurs in scattered localities and in low numbers.
Egypt	Dar et al., 2015b	To determine the capacity of three Egyptian *P. columella* populations to support larval development of *Fasciola hepatica* collected from cattle and sheep.	Experimental	330	Molecular	- The prevalence of *F. hepatica* infection in the three populations when infected with 5 miracidia of cattle origin: Beni-Suef 60.4%, Behaira 79.2% and Qalyubiyah 58.9%. - Prevalence of infection of snails infected with 2 miracidia of sheep 30.4% and cattle 42.2% origin and 5 miracidia of sheep 75.5% in Beni-Suef.

Dar et al., 2014	To determine the developmental pattern (normal or abnormal) of *F. hepatica* redial generations and specify the number of free rediae developing in snails according to their generation.	Experimental	400	-	- Most infected *P. columella* showed a normal development of redial generations. - The number of *F. hepatica* rediae present in *P. columella* was related to the number of fully grown sporocysts and the quantity of R1a rediae which developed into the snail body.
Grabner et al., 2014	Snails collected in irrigation canals were investigated for trematode occurrence with focus *P. columella* and the role it plays in the transmission of *F. gigantica.*	Field	296	Molecular	- *P. columella* was the most abundant snail in total numbers and the most dominant species in 10 sites and the second most dominant species in one site (out of the 21 sampled sites). - 38 of the 296 *P. columella* specimens collected in total at all sampling sites were positive for trematode infection and *F. gigantica* was detected in 10 *P. columella* of those.
Dar et al., 2015a	To determine the developmental pattern of redial generations and count free and live rediae according to their generation on juvenile *P. columella*, measuring 1 or 2 mm in height, subjected to 1 miracidium. *Galba truncantula* was used as the control.	Experimental	600	-	- Most infected snails showed a normal development of redial generations, whatever the lymnaeid species. - Total number of live rediae on day 49 was 24.6 in 1 mm and 34.6 in 2 mm group per infected *P. columella* snail.
Dar et al., 2016	To identify *P. columella* and *Radix natalensis* in Egypt using shell measurements and molecular data.	Field	-	Morphology and molecular	- Morphometric parameters overlapped indicating that they could not be used to differentiate between *P. columella* and *R. natalensis.* - There was an indication for the homogeneity of lymnaeid populations in Egypt since there was little intrasequence variations detected in the sequences of both gene loci.

Lofty and Lofty, 2015	To present an update on the list of Egyptian freshwater fauna.	Field	-	-	- Report on 28 freshwater snails inhabiting various habitats in Egypt. - In Egypt*, P. columella* has been found to be naturally infected with *F. gigantica*.
El-Kady et al., 2000	To survey and study seasonal dynamics of freshwater snails occurring in two irrigation and three drainage systems in Egypt.	Field	24	Morphology	- Twelve species of freshwater snails belonging to 9 families and two subclasses of class gastropoda were recorded. - Of all collected snails, *P. columella* and *G. truncatula* had the lowest abundance (0.1%).
Abd El-Wakeil et al., 2013	To survey benthic mollusc communities in River Nile and their branches in Assiut governorate, Egypt.	Field	46	Morphology	- There were 26 recorded species belonging to fifteen families. - P. *columella* constituted 29% of the sampled molluscs.

Table 2.2 Summary of studies included in the distribution of *Pseudosuccinea columella* and its role in the transmission of *Fasciola* spp. in South America for a period of 30 years (1990 – 2020).

Country	Author	Objective(s)	Type of study	Sample size (N)	Diagnostic method	Outcome(s)
Brazil	Coelho et al., 2008	To determine the influence of shell size on the infection rate and on the outcome of rediae and cercariae in *P. columella* infected with *F. hepatica.*	Experimental	600	-	- *P. columella* with larger shell sizes showed lower infection rates. - The smallest size class had both the highest number of infected snails and the highest number of immature stages per snail.
	Mendes et al., 2008	To investigate the influence of the definitive host on the development of *F. hepatica* in *P. columella* by infecting snails with miracidia derived from cattle or marmoset origin.	Experimental	600	-	- Survival rate of snails infected by cattle-derived miracidia was lower (41.0%) than that of snails infected by marmoset-derived miracidia (56.0%). - The percentage of positive snails from the marmoset group infections (28.0%) was higher than in those infected by the cattle group (25.3%).
	Cardoso et al., 2006	To investigate genetic variability among and within 9 Brazilian populations.	Field	205	Morphological and molecular	- There were low levels of genetic variability in the 9 populations and most of the genetic variation was interpopulational. - All screened *P. columella* were negative after shedding.
	D'Almeida et al., 2016	To monitor *P. columella* population density in various aquatic habitats and in drinking water in Brazil.	Field	1558	Morphology	- Of the total 2038 molluscs collected, 1558 of which were identified as *P. columella* that was the most abundant snail species in all sampling sites.

						- Seasonal changes had no significant impact on the relationship of specimens observed. - No *Fasciola* spp. and/or other digenean infections were detected.
	Pereira de Souza et al., 2002	To experimentally infect *P. columella* with *F. hepatica* and maintain the life cycle in the laboratory.	Experimental	87	-	- An overall infection prevalence of 40.2% was noted.
Argentina	Cucher et al., 2006	To develop a sensitive PCR assay for the specific detection of *F. hepatica* in naturally infected *Lymnaea* spp. snails by means of an optimised DNA extraction protocol.	Field	240	Molecular	- Successful development of PCR assay with a high specificity for *F. hepatica* in field-collected intermediate hosts. - *P. columella* showed an infection rate of 17.5% and 51.3% by direct examination and PCR, respectively.
	Martin et al., 2016	To report the first record of the freshwater snail *P. columella* in southern Pampas, Argentina and assess its future spread.	Field	-	Morphology	- *P. columella* was reported for the first time in southern Pampas, Argentina.
	Duffy et al., 2009	To record the first report of a real-time PCR approach used to identify main lymnaeid species from Argentina.	Field	-	Morphology and molecular	- Specific melting temperature peaks were obtained for the main lymnaeid species in Argentina which are *P. columella*, *G. truncatula*, *L. diaphana* and *L. viatrix.*
	Prepelitchi et al., 2011	To examine the dynamics, abundance and population structure of *P. columella* in the Ibera Macrosystem, Argentina.	Field	7851	Morphology	- *P. columella* specimens were found throughout the study period except during the drought. - This was the first report of *P. columella* in wetland types.
	Prepelitchi et al., 2003	To identify the species of snails collected in Berón de Astrada,	Field	601	Egg and adult fluke morphology	- All 601 collected snails were identified as *P. columella.*

		Argentina and to screen those snails for *F. hepatica* infections.			(orally infected rats)	- 8.8% of *P. columella* was exclusively naturally infected with *Fasciola hepatica.*
	Zarco et al., 2011	To report the first record of *P. columella* in central Argentina (Córdoba province).	Field	44	Morphology	- *P. columella* was reported for the first time in Córdoba province, central Argentina.
	Davies et al., 2014	To report the first record of *P. columella* from Salta province, northwest Argentina.	Field	98	Morphology	- *P. columella* was reported for the first time in Salta province, Argentina. - No trematode infections were recorded.
Uruguay	Magalhães et al., 2004	To design a pair of primers for the conserved and repetitive region of mitochondrial DNA from *F. hepatica* that could be used to detect infections by this trematode.	Experimental	24	Molecular	- Designed primers were able to detect the presence of *F. hepatica* in *P. columella* snails in the pre-patent period.
Colombia	Pereira et al., 2020	To identify the various lymnaeid snail species occupying different geographical regions of Santander and its bordering departments within Colombia.	Field	7	Molecular	- Four lymnaeid species are reported in the study area: *Galba cousin, G. truncatula, Galba schirazensis* and *P. columella*. - The freshwater snails *P. columella* and *G. schirazensis* were found free of infection.
	Salazar et al., 2006	To examine the effects of exposure to *F. hepatica* on life history traits of *Lymnanea cousini* and P*. columella*.	Experimental	100	-	- Infection rates of P*. columella* and *L. cousini* on at 4 weeks were 82.8 and 34.0%, respectively.
Venezuela	Pointier et al., 2009	To investigate the distribution of Lymnaeidae intermediate snail hosts of *Fasciola hepatica* in Venezuela.	Field	-	Morphology	- Four species were discovered in the duration of this study: *Galba cousin*, *G. cubensis*, *P. columella* and *G. truncatula*. - Susceptible *P. columella* was not found naturally infected by *F. hepatica*.
	Bargues et al.,2011	To attain a new baseline for fasciolosis in Venezuela.	Field	6	Morphology and Molecular	- *P. columella* specimens were confirmed using ribosomal and mitochondrial DNA markers.

	-There was no evidence to support *P. columella* as a source of the human fasciolosis cases reported in Venezuela.

Table 2.3 Summary of studies included in the distribution of *Pseudosuccinea columella* and its role in the transmission of *Fasciola* spp. in North America and the Caribbeans for a period of 30 years (1990 – 2020).

Country	Author	Objective(s)	Type of study	Sample size (N)	Diagnostic method	Outcome(s)
Cuba	Alba et al., 2018	To explore the effect of different parasite doses, successive exposures, and different parasite origins on the infection outcomes of susceptible and naturally resistant *P. columella* occurring in Cuba.	Experimental	120	-	- Resistant populations had zero parasite development. - High *F. hepatica* miracidial doses and serial exposures resulted in an overall redial burden and an increase in prevalence in susceptible snails. -There were differences in compatibility of susceptible snails related to the geographical scale.
	Gutiérrez et al., 2002	To analyze the influence of F. hepatica development on some of the life history traits of three Cuban isolates of *P. columella.*	Experimental	90	-	- Pargue Lenin and Punta Brava had 25/30 and 27/30 infected snails, respectively. - There were no infected snails in the La Palma population thus this constitutes of the first report of *F. hepatica* resistant *P. columella* in Cuba.
	Calienes, et al., 2004	To detect new *F. hepatica* resistant populations of *P. columella* collected in the western and central regions of Cuba using previously identified RAPD markers.	Experimental	100 (12 populations)	Molecular	- 9 out of the 12 *P. columella* populations were susceptible to F. hepatica infections. - A new natural population of resistant *P. columella* was identified in the locality of El Azufre in Pinar del Rio Province in addition to the other two resistant populations previously reported.
	Gutiérrez et al., 2011	To compare cellular reaction to miracidial development, shell morphometrics, mantle pigmentation pattern, and egg laying behaviour of isolates of Cuban *P.*	Field	100	Molecular	- resistant snails encapsulated and phagocytised miracidium early while in susceptible snails viable transforming miracidia was observed.

	columella susceptible and resistant to *Fasciola hepatica.*				- Susceptible snails laid eggs on container walls while non-susceptible ones laid eggs on the bottom of container. - Susceptible snails have a significantly more rounded shell and aperture than the non-susceptible ones.
Vázquez et al., 2014	To compare the compatibility of *Galba cubensis* and *P. columella* as intermediate hosts for *F. hepatica* in Cuba.	Experimental	150	-	- *G. cubensis* is a more compatible host for *F. hepatica* in Cuba when compared with *P. columella.*
Gutiérrez et al., 2003	To screen *P. columella* detected in a rice culture area at the El Pilon locality for *Fasciola* spp. natural infections.	Experimental	-	-	- First time reporting *P. columella* naturally infected with *F. hepatica* not only for Cuba but also for the Caribbean area.
Alba et al., 2019b	To present the ecology of resistant and susceptible *P. columella* in Cuba.	Field and experimental	329	Molecular	- No clear pattern of habitat types for non-susceptible *P. columella* populations were observed. - Low pH/TH affects *P. columella* negatively irrespective of whether is resistant or susceptible to Fasciola spp. infections.
Gutiérrez et al., 2001	To investigate life history traits of *Pseudosuccinea columella*.	Experimental	60	-	- Rates of increase (both finite and intrinsic) were lower in paired than in isolated snails. - Isolated snails showed the highest values of number of eggs per mass per individual.
Gutiérrez et al., 2005	To provide data on the variations of abundance of *F. hepatica* resistant and susceptible *P. columella* populations throughout the year under natural conditions.	Field	-	Morphology	- *P. columella* was the most abundant out of the gastropods sampled. - In El Azufre, resistant *P. columella* abundance was lower than the abundance attained by the susceptible strain.

Kansas	McKown and Ridley, 1995	To ascertain the existence of possible snail intermediate hosts of *Fasciola hepatica* within Kansas.	Experimental	-	-	- Of the five lymnaeid snails tested for susceptibility, only *Pseudosuccinea columella* and *Fossaria bulimoides* proved susceptible to experimental infection by *Fasciola hepatica.*
USA (Hawaii)	Cowie, 1998	To examine patterns in the introductions of non-indigenous slugs and freshwater snails in the Hawaiian.	Field	-	-	-Twenty-two species of freshwater snails and slugs have been reported in the wild in the Hawaiian Islands and *P. columella* is one of them introduced in 1950.

Table 2.4 Summary of studies included in the distribution of *Pseudosuccinea columella* and its role in the transmission of *Fasciola* spp. in Europe for a period of 30 years (1990 – 2020).

Country	Author	Objective(s)	Type of study	Sample size (N)	Diagnostic method	Outcome(s)
France	Pointier et al., 2007	To test the capacity of *P. columella* populations recovered along the banks of the Lot River in France to act as intermediate hosts for *F. hepatica.*	Experimental	26	-	- First record of *P. columella* in the wild in France. - Twenty-six experimentally infected *P. columella* had 100% prevalence of infection when exposed to *F. hepatica* miracidia.
	Dreyfuss et al., 2016	To determine the ability of *P. columella* to ensure complete larval development of *F. hepatica*, *Calicophoron daubneyi* or both parasites (co-infection).	Experimental	200	-	- The highest prevalence of *F. hepatica* infections was recorded in in the 4mm group. - Low frequencies were recorded in the co-infection groups and for *C. daubneyi* in the 3, 4 and 5mm groups.
	Vázquez et al., 2020	To study different intermediate and definitive hosts susceptibility to *Fasciola hepatica* using both field and experimental approaches.	Experimental	30	Molecular	- 60% of cattle were infected with *F. hepatica* but nutria and wild boars had 19% and 4.5% respectively. - All four snail species (*G. truncatula, P. columella, Lymnaea stagnalis* and *Radix balthica*) were susceptible *to F. hepatica* infection.
	Vignoles et al., 2018	To investigate the consequences of *P. columella* invasion on the dynamics of native lymnaeids living on the acid soils of central France.	Field and semi-experimental	75	-	- *P. columella* colonisation was more rapid in habitats with *G. truncatula* than those with *Omphiscola glabra*. - Colonisation of *P. columella* in these habitats lead to a decrease in the abundance of native lymnaeids in these ditches.

	Lounnas et al., 2017	To evaluate the distribution of genetic diversity in *P. columella* from 80 populations across 14 countries.	Field	1509	Molecular	- There was a lack of genetic polymorphism over thousands of kilometres. -There was a genetic distinction between liver fluke resistant and susceptible populations *of P. columella* in Cuba.
	Vignoles et al., 2015	To determine the better intermediate host for metacercarial production between *Galba truncatula* and *Pseudosuccinea columella* infected with *Fasciola hepatica*.	Experimental	200		- Overall, *P. columella* infections with *F. hepatica* resulted in higher metacercarial production than that noted with *G. truncatula.* - For *F. hepatica* infections, 38.2% and 33.3% prevalence were reported in the Egyptian and Italian *P. columella* populations, respectively.
Romania	Glöer and Sîrbu, 2005	To present the new freshwater snail species found in Romania in the last years as well as build an updated systematic catalogue of these freshwater molluscs.	Field	-	-	- *P. columella* was newly identified in Romania in this thermal lake.
Italy	Cianfanelli et al., 2007	To map-out the distribution of non-indigenous freshwater molluscs in Italy.	Field	-	-	- Report of *P. columella* in Italy.

2.4.1. Global distribution and abundance of *P. columella*

Pseudosuccinea columella was documented in 22 countries from Africa (Table 2.1), South America (Table 2.2), North America and the Caribbeans (Table 2.3), Europe (Table 2.4) and Oceania. However, the species was widely reported in African (n = 18) and South American (n = 16) countries. In the African continent, *P. columella* was documented in South Africa, Egypt, Madagascar, Cameroon, La Reunion, Zimbabwe, Namibia and Mayotte. However, the results also showed that this snail species was mostly reported and distributed in South Africa and Egypt. In South America, *P. columella* has been reported in Argentina, Uruguay, Brazil, Venezuela, Paraguay, Peru and Colombia. In this region, however, *P. columella* was shown to have a wide distribution in Argentina (n = 8), followed by Brazil (n = 5). In North America and the Caribbeans, *P. columella* has been documented in the USA, including Kansas and Hawaii states, Cuba and Guadeloupe. In Europe, *P. columella* has been recorded in France, Romania and Italy. Of all the continents, this freshwater snail is least distributed in Oceania, where it has only been reported in Australia and French Polynesia.

The results showed that *P. columella* inhabits a wide variety of natural and man-made freshwater systems (Table 2.5). These freshwater systems included riverbanks, ponds (some in botanical gardens), canals, irrigation systems, ditches, ravines, lakes, dams, drain channels, streams, areas of estuarine lake that have low salinity, wetlands and a thermal lake. Although rivers/riverbanks were the most common habitat for *P. columella* throughout the world, this lymnaeid snail typically inhabits a wide variety of habitats.

Pseudosuccinea columella were collected during all seasons, both in winter (dry) and summer (rainy), or only in summer (Table 2.5). There were no studies that were conducted during the winter season only. Reviewed studies showed that *P. columella* snails were collected in abundance during the summer (rainy) season (Cardoso et al., 2006; Bargues et al., 2011; Prepelitchi et al., 2011; D'Almeida et al., 2016) as compared to the winter season (Bargues et al., 2011; Prepelitchi et al., 2011; D'Almeida et al., 2016). Results also showed that South American countries recorded the highest number of collected *P. columella* specimens globally involving field studies. The highest number of *P. columella* were collected in the wetlands of Argentina (n = 7851) over a period of two years and four months (Table 2.2, Table 2.5) (Prepelitchi et al., 2011). Lounnas et al. (2017) collected 1509 *P. columella* individuals over 16 years in 14 countries (Table 2.4, Table 2.5). The lowest number of sampled *P. columella* (n = 5) was recorded in South Africa during the dry and rainy season of 2011 (Table 2.5) (Kemp et al., 2016).

Table 2.5 Global distribution of *Pseudosuccinea columella* between 1990 and 2020 (30 years).

Region	Country	Habitat(s)	Sampling size (N)	Season of collection	Author (Reference)
South America	Brazil	-	205	Summer and winter	Cardoso et al., 2006
		Drinking reservoirs for domestic ruminants and wetland	1558	All seasons	D'Almeida et al., 2016
	Venezuela	Irrigation canal	-	-	Pointier et al., 2009
		Irrigation and water canals	6	Both dry and rainy	Bargues et al.,2011
		Rivers, canals, irrigation systems, ravines, ditches, lakes, and ponds	57	-	Lounnas et al., 2017
	Colombia	Rivers, canals, irrigation systems, ravines, ditches, lakes, and ponds	497	-	Lounnas et al., 2017
		-	7	-	Pereira et al., 2020
	Peru	Rivers, canals, irrigation systems, ravines, ditches, lakes, and ponds	19	-	Lounnas et al., 2017
	Argentina	Rivers, canals, irrigation systems, ravines, ditches, lakes, and ponds	84	-	Lounnas et al., 2017
		-	-	Summer	Duffy et al., 2009
		Wetlands	7851	All seasons	Prepelitchi et al., 2011
		Riverbanks	44	Beginning of summer	Zarco et al., 2011
		River	98	Summer and Winter	Davies et al., 2014
		Riverbanks	-	Summer	Martin et al., 2016
	Paraguay	Rivers, canals, irrigation systems, ravines, ditches, lakes, and ponds	54	-	Lounnas et al., 2017
Africa	South Africa	Rivers and dams	100	-	Malatji et al., 2019
		Dams and rivers	-	-	Wolmarans and de Kock, 2006
		Rivers and dams	-	-	de Kock and Wolmarans, 2008

		River	-	-	de Kock and Wolmarans, 1998
		Rivers and dams	-	All seasons	de Kock et al., 2002
		Estuarine lake (in areas with low salinity)	-	-	Perissinotto et al., 2014
		Rivers, canals, irrigation systems, ravines, ditches, lakes, and ponds	23	-	Lounnas et al., 2017
		Rivers	5	Summer and winter	Kemp et al., 2016
	Cameroon	Riverbanks	13	All seasons	Tchakonté et al., 2014
	Mayotte	Rivers, canals, irrigation systems, ravines, ditches, lakes, and ponds	26	-	Lounnas et al., 2017
	La Reunion	Rivers, canals, irrigation systems, ravines, ditches, lakes, and ponds	51	-	Lounnas et al., 2017
	Namibia	River	-	-	Curtis, 1991
	Egypt	River	-	All seasons	Dar et al., 2016
		Irrigation canals, streams, small dams, and rivers	-	-	Lofty and Lofty, 2015
		Irrigation and drain channels	24	Summer	El-Kady et al., 2000
		River	46	All seasons	Abd El-Wakeil et al., 2013
	Zimbabwe	Lake banks	-	Dry and wet seasons	Carolus et al., 2019
	Madagascar	Rivers, canals, irrigation systems, ravines, ditches, lakes, and ponds	14	-	Lounnas et al., 2017
North America and the Caribbeans	Cuba	-	329	-	Alba et al., 2019b
		Temporary pond	60	-	Gutiérrez et al., 2001
		Small canal and a spring	-	All seasons	Gutiérrez et al., 2005
		Rivers, canals, irrigation systems, ravines, ditches, lakes, and ponds	244	-	Lounnas et al., 2017

	Guadeloupe	Rivers, canals, irrigation systems, ravines, ditches, lakes, and ponds	60	-	Lounnas et al., 2017
	USA	Rivers, canals, irrigation systems, ravines, ditches, lakes, and ponds	158	-	Lounnas et al., 2017
	USA (Hawaii)	-	-	-	Cowie, 1998
Europe	Romania	Thermal lake	-	-	Glöer and Sîrbu, 2005
	Italy	Botanical garden pond	-	-	Cianfanelli et al., 2007
	France	Rivers, canals, irrigation systems, ravines, ditches, lakes, and ponds	64	-	Lounnas et al., 2017
		Riverbanks	75	-	Vignoles et al., 2018
Oceania	Australia	Rivers	-	Dry season	Molloy and Anderson, 2006
	French Polynesia	Rivers, canals, irrigation systems, ravines, ditches, lakes, and ponds	98	-	Lounnas et al., 2017

2.4.2. Susceptibility of *P. columella* to *Fasciola* species infection

From the 56 reviewed studies, 28 assessed the epidemiological role played by *P. columella* in the transmission of *Fasciola* species, and these studies were reported in South America (Table 2.2, Table 2.6) North America and the Caribbeans (Table 2.3, Table 2.6), in Africa (Table 2.1, Table 2.6) and Europe (Table 2.4, Table 2.6). No studies reported on the infection of *P. columella* with *Fasciola* spp. in Oceania. Of these 28 studies, most studies (n = 19) assessed the susceptibility of *P. columella* to *Fasciola* spp. infection experimentally in all reviewed countries, with the exception of South Africa where natural infections were reported. Only nine studies assessed natural infections of *Fasciola* species in *P. columella*, and these studies were from Africa (South Africa and Egypt), South America (Argentina, Brazil and Columbia) and North America and the Caribbeans (Cuba).

The results showed that *P. columella*/*F. hepatica* infections were more prevalent, reported in 22 studies, as compared to *P. columella*/*F. gigantica* infections, which were reported in two studies (Table 2.6). *Pseudosuccinea columella* populations in Egypt were experimentally susceptible to both *F. hepatica* and *F. gigantica*. No *P. columella* populations have been found naturally infected with *F. hepatica* in the field in the African continent (Table 2.6). Experimental studies from Cuba (n = 2) showed populations of *P. columella* resistant to *F. hepatica* infections. Furthermore, field studies from Brazil and Argentina also did not report natural *F. hepatica* infections in *P. columella* populations collected from *F. hepatica*-endemic areas.

Table 2.6 Natural and experimental *Fasciola* infections intra-*Pseudosuccinea columella* reported between 1990 and 2020 (30 years).

Region	Country	Study type	No. of snails collected/used	No. infected (prevalence)	Detection method	Species of infection	Author (Reference)
Africa	South Africa	Field	100	100 (100%)	Molecular	*F. gigantica*	Malatji and Mukaratirwa, 2019
	Egypt	Experimental	330	178 (54%)	Molecular	*F. hepatica*	Dar et al., 2015b
		Experimental	400	145 (36.25%)	-	*F. hepatica*	Dar et al., 2014
		Field	296	39 (13.18%)	Molecular	*F. gigantica*	Grabner et al., 2014
		Experimental	100	38 (38%)	-	*F. hepatica*	Vignoles et al., 2015
		Experimental	600	115 (19. 17%)	-	*F. hepatica*	Dar et al., 2015a
Europe	France	Experimental	26	26 (100%)	-	*F. hepatica*	Pointier et al., 2007
		Experimental	200	107 (53.5%)	-	*F. hepatica*	Dreyfuss et al., 2016
		Experimental	30	17 (56.67%)	Molecular	*F. hepatica*	Vázquez et al., 2020
		Experimental	100	33 (33%)	-	*F. hepatica*	Vignoles et al., 2015
North America and the Caribbeans	Cuba	Experimental	120	-	-	*F. hepatica*	Alba et al., 2018
		Experimental	90	52 (57.8%)	-	*F. hepatica*	Gutiérrez et al., 2002
		Experimental	100 (12 populations)	9 populations	Molecular	*F. hepatica*	Calienes et al., 2004
		Experimental	-	Sus - 92%; Res -0%	-	*F. hepatica*	Gutiérrez et al., 2003
		Field	100	3 (3%)	Molecular	*F. hepatica*	Gutiérrez et al., 2011
		Experimental	150	-	-	*F. hepatica*	Vázquez et al., 2014
	Kansas	Experimental	-	-	-	*F. hepatica*	McKown and Ridley, 1995
South America	Brazil	Experimental	600	164 (27.33%)	-	*F. hepatica*	Mendes et al., 2008
		Field	1558	0	Cercaria shedding	-	D'Almeida et al., 2016

	Field	205	0	Cercaria shedding	-	Cardoso et al., 2006
	Experimental	87	35 (40.2%)	-	*F. hepatica*	Pereira de Souza et al., 2002
	Experimental	600	319 (53.17%)	-	*F. hepatica*	Coelho et al., 2008
Argentina	Field	240	123 (51.25%)	Molecular	*F. hepatica*	Cucher et al., 2006
	Field	500	44 (8.8%)	Cercaria shedding	*F. hepatica*	Prepelitchi et al., 2003
	Field	98	0	Cercaria shedding	-	Davies et al., 2014
Uruguay	Experimental	24	24 (100%)	Molecular	*F. hepatica*	Magalhães et al., 2004
Colombia	Experimental	100	82 (82%)	-	*F. hepatica*	Salazar et al., 2006
	Field	7	0	Observation of the cercaria, metacercariae and rediae.	-	Pereira et al., 2020

2.4.3. Prevalence of *Fasciola* species infection intra-*P. columella* populations

Although most studies did not indicate the methods they used to check for infection, 13 studies checked snail infections using either cercarial shedding (n = 4), molecular techniques (n = 8) or checking for any of the developmental stages of *Fasciola* inside the IH tissue under the microscope (n = 1) (Table 2.6).

The overall prevalence of *P. columella* infected with *Fasciola* spp. varied from 0 to 100%, the lowest (0%) recorded in Brazil (Cardoso et al., 2006; D'Almeida et al., 2016), Argentina (Davies et al., 2014) and Colombia (Pereira et al., 2020), and the highest (100%) recorded in South Africa (Malatji and Mukaratirwa, 2019), Uruguay (Magalhães et al., 2004) and France (Pointier et al., 2007). *Pseudosuccinea columella*/*Fasciola* spp. natural infections prevalence ranged from 0 to 100% (Table 2.6). Experimental *P. columella*/*F. hepatica* infections showed prevalence ranging from 19.7% in Egypt (Dar et al., 2015a) to 100% in France (Pointier et al., 2007) and Uruguay (Magalhães et al., 2004) (Table 2.6). No studies are reported on experimental F. gigantica intra-*P. columella* infections. *Pseudosuccinea columella* naturally infected with *F. hepatica* had a prevalence between 0 and 51.25%, with no infections documented in Brazil (Cardoso et al., 2006; D'Almeida et al., 2016), Argentina (Davies et al., 2014) and Colombia (Pereira et al., 2020), and a high infection rate of 51.25% was documented in Argentina (Cucher et al., 2006). The prevalence range for *F. gigantica* intra-*P. columella* natural infections was reported to be between 13.18 and 100% in Egypt (Grabner et al., 2014) and South Africa (Malatji and Mukaratirwa, 2019), respectively. The highest naturally occurring infections of *P. columella* with *F. hepatica* and *F. gigantica* were documented in Argentina (51.25%) (Cucher et al., 2006) and South Africa (100%) (Malatji and Mukaratirwa, 2019).

2.5. Discussion

Pseudosuccinea columella is thought to have originated from Central America, the Caribbean and the southern part of North America (Mas-Coma et al., 2005), and this review has shown that this species has been successfully introduced and established in other continents with varying environmental and ecological conditions. The results from this review showed that in addition to its native regions (Dar et al., 2014; Martin et al., 2016; Lounnas et al., 2017; Alba et al., 2018; Vignoles et al., 2018; Carolus et al., 2019), *P. columella* has been documented in Africa, Europe, South America (Martin et al., 2016; Lounnas et al., 2017; Alba et al., 2018) and Oceania (Martin et al., 2016; Lounnas et al., 2017; Alba et al., 2018; Vignoles et al., 2018; Alba et al., 2019b). The results further indicate that from these four newly invaded continents, this invasive freshwater snail has been reported in 19 countries but is now well established and widely distributed in Africa and South America, and is least distributed in Oceania

(Lounnas et al., 2017). However, abundance varied with localities and various environmental factors, such as the availability of suitable habitats and seasonal changes, amongst other factors. Although the reviewed studies showed that *P. columella* specimens were collected in abundance in native Argentina (Prepelitchi et al., 2011) and Brazil (D'Almeida et al., 2016), this invasive snail is considered the most abundant lymnaeid species in Egypt (Grabner et al., 2014), Brazil (D'Almeida et al., 2016) and in Kansas, USA (McKown and Ridley, 1995), and the second most abundant species in Zimbabwe (Carolus et al., 2019) in comparison to other freshwater lymnaeids.

According to de Kock et al. (2002), the availability of suitable water habitats largely influences the distribution of freshwater snails. Although an inspection of the frequency of habitats in reviewed studies showed that *P. columella* is commonly found on riverbanks, the results also showed that P. columella thrives in diverse freshwater habitats ranging from man-made, to natural, temporal and permanent habitats. In addition to freshwater habitats, this lymnaeid species has been found in ditches of acid soils that have water with low levels of calcium (Vignoles et al., 2018) and areas with low salinity (Perissinotto et al., 2014). Furthermore, it has been documented to occur in places with high altitudes and low temperatures (Bardales-Valdivia et al., 2021). The ability of *P. columella* to adapt to and inhabit almost all types of freshwater bodies, including thermal lakes and acidic soils, and tolerate a wide range of climatic conditions are some of the factors that make this snail such a successful invader (Prepelitchi et al., 2011; Vignoles et al., 2018) and the most widely distributed invasive freshwater snail species globally (Pointier et al., 2009; Lounnas et al., 2017).

The abundance of freshwater snails varies throughout the year with seasonal changes such as temperature, rainfall and water levels (Kleiman et al., 2007). According to D'Almeida et al. (2016), an increase in rainfall and temperature favours an increase in mollusc populations. This is consistent with the results from this review, as *P. columella* was noticeably more abundant during rainy seasons, and this was easily observed in studies that collected snails during all seasons of the year (de Kock et al., 2002; Abd El-Wakeil et al., 2013; Tchakonté et al., 2014; D'Almeida et al., 2016; Dar et al., 2016). Studies in Egypt (El-Kady et al., 2000) and Argentina (Kleiman et al., 2007) showed that the best time to collect snails when they are in their highest numbers was in spring and summer due to favourable temperature conditions and the availability of plenty of vegetation cover in the aquatic habitats. However, Prepelitchi et al. (2011) noted that the highest number and largest size of specimens of *P. columella* populations in the north of Corrientes province (Argentina) were found during winter. This is, however, inconsistent with Charlier et al. (2014) and Beesley et al. (2017), who stated that adult snails are known to be abundant in summer and spring seasons, while juvenile snails are mainly found in autumn. These results show that although *P. columella* populations can be found throughout the year and in different

seasons, the season in which *P. columella* may be found in abundance differs between and within countries due to varying habitats and climatic conditions.

The epidemiological importance of *P. columella* as an intermediate host for both *F. gigantica* and *F. hepatica* has been documented (de Kock et al., 1989; Grabner et al., 2014; Alba et al., 2019a; Carolus et al., 2019; Malatji et al., 2019). From the review, infections were commonly detected through shedding of cercariae from snails collected from their natural environments, observation of different developmental stages of the parasites after crushing or dissecting snails (Magalhães et al., 2004; Caron et al., 2008; Beesley et al., 2017) and through molecular techniques for better sensitivity (Magalhães et al., 2004; Cucher et al., 2006; Beesley et al., 2017). Reviewed studies showed that *P. columella* infections with *F. gigantica* have only been reported in Egypt (Grabner et al., 2014) and South Africa (Malatji and Mukaratirwa, 2019). Despite the high natural infections reported by Malatji and Mukaratirwa (2019) in South Africa, Grabner et al. (2014) still concluded that the maintenance of the natural life cycle of *F. gigantica* in *P. columella* remains uncertain. This conclusion may have been attributed to the low infection rate (13.18%) of *F. gigantica* observed in Egypt (Grabner et al., 2014). The limited information on the role played by *P. columella* in the transmission of *F. gigantica* in the invaded countries highlights the need for more field and experimental infection studies in these areas. The high prevalence of *F. gigantica* intra-*P. columella* infection (100%) reported in South Africa by Malatji and Mukaratirwa (2019) could be due to livestock drinking from seasonal ponds/small dams with no other source of drinking water and thereby snails getting exposed to heavy infections from hatching miracidia. There are plenty of such scenarios in the rural areas of South Africa and elsewhere in southern Africa, where animals drink and graze around these areas which are heavily contaminated by *Fasciola* spp. cercariae (Malatji and Mukaratirwa, pers. comm.).

The results for this review show that *F. hepatica* infections in *P. columella* were the most documented in five continents. This is not surprising, as according to Mas-Coma et al. (2005), this invasive lymnaeid species is responsible for the secondary spread of *F. hepatica*. The results also show that most *F. hepatica* intra-*P. columella* infection studies were conducted in the laboratory (experimental infections), as compared to field reports, which is a great concern as this does not give a full representation of what happens in the field. Additionally, the recorded infection rate in the reviewed studies was generally low in natural/field infection studies (Prepelitchi et al., 2003; Gutiérrez et al., 2011) as compared to experimental studies, with exception to that reported by Cucher et al. (2006) in Argentina. Although the observed high experimental infection rates of *P. columella* with *F. hepatica* show the importance of this invasive snail as one of the vectors responsible for the transmission of fascioliasis (Dar et al., 2015b), there is still a need for field-based studies to further determine not only

the prevalence of *Fasciola* spp., but also the competence of different populations of *P. columella* in maintaining and transmitting both Fasciola species globally.

Literature has shown that geographical variations in *Lymnaea* species influence their susceptibility to infections by *F. hepatica* (Gasnier et al., 2000; Coelho et al., 2008). This has been observed by Gutiérrez et al. (2011) and Alba et al. (2018), who reported on variations within *P. columella* species that influenced their susceptibility to *F. hepatica* infections. In Cuba, two different phenotypes of *P. columella* populations have been identified and reported to show either resistance or susceptibility to *F. hepatica* infections (Alba et al., 2018). These authors have shown that the resistant field-occurring *P. columella* phenotypes are characterized by their ability to encapsulate and phagocytize miracidia using their (host's) immune cells (Gutiérrez et al., 2003, 2011; Alba et al., 2018).

Although the presence of resistant *P. columella* phenotypes has not been extensively studied in most countries, the inability of some phenotypes of this invasive snail to be infected and transmit particularly *F. hepatica* in endemic areas may lead to the assumption that this phenotype is unknowingly widely distributed. Such cases have been reported in Australia (Molloy and Anderson, 2006), Venezuela (Pointier et al., 2009; Bargues et al., 2011), Colombia (Pereira et al., 2020), north-west Argentina (Davies et al., 2014) and Brazil (Cardoso et al., 2006; D'Almeida et al., 2016), where *P. columella* phenotypes collected in *Fasciola*-endemic areas were found with no infections. Additionally, *P. columella* phenotypes collected in locations in South Africa where both *F. hepatica* and *F. gigantica* overlap were only found infected with *F. gigantica* (Malatji and Mukaratirwa, 2019). This, therefore, raises concerns about the existence and geographical distribution of the different phenotypes of *P. columella*. As a result, there is still a need to conduct both an experimental and field assessment of the infection of *P. columella* phenotypes with both *Fasciola* species, especially in countries where this invasive snail has been reported with no clear epidemiological role in the transmission of Fasciola species, and in areas where both *F. hepatica* and *F. gigantica* overlap. This will help determine the geographical expansion of *P. columella* phenotypes resistant to *F. hepatica* infections, as well as the competence of the susceptible species in the transmission of both *Fasciola* species in fasciolosis-endemic areas.

2.6. Conclusion

It is evident that intensive research still needs to be conducted focusing on (1) assessing the global distribution and importance of this invasive snail in the transmission of both *Fasciola* species, especially in the field/natural environment, as there seem to be more countries where the snail is present and yet to be documented; (2) assessing the susceptibility of different *P. columella* phenotypes to *Fasciola* spp. populations to differentiate susceptible and resistant phenotypes, in countries where the species

has been reported; (3) the experimental infection of *P. columella* populations not found with infections but collected in fasciolosis-endemic areas; and (4) studies to compare the competence of *P. columella* in the transmission of fasciolosis with other lymnaeid IHs native to those particular areas where fasciolosis is endemic. Additionally, this review has pointed out the importance of researchers to describe the ecological parameters of habitats where species have been collected from, as this could assist in explaining questionably high rates of documented *P. columella* naturally infected by either *Fasciola* species. A limitation in this review might be that a number of studies published in languages other than English might not have been included and that the study only considered articles published in the last 30 years.

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Chapter 3

Susceptibility of lymnaeid snails to *Fasciola hepatica* and *Fasciola gigantica* (Digenea: Fasciolidae): A systematic review and meta-analysis

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3.1. Abstract

Fasciolosis is a food-borne disease that causes major economic losses, globally. This zoonotic disease is caused by *Fasciola hepatica* and *Fasciola gigantica* species which employ freshwater snails from the family Lymnaeidae as their intermediate hosts. Thus, a key aspect of understanding the epidemiology of the disease lies in understanding the transmission ecology of the parasite. Therefore, this systematic review and meta-analysis were conducted to assess the experimental susceptibility and prevalence of natural infections of *F. hepatica* and *F. gigantica* in lymnaeid snails. Relevant peer-reviewed articles published in the past 20 years (2004-2023) were searched and appraised. Prevalence and infection rate estimates were based on 41 studies that met the inclusion criteria. Results showed that 5575 lymnaeid snails were subjected to experimental infections and 44002 were screened for natural infections. The overall pooled infection rate was higher in experimental infections 50% (95% CI: 42-58%) compared to natural infections of field-collected snails 6% (95% CI: 0-22%). The highest pooled infection rate was recorded in South America at 64% (95% CI: 48-78%) for experimental infections while the lowest was recorded for natural infections at 2% (95% CI: 0-6%) in Europe and 2% (95%: CI 0-17%) in Asia. In experimental studies, *F. gigantica* recorded the highest pooled prevalence at 73% (95% CI: 61-84%) compared to *F. hepatica* which recorded 47% (95% CI: 38-56%). For natural infections, however, *F. hepatica* had the highest prevalence (12% (95% CI: 0-30%)) while the lowest was noted for naturally infected *F. gigantica* at 2% (95% CI: 0-18%). Based on the snail species, the highest pooled prevalence was recorded for *Pseudosuccinea columella* infected with *F. hepatica* and *F. gigantica* at 47% (95% CI: 33-61%) while the lowest was recorded for *F. hepatica* naturally infected *Galba truncatula* at 4% (95% CI: 0-10%). Natural *Fasciola* spp. infections in intermediate snail hosts decreased in prevalence while experimental infections have increased in prevalence over the past 20 years. This might be attributed to improvement in control strategies and/or climate change might be affecting the transmission of fasciolosis. While there seems to be a strong intermediate host specificity between the two *Fasciola* spp., experimental infection results showed that *G. truncatula* and *R. natalensis* are susceptible to *F. hepatica* and *F. gigantica*, respectively.

Keywords: *Fasciola hepatica*, *Fasciola gigantica*, intermediate hosts, lymnaeids, experimental infections, natural infections, prevalence.

3.2. Introduction

Fasciolosis is a zoonotic food-borne disease of livestock and wild ruminants, and humans caused by the digenean liver flukes *Fasciola hepatica* Linnaeus, 1758 and *Fasciola gigantica* Cobbold, 1855 (Kithuka et al., 2002; Chen et al., 2013; Vázquez, et al., 2019a; Ahmad et al., 2022). This parasitic disease causes economic losses in the livestock industry (Gutierrez et al., 2001) resulting from reduced productivity, liver condemnation, mortality, and expenditures for anthelmintics (Kithuka et al., 2002; Kleiman et al., 2007; Mucheka et al., 2015). These global economic losses have been estimated to exceed 2 billion dollars annually (Spithill et al., 1999; Medeiros et al., 2014; Lalor et al., 2021).

Fasciolosis has been recorded in more than 70 countries, across all inhabited continents (Lalor et al., 2021). *Fasciola hepatica* has been reported in temperate regions of Europe, Asia, Africa, Australia, and Americas (Mas-Coma, 2004; Lalor et al., 2021; Ahmad et al., 2022). Conversely, *F. gigantica* is mostly distributed in tropical and subtropical regions of Africa, Asia (Mas-Coma, 2004; Shoriki et al., 2014; Mucheka et al., 2015; Lalor et al., 2021; Ahmad et al., 2022), and the Middle East (Lalor et al., 2021). Furthermore, the overlapping geographical distribution of both species has been reported in many parts of Africa (Aleixo et al., 2015; Ahmad et al., 2022; Nukeri et al., 2022) and Central Asia where hybrid forms of the parasite have been recorded (Shoriki et al., 2014; Aleixo et al., 2015).

According to McKown and Ridley (1995), the presence of a compatible snail intermediate host (IH) in fasciolosis endemic areas is crucial to complete the life cycle of the parasite. The transmission of *Fasciola* spp. in a specific geographical region mostly depends on the presence of vector snails from the family Lymnaeidae Rafinesque, 1815 (Alba et al., 2019). As Vinarski (2013) reported, this family consists of two subfamilies, Radicinae and Lymnaeinae, with about 26 genera collectively. Species from the genera *Galba* (Cruz-Mendoza et al., 2004; Gutierrez et al., 2011; Alemu, 2019), *Lymnaea* (Kim et al., 2014; Alemu, 2019), *Pseudosuccinea* (Cucher et al., 2006; Alemu, 2019; Ngcamphalala et al., 2022), *Forassia* (Cruz-Mendoza et al., 2004; Alemu, 2019), *Radix* (Bargues et al., 2011; Imani-Baran et al., 2012; Huang et al., 2019*), Austropeplea* (Dung et al., 2013; Kim et al., 2014), and *Omphiscola* (Correa et al., 2017; Rondelaud et al., 2022) act as the IHs for *Fasciola* species. Although approximately 1200 lymnaeid snail species have been described globally (Vázquez et al., 2019b), only 30 species are known as IHs of *Fasciola* spp. (Alba et al., 2019; Vázquez, et al., 2019a). These species are distributed worldwide (Prepelitchi et al., 2011; Vinarski, 2013) and have been reported to extend from tropical to temperate regions with some occurring at extremely cold latitudes (Vázquez et al., 2019b).

Like most trematodes, *Fasciola* spp. show a marked snail host specificity (Bargues and Mas-Coma, 2005; Bargues et al., 2011). *Fasciola gigantica* is mainly transmitted by snail species from the genus *Radix,* particularly those belonging to Hubendick's (1951) superspecies of *Radix auricularia* (Linnaeus,

1758), which includes *R. natalensis* (Krauss, 1848) in Africa, *R. rubiginosa* (Michelin, 1831) in Asia (Brown, 1994) and *R. auricularia* in Europe (Mas-Coma et al., 2005). Recently, *Pseudosuccinea columella* has been shown to transmit this species in Africa (Grabner et al., 2014, Carolus et al., 2019; Malatji and Mukaratirwa, 2019). *Fasciola hepatica* is, however, transmitted by snails from various genera, but *Galba* species are the main IHs of this species globally (Bargues and Mas-Coma, 2005). According to Vázquez et al. (2019a), research on the compatibility of snail and parasite populations may serve to understand better and predict the transmission of fasciolosis globally. Additionally, a key aspect of understanding the epidemiology of a disease lies in understanding the transmission ecology of the parasite (Vázquez et al., 2015). Furthermore, understanding the susceptibility of the IHs to *Fasciola* spp. in a given locality may assist with the development of strategic control programs. Therefore, this study reviewed and analysed the results of peer-reviewed research reporting on the global experimental susceptibility/infectivity and natural infections of *F. hepatica* and *F. gigantica* in lymnaeid snail species in the past 20 years (2004-2023).

3.3. Methodology

3.3.1. Search strategy

A systematic literature search was conducted on PubMed, Web of Science, and Google Scholar databases, and a combination of the following search terms and Boolean operators (OR, AND) were used: *Fasciola hepatica* OR *Fasciola gigantica* AND intermediate hosts OR lymnaeids OR Lymnaeidae OR *Pseudosuccinea OR Galba OR Fossaria OR Lymnaea* OR *Omphiscola* OR *Austropeplea* OR *Radix* AND natural infection OR experimental infection OR prevalence OR infectivity. Peer-reviewed articles published in the last 20 years (2004-2023) were retrieved and appraisedAdditional articles were identified by cross-referencing selected articles' biographies (snowballing). EndNote reference manager version X8 (Clarivate Analytics, Philadelphia, PA, USA) was used to retrieve and manage full-text articles.

3.3.2. Inclusion and exclusion criteria

The following inclusion criteria were used to select articles for the systematic review and meta-analysis: (i) clearly stated the number of lymnaeid snails screened and/or infected with *F*. *hepatica* and *F. gigantica,* (ii) identified and reported the *Fasciola* species up to species level, (iii) identified the intermediate host snails up to species level, (iv) reported prevalence based on natural infections or infection rate based on experimental infections, and (v) indicated the detection method used.

Studies were excluded if they reported only on *Fasciola* spp. infection in definitive hosts, identification and distribution of the intermediate hosts without *Fasciola* infections, and articles written in other languages besides English.

3.3.3. Data extraction

Based on the study design, PIN and IN screened the titles and abstracts of articles, and relevant articles were retrieved. Full texts of retrieved articles were thoroughly reviewed and those that did not meet the inclusion criteria were excluded. Microsoft (MS) Excel spreadsheet was used to capture data from the text, tables, and figures for meta-analysis. Data extracted from relevant articles included author's names, year of publication, continent, country where the study was conducted, *Fasciola* species, snail host, sample size, number of infected host snails, prevalence, and detection method.

3.3.4. Quality assessment

The overall quality of the articles for meta-analysis was assessed using the Grading of Recommendations Assessment, Development, and Evaluation (GRADE) approach (Guyatt et al., 2008; Doi and Thalib, 2008). The quality of all studies included was assessed by scoring one point for each inclusion criterion that was fulfilled and a 0 was given for each unfulfilled criterion. As a result, each selected study was assigned a score ranging from 0 to 5. Studies with an index score of 5 were deemed high quality, 4 as moderate, and those that scored ≤ 3 were considered low quality and excluded from the analysis (Doi and Thalib, 2008). The total quality scores for all included studies ranged from moderate to good quality (Table 3.1, Table 3.2).

Table 3.1 Experimental infection of snail intermediate hosts with *Fasciola spp.* reported in the period 2004-2023.

Continent	Country	Host species	Parasite species	Origin of strain	No. examined	No. infected	% infectivity	Detection technique	Index score	References
South America	Argentina	*Galba (G.) neotropica*	*Fasciola hepatica*	Argentina	159	50	79.5	Dissection	5	Sanabria et al., 2012
South America	Argentina	*G. truncatula*	*F. hepatica*	Argentina	137	67	48.9	Dissection	5	Sanabria et al., 2012
South America	Colombia	*G. cousini*	*F. hepatica*	Colombia	100	34	34	Dissection	5	Salazar et al., 2006
South America	Colombia	*Pseudosuccinea (P.) columella*	*F. hepatica*	Colombia	100	83	83	Dissection	5	Salazar et al., 2006
South America	Cuba	*P. columella*	*F. hepatica*	Cuba	180	143	79.4	Dissection	5	Alba et al., 2018
South America	Cuba	*G. cubensis*	*F. gigantica*	Cuba	270	217	80.4	Dissection	5	Vazquez et al., 2014
South America	Cuba	*P. columella*	*F. gigantica*	Cuba	145	97	66.9	Dissection	5	Vazquez et al., 2014
Africa	Egypt	*Radix (R.) natalensis*	*F. hepatica*	France	263	62	23.6	Shedding	5	Dar et al., 2010
Africa	Egypt	*R. natalensis*	*F. hepatica*	France	45	24	53.3	Dissection	5	Dar et al., 2014a
Africa	Egypt	*P. columella*	*F. hepatica*	Egypt	379	117	30.9	Dissection	5	Dar et al., 2015a
Africa	Egypt	*P. columella*	*F. hepatica*	France	210	73	34.8	Dissection	5	Dar et al., 2015b
Africa	Egypt	*G. truncatula*	*F. hepatica*	France	477	302	63.3	Dissection	5	Dar et al., 2015b
Africa	Egypt	*P. columella*	*F. hepatica*	France	261	132	50.6	Dissection	5	Dar et al., 2014b
Africa	Egypt	*G. truncatula*	*F. hepatica*	France	55	49	89.1	Dissection	5	Dar et al., 2014b
Africa	Egypt	*G. truncatula*	*F. hepatica*	France	236	170	72.0	Shedding	5	Vignoles et al., 2015
Africa	Egypt	*P. columella*	*F. hepatica*	France	254	165	65	Shedding	5	Vignoles et al., 2015

Europe	France	*G. truncatula*	*F. hepatica*	Egypt + France	498	252	50.6	Dissection	5	Dar et al., 2013
Europe	France	*P. columella*	*F. hepatica*	France	319	104	32.6	Shedding	5	Dreyfuss et al., 2016
Europe	France	*P. columella*	*F. hepatica*	France	26	26	100	Dissection	4	Pointier et al., 2007
Europe	France	*G. truncatula*	*F. hepatica*	France	77	62	80.5	Dissection	5	Rondelaud et al., 2004
Europe	France	*G. truncatula*	*F. gigantica*	Egypt	83	41	49.4	Dissection	5	Rondelaud et al., 2004
Europe	France	*Omphiscola (O.) glabra*	*F. hepatica*	France	103	53	51.5	Dissection	5	Rondelaud et al., 2015
Europe	France	*G. truncatula*	*F. hepatica*	France	68	53	77.9	Dissection	5	Rondelaud et al., 2015
Europe	France	*G. truncatula*	*F. hepatica*	Argentina	370	112	30.3	Dissection	5	Sanabria et al., 2013
Asia	Iran	*R. auricularia*	*F. gigantica*	Iran	294	115	39.1	Shedding	5	Ashrafi and Mas-Coma, 2014
Europe	Sweden	*G. truncatula*	*F. hepatica*	Sweden	90	51	56.7	Dissection	5	Novobilsky et al., 2013
Europe	Sweden	*Lymnaea (L.) fuscus*	*F. hepatica*	Sweden	114	9	7.9	Dissection	5	Novobilsky et al., 2013
Europe	Sweden	*L. palustris*	*F. hepatica*	Sweden	119	40	33.6	Dissection	5	Novobilsky et al., 2013

Table 3.2 Reports of naturally infected snail intermediate hosts with *Fasciola* spp. in the period 2004-2023.

Continent	Country	Host species	Parasite species	No. examined	No. infected	Prevalence (%)	Detection technique	Index score	References
Europe	Ireland	*R. peregra*	*F. hepatica*	167	62	37	Molecular	5	Relf et al., 2009
Europe	Sweden	*L. fuscus*	*F. hepatica*	130	0	0	Molecular	5	Novobilsky et al., 2013
Europe	Sweden	*L. palustris*	*F. hepatica*	668	1	0.15	Molecular	5	Novobilsky et al., 2013
Europe	Spain	*G. truncatula*	*F. hepatica*	230	88	38.3	Dissection	5	Martinez-Ibeas et al., 2013
Europe	Poland	*G. truncatula*	*F. hepatica*	192	51	26.6	Molecular	5	Kozak and Wedrychowicz, 2010
Europe	Spain	*G. truncatula*	*F. hepatica*	1141	50	4.38	Dissection	5	Iglesias-Pineiro et al., 2016
Europe	France	*O. glabra*	*F. hepatica*	130	28	21.5	Molecular	5	Correa et al., 2017
Europe	France	*G. truncatula*	*F. hepatica*	410	49	12	Molecular	5	Correa et al., 2017
Europe	France	*O. glabra*	*F. hepatica*	1950	19	0.9	Dissection	5	Rondelaud et al., 2022
Europe	France	*G. truncatula*	*F. hepatica*	1948	16	0.6	Dissection	5	Rondelaud et al., 2022
Europe	France	*O. glabra*	*F. hepatica*	3900	55	1.4	Dissection	5	Rondelaud et al., 2022
Europe	France	*G. truncatula*	*F. hepatica*	3897	82	2.1	Dissection	5	Rondelaud et al., 2022
Africa	South Africa	*P. columella*	*Fasciola gigantica*	100	100	100	Molecular	5	Malatji and Mukaratirwa, 2020
Africa	Egypt	*P. columella*	*F. gigantica*	296	10	3.38	Molecular	5	Grabner et al., 2014
Africa	Egypt	*G. truncatula*	*F. hepatica*	731	71	9.7	Molecular	5	Arafa et al., 2018
Asia	Iran	*R. auricularia*	*F. gigantica*	496	12	2.42	Molecular	5	Yakhchali et al., 2014
Asia	India	*R. acuminata*	*F. gigantica*	400	161	40.25	Molecular	5	Sunita et al., 2021
Asia	India	*R. acuminata*	*F. gigantica*	2077	89	4.3	Molecular	5	Rajanna et al., 2018
Asia	South Korea	*Austropeplea (A.) viridis*	*F. hepatica*	12	1	8.33	Molecular	4	Kim et al., 2014
Asia	South Korea	*L. ollula*	*F. hepatica*	15	5	42.7	Molecular	4	Kim et al., 2014

Asia	Iran	*R. gedrosiana*	*F. gigantica*	2543	298	11.72	Molecular	5	Imani-Baran et al., 2012
Asia	China	*R. cucunorica*	*F. hepatica*	409	179	43.76	Molecular	5	Huang et al., 2019
Asia	Vietnam	*A. viridis*	*F. gigantica*	15364	124	0.8	Molecular	5	Dung et al., 2013
South America	Colombia	*G. cousini*	*F. hepatica*	521	68	13	Dissection	5	Pereira et al., 2020
South America	Colombia	*G. truncatula*	*F. hepatica*	68	1	1.4	Dissection	5	Pereira et al., 2020
South America	Cuba	*P. columella*	*F. hepatica*	100	3	30	Molecular	5	Gutierrez et al., 2011
South America	Ecuador	*G. schirazensis*	*F. hepatica*	37	7	19	Molecular	5	Geli-Erazo et al., 2020
South America	Ecuador	*G. schirazensis*	*F. hepatica*	1480	89	6	Molecular	5	Caron et al., 2017
South America	Argentina	*P. columella*	*F. hepatica*	480	165	51.3	Molecular	5	Cucher et al., 2006
South America	Argentina	*G. viatrix*	*F. hepatica*	68	22	61.8	Molecular	5	Cucher et al., 2006
North America	Mexico	*G. humilis*	*F. hepatica*	3372	2537	75.2	Dissection	5	Cruz-Mendoza et al., 2004
North America	Mexico	*G. bulimoides*	*F. hepatica*	670	515	76.9	Dissection	5	Cruz-Mendoza et al., 2004

3.3.5. Data analysis

The double arcsine approach was used to transform the prevalence data to avoid overestimating the weight of individual studies (Barendregt et al., 2013). This approach utilizes the arcsine transformation twice to the prevalence values to account for the heterogeneity caused by studies with extreme proportions or smaller sample sizes (Barendregt et al., 2013). MetaXL add-in for Microsoft Excel (www.epigear.com) was employed to compute a quality effects model to account for the heterogeneity (Barendregt et al., 2013). The level of heterogeneity between estimates was evaluated using inverse variance statistic (I^2 index), and the differences were accounted for using Cochrane's Q test (Barendregt and Doi, 2016). The I^2 index score was interpreted as low heterogeneity if it was <25%, moderate at 50%, and high heterogeneity at >75% (Higgins et al., 2003). The estimated prevalence and the 95% confidence interval (CI) of *Fasciola* species infections in lymnaeid snails were demonstrated on forest plots. Subgroup analysis was done to assess heterogeneity and factors that could influence the observed pooled prevalence estimates (PPE); thus, the data was grouped according to the region/continent on which studies were conducted, snail species involved, *Fasciola* species, method of detection, and period covered by the studies and publication bias was evaluated using funnel plots. Meta-regression was conducted using IBM SPSS Statistics 28.0 to identify the sources of heterogeneity. Meta-regression was performed with continents, diagnostic tests, and *Fasciola* species and study period fixed as independent factors. The meta regression was treated as linear model on the logit transformed prevalence data. The linear regression analysis was conducted to evaluate publication bias using Egger's test.

3.4. Results

3.4.1. Search results

Of the 861 articles obtained after going through the search databases and snowballing, 774 articles were excluded because they were either duplicates or deemed ineligible based on the title and/or abstract contents (Figure 3.1). Full-texts of 46 studies were retrieved and assessed for eligibility based on the predetermined inclusion criteria and five articles were deemed ineligible. The remaining 41 studies met the inclusion criteria and quality assessment. These were distributed across Europe (13/41; 31.7%), Africa (10/41; 24.4%), South America (9/41; 21.9%), Asia (8/41; 19.5%), and North America (1/41; 2.43%). Of these studies, 46.3% (19/41) reported experimental infection while 53.7% (22/41) reported natural infections.

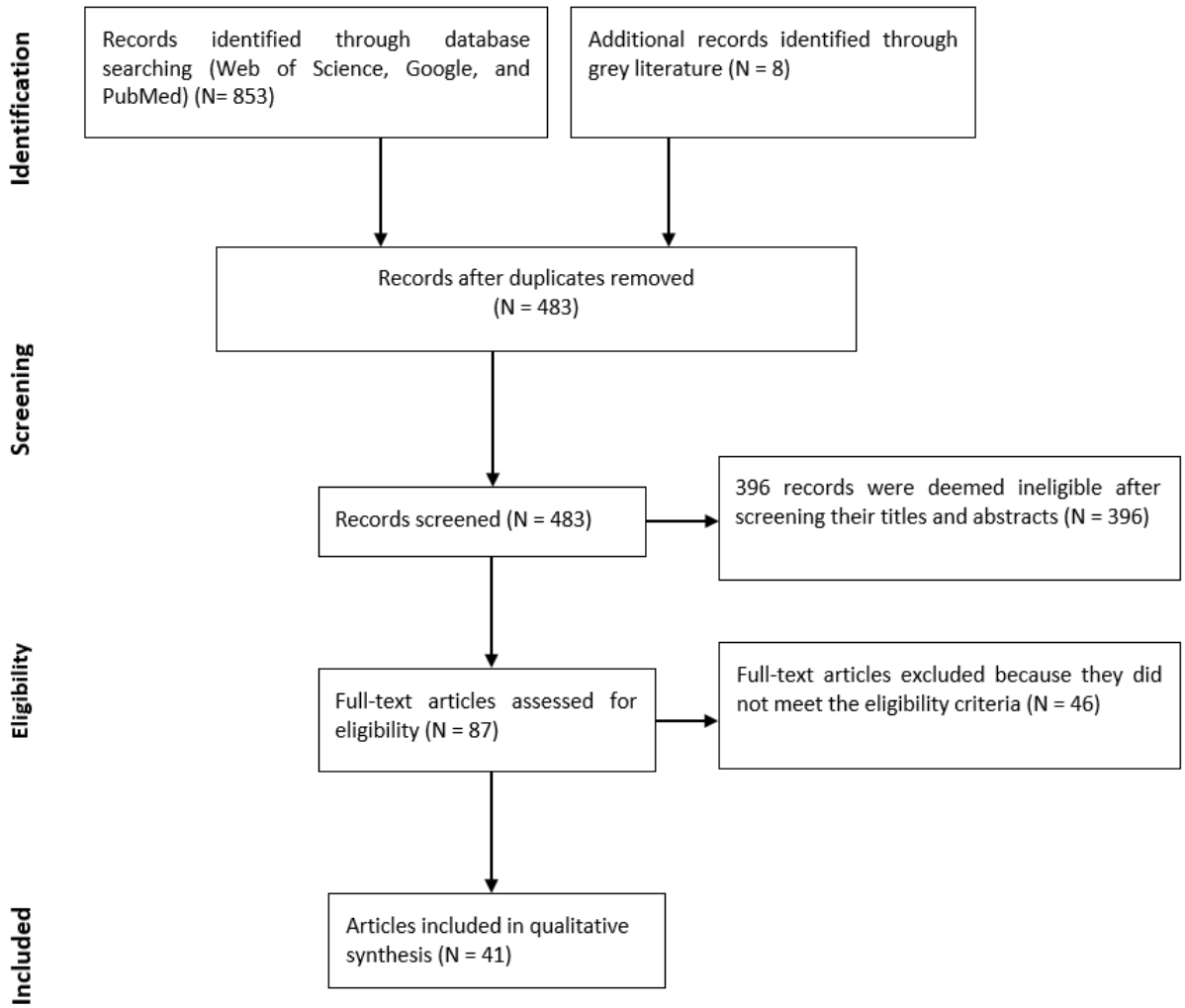

Figure 3.1 PRISMA diagram.

3.4.2. Overall experimental susceptibility of lymnaeid snails to *Fasciola* spp.

Experimental infections of *Fasciola* species in lymnaeid snails were conducted in Europe (France and Sweden), and in Africa (Egypt). In Asia, experiments were conducted in Iran while in South America experiments were conducted in Argentina, Colombia, and Cuba (Table 3.1). A total of 5575 freshwater lymnaeid snails were collected to be subjected to experimental infections with *Fasciola* spp. between 2004 and 2023. Of these freshwater snails, 2815 (50.45%) were infected with either *F. hepatica* or *F.*

gigantica. The lymnaeid snail species involved in the experiments were *Galba (G.) truncatula, G. cubensis, G. cousini, G. viatrix var ventricose, G. neotropica, Pseudosuccinea (P.) columella, Radix (R.) auricularia, R. natalensis, Lymnaea (L.) fuscus, L. palustris,* and *Omphiscola (O.) glabra*. Experimental infection rates ranged from 7.89% in *L. fuscus* infected with *F. hepatica* to 80.37% in *G. cubensis* infected with *F. gigantica* (Table 3.3). Of the 11 snail species mentioned above, nine were successfully infected with *F. hepatica* and four with *F. gigantica*. Only *P. columella* and *G. truncatula* had records of successful experimental infections with both *Fasciola* species (Table 3.3). The overall pooled experimental infection rate of lymnaeid snails with *Fasciola* spp. was 50% (95% CI: 42-58%) (Figure 3.1). High heterogeneity in the results was revealed by the quality effects model (Q = 1425, p<0.001), with I^2 = 96% (Figure 3.1). The meta-regression model demonstrated a statistically significant overall p-value of 0.05 for experimental investigations, suggesting that the predictors collectively account for the variation in prevalence (Table 3.4). The R-squared change was 0.119, indicating that 11.9% of the variance in the prevalence data could be explained by the predictors. However, the prevalence level was not significantly impacted by individual factors (Table 3.4).

Table 3.3 Frequency of lymnaeid snails experimentally infected with *Fasciola gigantica* and *F. hepatica* in the past 20 years.

Snail species	No. of studies	No. infected	No. examined	Diagnostic tool (%)		Species of infection		Overall prevalence (%)
				Shedding	Dissection	*Fasciola hepatica*	*Fasciola gigantica*	
Galba (G.) truncatula	10	1196	2155	72.03	53.47	1155	41	55.49
G. cubensis	1	217	270	-	80.37	-	217	80.37
G. cousini	1	34	100	-	34.00	34	-	34.00
G. neotropica	1	50	159	-	31.45	50	-	31.45
Radix (R.) auricularia	1	115	151	76.16	-	-	115	76.16
R. natalensis	2	83	308	-	26.95	83	-	26.95
Pseudosuccinea (P.) columella	8	945	1963	40.71	52.02	848	97	48,14
Lymnaea (L.) fuscus	1	9	114	-	7.89	9	-	7.89
L. palustris	1	40	119		33.61	40	-	33.61
L. viatrix var. ventricosa	1	73	133	-	54.89	73	-	54.89
Omphiscola (O.) glabra	1	53	103	-	51.46	53	-	51.46
Total	-	2815	5575	52.74	49.81	2386	429	50.45

Table 3.4 Meta-regression of overall and subgroups for individual variables on prevalence of *Fasciola* infections in snail intermediate host in the past 20 years.

Model		Unstandardized Coefficients		Standardized Coefficients			R^2	95.0% Confidence Interval for B	
		B	Std. Error	Beta	t	Sig.		Lower Bound	Upper Bound
Natural infection	Continents	.124	.364	.069	.341	.736		-.622	.870
	*Fasciol*a sp.	1.616	.964	.362	1.676	.105		-.362	3.595
	Diagnostic test	-.411	.617	-.116	-.666	.511		-1.677	.855
	period	-1.883	.639	-.458	-2.947	.007		-3.195	-.572
	Combined effect					.001	.474		
Experimental infection	Continents	.167	.136	.234	1.227	.226		-.107	.441
	Fasciola sp.	-.533	.476	-.182	-1.120	.269		-1.493	.427
	Diagnostic test	-.033	.344	-.015	-.097	.923		-.727	.661
	period	.672	.332	.391	2.021	.050		.002	1.343
	Combined effect					.05	.194		

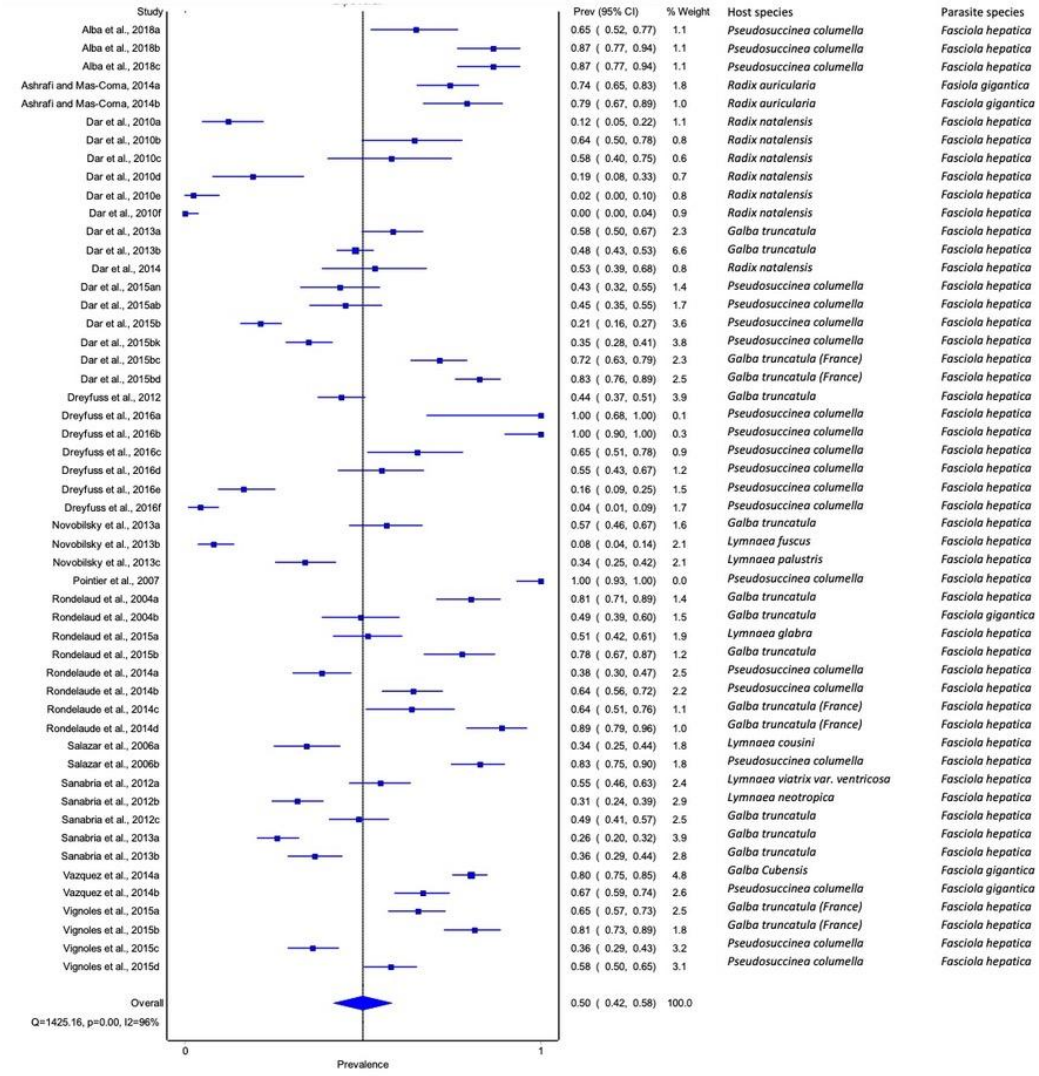

Figure 3.2 Forest plot showing the overall experimental infection rates of *Fasciola hepatica* and *F. gigantica* in lymnaeid snails.

3.4.2.1. Experimental infectivity of lymnaeid snails by *Fasciola* spp. by continent

The highest pooled infection rate for *Fasciola* spp. (Figures 3.3A-C) was 64% recorded in South America (95% CI: 48-78%, Figure 3.3C), followed by Africa at 48% (95% CI: 35-61%, Figure 3.3B) and Europe at 42% (95% CI: 28-56%, Figure 3.3A). Asia did not qualify for the meta-analysis. Heterogeneity was recorded at I^2= 96% for all three reported continents.

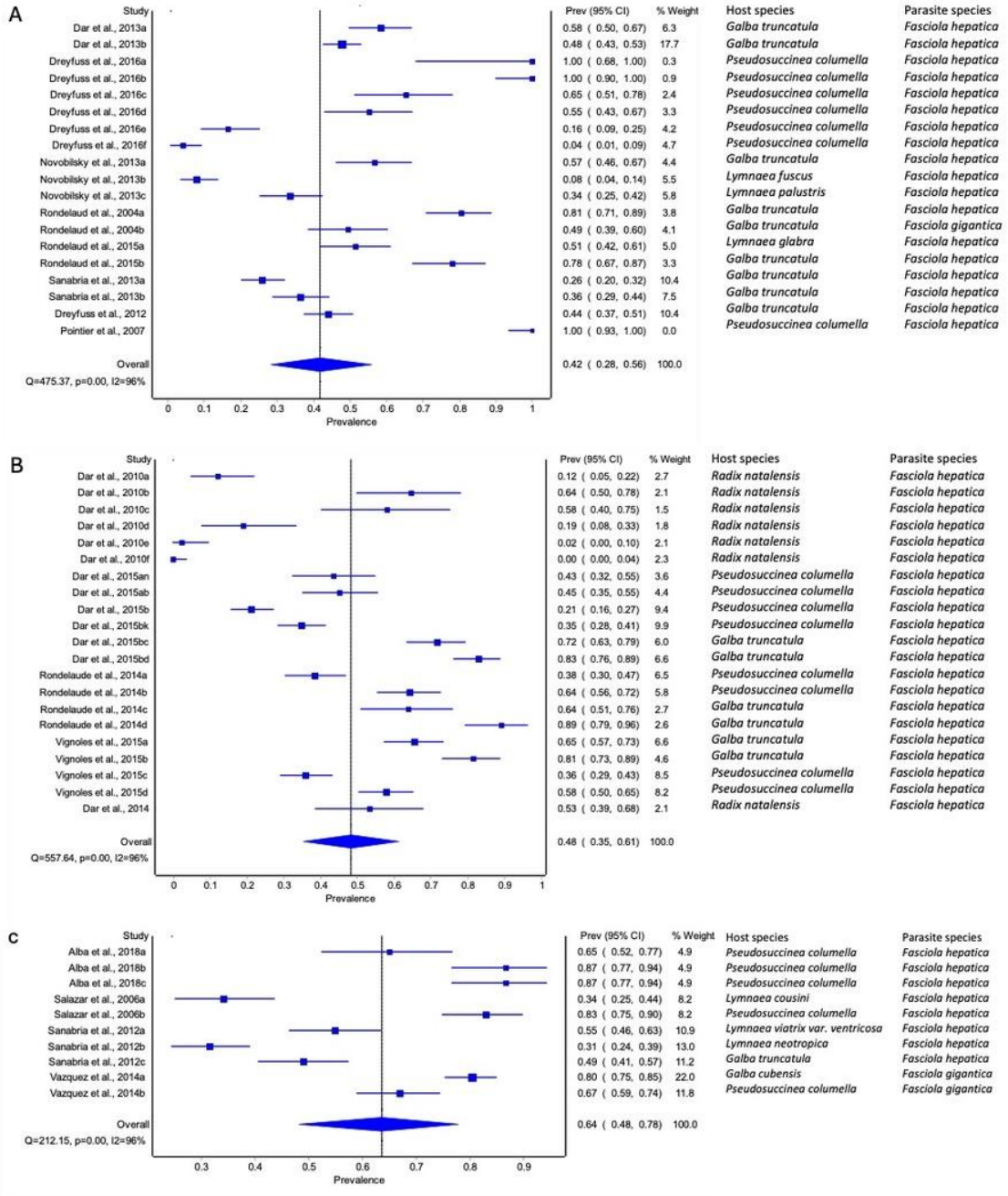

Figure 3.3 Forest plots of experimental infection rates of *Fasciola hepatica* and *Fasciola gigantica* in lymnaeid snails from (A) Europe, (B) Africa, and (C) South America.

3.4.2.2. Experimental infection rate of lymnaeid snails by *Fasciola* spp. per snail species

Only three of the 11 recorded lymnaeid snail species qualified for meta-analysis, viz *G. truncatula*, *R. natalensis,* and *P. columella*. The estimated pooled infection rate of lymnaeid snails infected with *F. hepatica and F. gigantica* is illustrated in figures 3.4A-C. *Galba truncatula* infected with *F. hepatica* (96.57%, 1155/1196) and *F. gigantica* (3.43%, 41/1196) (Table 3.3) showed a pooled infection rate of

37% (95% CI: 17-59%, Figure 3.4A), *R. natalensis* with *F. hepatica* with a prevalence of 21% (95% CI: 03-48%, Figure 3.4C) and *P. columella* with both *F. hepatica* (43.20%, 848/1963) and *F. gigantica* (4.64%, 91/1963) (Table 3.3) with a pooled infection rate of 47% (95% CI: 33-61%, Figure 3.4B). *Galba truncatula* and *P. columella* showed a heterogeneity of I^2 = 97% while *R. natalensis* demonstrated a heterogeneity of I^2 = 95%.

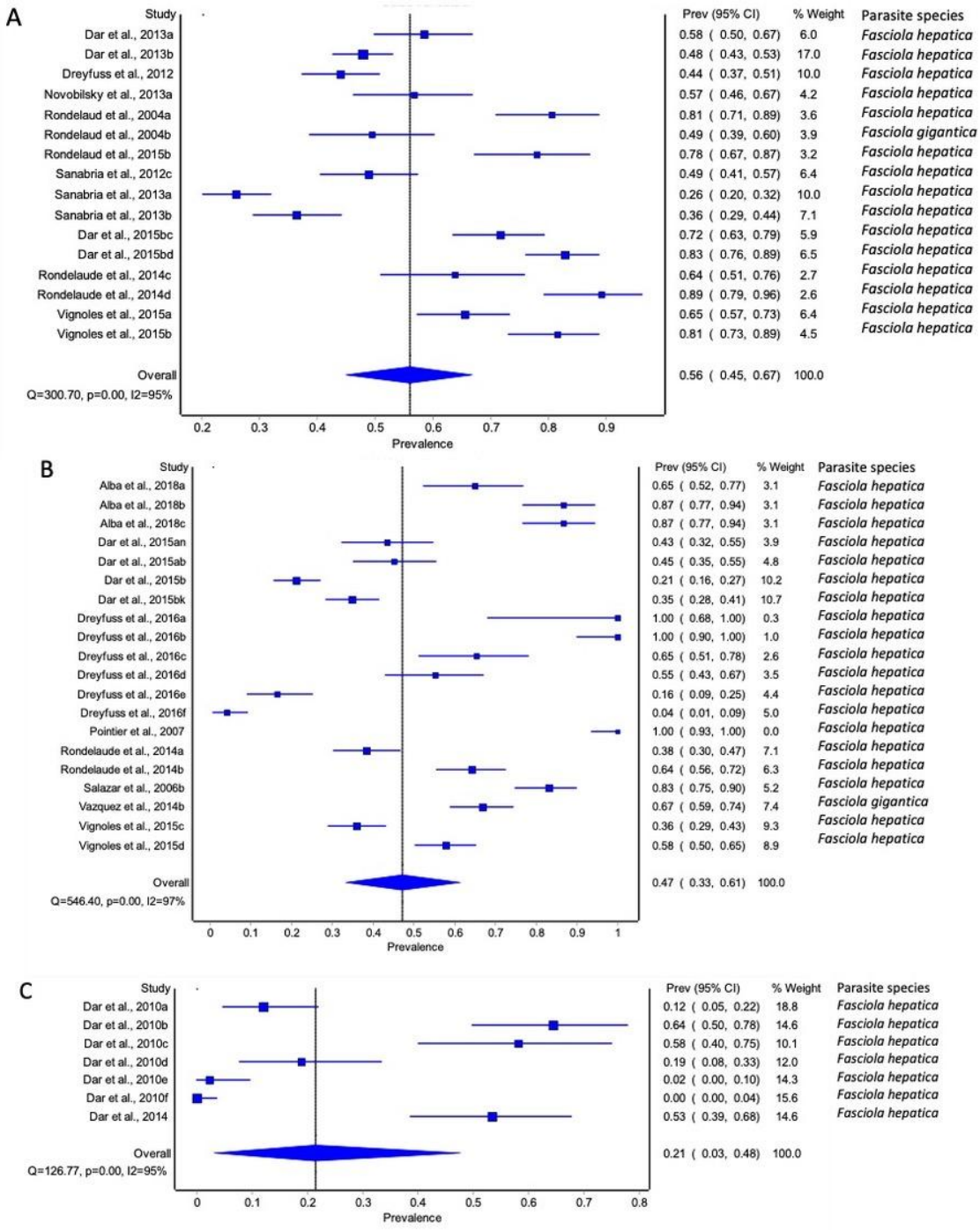

Figure 3.4 Forest plots of experimental infection rates of *Fasciola* species based on the intermediate hosts: (A) *Galba truncatula*, (B) *Pseudosuccinea columella,* and (C) *Radix natalensis.*

3.4.2.3. Experimental infection rate of lymnaeid snails by parasite species

The pooled infection rate of *Fasciola* spp. to lymnaeid snails was high with *F. gigantica* at 73% (95% CI: 61-84%) and low in *F. hepatica* at 47% (95% CI: 38-55%). However, heterogeneity was higher for *F.*

hepatica (Q=1234.09, *p*<0.001; I^2 = 96%, Figure 3.5A) compared to *F. gigantica* (Q=31.73, *p*<0.001; I^2 = 87%, Figure 3.5B).

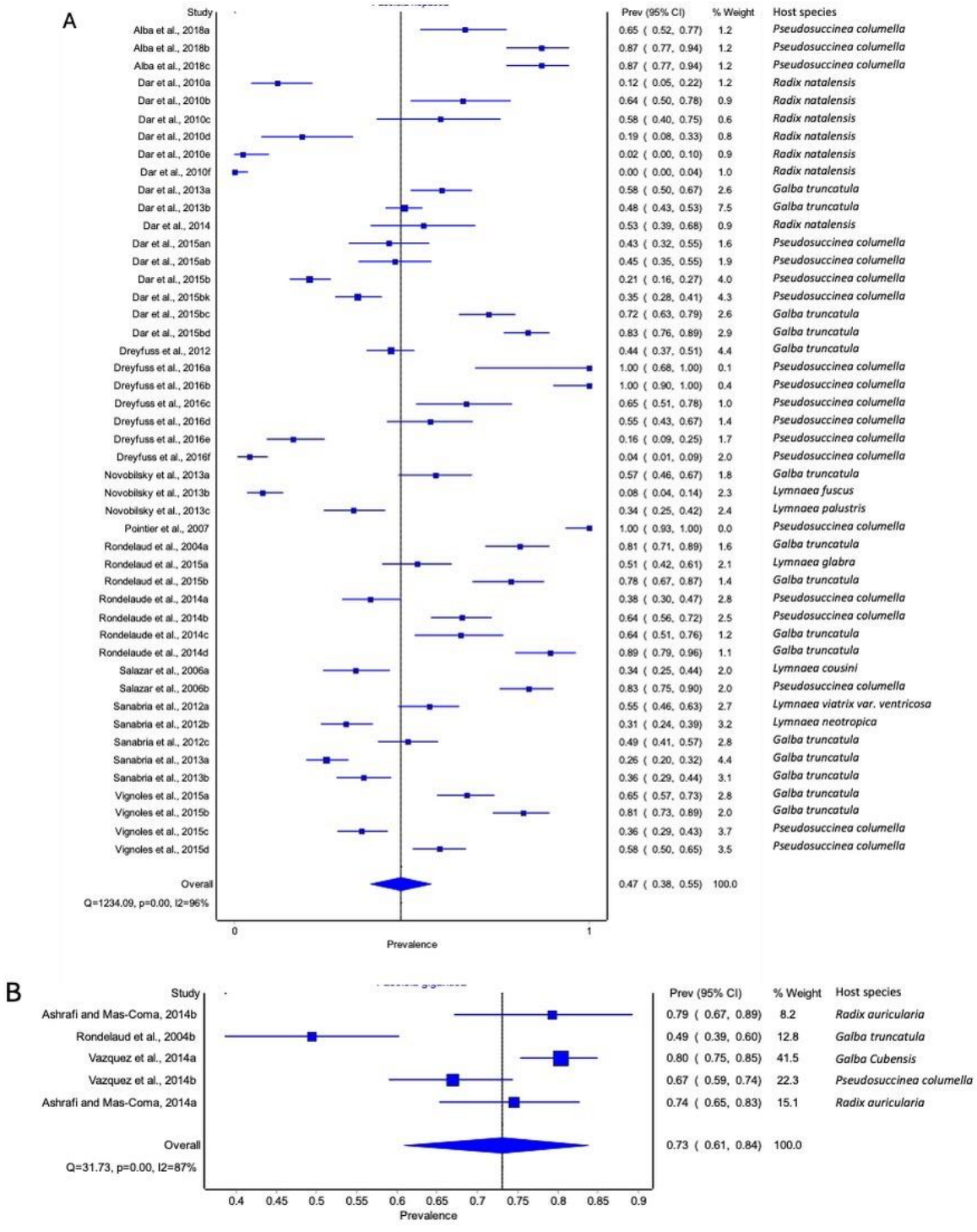

Figure 3.5 Forest plots showing experimental infection rates in lymnaeid snails based on *Fasciola* species: (A) *Fasciola hepatica* and (B) *Fasciola gigantica.*

3.4.2.4. Experimental infectivity of lymnaeid snails by *Fasciola* spp. per method of detection

The pooled experimental infection rate of *Fasciola* snails in lymnaeid snails was higher using cercariae shedding 52% (95% CI: 32-72%, Figure 3.6A; Table 3.3) compared to 49% (95% CI: 40-59%, Figure 3.6B; Table 3.3) using snail dissection. Heterogeneity was documented as I^2 = 97% and I^2 = 96% for shedding and dissection, respectively.

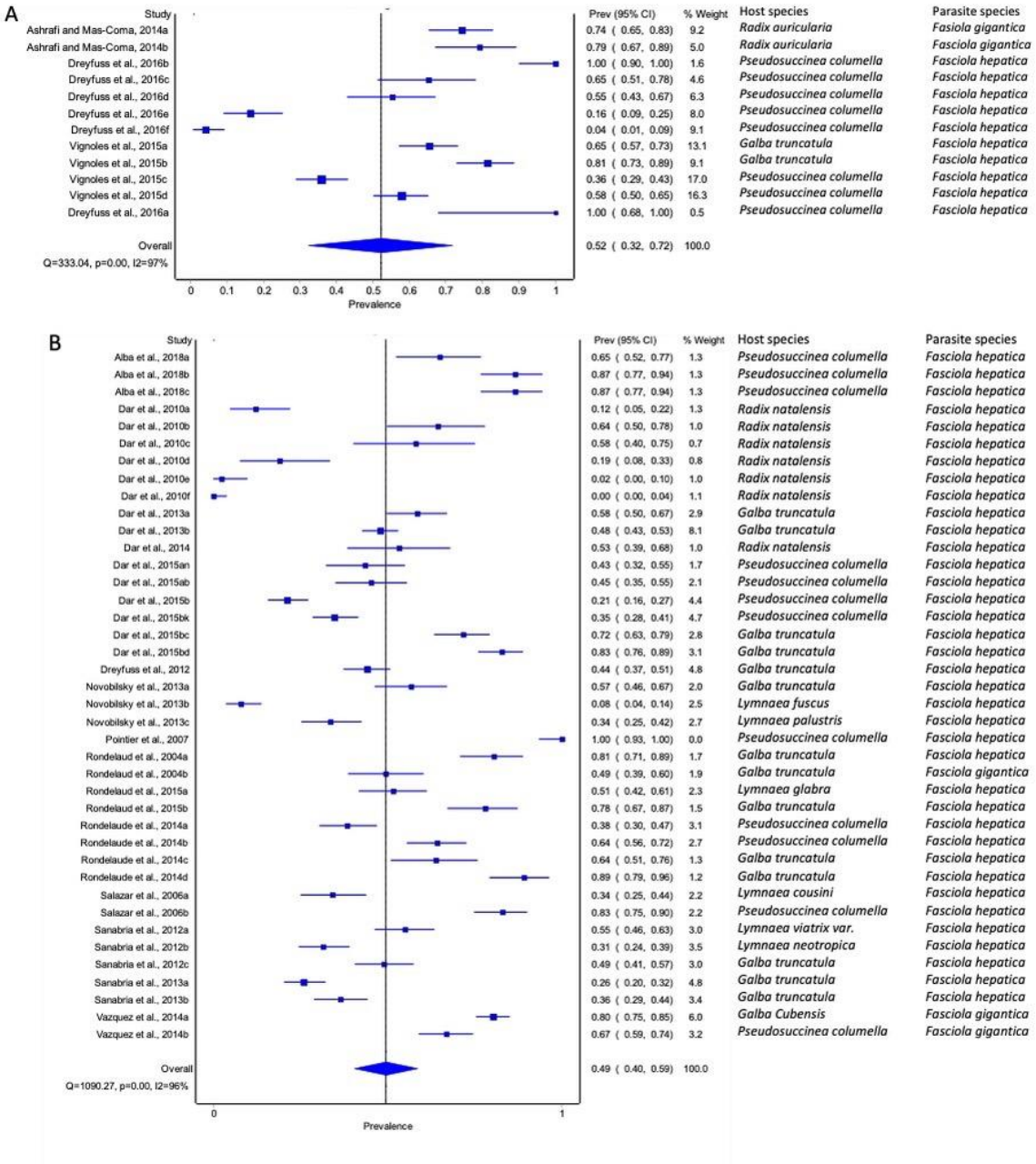

Figure 3.6 Forest plots of experimental infection rates of *Fasciola hepatica* and *Fasciola gigantica* based on detection technique (A) shedding and (B) dissection.

3.4.2.5. Experimental infection rate of lymnaeid snails by *Fasciola* spp. based on years

The estimated pooled infection rate of *Fasciola* spp. experimentally infected snails for 20 years is shown in figure 3.7 The pooled infection rate in the decade 2004-2013 was 41% (95% CI: 29-53%, Figure 3.7A), which was lower than the 57% (95% CI: 47-68%, Figure 3.7B) pooled infection rate documented between 2014-2023. Heterogeneity was I^2 = 96% for both periods (Figures 3.7A-B).

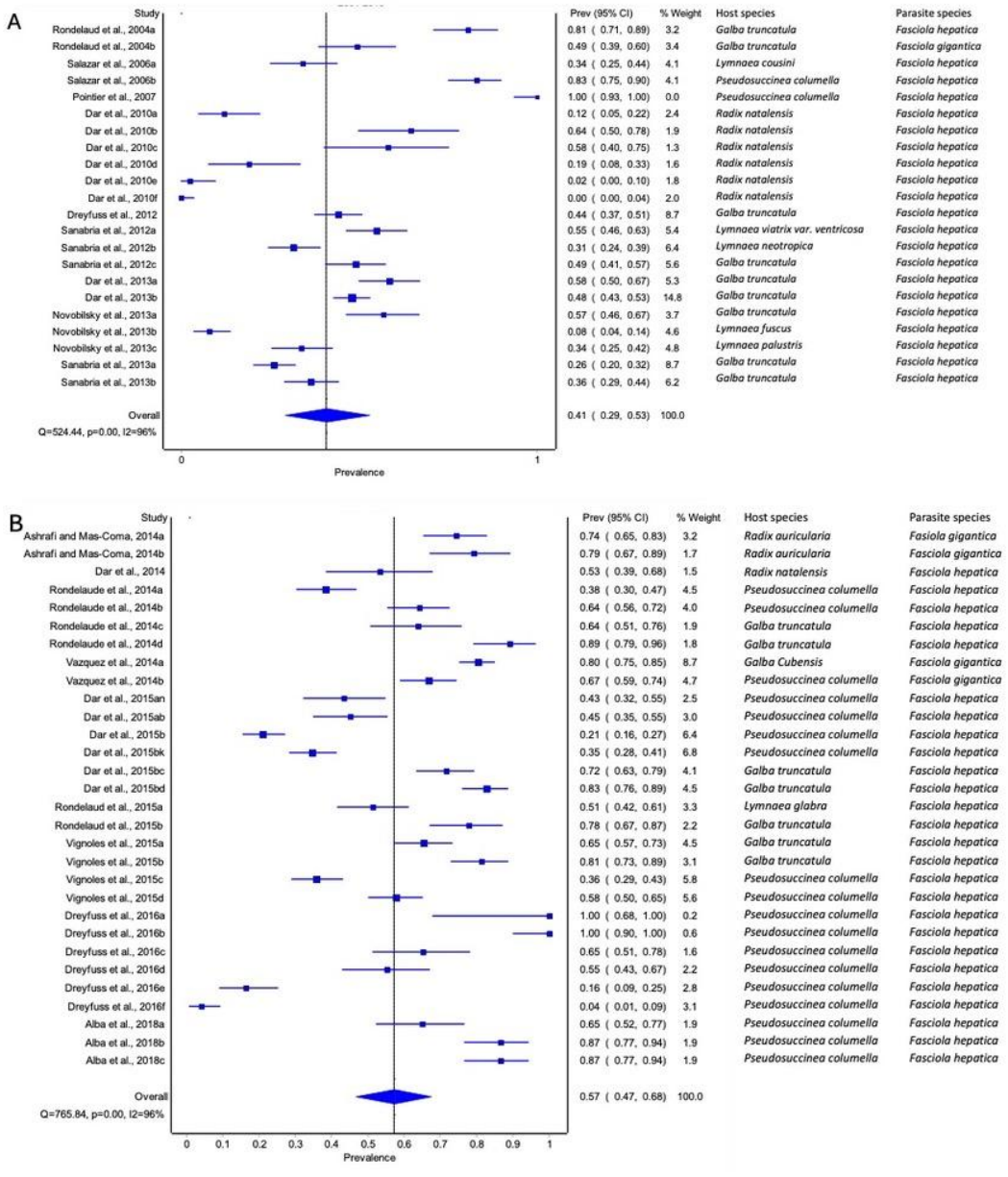

Figure 3.7 Forest plots showing experimental infection rate of lymnaeid snails with *Fasciola* species from (A) 2004-2013 and (B) 2014-2023.

3.4.2.6. Publication bias of studies reporting on experimental infections of *Fasciola* spp. in lymnaeid snails

Figure 3.8 shows the funnel plot which is asymmetrical in shape and depicts publication bias which may result from either a small sample size or publication bias within articles.

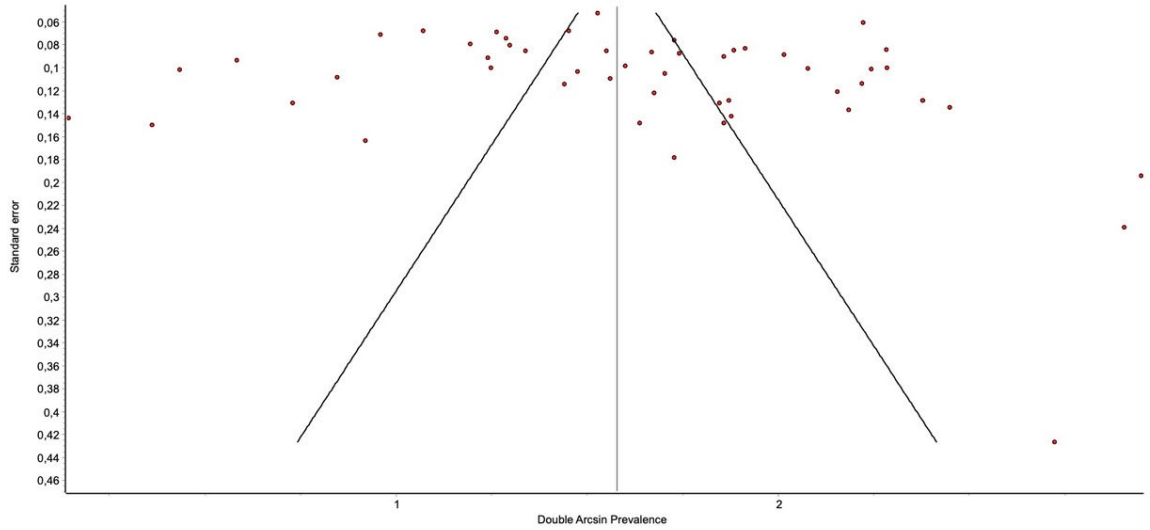

Figure 3.8 Funnel plot showing overall publications on experimental infections of *Fasciola* species in their intermediate host snail.

3.4.3. Natural infections of lymnaeid snails by *Fasciola* spp.

Field prevalence data for lymnaeid snails infected by *Fasciola* spp. was recorded in South America (Colombia, Cuba, Ecuador, and Argentina), North America (Mexico), Africa (Egypt and South Africa), Europe (Ireland, Sweden, Spain, Poland, and France) and Asian (Iran, India, South Korea, China, and Vietnam) (Table 3.2). Of the 44002 field-collected lymnaeid snails, 5656 were positive for *Fasciola* infections with an overall prevalence of 12.85%. Prevalence of lymnaeid snails naturally infected with *F. hepatica* and *F. gigantica* ranged from 0-76.9% in *L. fuscus* and *G. bulimoides*, respectively (Table 3.5). The 17 infected snail species were *G. truncatula, G. cousini, G. viatrix, G. humilis, G. schirazensis, G. bulimoides, P. columella, R. cucunorica, R. gedrosiana, R. peregra, R. auricularia, R. acuminata, L. palustris, L. ollula, L. fuscus, O. glabra, and Austropeplea (A.) viridis* (Table 3.5). Only *P. columella* and *G. truncatula*, however, qualified for meta-analysis. The estimated overall pooled prevalence for natural infections in lymnaeid snails was recorded at 6% (95% CI: 0-22%) (Figure 3.9). A significantly high heterogeneity was recorded Q = 15220.37 (p<0.001), with I^2 = 100% (Figure 3.9).

The R-squared change was 0.474, and the p-value was significant at 0.001 (Table 3.4). This suggests that the predictors have a greater impact and that the model accounts for 47.4% of the variation in prevalence. Regarding each of the specific factors, the p-values for the continents, diagnostic tests, and *Fasciola* species were all greater than 0.05 for natural snail infection by *Fasciola*, indicating no significant effects. Nonetheless, the p-value for the time frame was 0.007, suggesting that time had a significant effect on the prevalence of *Fasciola* infection (Table 3.4).

Table 3.5 Frequency of lymnaeid snails naturally infected with *F. gigantica* and *F. hepatica* in the past 20 years.

Snail species	No. of studies	No. examined	No. infected	Diagnostic tool (%)			Species of infection		Overall prevalence (%)
				Dissection	Molecular	Shedding	*Fasciola hepatica*	*Fasciola gigantica*	
Galba (G.) truncatula	7	8617	408	3.25	12.87	-	408	-	4.73
G. bulimoides	1	670	515	76.87	-	-	515	-	76.87
G schirazensis	2	1517	96	-	6.33	-	96	-	6.33
G. humilis	1	3372	2537	75.24	-	-	2537	-	75.24
G. viatrix	1	68	22	-	61.76	2.94	22	-	32.35
G. cousini	1	521	68	13.05	-	-	68	-	13.05
Radix (R.) acuminata	2	2477	250				-	250	10.09
R. auricularia	1	496	12	-	2.45	-	-	12	2.45
R. peregra	1	167	62	-	37.13	-	62	-	37.13
R. gedrosiana	1	2543	298	-	11.72	-	-	298	11.72
R. cucunorica	1	409	179	-	43.77	-	179	-	43.77
Pseudosuccinea (P.) columella	4	976	278	-	32.51	17.25	168	110	28.48
Lymnaea (L.) fuscus	1	130	0	-	0	-	-	-	0
R. ollula	1	15	5	-	33.33	-	5	-	33.33
L. palustris	1	668	1	-	0.15	-	1	-	0.15
Omphiscola glabra	2	5980	102	1.26	21.54	-	102	-	1.71
Austropeplea (A.) viridis	2	15376	125	-	0.81	-	1	124	0.81
Total	**-**	**44002**	**5656**	**20.72**	**4.31**	**15.69**	**4576**	**1080**	**12.85**

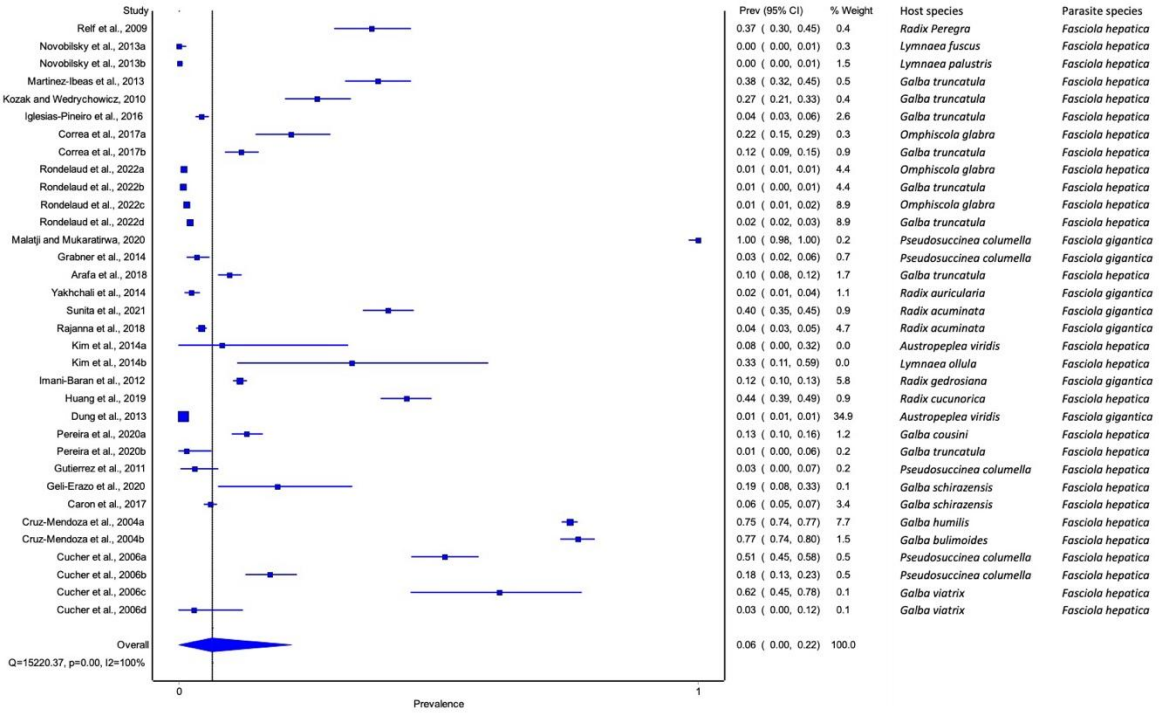

Figure 3.9 Forest plot showing the overall rates of natural infections of lymnaeid snails with *Fasciola* spp. worldwide.

3.4.3.1. Prevalence of natural infections of lymnaeid snails by *Fasciola* spp. per continent

The estimated pooled prevalence estimates for lymnaeid snails naturally infected by *Fasciola* spp. globally is illustrated in figures 3.10A-C. The prevalence recorded per continent was 2% in Asia (95% CI: 0-17, figure 3.10A), 2% in Europe (95% CI: 0-6%, Figure 3.10B), and 11% in South America (95% CI: 0-29%, Figure 3.10C). Africa and North America data did not qualify for meta-analysis. Heterogeneity results were I^2 = 98%, I^2 = 99%, and I^2 = 100% for South America, Europe, and Asia, respectively (Figures 3.10A-C).

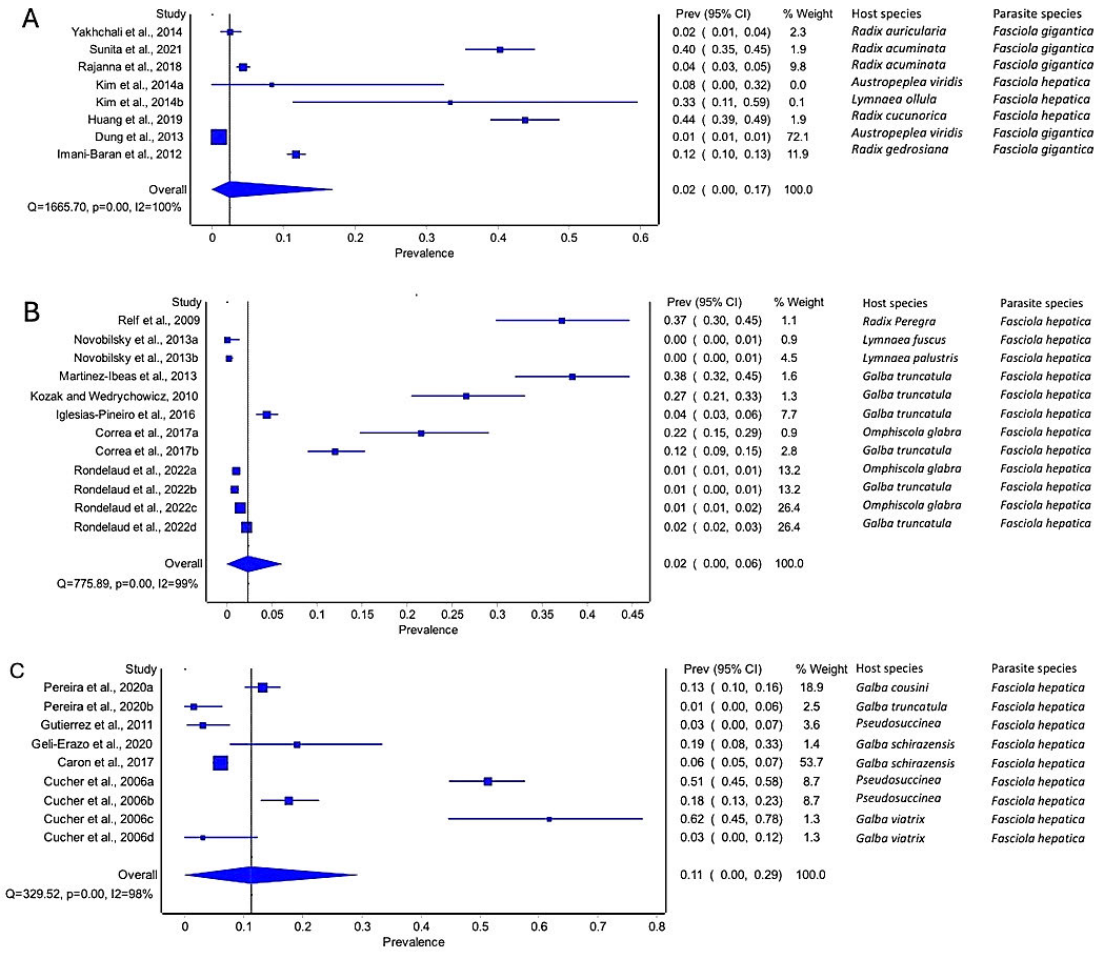

Figure 3.10 Forest plots of the infection rates of natural infections of *Fasciola* species in lymnaeid snails based on continents: (A) Asia, (B) Europe, and (C) South America.

3.4.3.2. Prevalence of natural infections of lymnaeid snails by *Fasciola* spp. per snail species

Pseudosuccinea columella and *G. truncatula* were the only snail species that qualified for meta-analysis. The average prevalence of natural infections of *P. columella* infected with *F. hepatica* was 17.21% (168/976) and *F. gigantica* was 11.27% (110/976) (Table 3.5), with an overall pooled prevalence of 26% (95% CI: 0-72%, Figure 3.11A). *Galba truncatula* infected by *F. hepatica* recorded a pooled prevalence of 4% (95% CI: 0-10%, Figure 3.11B). Heterogeneity results were documented as I^2 = 99% for both lymnaeid species.

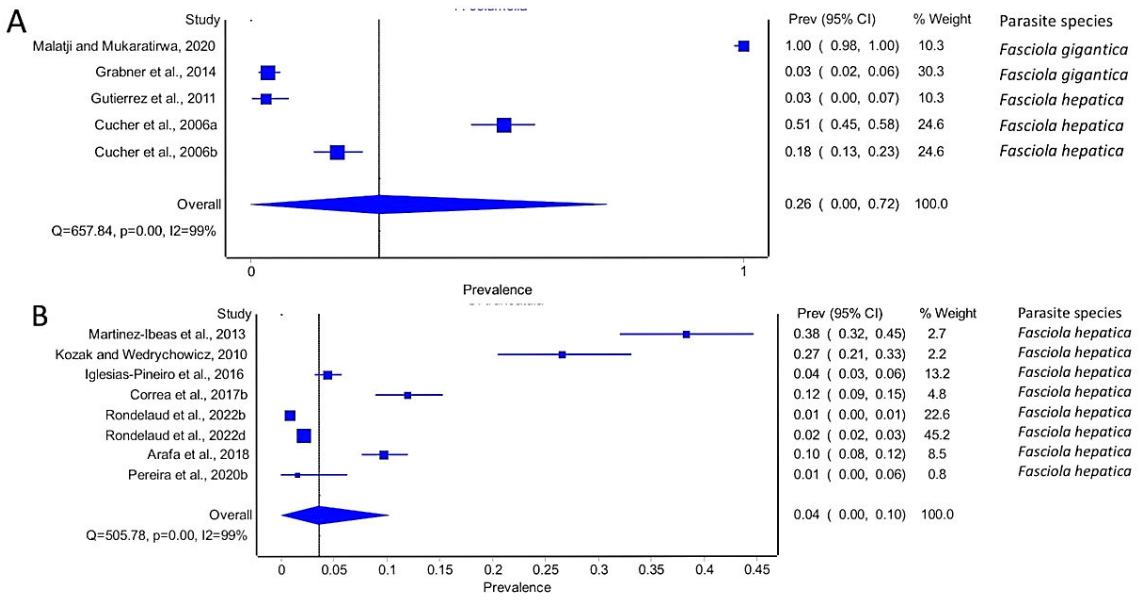

Figure 3.11 Forest plots of rates of natural infections of *Fasciola* species based on intermediate snail host (A) *Pseudosuccinea columella* and (B) *Galba truncatula.*

3.4.3.3. Prevalence of natural infections of lymnaeid snails by *Fasciola* spp. per parasite species

The estimated pooled infection rates of the individual *Fasciola* spp. naturally infecting lymnaeid snails were reported in figures 9A-B. *Fasciola gigantica* showed a prevalence of 2% (95% CI: 0-18%, Figure 3.12A) and figure 3.12B shows a prevalence of 12% (95% CI: 0-30%) for *F. hepatica* natural infections. The heterogeneity was significantly high for both *F. gigantica* (Q = 1873.85, p<0.001, I^2 = 100%) and *F. hepatica* (Q = 11616.37, p<0.001, I^2 = 100%) natural infections (Figures 3.12A-B).

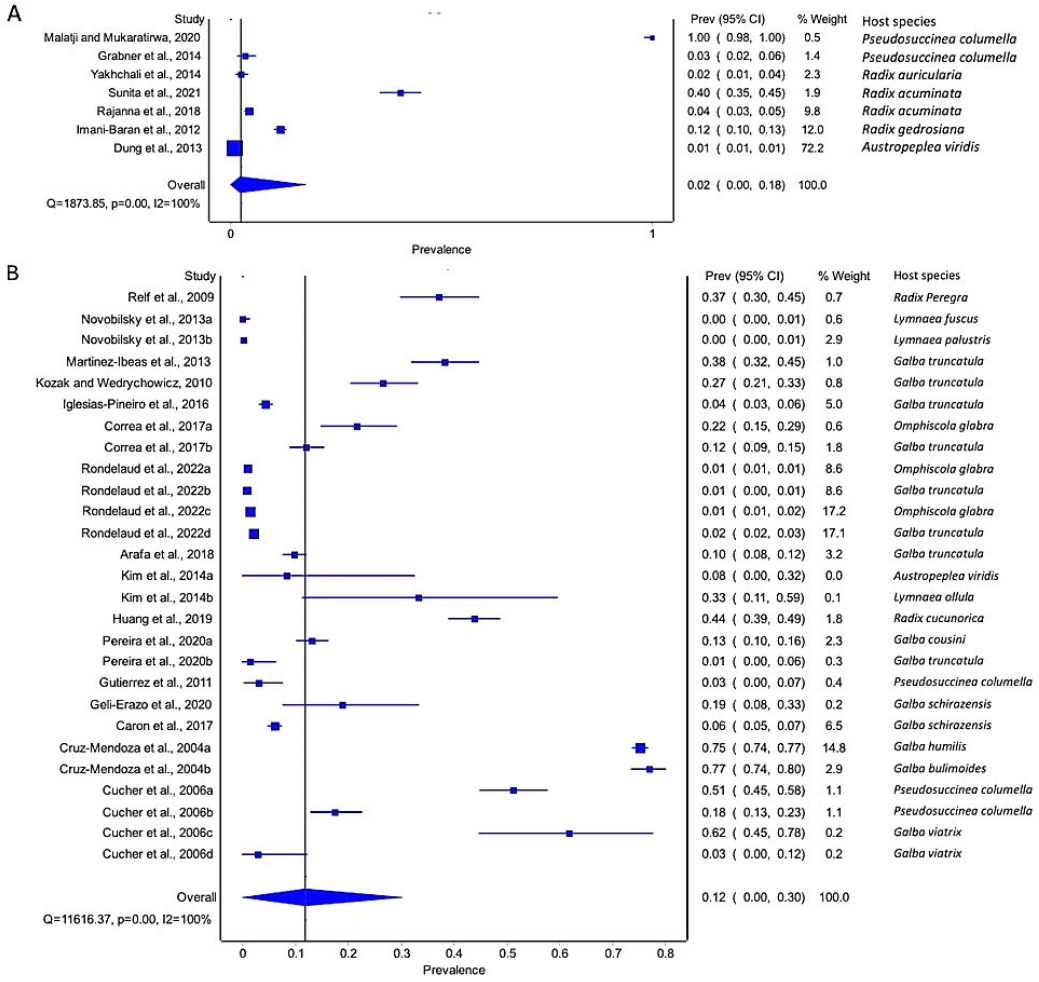

Figure 3.12 Forest plots showing the rates of infection of *Fasciola* spp. in naturally infected lymnaeid snails based on species (A) *Fasciola gigantica,* and (B) *Fasciola hepatica.*

3.4.3.4. Prevalence of natural infections of lymnaeid snails by *Fasciola* spp. per method of detection

Detection of natural *Fasciola* spp. infections in lymnaeid snails was based on molecular (Polymerase chain reaction (PCR) and Restriction fragment length polymorphism (RFLP)), dissection, and cercariae shedding techniques (Table 3.2; Table 3.5). Prevalence based on the diagnostic tool utilized raged from 20.72% by dissection to 2.45% by RFLP (Table 3.5). Molecular and dissection techniques were the only techniques that qualified for meta-analysis (Figures 3.13A-B). Low pooled prevalence was recorded with molecular techniques (4%, 95% CI: 0-17%) (Figure 3.13) compared to the dissection technique at 12% (95% CI: 0-40%) (Figure 3.13). Recorded heterogeneity was I^2 = 100% for dissection and I^2 = 99% for molecular technique (Figure 3.13).

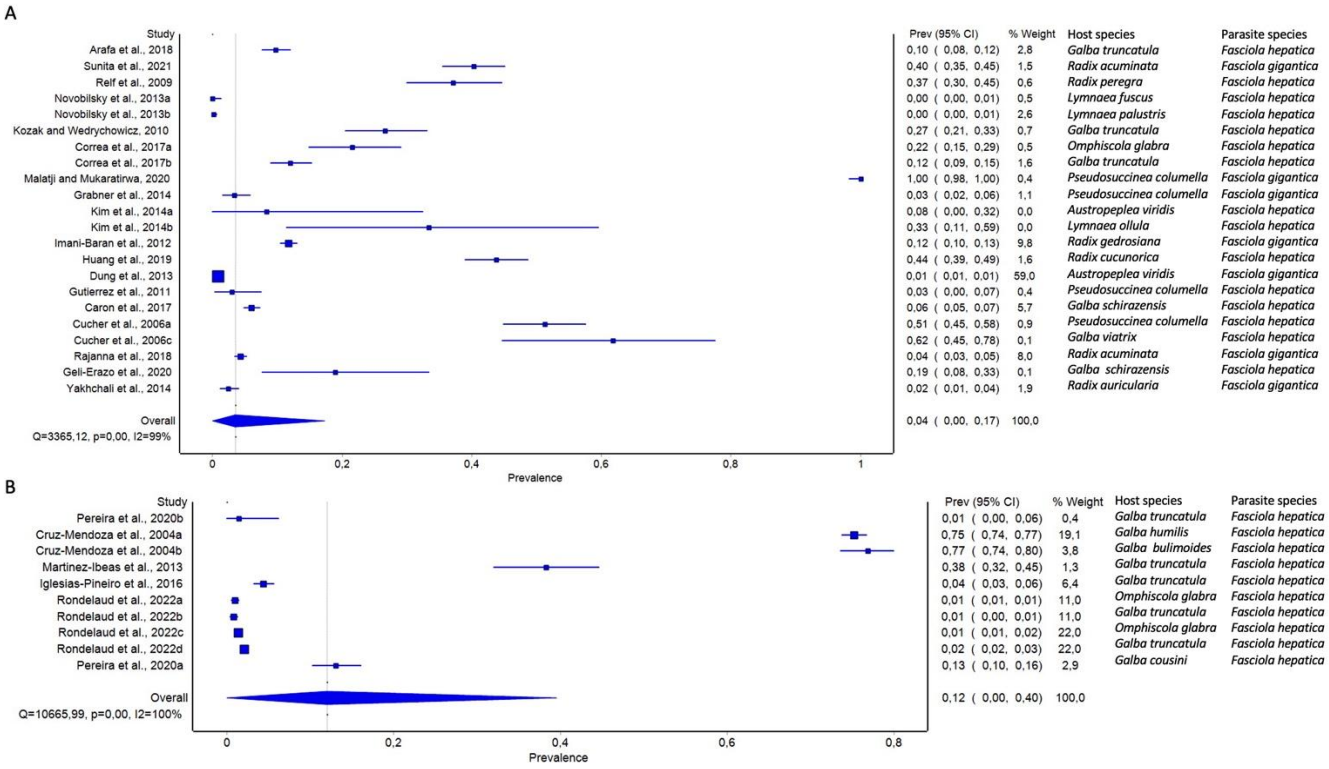

Figure 3.13 Forest plots showing the rates of natural infections of *Fasciola* species in lymnaeid snails recorded using (A) molecular techniques and (B) dissection.

3.4.3.5. Prevalence of natural infections of lymnaeid snails by *Fasciola* spp. by years

Pooled prevalence for natural *Fasciola* spp. infections in their IHs hosts was 9% (95% CI: 0-70%, Figure 3.14A) in the decade 2004-2013, which was higher than 3% (95% CI: 0-9%) (Figure 3.14B) in 2014-2023 (Figures 3.14A-B). Heterogeneity was I^2 = 100% for 2004-2013 (Figure 3.14B) and I^2 = 99% for 2014-2023 (Figure 3.14B).

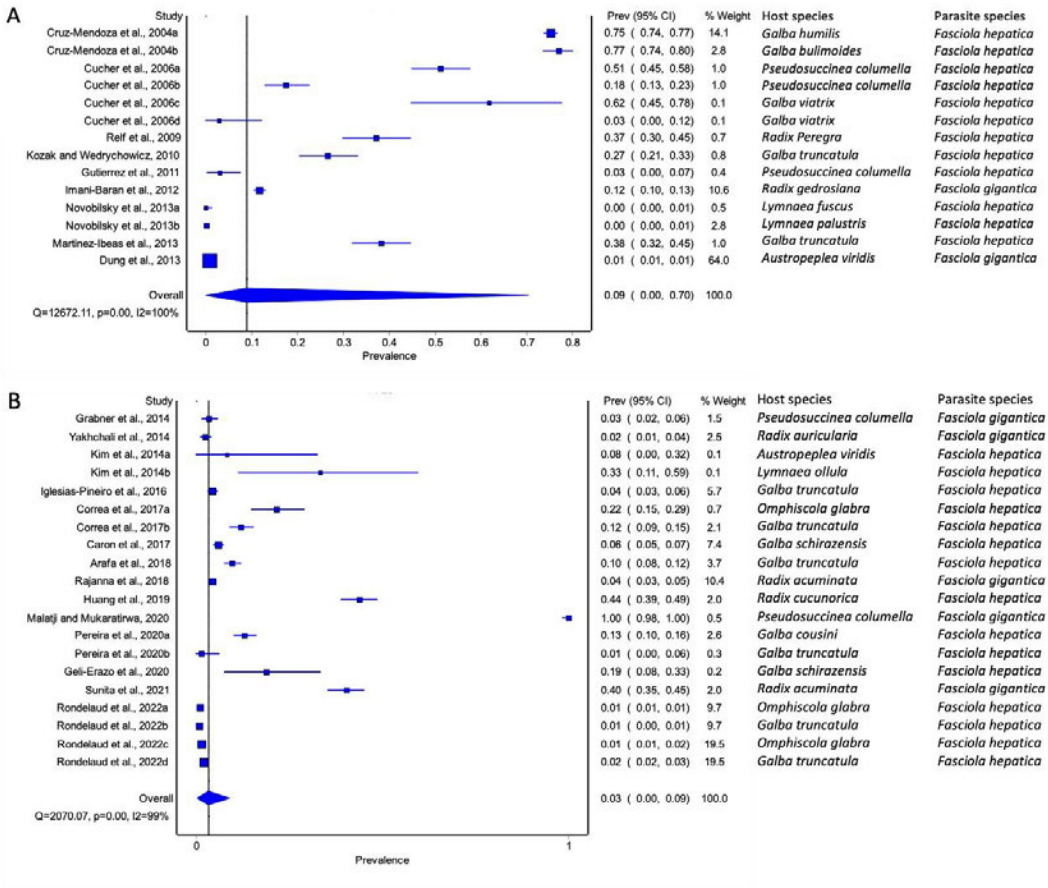

Figure 3.14 Forest plots of the rates of infection of lymnaeid snails naturally infected with *Fasciola* species from (A) 2004-2013 and (B) 2014-2023.

3.4.3.6. Publication bias of studies reporting on the natural infections of *Fasciola* spp. in lymnaeid snail spp.

Funnel plots showed an asymmetric funnel shape (scattered points) (Figure 3.15), which indicates the presence of publication bias which may be due to content in the articles or small sample size.

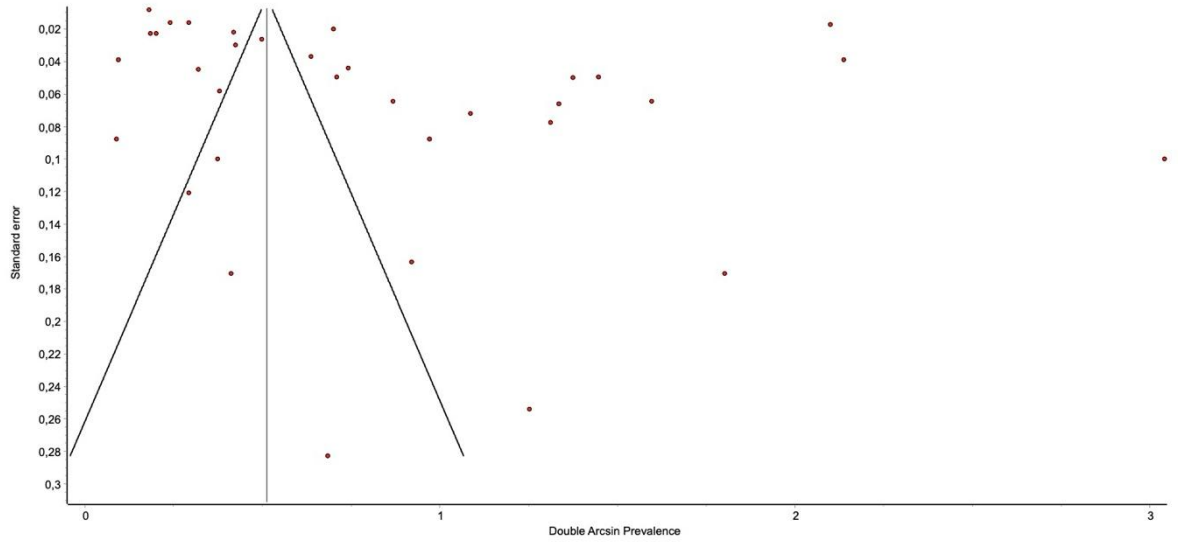

Figure 3.15 Funnel plot of overall publications on natural *Fasciola* spp. on their intermediate snail hosts.

3.5. Discussion

The results showed that the overall pooled prevalence of natural infections of *Fasciola* species in lymnaeids was significantly lower than the infection rate recorded based on experimental infections. This was to be expected as conditions for experimental infections are made optimum and controlled in laboratory settings compared to natural environments where various uncontrolled variables and stressors may hinder successful infection of the intermediate host. Infections under laboratory settings have been shown to be a valuable approach in the investigation of compatibility differences in IHs since they allow for the control and management of variables that can influence the infection outcome (Sorensen and Minchella, 2001; Vázquez et al., 2019a). These variables include snail shell size (Dar et al., 2014a; Dreyfus et al., 2016), the infective parasite dose (Sorensen and Minchella, 2001; Pointier et al., 2007), optimum conditions (optimum temperature, constant light/dark periods, abundant food, pollution free water, dissolved essential minerals) (de Kock et al., 1986; Dar et al., 2014a; Dar et al., 2015a; Dreyfus et al., 2016), deliberate exposure to miracidia (Dreyfus et al., 2012; Ashrafi and Mas-Coma, 2014) amongst others. This control of variables leads to a higher infection rate as the probability of one snail getting infected strictly depends on its suitability as IH and its surrounding ecology (Vázquez et al., 2015). Prepelitchi et al. (2011) also noted that snail populations are subjected to rigorous ecological constraints due to large environmental temporal fluctuations. Additionally, parasites may die before finding an appropriate IH in a natural environment, especially those with a narrow tolerance to specific physicochemical factors (Vázquez et al., 2015).

Overall, while the model for natural infection demonstrates stronger explanatory power (R-square = 0.474), the mixed results point to the complexity of factors influencing *Fasciola* infection prevalence in snails, which requires further investigation with more refined models or additional data. Nyagura et al. (2024) claimed that when several factors are combined, the variability becomes much more prevalent, suggesting that understanding how these factors interact is essential to comprehending the complexity of epidemiological results. Instead of a single determinant, the results of this study corroborate the idea that the epidemiology of snail-borne parasites is typically driven by a confluence of factors that interact significantly (Hajipour et al., 2021).

The wide global distribution of lymnaeid snails is of great concern as the geographic distribution of these *Fasciola* spp. depends on the availability and ecological needs of their respective intermediate host species (Malatji et al., 2019). As expected, this review showed a range of lymnaeid snails that were implicated in the transmission of *Fasciola* species in the field and in experimental setting in five of the six inhabited continents. These results are consistent with previous reports as this snail family has been reported in all continents except Antarctica (Vázquez et al., 2019b). For both experimental infections and natural infections, South America recorded the highest pooled prevalence, and the lowest prevalence was recorded in Europe and Asia. While all three continents recorded multiple snail species involved in the natural transmission of *F. hepatica*, *G. truncatula* and *O. glabra* contributed more to the pooled prevalence. Mas-Coma et al. (2005) implicated the geographical expansion of *G. truncatula* and *P. columella* in the dispersal of *F. hepatica* from Europe to other continents. However, though infections in *G. truncatula* were also noted in South America, other *Galba* species (*G. schirazensis*) proved to contribute more to the pooled prevalence. The latter results may be a misinterpretation however, as *G. schirazensis* had been frequently confused with *G. truncatula*. A reason for this may be that *G. schirazensis* and *G. truncatula* can be considered as cryptic species as they very similar in anatomical variation and shell morphology (Vázquez et al., 2019a; Correa et al., 2011). Furthermore, South America comparatively recorded the highest infections (76.9%) of *Fasciola* spp. (Cruz-Mendoza et al., 2004) compared to Europe (38.3%) (Martinez-Ibeas et al., 2013).

Fasciola gigantica recorded a high pooled prevalence in experimental infections, however, in the natural environment, the pooled prevalence was higher with *F. hepatica.* Furthermore, the later species showed a wider geographical expansion, recorded in five continents while *F. gigantica* showed restriction to two continents. Contributing to this may be the wide range of snail species involved in the natural transmission of *F. hepatica*, which explains its geographical expansion compared to *F. gigantica*. Furthermore, Mas-Coma et al. (2005) linked the smaller geographical distribution of *F. gigantica* to its IHs having a weaker diffusion capacity.

Our analysis showed that while various snail species have been subjected to experimental infections and assessed for natural infection of *Fasciola* spp., most studies were conducted on *G. truncatula*, *R. natalensis*, and *P. columella*. Our results further showed that the invasive snail, *P. columella,* which recorded a high pooled prevalence was infected by both *F. hepatica* and *F. gigantica* for both experimental and natural infections, despite *G. truncatula* and *R. natalensis* being the main IHs of *F. hepatica* (Gasnier et al., 2000; Cruz-Mendoza et al., 2004; Bargues and Mas-Coma, 2005; Pointier et al., 2009; Kim et al., 2014; Beesley et al., 2018; Alemu, 2019) and *F. gigantica* (Bargues and Mas-Coma, 2005; Mas-Coma et al., 2005; Rajanna et al., 2018; Alemu, 2019; Nyagura et al., 2022), respectively. The ability of *P. columella* to transmit both *F. hepatica* and *F. gigantica* in a natural environment has been documented in many countries (Grabner et al., 2014; Carolus et al., 2019; Alba et al., 2019; Malatji and Mukaratirwa, 2019; Ngcamphalala et al., 2022). Furthermore, according to Mas-Coma et al. (2005), *P. columella* has been linked to the secondary transmission of *F. hepatica*. Additionally, *A. viridis* was shown to transmit both *F. hepatica* (Kim et al., 2014) and *F. gigantica* (Dung et al., 2013) in nature even though the prevalence of both infections was significantly low. However, it has been previously noted that even low prevalence in naturally infected intermediate snails matters (Vázquez et al., 2015) as a single miracidium infection can produce approximately 4000 metacercariae leading to substantial environmental contamination (Andrews, 1999; Nguyen et al., 2012).

Experimental studies recorded a high pooled prevalence based on cercariae shedding, which is the most used and affordable detection method to assess trematode infection in snails. However, this method tends to underestimate the true prevalence of infection as it mainly detects patent infections, and infections that are still at the prepatent stage are regarded as negatives (Curtis and Hubbard, 1990; Born-Torrijos et al., 2014; Tigga et al., 2014). Whilst microscopic dissection recorded the lowest pooled prevalence in experimental infections, this method however had the highest prevalence in natural infections with molecular detection recording the lowest. Contributing to the lowest prevalence of molecular detection (polymerase chain reaction (PCR) and Restriction Fragment Length Polymorphism (RFLP)) might have been due to the low number of studies using this technique to detect *Fasciola* spp. infections in IH snails. Furthermore, most studies might have opted not to use molecular techniques due to the costs involved equipment, consumables, and sequencing (Caron et al., 2010; Tigga et al., 2014), and the lack of skilled personnel to carry out the PCR (Tigga et al., 2014). Several authors emphasized investing in PCR as a supplementary/confirmation method as it has been shown to detect *Fasciola* spp. infections (DNA) in IH snails after they had been deemed negative by shedding and/or dissection (Caron et al., 2017; Rajanna et al., 2018; Geli-Erazo et al., 2020; Malatji and Mukaratirwa, 2019).

Experimental infections showed a high pooled infection rate of *Fasciola* spp. infections in the most recent decade (2014-2023) which might be attributed to the increased number of experimental studies conducted to better understand the host-parasite interaction and transmission of these important zoonotic parasites. However, prevalence data for natural infections showed a decline in prevalence for the past 20 years, with the decade (2014-2023) recording the lowest pooled prevalence. The natural infection model's significant results for the period ($p = 0.007$) indicate that temporal factors, such as seasonal variation or changes in environmental conditions, may be crucial in influencing prevalence, despite the overall heterogeneity seen across the various factors (continents, diagnostic tests, and *Fasciola* species). This decline in natural infections may be attributed to climate change as the primary determinant of transmission efficiency is the relationship between rainfall and temperature (Fox et al., 2011) both of which have either positive or negative effects on the distribution of the intermediate snail hosts and survival of free-living stages of the *Fasciola* spp. parasite (Madsen and Stauffer, 2022). Another reason for the decline in natural infections prevalence may be the effectiveness of control strategies targeting infections in definitive hosts and IHs such as the use of anthelmintics and controlling the snail IH using molluscicides (Madsen and Stauffer, 2022).

There could be a number of reasons for the continents, diagnostic tests, and *Fasciola* species' lack of significance, such as the fact that these variables may not vary sufficiently between studies or that other unmeasured confounders may obscure their impact. The observed high level of heterogeneity may potentially indicate that the results are being influenced by other factors that were not taken into account in the model, such as methodological or regional variances.

The limitation of this review is that only articles written in English were included to ensure that there was no misrepresentation of methodologies and results in cases where there could be incorrect translations from other languages to English. Additionally, publication bias was detected for both field and experimental studies. Despite using a standardized analysis process, it was difficult to achieve consistent meta-analysis due to the differences in study design, detection, and quantification methods in the different studies. Furthermore, several studies failed to provide complete information on the prevalence of *Fasciola* species amongst freshwater snails, and those that had all the information were not evenly distributed across continents. Hence, meta-analysis could not be conducted for some IH species and some continents. As a result, the prevalence data presented in this review do not fully represent the prevalence of *Fasciola* spp. infections amongst freshwater snail species globally.

3.6. Conclusion

The review highlighted crucial information on the prevalence of *Fasciola* spp. infection in their intermediate snail hosts across the globe. Although numerous IHs have been confirmed to transmit

Fasciola spp. either experimentally or naturally, however, *G. truncatula*, *R. natalensis,* and *P. columella* emerged as the main IHs, globally. Natural infection results showed a strong intermediate host specificity between the two *Fasciola* spp., where *G. truncatula* and *R. natalensis* are susceptible *to F. hepatica* and *F. gigantica* respectively, whilst *P. columella* is able to transmit both species. This information is important in determining and estimating the species-specific distribution and transmission, which can be used as baseline data for interrupting the life cycle of fasciolosis in a given area.

We, therefore, recommend continuous surveillance and monitoring of the dispersal of the lymnaeid snail species involved in the transmission of specific *Fasciola* spp. Additionally, to employ the use of molecular detection methods to supplement classic parasitological methods, to confirm the detection of infection and identity of species. Moreover, focus on developing and using other protocols such as the Loop-mediated isothermal amplification (LAMP) and other PCR-based protocols that can detect and identify species without the extra sequencing costs is required.

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Chapter 4

Molecular identification of intermediate hosts of *Fasciola* spp. from selected localities in South Africa

4.1 Abstract

Freshwater snails from the family Lymnaeidae act as intermediate hosts for at least 70 trematode species from 13 different families. Despite the known and elucidated medical and veterinary importance of these snails, there is a paucity of information on the molecular identification and genetic diversity of these snails in various provinces of South Africa. This study therefore identified and assessed the genetic diversity of lymnaeid snails from selected habitats in five provinces of South Africa. Based on the mitochondrial COI and 16S ribosomal DNA markers, *Pseudosuccinea columella* and *Radix natalensis* were identified. Genetic diversity analysis showed lack of genetic variation between the *P. columella* populations from the five provinces. These snails formed one clade, consistent with one novel haplotype consisting of all isolates based on both COI and 16S rDNA, indicating a common ancestor. On the other hand, *R. natalensis* populations formed different subclades, representing different geographical locations as represented by provinces. Haplotype analysis of COI showed genetic variations, with two distinct haplotypes formed, representing provinces. The 16S gene had three *R. natalensis* haplotypes, of which two represented Mpumalanga province showing intrapopulation variation. *Radix natalensis* populations exhibited a moderate haplotype diversity (Hd) of 0.467 and low nucleotide diversity of 0.014 with the COI gene, highlighting low level of gene flow. Furthermore, negative Tajima's D indicated excess of rare variation, which is consistent with population growth or positive selection, but not statistically significant. However, the positive Fu's Fs value of 10.383 indicate allele deficiency, which may be due to recent population bottleneck. The 16S gene showed low levels of gene flow as observed by the moderate haplotype diversity (Hd) of 0.618 and low nucleotide diversity of 0.00685. The Tajima's D for the 16S gene was positive and significant Tajima's D (2.20815) signifying a deviation from evolutionary neutrality. Thus, suggesting that the recent population expansion was restricted to one area. These results highlight the need to continuously monitor the changes in biodiversity, distribution and genetic diversity of lymnaeid snail species in South Africa.

Keywords: Genetic diversity, *Pseudosuccinea columella*, *Radix natalensis*, intermediate hosts, *Fasciola gigantica*, *Fasciola hepatica*, South Africa.

4.2 Introduction

Freshwater gastropods from the family Lymnaeidae are of major medical and veterinary significance as they are implicated in the transmission of *Fasciola* spp. worldwide (Bargues and Mas-coma, 1997; Stothard et al., 2000; Dar et al., 2016; Alba et al., 2019; Min et al., 2022; Dumidae et al., 2024). *Fasciola* spp. infections severely affect millions of people, livestock especially domestic ruminants and cause considerable economic losses worldwide (Standley et al., 2013; Mahulu et al., 2019; Dumidae et al., 2024). For the completion of their life cycle, *Fasciola* spp. utilize a variety of lymnaeid snails as the IHs for developing miracidium, sporocyst, redia, and cercaria stages (Dumidae et al., 2024).

More than 30 lymnaeid species have been described and confirmed as IHs of *Fasciola* spp. globally (Alba et al., 2019; Vázquez, et al., 2019a). These lymnaeid snails act as IHs for at least 70 trematode species from 13 different families (Correa et al., 2010; Dumidae et al., 2024), and are distributed worldwide, extending from tropical to temperate regions with some occurring at extremely cold latitudes (Prepelitchi et al., 2011; Vinarski, 2013; Vázquez et al., 2019b). A solid phylogenetic framework of lymnaeid snail species is thus required to identify and characterize regions of epidemiological risk and to enhance understanding of the host-parasite interaction between lymnaeids and *Fasciola* spp. (Correa et al., 2010). However, identification of lymnaeid snails has been one of the most challenging issues when establishing fasciolosis epidemiological risk maps and studying the diversity of this taxon (Correa et al., 2011).

The taxonomy of lymnaeid snail species, especially at a species level, is debatable due to the intraspecific uniformity of their anatomy and their diversity in shell morphology due to their environmental habitat conditions (Dung et al., 2013; Standley et al., 2013; Dumidae et al., 2024). The taxonomic classification of freshwater Lymnaeidae based solely on morphological characteristics is not always reliable and the nomenclature of lymnaeids on this basis is controversial (Dung et al., 2013). This has subsequently resulted in numerous morphological species being described, leading to more complex classifications (Bargues and Mas-Coma, 1997; Pfenninger et al., 2006). Therefore, accurate identification of lymnaeid gastropods is critical, in order to map the potential geographical distribution of fasciolosis and competence of different IHs in transmitting *Fasciola* spp. (Mahulu et al., 2019).

In recent years, molecular taxonomy and phylogenetics have been utilized to identify numerous organisms globally (Dumidae et al., 2024). Several molecular markers have been used to investigate the taxonomy, phylogeny, population genetics, and molecular evolution for lymnaeid snails, (Virnaski et al., 2020; Dumidae et al., 2024). Mitochondrial cytochrome c oxidase subunit I (COI) and 16S rRNA, and the nuclear 18S and ITS DNA regions have been commonly used for genetic analysis in freshwater lymnaeids (Bargues et al., 2011; Standley et al., 2013; Dar et al., 2016; Dumidae et al., 2024). The

application of molecular phylogenetics resulted in the taxonomy of *Fasciola* spp. IHs that had been combined into genus *Lymnaea* Lamarck, 1799 to be classified into the genus *Radix* and *Galba* which is being used today (Mahulu et al., 2019). Despite this revised classification, the phylogeny and generic classification of freshwater lymnaeid snails continue to be debated (Mahulu et al., 2019).

The geographical distribution of lymnaeid snails in South Africa has been documented, with five species identified to be present (Malatji et al., 2019). However, studies on the genetic diversity of these snails are limited to few studies which has led to a lack of representation and comparable sequences in the GenBank database (Molaba et al., 2023). Thus, this study aimed to determine the genetic diversity of lymnaeid snails from five provinces of South Africa using mitochondrial COI and 16S rDNA genetic markers.

4.3 Materials and methods

4.3.1 Ethical consideration

The study was approved by the Animal Ethics committee of the University of KwaZulu-Natal (AREC/020/020P), the DALRRD section 20 (P2020-13) and the SANBI-NZG Research and Scientific committee (SANBI/RES/P2020/13) (Appendices 1 - 3).

4.3.2 Sample collection

A snail survey with focus on Lymnaeid snails was conducted in Mpumalanga, Gauteng, KwaZulu Natal, Eastern Cape and Limpopo provinces of South Africa, between June 2021 and May 2024 (Figure 4.1). One thousand and fifty-nine (1059) freshwater lymnaeid snails were collected from natural (n = 492) and man-made (n = 567) water bodies frequented by animals and/or human for drinking water or other anthropogenic activities. The snails were collected using the scooping method and/or hand picking as described by (Appleton and Miranda, 2015a). Once collected, the snails were kept in plastic containers filled with water from their habitat or distilled water for transportation to the laboratory located at the Biomedical Resource Unit (BRU), University of KwaZulu-Natal, Westville Campus, Durban, South Africa.

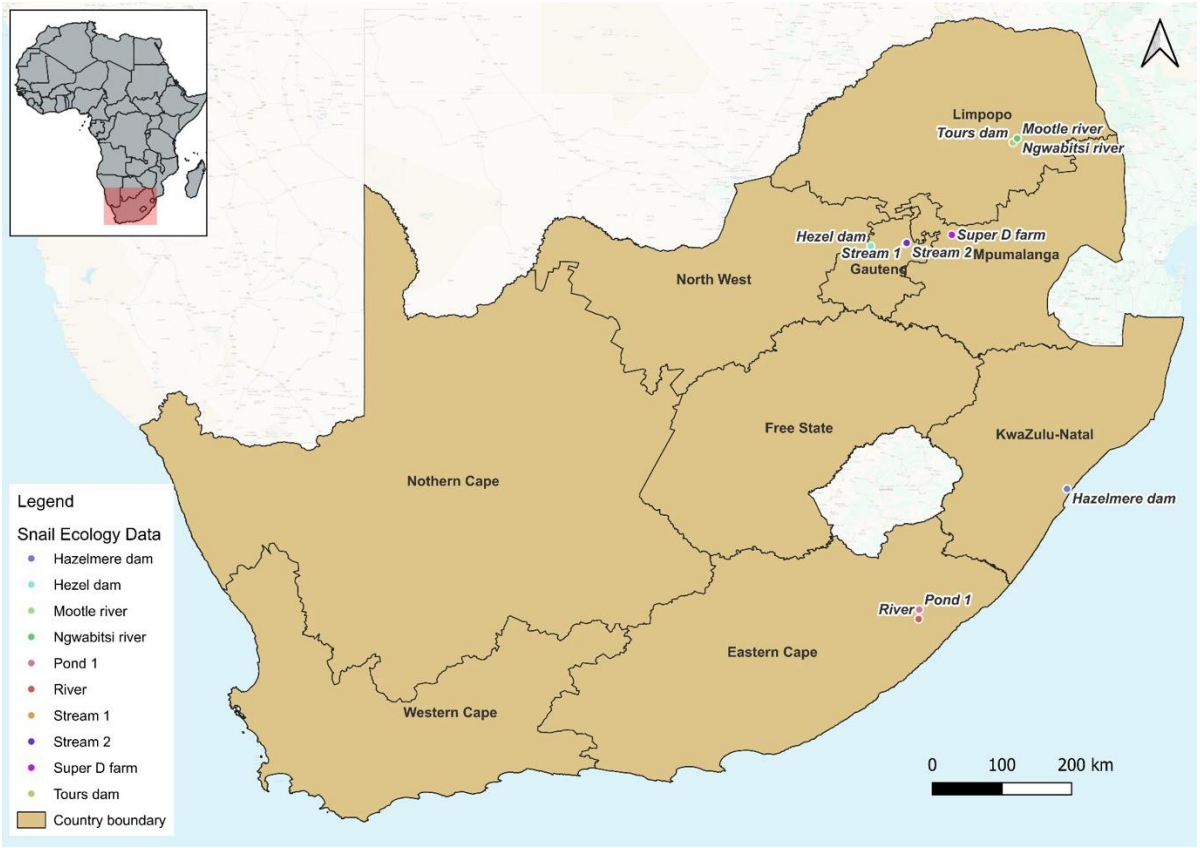

Figure 4.1 Localities from where freshwater lymnaeid snails were collected in South Africa.

4.3.3 DNA extraction and molecular analysis

DNA was extracted from snail foot or whole tissue using Genomic DNA™ -Tissue MiniPrep (Zymo Research Corporation) according to the manufacturer's instructions. Genomic DNA was quantified using the Nanodrop. The mitochondrial region of the snail's DNA was amplified using the universal COI primers (LCO1490: 5'-GGTCAACAAATCATAAAGATATTGG-3' and HCO2198: 5'-TAAACTTCAGGGTGACCAAAAAATCA-3') (Folmer et al., 1994) and the ribosomal gene 16S rDNA (16S F: 5'-CGCCTGTTTATCAAAAACAT-3' and 16S R: 5' -CCGGTCTGAACTCAGATCACGT-3') (Palumbi, 1991). Amplifications were performed in a reaction mixture containing 12 µL 2× Taq DNA Polymerase Master Mix RED™, 1 µL of each primer (10 µM), ~50-100 µg/ µL of template DNA and double-distilled water (ddH_2O) was added to the final volume of 25 µL. Thermal cycling were set at 95°C for 5 mins, 40 cycles of (60s at 95°C, 70s at 50°C, 60s at 72°C) and final extension 72°C for 10 mins for COI, and 94 ˚C for 4 min; followed by 35 cycles of (60s at 94 ˚C, 60s at 61 ˚C, 60s at 72 ˚C) and final extension for 10 min at 72°C for 16S. Fragments were separated using 2% agarose gels stained with SYBR Safe DNA Gel Stain (Applied Biosystems, USA) and visualised using Uvitec Uvidoc HD6. The successful amplification had bands at 710 bp and 434 bp for COI and 16S, respectively.

4.3.4 Sequencing

Amplicons were cleaned and purified using a combination of 2 µL FastAP™ and 1 µL Exonuclease (Applied Biosystems, USA) enzymes, the purification reaction ran for one cycle at 37 °C for 15 mins and 85 °C for 15 mins. Cycle sequencing was performed in a final reaction volume of 10 µL containing 0.7 µL BigDye, 2.55 µL sequencing buffer, 0.75 µL ddH2O, 1 µL of primer and 5 µL of the PCR product, with the following cycling conditions: one cycle at 95 °C for 2 min; 40 cycles at 85 °C for 10s, 53°C for 10s and 60°C for 2:30s. Using the same primers for PCR, both forward and reverse directions were sequenced using the BigDye Terminator™ v3.1 cycle sequencing kit (Thermofisher Scientific, California, USA). The Sanger chain termination method was run on the ABI PRISM 3500™ automated DNA sequencer (Applied Biosystems, USA).

4.3.5 Sequence and haplotype analysis

Sequencing Analysis software (SeqA™) (Applied Biosystems, USA) was used to visualise, base call and score the sequences. Sequences that passed quality check were then edited, consensus sequences created, and assembled using BioEdit program (Hall, 1999). The sequence datasets were aligned with homologous sequences obtained from the GenBank using the Clustal W (Thompson et al., 1997) and trimmed to common length of 399 and 600 nucleotide length for 16S and COI, respectively. The General Time Reversible models (GTR+G and GTR+I+G) were selected as the best fit models for our dataset for 16S and COI using jModeltest 2.3 (Posada, 2008), respectively. Maximum likelihood (ML) and Neighbour-joining trees were generated on both PAUP* 4.0 (Swofford, 2002), and the nodal supports were estimated using 1000 bootstrap pseudo-replicates. Bayesian inference analysis was executed on MrBayes 3.1.2 (Huelsenbeck and Ronquist, 2001) using the four Markov chains, ran for five million generations, and the first 500 000 trees were discarded as burn-in. The analysis was run until the standard deviation of the split frequencies was less than 0.01. DNA Sequence Polymorphism (DnaSP) (v 5.10.1) (Rozas et al., 2003) was used to generate haplotypes and calculate genetic diversities. TCS (Templeton, Crandall, and Singh) haplotype network was generated using PoPArt (Clement et al., 2002).

4.4 Results

4.4.1 Molecular identification and phylogenetic relationship of Lymnaeidae snails based on the COI and 16S genes

Pseudosuccinea columella and *Radix natalensis* were identified in the sampled areas (Figure 4.1 and 4.2). *Pseudosuccinea columella* was found in all five provinces (Figure 4.1), whereas *R. natalensis* was limited to Limpopo and Mpumalanga provinces. The phylogenetic tree showed that these Lymnaeidae species formed monophyletic sister clades, showing a clear separation between *P. columella*, *Galba truncatula* and other *Radix* spp., though not supported. Thirty-nine sequences from five provinces

identified as *P. columella* showed a homology of 100% with other South African sample (PP228863.1) based on the COI gene (Figure 4.2). These samples further formed a strongly supported clade (100/100/1.0) with other GenBank isolates (Figure 4.2; Table 4.1), indicating a lack of intraspecies variation between all *P. columella* isolates, with an overall genetic distance of 0% (Figure 4.2). Furthermore, the individual sub-cladding of these species showed a distant relationship between these species and other Lymnaeidae species. *Radix natalensis* isolates formed a moderately supported sister clade with other *Radix* species, *R. auricularia* and *R. rubiginosa*. However, these species showed somehow more closer relationship with *R. auricularia*. These *R. natalensis* isolates formed strongly supported sub-clades, showing intraspecific variation and provincial clustering between Mpumalanga and Limpopo samples, with an average genetic distance of 3% (Figure 4.2). These sequences were submitted to GenBank under the accession numbers PQ898647 – PQ898695

The 16S gene analysis was based on isolates from Limpopo, Mpumalanga and Gauteng (accession numbers PQ898568 – PQ8988600). The structuring of the phylogenetic tree was to an extent similar to that of the COI. The tree formed non-supported monophyletic clades, clustering *Radix* spp. together, separate for the *Pseudosuccinea*/*Galba clade*. Twenty-two experimental sequences from all three provinces identified as *P. columella* and formed a clade with GenBank derived sequences from Brazil, France, and New Mexico, with a bootstrap value of 100% (100/100/1.0) (Figure 4.3). Like with COI, the *P. columella* populations did not show intraspecies variation, with no genetic distance existing among the isolates. However, these species formed a weakly supported sister clade with *Galba truncatula* (57/82/0.53), which deviated from what was observed with COI. Furthermore, *R. natalensis* formed a strongly supported clade (99/100/1.0) sister clade with *Radix auricularia* and *Radix rubiginosa*. Corresponding with COI, the 16S *R. natalensis* isolates also diverged to form two moderately supported sub-clades representing Mpumalanga and Limpopo provinces (Figure 4.3). However, specimens from Mpumalanga further showed separation of the MPHG010 isolate from the other isolates, with a genetic distance of 0.25%.

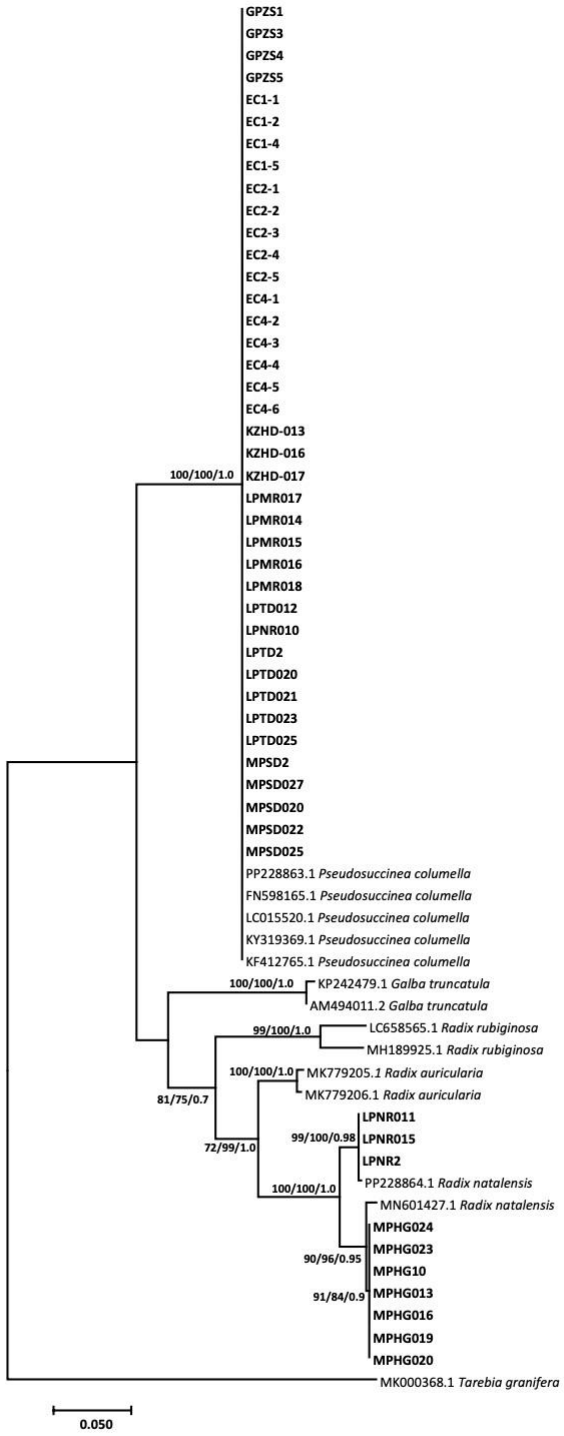

Figure 4.1 Maximum likelihood tree showing the relationship between Lymnaeidae snails based on COI region. The bootstrap values are shown on the node in the order ML/NJ/BI, and 50% majority-rule is applied. Samples from the study are presented in bold with prefix; LP – Limpopo province, GP – Gauteng province, MP – Mpumalanga province, KZ – KwaZulu-Natal province, EC – Eastern Cape province.

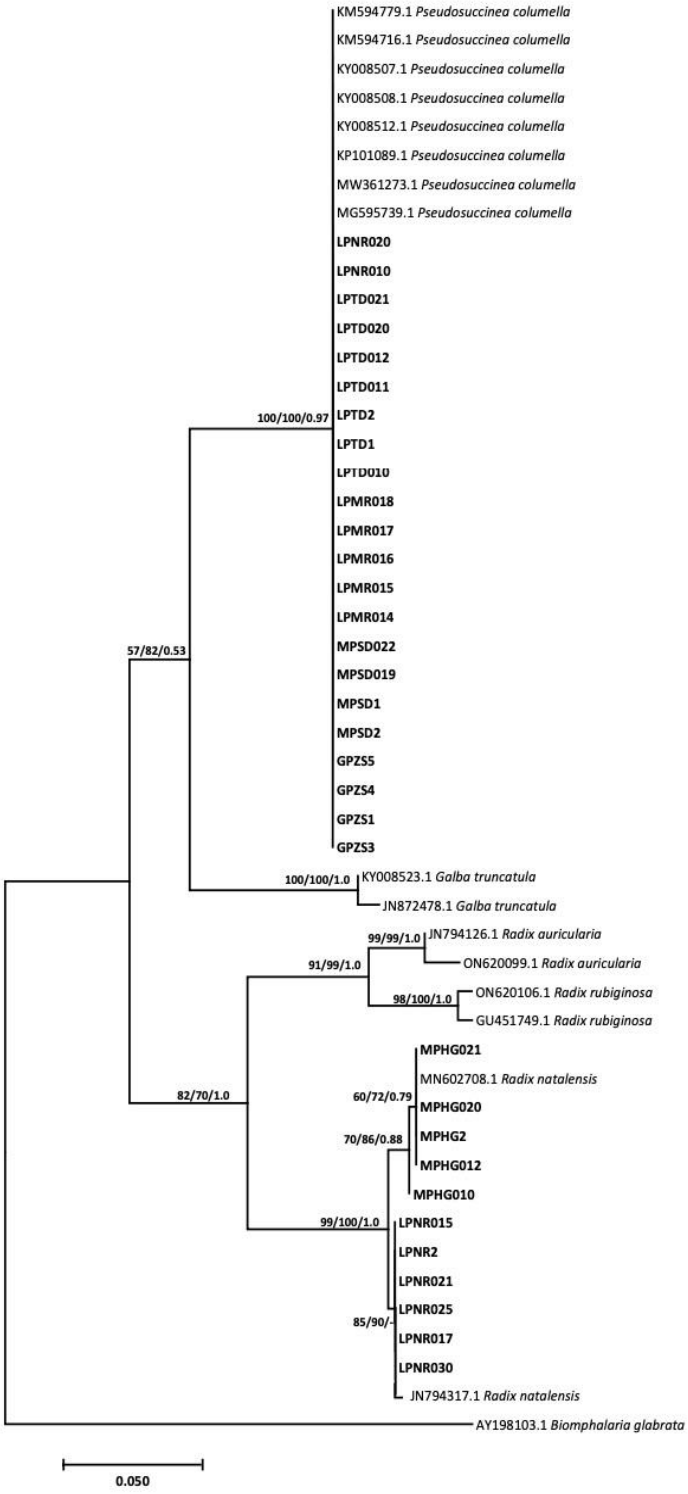

Figure 4.3 Maximum likelihood tree showing the relationship between Lymnaeidae snails based on 16S rDNA region. The bootstrap values are shown on the node in the order ML/NJ/BI, and 50% majority-rule is applied. Samples from the study are presented in bold with prefix; LP – Limpopo province, GP – Gauteng province, MP – Mpumalanga province.

Table 4.1 Haplotype status of the GenBank and studied isolates from five provinces of South Africa based on the COI gene.

Haplotype	Frequency	Species	Sequence ID	
			Study sequence ID	GenBank
Hap 1	44	*P. columella*	GPZS1, GPZS3, GPZS4, GPZS5, EC1-1, EC1-2, EC1-4, EC1-5, EC2-1, EC2-2, EC2-3, EC2-4, EC2-5, EC4-1, EC4-2, EC4-3, EC4-4, EC4-5, EC4-6, KZHD-013, KZHD-016, KZHD-017, LPMR017, LPMR014, LPMR015, LPMR016, LPMR018, LPTD012, LPNR010, LPTD2, LPTD020, LPTD021, LPTD023, LPTD025, MPSD2, MPSD027, MPSD020, MPSD022, MPSD025	PP228863.1 (South Africa), FN598165.1 (Colombia), LC015520.1 (Egypt), KY319369.1 (New Mexico), KF412765.1 (Egypt)
Hap 2	3	*R. natalensis*	LPNR2, LPNR011, LPNR015	-
Hap 3	7	*R. natalensis*	MPHG024, MPHG023, MPHG10, MPHG013, MPHG016, MPHG019, MPHG020	-
Hap 4	1	*Galba truncatula*	-	KP242479.1
Hap 5	1	*G. truncatula*	-	AM494011.2
Hap 6	1	*R. natalensis*	-	MN601427.1
Hap 7	1	*R. natalensis*	-	PP228864.1 (South Africa)
Hap 8	1	*Radix rubiginosa*	-	LC658565.1
Hap 9	1	*R. rubiginosa*	-	MH189925.1
Hap 10	1	*R. auricularia*	-	MK779205.1
Hap 11	1	*R. auricularia*	-	MK779206.

4.4.2 Haplotype and genetic diversity of Lymnaeidae snail species based on the COI gene

The COI sequence alignment yielded 11 haplotypes, of which three haplotypes represented the study samples (Table 4.1, Figure 4.4). Consistent with the phylogenetic tree, all *P. columella* samples formed a novel haplotype (Hap_1). The *R. natalensis* samples from this study formed two distinct haplotypes (Hap_2 and Hap_3), also confirming the provincial clustering as also indicated on the phylogenetic tree. Haplotype Hap_2, which consisted of samples from Limpopo province, separated from the Mpumalanga haplotype Hap_3 by 18 mutational steps (Figure 4.2). These *R. natalensis* populations showed a moderate haplotype diversity of 0.467, and low nucleotide diversity of 0.014. The neutrality analysis (Fu's Fs value = 10.383; Tajima's D = 1.4948) indicated allele deficiency, which may be due to recent population bottleneck, though not statistically significant (Table 4.2).

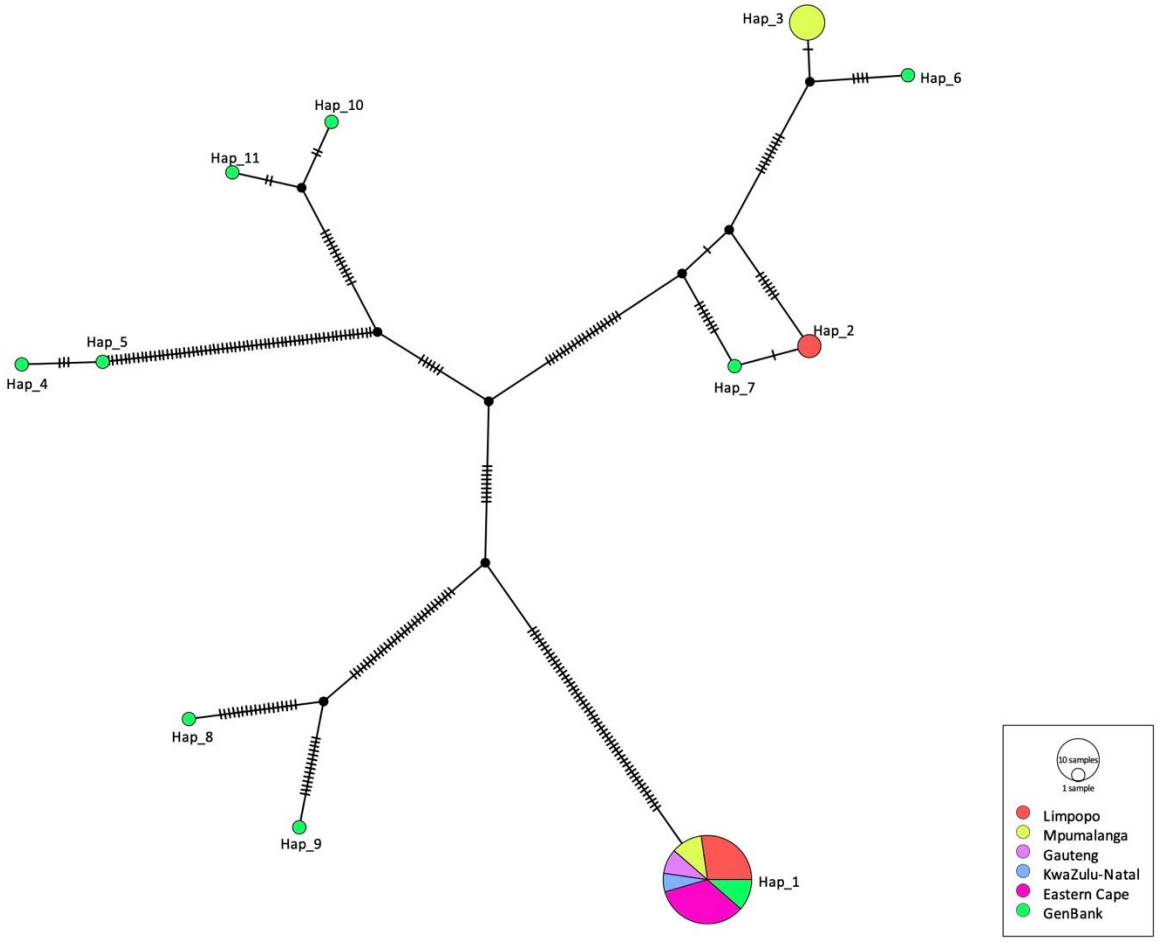

Figure 4.4 Haplotype network of Lymnaeidae snail species from five provinces of South Africa based on the COI gene. The circle size is proportional to the frequency of each haplotype and the mutational steps are indicated by a dash.

Table 4.2 Overall genetic diversities of *Pseudosuccinea columella* and *Radix natalensis* samples from five provinces of South Africa.

Parameters	**COI**		**16S**	
	P. columella	*R. natalensis*	*P. columella*	*R. natalensis*
N	39	10	22	11
No. of populations	5	2	3	2
Haplotypes generated (h)	1	2	1	3
Haplotype diversity (Hd±Std)	0	0.467±0.132	0	0.618±0.104
Number of segregating sites (S)	0	18	0	5
Nucleotide diversity (Pi±Std)	0	0.014±0.00395	0	0.00685±0.0009
Average number of nucleotide differences (k)	0	8.40	0	2.6909
Tajima's D	0	1.4948	0	2.20815*
Fu's Fs	0	10.383	0	2.997

* - statistically significant; N – total number of samples

4.4.3 Haplotype and genetic diversities of Lymnaeidae species based on the 16S rDNA gene

16S rDNA sequence alignment yielded 10 haplotypes, of which four consisted of studied isolates (Table 4.3, Figure 4.5). The 16S *P. columella* samples formed one haplotype (Hap_8), further confirming lack of interpopulation variability (Table 4.2). *Radix natalensis* formed three haplotypes of with a haplotype diversity (Hd) of 0.618 and nucleotide diversity of 0.00685. Samples from Mpumalanga province showed intrapopulation variation, and the samples formed Hap_1 and Hap_3 haplotypes. Hap_3 haplotype represented isolate MPHG010, which was separated from other samples (Hap_2) with one mutational step. Hap_2 haplotype represented isolates from Limpopo province and shared the same ancestor with an isolate from China (JN794317.1). The *R. natalensis* population showed a significantly positive Tajima's D (2.20815, $p < 0.05$), suggesting a deviation from evolutionary neutrality. The Fu's Fs value was positive 2.997 (Table 4.2) which indicated allele deficiency, which may be due to a recent population bottleneck.

Table 4.3 Haplotype status of lymnaeid species from the GenBank and study isolates from five provinces of South Africa based on the 16S rDNA gene.

Haplotype	Frequency	Species	Sequence ID	
			Study sequence ID	GenBank
Hap 1	5	*R. natalensis*	MPHG012, MPHG2, MPHG020, MPHG021	MN602708.1 (Kenya)
Hap 2	7	*R. natalensis*	LPNR015, LPNR2, LPNR021, LPNR025, LPNR017, LPNR030	JN794317.1 (China)
Hap 3	1	*R. natalensis*	MPHG010	
Hap 4	1	*Radix auricularia*	-	JN794126.1
Hap 5	1	*R. auricularia*	-	ON620099.1
Hap 6	1	*Radix rubiginosa*	-	ON620106.1
Hap 7	1	*R. rubiginosa*	-	GU451749.1
Hap 8	30	*P. columella*	GPZS3, GPZS1, GPZS4, GPZS5, MPSD2, MPSD1, MPSD019, MPSD022, LPMR014, LPMR015, LPMR016, LPMR017, LPMR018, LPTD010, LPTD1, LPTD2, LPTD011, LPTD012, LPTD020, LPTD021, LPNR010, LPNR020	MG595739.1 (New Mexico), MW361273.1 (New Mexico), KP101089.1 (New Mexico), KY008512.1 (Brazil), KY008508.1 (Brazil), KY008507.1(Brazil), KM594779.1 (France), KM594716.1 (France)
Hap 9	1	*Galba truncatula*	-	KY008523.1
Hap 10	1	*G. truncatula*	-	JN872478.1

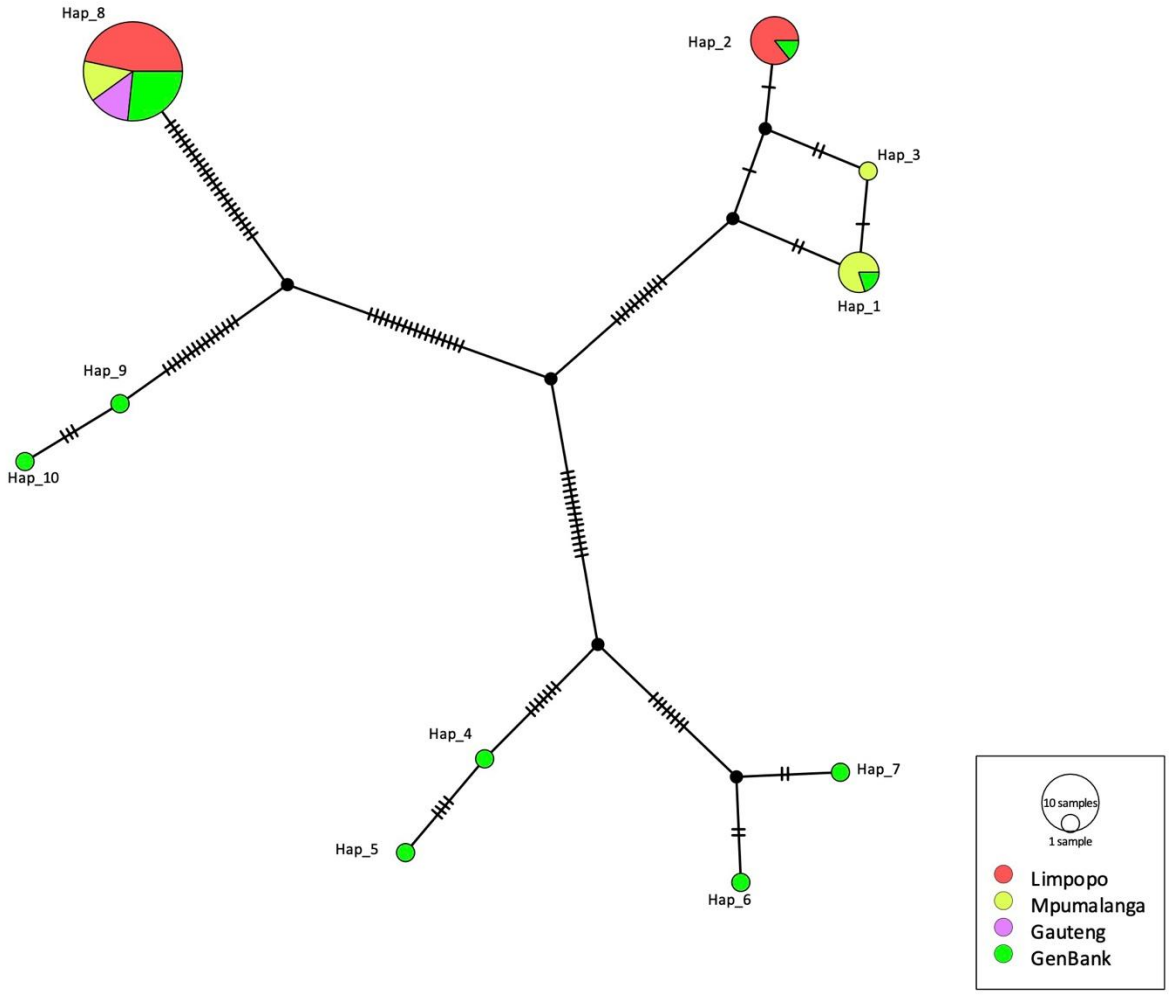

Figure 4.5 Haplotype network of lymnaeid snail species from five provinces of South Africa based on the 16S rDNA gene. The circle size is proportional to the frequency of each haplotype and the mutational steps are indicated by a dash.

4.5 Discussion

Previous studies have documented the presence of five lymnaeid species in South Africa (Appleton and Miranda, 2015b; Malatji et al., 2019; Nyagura et al., 2022). However, the present study identified *R. natalensis* and *P. columella*, of which the former was documented in Limpopo and Mpumalanga provinces and reiterated the widespread of *P. columella* which was found in all five sampled provinces. This was not surprising as this invasive snail species was previously ranked the third most widespread freshwater snail in a snail survey carried out in South Africa (de Kock et al., 1989) and has been implicated in transmission of *F. hepatica* (Molaba et al., 2023) and *F. gigantica* (Malatji and Mukaratirwa, 2019). The documentation of *R. natalensis* in two provinces was surprising as this species

has been previously recorded as one of the most distributed species (de Kock et al., 1989), and the main IHs of *F. gigantica* in Africa (Malatji et al., 2019). This observed restricted distribution may have been attributed to the limitations in sampling areas, which may have underrepresented the dispersal of this species. *Galba truncatula* was not found in our study and the most plausible reason is the difficulty in finding these snails as they are small and prefer low temperature regions and habitats with shallow water and clay soils, banks of shallow streams, swamps and reeds (Knubben-Schweizer and Torgerson, 2015).

Despite *P. columella* being documented across five provinces in South Africa, with differences in agro-ecological climates, this species did not show any genetic variability within or between populations based on both mitochondrial markers. The isolates clustered together along with the GenBank sequences, and further formed one novel haplotype, indicating that the isolates from our study might have originated from the same parental generation, and predominantly use selfing as their main mode of reproduction. *Pseudosuccinea columella* populations from Mpumalanga, KwaZulu-Natal and Eastern Cape provinces, which have different climatic conditions were all monomorphic, and all formed a single allele at 210bp (Malatji and Mukaratirwa, unpublished data). The lack of variability in *P. columella* populations in this study corresponded to the extremely low genetic diversity in *P. columella* populations at a global scale, which shifted from the Hardy-Weinberg equilibrium and exhibits strong genotypic disequilibrium as reported by Lounnas et al. (2017). According to Dumidae et al. (2024) the low levels of genetic diversity may be due to prolonged or severe repeated periods of population bottlenecks caused by natural environmental changes, particularly changes stemming from seasonal changes. While the phenotypic identification of the mantle was not conducted, it can be concluded that the population circulating in South Africa is susceptible to *F. hepatica*, based on recent reports of infections (Molaba et al., 2023) and *F. gigantica* and suspected *Fasciola* hybrid (Malatji and Mukaratirwa, 2019).

Analysis of both the 16S rDNA and COI genes showed a sub-clustering of the native *R. natalensis* samples based on their province of origin. This genetic variation was further confirmed by the haplotype analysis whereby the samples from these different provinces formed different haplotypes, thus indicating geographical divergence. This was not expected as populations of the same species are assumed to have little or negligible amounts of variations within or between them (Stothard et al., 2002), and furthermore, Limpopo and Mpumalanga provinces are in the same agro-climatic region as part of the tropical and subtropical regions of South Africa (de Kock et al., 2002. Occurrence of the genetic variations in populations can be influenced by local differences in temperature and diurnal temperature cycles within waterbodies resulting in numerous microhabitats having different ecological conditions, which may have been the case for this study (Hubendick, 1958).

Despite the interpopulation variability observed, the low mean nucleotide diversity and haplotype diversity observed reflect low levels of genetic variation within *R. natalensis* populations. Contributing to this may be that *R. natalensis* are hermaphrodites, and reproduce predominantly by selfing, which increases homozygosity and subsequently loss of variability in populations (Jarne et al., 1993; Meunier et al., 2001). The neutrality tests (Tajima's D and Fu's Fs) also suggested that the population had experienced a historical bottleneck. Tajima's D for the COI gene was not significant, indicating that the expansion of these snails may have been restricted to specific areas (Dumidae et al., 2024). Availability of aquatic habitats is one of the seasonal variabilities that would lead to population bottlenecks leading to the reduction in the size of the population and losses in genetic differences due to genetic drift (Weber et al., 2004; Dumidae et al., 2024). The *R. natalensis* populations in this study were predominantly found in dams, where there is less possibility of exchange of genetic materials, which lead to inbreeding. Conversely, the Tajima's D for the 16S was significant signifying population contraction might have occurred (Tajima, 1989). Sudden reduction in population size may be due changes in climatic factors and physicochemical parameters in waterbodies which have been shown to significantly affect species diversity, distribution and abundance (Mereta et al., 2012; Min et al., 2022; Nwoko et al., 2022). Nwoko et al. (2022) reported that rainfall negatively affected *R. natalensis* populations in KwaZulu-Natal province of South Africa during the rainy season as heavy rains washed away the snails from those habitats.

The limitation of this study was the limited location coverage, with only a few areas sampled within provinces, and usually within the same district, which may have contributed to the lowered nucleotide diversity observed. The expanded sampling location would have provided more insight into the *R. natalensis* sub-clustering, and an indication of whether this genetic variability occurs between all provinces, showing a high level of genetic variation. Furthermore, the 16S sequences only covered three provinces, though the remaining provinces only recorded *P. columella*, which showed no genetic variability. Factoring the similarities in COI and 16S results, the authors could have included a nuclear marker (ITS-2) to confirm the observed relationship.

4.6. Conclusion

Radix natalensis populations from Mpumalanga and Limpopo provinces of South Africa showed genetic variations within populations. However, *P. columella* did not show any genetic variability within and between populations from five provinces. This might be an indication that these populations are derived from the same parental generation and are expanding in their geographical distribution in South Africa. Future studies are recommended to expand and cover a larger snail sampling localities, including various districts in all provinces of South Africa, to further understand the provincial sub-

cladding of the *R. natalensis* populations and application of metagenomics to elucidate the molecular adaptation of *P. columella* to various environmental and climatic conditions.

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Chapter 5

Geographical distribution, ecology and infection status of hosts of *Fasciola* species from selected localities in South Africa

5.1. Abstract

Four hundred of the 5000 freshwater snail species documented worldwide have been reported to be on the African continent. Although freshwater snails have a wide distribution and are of veterinary and medical importance, information on their identity and geographic distribution remain poorly understood and documented in many African countries. This study, therefore, aimed to assess the distribution, ecology and infection status of lymnaeid snails which are the main intermediate hosts of Fasciola spp., from selected localities in six provinces of South Africa. To achieve this, freshwater snail survey was conducted in six South Africa provinces between 2021 and 2022, targeting water habitats where either human and/or animal activities were occurring. Snails were collected using a scoop and/or by hand picking, and information on the localities such as location coordinates, water body types, water chemistry and snail data were recorded using pre-designed forms. A total of 3417 freshwater snails across 10 habitats were collected during this period, of which 1059 were lymnaeids. *Radix natalensis* and *Pseudosuccinea columella* were identified and the later was the most widely distributed lymnaeid snail, found in five out of six surveyed provinces. Although *R. natalensis* was the most abundant lymnaeid species (649/1059), and collected from Mpumalanga and Limpopo provinces, the differences in abundance when compared to *P. columella* was not statistically significant ($p > 0.05$). Limpopo province recorded the highest number of lymnaeid specimens (746/1059), and both species occurred. Results showed that lymnaeid snails favoured perennial water bodies (dams and rivers) with muddy substrate and abundant aquatic grass. While *R. natalensis* occurred only in grassy aquatic habitats, *P. columella* utilized diverse habitats including habitats with algae, sandy and rocky substrates. There were no statistical differences in the abundance of lymnaeid snail species based on the habitat type (perennial and seasonal sites), or between activities occurring on site (human and animal vs animal only) ($p > 0.05$). The two lymnaeid species mostly cohabitated together, or with other snail species such as *Bulinus globosus*, *B. tropicus*, *B. forskaii*, *Biomphalaria pfeifferi*, *Gyraulus conollyi*, *Physella acuta*, and *Tarebia granifera*. The generalized linear models showed that the province, site type, substrate, activity, and pH were all important indicators of *P. columella* abundance while substrate and activity were the important indicators of the abundance of *R. natalensis*. Snails were shed for cercariae, and infections were screened from the snail tissue and identification of cercariae based on 28S marker. The following trematode infection rates were recorded in *P. columella* (12.33%), *B. truncatus* (33.33%), *Ph. acuta* (33.33%) and *R. natalensis* (36%). However, none of the sequences matched *Fasciola* spp. and many of them had no matches on the GenBank.

Thus, the results highlight a need for the design of sensitive *Fasciola* spp. specific PCR primers. Furthermore, there is a need to expand the survey on freshwater lymnaeids to include the other regions of South Africa to understand the distribution and locality biodiversity of these snails, and their future risk in expanding the spread of *Fasciola* spp.

Keywords: Lymnaeids*, Pseudosuccinea columella, Radix natalensis, Fasciola* species, geographic distribution, ecology, abundance, South Africa.

5.2. Introduction

Molluscs are the second most diverse animal phylum in the world (Lydeard et al., 2004; Kemp et al., 2016). Approximately 5000 freshwater snail species have been documented worldwide (Olkeba et al., 2020; Min et al., 2022), and 400 of which have been recorded in African countries (Brown, 1994; Moema et al., 2008). Freshwater snails have been recognised to play a role as first or second intermediate hosts (IHs) of trematodes and some nematodes, and this includes certain parasitic diseases of public health and veterinary importance such as schistosomiasis, clonorchiasis, fascioliasis, and opisthorchiasis (Strong et al., 2008; Madsen and Hung, 2014; Lu et al., 2018). Despite this, snail-borne infections remain some of the major parasitic diseases with persisting global public health issues, particularly in poor countries (Lu et al., 2018; Luviano et al., 2022).

Approximately 350 freshwater snail species have been estimated to be possible intermediate hosts of pathogens that affect both animals and humans (Lofty and Lofty, 2015). One of such snail borne infections of veterinary and medical importance is fasciolosis, a parasitic disease caused by digenean liver flukes from the family Fasciolidae (Admassu et al., 2015; Molaba et al., 2023). Freshwater snails from the family Lymnaeidae have been implicated as the primary vectors of *Fasciola* spp. globally (Molaba et al., 2023). The transmission and epidemiology of fasciolosis depends on the availability and ecological needs of their respective intermediate host species (Correa et al., 2010; Malatji et al., 2019a; Nyagura et al., 2022), and in turn primarily influenced by the availability of suitable habitats (de Kock and Wolmarans, 1998). Furthermore, Malatji et al. (2019b) reported that ecological factors (rainfall, soil stratum type, type of habitat and temperature) and climate are some of the determining factors in the dispersion of lymnaeid snails and their capacity to sustain the developmental stages of trematode species.

The distribution of molluscs is highly dependent on the availability of aquatic biotopes (de Kock et al., 2002a; Kemp et al., 2016) coupled with vegetation and sediment (Kemp et al., 2016). Lymnaeidae snails have been shown to occur in a wide range of habitat types worldwide (Hubendick, 1951; Alba et al., 2019) as they are an ecologically diverse family (McCraw, 1959). The most successful alien invasive species, *P. columella* (de Kock et al., 2002a; de Kock and Wolmarans, 2007), for example, can

be found in both transformed/man-made and natural habitats (Alba et al., 2019; Ngcamphalala et al., 2022). This snail species is amphibious; thus, it could be easily affected by the chemical and physical factors of water (Alba et al., 2019). The African native *R. natalensis* (Appleton and Miranda, 2015; Malatji et al., 2019b) has been shown to occur in both standing and flowing waterbodies (Appleton and Miranda, 2015) and it thrives at lower altitudes (Chikowore et al., 2019). The distribution of *G. truncatula* unlike its counterparts is confined to higher altitudes as it prefers cooler habitats (Malatji et al., 2019b; Mahulu et al., 2019) and can withstand droughts and survive in unsuitable waterbodies with harsh environmental conditions (McCraw, 1959; Mahulu et al., 2019).

In South Africa, *Fasciola* spp. infections have been reported to occur in Limpopo, Mpumalanga, Eastern Cape, Western Cape, KwaZulu-Natal and Gauteng Provinces (Nyagura et al., 2022). The authors also report that the transmission pattern of fasciolosis coincided with that of the freshwater snail hosts implicated in the transmission of this trematode species. Five snail species (*Galba truncatula*, *Radix natalensis, Radix auricularia*, *R. rubiginosa*, and *Pseudosuccinea columella*) that have been implicated in the transmission of fasciolosis have been documented in this country (Malatji et al., 2019a; Nyagura et al., 2022). *Fasciola gigantica* is found in tropical/warmer regions and is transmitted by *R. natalensis* in South Africa (Mahulu et al., 2019; Malatji et al., 2019a). Cases of *F. gigantica* have been documented in Mpumalanga and KwaZulu-Natal provinces where *R. natalensis* was also documented (Nyagura et al., 2022). *Fasciola hepatica* on the other hand is mostly found in subtropical/cooler regions and *G. truncatula* acts as the IH for this species (Mahulu et al., 2019; Malatji et al., 2019a). In South Africa, this snail species and *F. hepatica* have been reported in KwaZulu-Natal, Limpopo, Gauteng and Mpumalanga provinces (Nyagura et al., 2022). The extremely invasive *P. columella,* a North American native snail, has been documented to occupy many freshwater habitats in the African continent (Malatji and Mukaratirwa, 2019; Jones et al., 2024). In South Africa, *P. columella* has enhanced the transmission of fasciolosis as it has been found naturally infected with *F. hepatica* in Gauteng (Molaba et al., 2023) and *F. gigantica* in KwaZulu-Natal and Eastern Cape provinces (Malatji and Mukaratirwa, 2019).

Due to environmental heterogeneity, it is essential to conduct local surveys to determine the habitat preference of snail IHs, as the relative importance of environmental factors differs considerably in different geographic regions (Liu et al., 2021; Min et al., 2022). Furthermore, monitoring the biodiversity changes in the water bodies due to anthropogenic activities is important (Darwall et al., 2009). Additionally, to identify factors that influence the habitat preference and distribution of snails is crucial for the development of prevention and control strategies for snail-borne diseases (McCraw, 1959; Olkeba et al., 2020). Although the occurrence of lymnaeid snails and *Fasciola* spp. in definitive hosts have been adequately reported in South Africa, the distribution data is over three decades old

and does not represent the current status. Furthermore, the potential epidemiological role played by these freshwater snails in the transmission of *F. hepatica* and *F. gigantica* has not been extensively studied. This study, therefore, aimed to assess the distribution and ecology of freshwater lymnaeid snails and their infection status from selected localities in six provinces of South Africa.

5.3. Methodology

5.3.1. Ethical consideration

The study was approved by the Animal Ethics committee of the University of KwaZulu-Natal (AREC/020/020P) and the DALRRD section 20 (P2020-13) (Appendices 1 and 2).

5.3.2. Sample collection

Lymnaeid snails were surveyed in all seasons from 2021-2022 across six provinces (Mpumalanga, Gauteng, KwaZulu-Natal, Free State, Eastern Cape and Limpopo) of South Africa. The study followed a stratified random sampling method (Coppolino, 2010; Nwoko et al., 2023). Firstly, study areas were stratified by province and by locality which was then followed by simple random sampling (Nwoko et al., 2023). Study provinces were selected based on the presence of recent fasciolosis cases in the province, previous and local knowledge of freshwater snail collection and biodiversity, permanent and temporal habitats (Malatji et al., 2019b; Malatji and Mukaratirwa, 2020; Nyagura et al., 2022; Molaba et al., 2023), activities occurring in the sites such as washing and bathing points, fishing and animal water points (Nwoko et al., 2023). Freshwater snails were collected within one meter into the water using a metal scoop or through hand picking from the substrate and/or vegetation and preserved in 70% ethanol. Thirty to forty minutes were spent at each site, and the number of snails collected were considered as snail abundance, and the number of identified snail species per site were used to determine species diversity. Collected snails were kept and transported to the laboratory in plastic containers. Upon return to the laboratory, snails were individually shed to screen for natural infections by placing them into tot cups containing distilled water and exposing them to light for 1 hour and again for 1 hour in the dark. Shed cercariae were preserved in 70% ethanol for future molecular analysis.

Information collected included general locality information (locality name, type of habitat, GPS coordinates), and water temperature and pH were recorded on pre-designed forms. Ecological data (substrate, vegetation, animal contact, human contact), and snail data (snail species, abundance) were recorded. Representative photographs of different types of sampled habitats were taken (Figure 5.1).

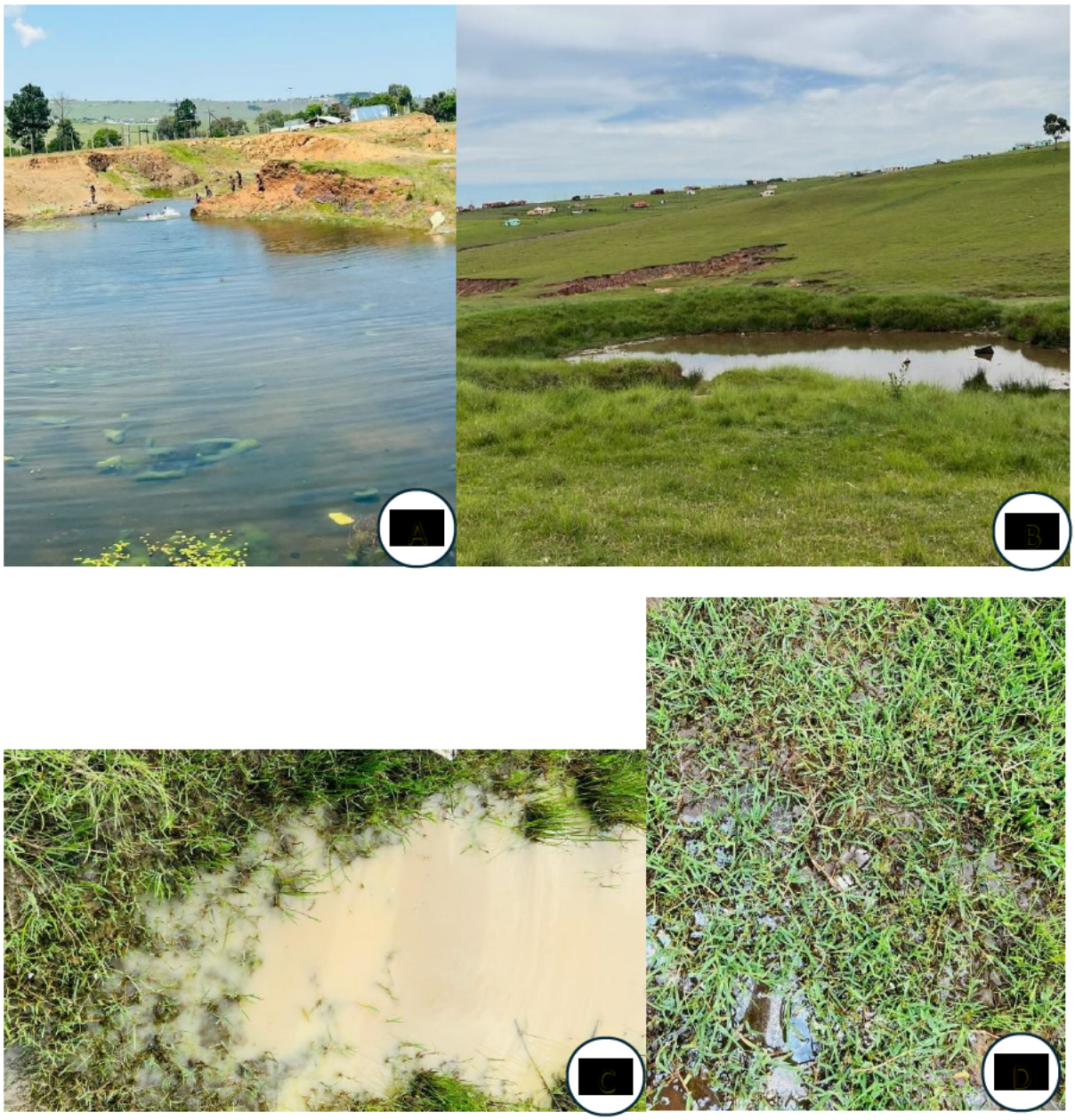

Figure 5.1 Images depicting site choices for freshwater snail surveys. A – habitats with human activities (swimming, washing of clothes and car wash) in Mthatha, Eastern Cape province; B to D– habitats near communities where there is both animal and human activities in Harrismith, Free State province; (c) Verulam, KwaZulu-Natal province and (d) Tzaneen, Limpopo province.

5.3.3. DNA extraction and molecular analysis

Genomic DNA was extracted from snail tissue and cercariae using DNeasy® Blood and Tissue Kit (Qiagen), according to the manufacturer's instructions. Resulting DNA was quantified using the Nanodrop. The 28S rRNA gene and primers (forward: 5'-A CGTGATTACCCGCTGAACT-3' and reverse: 5'-C TGAGAAAGTGCACTGACAAG-3') (Iman-Baran et al., 2012) were used for the detection and identification of *Fasciola* spp. infections in snails and cercariae. Genomic DNA from *F. hepatica* adult

flukes was used as a positive control. Amplifications were performed in a 25 µL PCR reaction mixture following the thermal cycling conditions 94°C for 3 min; 30 cycles of (30s at 94°C, 30s at 60°C, 60s at 72°C) and final extension for 10 min at 72°C. Fragments were separated using 2% agarose gels stained with SYBR Safe DNA Gel Stain (Applied Biosystems, USA) and visualised using Uvitec Uvidoc HD6. Successful results were identified by a band at 618 bp. Positive amplicons were sent for sequencing at Inqaba Biotechnical Industries (Pty) Ltd., Pretoria, South Africa for sanger sequencing. Resulting sequences were edited, consensus sequences created, and assembled using the Clustal W (Thompson et al., 1997) option of the BioEdit program (Hall, 1999). The resulting dataset was BLAST searched on the NCBI GenBank database to identify the closest matches.

5.3.4. Data analysis

Raw data of the ecological factors was captured on Microsoft Excel spread sheet. To evaluate the normality of the snail abundance data, the Shapiro-Wilk test was used. Non-parametric tests were used for additional analysis as the data did not follow a normal distribution. While the Kruskal-Wallis test was used to evaluate variations in snail abundance across habitat types and provinces, the Mann-Whitney U test was used to compare the abundance of *P. columella* and *R. natalensis* between perennial and seasonal habitats, and different activities occurring on site (human and animal versus animal-only).

Additionally, Generalised Linear Models (GLMs) were used to ascertain how different ecological and environmental factors—such as temperature, pH, activity, substrate, site type, and province—affect snail abundance. Since snail abundance is count data, the interaction between the predictor variables and snail abundance was examined using a Poisson log-linear model. The Omnibus test, Pearson's chi-square, and Akaike's Information Criterion (AIC) were used to evaluate the models' goodness of fit. In addition, parameter estimations were analysed to find important snail abundance predictors.

5.4. Results

5.4.1. Distribution and abundance of lymnaeid snail species

A total of 3417 freshwater snails were collected from the 26 of 43 surveyed habitats between 2021-2022, from six of 16 localities in all six provinces (Figure 5.2). Of these, 1059 snails belonged to the Lymnaeidae family and were collected from five of the six surveyed provinces. These were identified as *Radix natalensis* and *Pseudosuccinea columella* and were collected from 10 habitats out of the 26 surveyed habitats, in Mpumalanga, Limpopo, Gauteng, KwaZulu-Natal and Eastern Cape provinces of South Africa (Figure 4.3 (Chapter 4)). *Radix natalensis* was documented in three sites from Limpopo (n = 2) and Mpumalanga (n=1) provinces (Table 5.1). *Pseudosuccinea columella* was found in all five provinces indicating a wider geographical distribution. Of the 1059 lymnaeid snails collected, the

highest number of snails were collected in Tzaneen, Limpopo province, which contributed 64% (746/1159) to the total collected specimens (Table 5.1). *Radix natalensis* was the most abundant snail species (n = 649) (Table 5.1), of which majority were collected from Tzaneen (n = 573). This area also documented 42% (173/410) of the total *P. columella* collected, and the remaining 58% (n = 237) were scattered across four provinces (Table 5.1). Inversely, Mpumalanga and Gauteng province recorded the lowest number of *R. natalensis* (n = 76) and *P. columella* (n = 7), respectively. The Shapiro-Wilk test revealed that the snail abundance data did not follow a normal distribution ($p < 0.05$) (Table 5.2). Furthermore, the non-parametric tests revealed no significant difference in snail abundance between *P. columella* and *R. natalensis* ($p > 0.05$) (Table 5.3). When comparing snail abundance among provinces using the Kruskal-Wallis test, no significant differences were detected ($p > 0.05$) (Table 5.4, Table 5.5).

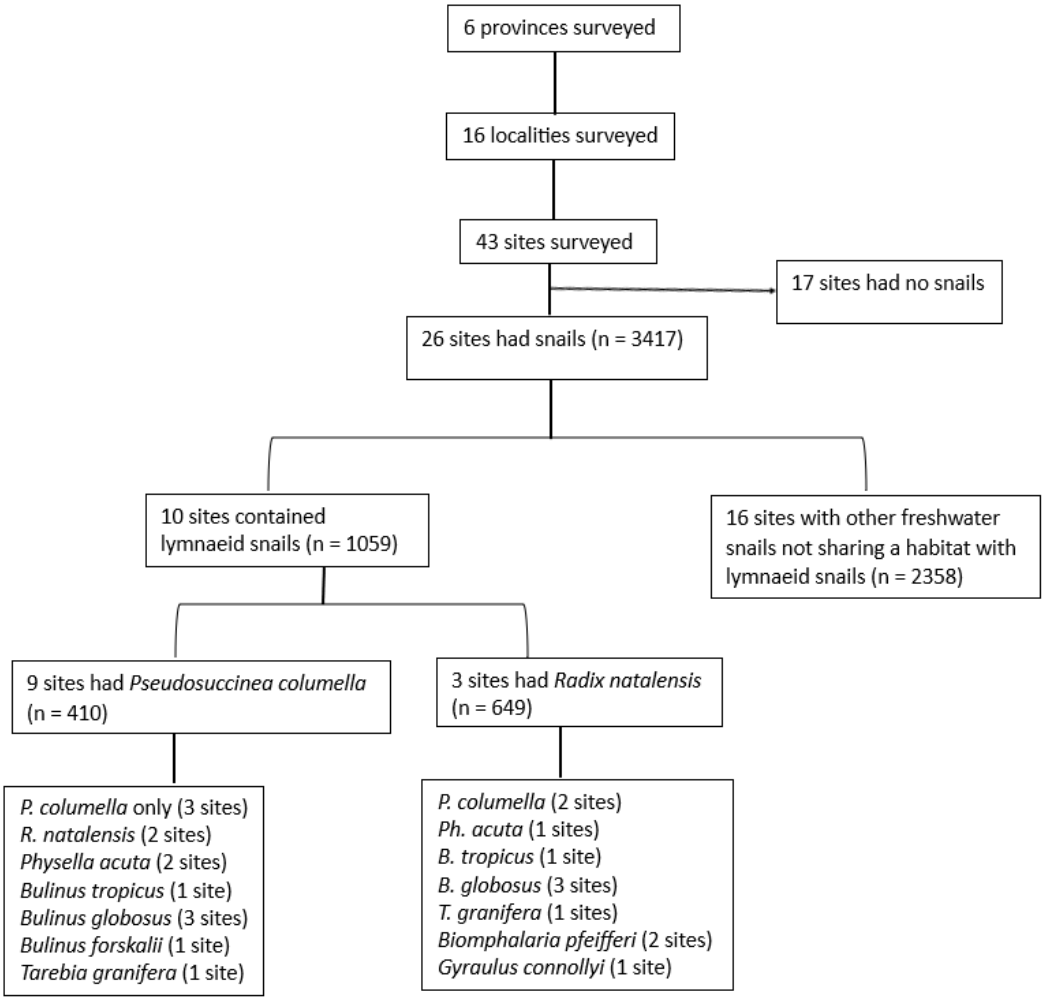

Figure 5.2 Flow diagram overall data of surveyed water bodies and freshwater snail species in 5 provinces, South Africa between 2021-2022.

Table 5.1 Abundance of *P. columella* and *R. natalensis* collected from different localities in five provinces, South Africa.

Province	Locality	No. of sites	Abundance		Total
			Pseudosuccinea columella	***Radix natalensis***	
Limpopo	Tzaneen	3	173	573 (2)	746
Mpumalanga	Middleburg	1	46	0	46
	Mbombela	1	0	76	76
Gauteng	Culinan	2	7	0	7
Eastern Cape	Mthatha	2	63	0	63
KwaZulu-Natal	Durban	1	121	0	121
Total		**10**	**410**	**649**	**1059**

* The number in brackets depicts the number of sites from which *Radix natalensis* was found.

Table 5.2 Normality tests on the abundance recorded for *Pseudosuccinea columella* and *Radix natalensis* from five provinces, South Africa.

Snail species	Kolmogorov-Smirnov[a]			Shapiro-Wilk		
	Statistic	df	Significance	Statistic	df	Significance
P. columella	0.300	10	0.011	0.763	10	0.005
R. natalensis	0.364	10	<0.001	0.499	10	<0.001

[a] Lilliefors significance correction

Table 5.3 Abundance based on the Mann-Whitney U test for snail species, type of site and activity occurring on site.

Grouping variable	*P. columella*		*R. natalensis*	
	Statistic	Significance	Statistic	Significance
Species	247.000	0.090	247.000	0.090
Perennial and seasonal	4.500	0.170	6.000	0.207
Activity on site	12.000	1.000	4.000	0.087

Table 5.4 Abundance based on the Kruskal-Wallis H test for lymnaeid snail habitat preference.

Snail species	Statistic	df	Significance
P. columella	3.656	4	0.455
R. natalensis	8.670	4	0.070

Table 5.5 Snail abundance summary of parameters tested for *Pseudosuccinea columella* and *Radix natalensis.*

Variables	***Pseudosuccinea columella***			***Radix natalensis***		
	β estimates	**Std. Error**	**Sig.**	**β estimates**	**Std. Error**	**Sig.**
(Intercept)	-1.666E-14	1.6653E-14	.317	-21.593	1.0000	<.001
KwaZulu-Natal	-31.191	1.8003E-12	<.001	.000	1.9998	1.000
Mpumalanga	-29.339	2.0545E-12	<.001	-.076	1.4139	.957
Limpopo	-4.422	1.2254E-11	<.001	.000	1.4139	1.000
Eastern Cape	-29.255	9.0030E-13	<.001	-7.229E-5	1.9375	1.000
Perennial	4.167	9.0015E-13	<.001	2.311E-5	1.4142	1.000
Grass	2.452	1.1354E-11	<.001	26.000	.9999	<.001
Sandy	1.792	1.6653E-14	<.001	-3.402E-5	1.4142	1.000
Human and animal	2.820	9.0013E-13	<.001	1.790	.0000	<.001
pH	-1.770	.8849	.045	-1.679	2.0696	.417
Water Temperature	.016	.0395	.680	-.059	.0566	.294

5.4.2. Habitat characteristics and preferences of lymnaeids and co-habitation with other snail species

The 10 habitats with records of lymnaeid snails were both perennial (dam and rivers) and temporal (streams and ponds) habitats (Table 5.6; Table 5.7). *Radix natalensis* was found in perennial habitats, i.e. dams (n =2) and river (n = 1) (Table 5.7). *Pseudosuccinea columella* was found in four habitat types: rivers (n =3), dams (n =2), streams (n=2) and ponds (n =2). A high number of lymnaeid snails were recorded in perennial habitats for both *R. natalensis* (n = 649) and *P. columella* (n = 344) (Table 5.6). However, both snail species were collected in abundance in dams, which contributed 79.23% (839/1059) to the total number of snails collected at any of the other water bodies with both *P. columella* (25.68%) and *R. natalensis* (53.54%) (Table 5.7). Streams had the lowest number of lymnaeid snails with 66% (7/1059) *P. columella* recorded (Table 5.7). There was no significant difference in snail abundance ($p > 0.05$) based on perennial and seasonal sites (Table 5.3). All *R. natalensis* specimens were collected from habitats with abundant plants (grass) (Table 5.6; Figure 5.1D). Three hundred and ninety-one (95.37%) *P. columella* specimens were collected from habitats that were both muddy and contained abundant vegetation. Algae-rich habitats had the lowest recorded abundance of *P. columella* (n = 1) (Table 5.6). Results from the Kruskal-Wallis test revealed no significant difference ($p > 0.05$) when comparing snail abundance among habitat types (Table 5.4).

Table 5.6 Habitat characteristics of the sites from which *Pseudosuccinea columella* and *Radix natalensis* were found and the number of samples collected.

Habitat condition	***P. columella***		***R. natalensis***	
	No. of sites	**No. of samples**	**No. of sites**	**No. of samples**
Wet supply				
Perennial	5	344	3	649
Seasonal	4	66	0	0
Substrate				
Sandy	1	6	-	-
Grass (rotten/fresh)	0	-	3	649
Muddy + grass	6	391	-	-
Rocky	1	13	-	-
Algae	1	1	-	-
Activities				
Human	4	298	3	649
Animal	8	259	3	649

Table 5.7 Habitat preference of *Pseudosuccinea columella* and *Radix natalensis.*

Water body	*P. columella*		*R. natalensis*	
	No. of habitats	**No. of snails collected**	**No. of habitats**	**No. of snails collected**
Pond	2	59	0	0
River	3	72	1	82
Stream	2	7	0	0
Dam	2	272	2	567
Total	9	410	3	649

Seven other snail species were recorded cohabitating with the lymnaeid snails while other snail species were recorded in the remaining 16 surveyed sites (Figure 5.2). The freshwater snails which shared habitats with lymnaeids included the planorbids *Bulinus forskalii, B. globosus, B. tropicus, Biomphalaria pfeifferi, and Gyraulus connollyi,* and the invasive *Physella acuta* and *Tarebia granifera*. (Figure 5.2). In 90% (9/10) of these sites, *Pseudosuccinea columella* was found alone in 3 habitats and cohabitating with *R. natalensis*, *Ph. acuta*, *B. tropicus*, *B. globosus*, *B. forskalii,* and *T. granifera* in the other 6 sites. *Radix natalensis* was found in 30% of the sites cohabitating with *P. columella*, *Ph. acuta*, *B. tropicus*, *B. globosus*, *T. granifera*, *Bio. pfeifferi*, and *G. connollyi* (Figure 5.2).

5.4.3. Factors related to the abundance of *P. columella* and *R. natalensis*

Relative to the other categories, both lymnaeid species were more abundant in habitats with both human and animal activities (Figure 5.3). *Pseudosuccinea columella* and *R. natalensis* were more abundant in habitats that had both animal and human activities, while *P. columella* was found in low densities in habitats with animal consumption and other human activities like bathing and washing. *Radix natalensis* was not recorded in habitats, with both animal activities and fishing (Figure 5.3). The Mann Whitney U test showed no significant difference in snail abundance between activity types (human and animal vs animal only) ($p > 0.05$) (Table 5.3) Additionally, this can be seen on summary Tables 5.5. Water temperatures ranged from 11.1±1˚C in Culinan, Gauteng to 37.2±1 ˚C in Mthatha, Eastern Cape with a mean temperature of 23.96±1 ˚C (Table 5.8). *Pseudosuccinea columella* was found in all these water temperature ranges, however, *R. natalensis* was found in areas where the water temperature ranged from 17.7±1 to 28.7±1. The water pH ranges where lymnaeid snails were neutral to basic, and these were 7.22 in Tzaneen, Limpopo to 8.94 in in Culinan, Gauteng with a mean pH of 7.82 (Table 5.8). *Pseudosuccinea columella* was found in both neutral and strong basic pH, but *R. natalensis* was found in habitats with neutral pH. Lymnaeid snails were collected in abundance in habitats with water temperature of 19.2±1˚C and pH = 7.22 for both *P. columella* (n = 151) and *R. natalensis* (n = 491) from Tzaneen, Limpopo province. The lowest lymnaeid abundance was recorded for *P. columella* (n = 1) at 15.5±1˚C and pH = 8.94 (Table 5.8). ANOVA regression and residual showed

these factors did not significantly influence the abundance of both lymnaeid species ($p > 0.05$) (Table 5.9).

Table 5.8 Water temperature and pH in in waterbodies inhabited by *Pseudosuccinea columella* and *Radix natalensis.*

Locality	**Site**	***P. columella***	***R. natalensis***	**pH**	**Water Temperature (˚C)**
Durban	Hazelmere dam	121	0	7.51	27.7 ±1
Mbombela	Hezel Dam	0	76	7.62	28.7±1
Middleburg	Pond 1	46	0	7.47	24.7±1
Tzaneen	Tours dam	151	491	7.22	19.2±1
	Mootle river	13	0	7.68	22.2±1
	Ngwabitsi river	9	82	8.79	17.7±1
Mthatha	Pond 1	13	0	7.48	37.2±1
	River	50	0	8.12	35.6±1
Culinan	Stream 1	6	0	7.34	11.1±1
	Stream 2	1	0	8.94	15.5±1
Mean		-	-	7.82	23.96±1

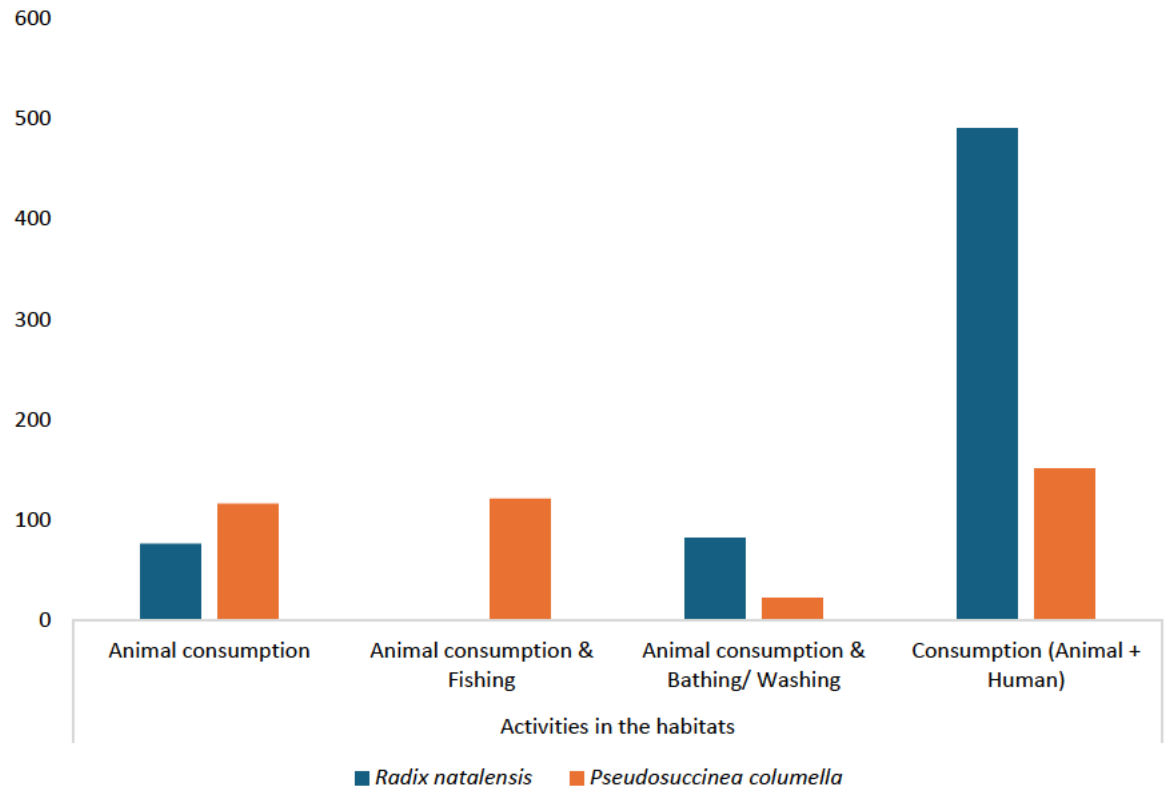

Figure 5.3 Abundance based on the types of activities occurring at the habitat preferred by *Radix natalensis* and *Pseudosuccinea columella*.

Table 5.9 Regression analysis on habitat water temperature and pH in lymnaeid snail habitats from five provinces, South Africa.

Snail species	Model	Sum of squares	df	Mean square	F	p-value
P. columella	Regression	4953.294	2	2476.647		
	Residual	20750.706	7	2964.387	0.835	0.473
	Total	25704.000	9			
R. natalensis	Regression	32058.952	2	16029.476		
	Residual	179401.948	7	25628.850	0.625	0.562
	Total	211460.900	9			

5.4.4. Generalized linear models (GLM) for snail abundance

A Poisson log-linear model was used to fit the GLM for *P. Columella*. A significant Likelihood Ratio chi-square of 568.537 ($p < 0.00$) was found by the Omnibus test, suggesting that the model adequately described the variation in snail abundance (Table 5.10). According to the parameter estimations, province, site type, substrate, activity, and pH all had a substantial impact on *P. columella* abundance. With a significant Likelihood Ratio chi-square of 2049.525 ($p < 0.00$) for *R. natalensis*, the GLM appears

to have sufficiently explained the variation in snail abundance. Substrate and activity were important indicators of *R. natalensis* abundance (Table 5.10).

Table 5.10 Omnibus test based on province, type of site, substrate type, activity on site, and water temperature and pH collected for *P. columella* and *R. natalensis*.

Snail species	Likelihood Ratio Chi-Square	df	Significance
P. columella	568.537	9	<.001
R. natalensis	2049.525	9	<.001

5.4.5. Molecular identification of *Fasciola* spp. infections in freshwater snails

A representative of 134 freshwater snails were screened for *Fasciola* spp. infections, and 22.39% (30/134) of the *R. natalensis, P. columella, Physella acuta* and *B. truncatus* showed a band at the expected size (618 bp). None of the *P. columella* specimens from Mthatha, Eastern Cape were infected (Table 5.11). However, upon sequencing, none of the sequences matched with *Fasciola* spp., 12/30 matched with *Orientocreadium* spp. (11/12) and *Trichobilharzia ocellata* (1/12) with similarities ranging from 98.19% to 99.65% (Table 5.11). *Orientocreadium* spp. *Orientocreadium* spp. DNA was detected in *P. columella, R. natalensis* and *Physella acuta* from Gauteng. *Trichobilharzia ocellata* was only detected in *P. columella* (Table 5.11). The sequences from the remaining 18/30 of the sequences failed quality control.

Table 5.11 Species identification of infections in freshwater snails from five provinces, South Africa.

Province of Origin	Sample ID	Host	Species of infection	% similarity
Gauteng	F6	*Fasciola hepatica* adult fluke	*F. hepatica*	99.65
	ACPH1	*Physella acuta*	*Orientocreadium* spp.	98.37
	ACPH2	*Ph. acuta*	*Orientocreadium* spp.	98.73
	ACPH4	*Ph. acuta*	*Orientocreadium spp.*	98.56
	ACPH5	*Ph. acuta*	*Orientocreadium* spp.	98.56
Limpopo	RT1	*Radix natalensis*	*Orientocreadium* spp.	98.19
	RT4	*R. natalensis*	*Orientocreadium* spp.	98.73
	RT6	*R. natalensis*	*Orientocreadium* spp.	99.28
	RT7	*R. natalensis*	*Orientocreadium* spp.	98.57
	RT8	*R. natalensis*	*Orientocreadium* spp.	99.10
	RT9	*R. natalensis*	*Orientocreadium* spp.	99.28
	RT10	*R. natalensis*	*Orientocreadium* spp.	99.46
KwaZulu-Natal	22PC	*Pseudosuccinea columella*	*Trichobilharzia ocellata*	99.31

5.5. Discussion

Pseudosuccinea columella has been documented as the third most widely distributed freshwater snail species in this country after *R. natalensis* and *B. tropicus*, respectively (de Kock et al., 1989, Appleton, 2003, de Kock and Wolmarans, 2008; Malati and Mukaratirwa, 2019). Data from this study, however, showed that this invasive lymnaeid snail was more widely distributed than both previously mentioned freshwater species. Despite the confined distribution of the native *R. natalensis* to Limpopo and Mpumalanga provinces, it occurred in high numbers compared to *P. columella*, although this was not statistically significant. This confirms previous reports that this native snail species is one of the most abundant freshwater snail species in South Africa (Appleton, 2003; Moema et al., 2008).

Of the five provinces where these two lymnaeid snail species were recorded, Limpopo province recorded the highest number of snails, followed by KwaZulu-Natal and then Mpumalanga provinces, but the differences in abundance between the provinces were not statistically significant. This was not surprising as these three provinces represent the tropical and sub-tropical regions of South Africa, and freshwater snails thrive in these regions (de Kock et al., 2002b). Despite previous reports showing the presence of five snail species in KwaZulu-Natal province (de Kock et al., 2003; Perissinotto et al., 2014; Appleton & Miranda, 2015; Malatji et al., 2019a), this study documented *P. columella* in Durban, both *P. columella* and *R. natalensis* recorded in Limpopo and Mpumalanga provinces. Furthermore, previous reports have also shown that in addition to these lymnaeid snail species recorded, there have been reports of *G. truncatula* (de Kock et al., 2003; Molaba et al., 2023), and *R. natalensis* (de Kock et al. 2001; Kemp et al., 2016; Moema et al. 2019; Malatji et al., 2019b) in all these surveyed provinces.

Both *R. natalensis* and *P. columella* showed the ability to cohabitate with each other and other snail species. Of the three habitats where *R. natalensis* was found, co-habitated with *P. columella* in two of the habitats, and both were found in higher numbers compared to other habitats, but *R. natalensis* was more dominant. Co-habitation of these lymnaeid snail species was common in areas where both species occur and was previously reported in Mpumalanga province (Malatji et al., 2019b). These species also shared habitats with predominantly Planorbidae species such as *B. globosus*, *B. tropicus*, *B. forskalii*, *Bio. pfeifferi*, and *G. connolyi*, and species from other families such as *Ph. acuta* and *T. granifera*. Although lymnaeid species were more abundant in most cases, there was an instance where *Bio. pfeifferi* was more abundant than *R. natalensis* in one habitat in Mpumalanga where *P. columella* was the only lymnaeid identified previously reported in the same habitat (Malatji et al., 2019b), and also in higher numbers. Similar observations were made in Hazelmere dam where our previous surveys found *P. columella* in higher numbers and as the only species in the site, but the current survey showed the presence of both *P. columella* and *Ph. acuta* and later species was more abundant than the former with n = 121 and n = 394 (results not presented) specimens, respectively.

Lymnaeids are largely dependent on atmospheric oxygen thus most of the species are found in ponds or streams that are semi-permanent or permanent (McCraw, 1959). In accordance with this, both lymnaeid species were found in abundance in dams over any other waterbody. However, *R. natalensis* was restricted to perennials (dams and rivers) which was consistent with other findings that this species prefers perennial habitats (de Kock et al., 1989; Brown, 13994; Malatji et al., 2019b). The success and high abundance of *R. natalensis* and *P. columella* can thus be attributed to their affinity to perennial water bodies as climatic change towards warming has led to the destruction of seasonal water bodies and consequently a decline in snails that favour these habitats (Zhytova et al., 2022). *Pseudosuccinea columella* proved to have a wider range of habitat utility and can tolerate a wider range of habitat conditions than its counterpart, *R. natalensis*. This invasive lymnaeid snail inhabited seasonal water bodies ranging from streams, to ponds, and perennial rivers and dams. This was to be expected as *P. columella* has been reported to thrive in various freshwater bodies including both permanent and temporal waterbodies (Appleton, 2003; Prepelitchi et al., 2011; Alba et al., 2019; Ngcamphalala et al., 2022), and its invasiveness has been reported to aid it to establish itself in both suitable habitats and in relatively unsuitable environmental conditions (de Kock et al., 1989).

From this study, the type of substrate influenced the habitat distribution and abundance of freshwater species, particularly for *P. columella*. Both *P. columella* and *R. natalensis* were found in abundance in grassy habitats (fresh and/or rotten plant materials). Their affinity to vegetation was expected as biological factors such as macrophytes have an impact on the population dynamics and ecology of freshwater snails (Ofoezie, 1999), by serving as shelter, a source of food, protection from direct radiation, prevention from wash off by high water current, and a substrate for egg laying (Appleton and Madsen, 2012; Oso et al., 2023). Conversely, the lower abundance of lymnaeid snails in habitats with a sandy substrate could be due to the poor stability (sand easily washes away) of the sandy substrate which severely disturbs any benthic dwelling invertebrates (Min et al., 2022).

Both lymnaeid species were found in habitats with different types of human activities but were found in abundance in habitats which were used by animal for grazing surrounding vegetations, drinking, cooling-off inside the water and defecating in the water. This finding is consistent with previous reports as freshwater snails tend to occur at high frequency and abundance in polluted waters as the water contains high concentrations of ions and organic matter which favour snail growth and propagation (Olkeba et al., 2020; Min et al., 2022). The authors suggested that reducing anthropogenic activities from disturbed water bodies could be used as a control strategy for snail-borne diseases (Olkeba et al., 2020; Min et al., 2022). According to Van Someren (1946), water pH is not a major factor in snail distribution as it does not serve to differentiate between habitats preferred by lymnaeid snails. Members from this species have been shown to tolerate pH ranges between 6.0 – 9.5 (Van Someren,

1946), as it was observed in this study. Temperature has been shown to be one of the important ecological factors in the distribution of freshwater snails as it affects the metabolic processes of the snail thus affecting snail reproduction, growth and survival (Hubendick, 1958; Mereta et al., 2023). Additionally, temperature greatly affects water properties such as the oxygen content of the waterbody through photosynthesis and bacterial decomposition (Hubendick, 1958).

In Africa, *Galba truncatula* and *R. natalensis* are long known as IHs of *F. hepatica* and *F. gigantica*, respectively (Bargues and Mas-Coma, 2005; Alemu, 2019). Recent reports have shown that the invasive *P. columella* can transmit *F. gigantica* (Malatji and Mukaratirwa, 2019) and *F. hepatica* (Molaba et al, 2023). However, Molaba et al. (2023) recently detected *F. hepatica* DNA in *Ph. acuta* and *B. truncatus* and this finding does not confirm natural infection. None of the *R. natalensis*, *P. columella*, *B. truncatus* and *Ph. acuta* screened for in this study were detected with *Fasciola* spp. DNA. The resulting sequences had no matches on the GenBank or matched with *Orientocreadium* spp. and/ or *Trichobilharzia ocellata*. Beesley et al. (2017) reported that one of the drawbacks with PCR was reproducibility between laboratories, stating that published methods often not work in other laboratories which might have been the case in this study. Furthermore, sensitivity of PCR has been shown to be reduced by inhibitory factors present within the intermediate snail host (Cucher et al., 2006; Beesley et al., 2017). Another issue might have been the sensitivity and specificity of the primers used in this study as it is difficult to make sure that PCRs are *F. hepatica* specific (Beesley et al., 2017). Beesley et al. (2017) noted this by observing that there were little or zero published DNA sequences available for the many trematode species isolated from *G. truncatula* making it hard to compare sequences obtained from various trematodes. Furthermore, the authors noted that most of the published *F. hepatica* PCRs have not been validated for snail tissue amplification.

5.6. Conclusion

The findings of this study can, therefore, be useful in the integration of prevention and control measures for snail-borne diseases. The results highlighted the need to design genetic primers that are more sensitive and specific for detection of infection in snails. *Fasciola* spp. Additionally, there is a need to screen freshwater snails for all digenean trematodes to understand the extent of the role played by individual snails of in the epidemiology of trematode infections of veterinary and medical importance. The limitation of this study was that the surveys were conducted on six of the nine provinces of South Africa, and selected sites and localities and hence, the results cannot be extrapolated for the entire province. There is, therefore, a need for future studies to expand the study areas into other provinces of the country, covering more localities. Furthermore, continuously monitoring of both native and invasive freshwater snail species to not only monitor diseases, but also

understand the changes in biodiversity and improve on control measures. Additionally, for future studies to include other environmental parameters such as dissolved oxygen, total dissolved solids, water velocity, and amount of minerals in the water to better understand habitat preference of these freshwater snails. Future study should also focus on potential use of non-lymnaeid snails as biological control agents through competition for food and habitats.

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Chapter 6

6.1 General conclusion

The comparison of experimental and field studies showed that overall pooled prevalence of experimental infections was significantly higher than that of the natural infections. *Pseudosuccinea columella* and *Galba truncatula* qualified for meta-analysis with pooled prevalences for natural infections of both *Fasciola* spp. and only *F. hepatica*, respectively. In experimental infections, *G. truncatula* and *P. columella* qualified with pooled prevalences both *Fasciola* spp., and *R. natalensis* for *F. hepatica*. Thus, the results of this study showed that *Fasciola* spp. use a wide range of lymnaeid species as their first IHs but *G. truncatula*, *R. natalensis,* and *P. columella* emerged as the main IHs, globally. While there seems to be a strong IH specificity between the two *Fasciola* spp., experimental infection results showed that *G. truncatula* and *R. natalensis* are susceptible to *F. gigantica* and *F. hepatica*, respectively. For both experimental and natural infections, *P. columella* was shown to be the better transmitter for fasciolosis.

Radix natalensis and *P. columella* were the only lymnaeids collected and identified, despite five lymnaeid species previously documented in South Africa (Appleton and Miranda, 2015; Malatji et al., 2019a; Malatji et al., 2019b). *Radix natalensis* samples showed intra-species diversity between and within populations. Though it may be concluded that this snail species is starting to diverge based on locality, however, a larger samples size and more localities from all provinces should have to be surveyed to reach a scientifically solid conclusion. *Pseudosuccinea columella* had the widest distribution as this snail species was found in all five surveyed provinces (KwaZulu-Natal, Limpopo, Eastern Cape, Mpumalanga and Gauteng). *Radix natalensis* was recorded as the snail species with the highest abundance even though its distribution was confined to grassy perennial waterbodies in Mpumalanga and Limpopo. Additionally, the overall highest abundance of lymnaeid snails was recorded in the Limpopo province which came as no surprise as this province is part of the tropical and subtropical regions of South Africa (de Kock et al., 2002).

Pseudosuccinea columella had only been proven to transmit *F. gigantica* (Grabner et al., 2014; Malatji and Mukaratirwa, 2019) and *F. hepatica* in South Africa (Molaba et al., 2023). However, these reports were limited to few locations. Assessment of lymnaeid snails from five provinces study however detected other trematode species like *Orientocreadium* spp. and *Trichobilharzia* spp. in *R. natalensis* and *P. columella*, respectively. These findings lead to the conclusion that there was need to design *Fasciola* spp. specific primers that were more sensitive and specific.

6.2 Limitation and recommendations for future research

The limitation of this study was the low number of localities and habitats surveyed, and thus the results can be for only that area and cannot be extrapolated to represent the whole province. Additionally, the sample sizes for specimens used in this study were small thus drawing conclusions on infection rates, type of trematodes that caused the infection and the genetic diversities of lymnaeid snails would be biased as these results do not represent a large group of the freshwater snails. Furthermore, the primers used in this study for the detection and characterisation of snail infections did not yield positive results and it might be that they were not specific to *Fasciola*, and hence identified other trematodes. Therefore, future research must focus on;

- Countrywide survey on freshwater snails to map out their distribution, habitat preferences, identify factors influencing their distribution and their abundance.
- Short- and long-term monitoring of freshwater snails' biodiversity and the trematodes they transmit, in order to record and understand any changes that may occur.
- Assess genetic diversity within and across lymnaeid snail populations from all nine provinces of South Africa
- Designing species specific primers that are highly sensitive and can easily detect and distinguish even between closely related trematode species from freshwater snail species.
- Development and application of eDNA protocols based on water samples, which will be crucial for detection of natural infections and monitoring of habitats with continuous *Fasciola* spp. infections in surveys to better understand the geographical extension of fasciolosis and areas with potential high risk of infections in both animals and human.
- There is also a need to screen other freshwater snail species, especially invasive snail species which are abundant in fasciolosis endemic areas, for their potential involvement in the distribution of *F. hepatica* and *F. gigantica*.

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Appendix

UNIVERSITY OF KWAZULU-NATAL
INYUVESI YAKWAZULU-NATALI

03 December 2020

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Dear Dr Malatji,

Protocol reference number: AREC/020/020P
Project title: Mapping Fasciola species and their intermediate hosts in South Africa.

Full Approval – Research Application

With regard to your revised application received on 12 October 2020, the Animal Research Ethics Committee has accepted the documents submitted and **FULL APPROVAL** for the protocol has been granted.

Please note: There must be adherence to national and institutional COVID-19 regulations and guidelines at all times. Researchers will be personally responsible and liable for non-adherence to national regulations. If in doubt, please contact the Research Ethics Chair and/or the University Dean of Research for advice.

Please note: Any Veterinary and Para-Veterinary procedures must be conducted by a SAVC registered VET or SAVC authorized person.

Any alteration/s to the approved research protocol, i.e Title of Project, Location of the Study, Research Approach and Methods must be reviewed and approved through the amendment/modification prior to its implementation. In case you have further queries, please quote the above reference number.

Please note: Research data should be securely stored in the discipline/department for a period of 5 years.

The ethical clearance certificate is only valid for a period of one year from the date of issue. Renewal for the study must be applied for before 02 December 2021.

Attached to the Approval letter is a template of the Progress Report that is required at the end of the study, or when applying for Renewal (whichever comes first). An Adverse Event Reporting form has also been attached in the event of any unanticipated event involving the animals' health / wellbeing.

I take this opportunity of wishing you everything of the best with your study.

Yours faithfully

[redacted]

Dr Sanil D Singh, BVSc, MS, PhD
Chair: Animal Research Ethics Committee

/kr

cc Supervisor: Prof Samson Mukaratirwa
cc BRU Manager: Dr Jaca

Animal Research Ethics Committee (AREC)
Ms Karen Reinertsen (Administrator)
Westville Campus, Govan Mbeki Building
Postal Address: Private Bag X54001, Durban 4000
Telephone: +27 (0) 31 260 8850 **Facsimile:** +27 (0) 31 260 4609 **Email:** animalethics@ukzn.ac.za
Website: http://research.ukzn.ac.za/Research-Ethics/Animal-Ethics.aspx

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Appendix 1 University of KwaZulu-Natal ethical clearance letter.

agriculture, land reform
& rural development
Department:
Agriculture, Land Reform and Rural Development
REPUBLIC OF SOUTH AFRICA

Directorate Animal Health, Department of Agriculture, Land Reform & Rural Development
Private Bag X138, Pretoria 0001

Enquiries: Ms Marna Laing • Tel: +27 12 319 7442 • Fax: +27 12 319 7470 • E-mail: MarnaL@dalrrd.gov.za
Reference: 12/11/1/1/18 (1866 LJvR)

Dr Mokgadi Pulane Malatji
National Zoological Gardens of South Africa
232 Boom Street
Pretoria
0001
Email: M.Malatji2@sanbi.org.za / pulanemalatji@gmail.com

Dear Dr Malatji,

RE: PERMISSION TO DO RESEARCH IN TERMS OF SECTION 20 OF THE ANIMAL DISEASES ACT, 1984 (ACT NO. 35 OF 1984)

Your application sent per email on 5 March 2021, requesting permission under Section 20 of the Animal Disease Act, 1984 (Act No. 35 of 1984) to perform a research project or study, refers. I am pleased to inform you that permission is hereby granted to perform the following study, with the following conditions:

Conditions:

1. This permission does not relieve the researcher of any responsibility which may be placed on him by any other act of the Republic of South Africa;
2. The study is approved as per the application form dated 1 March 2021 and the correspondence thereafter. Written permission from the Director: Animal Health must be obtained prior to any deviation from the conditions approved for this study under this Section 20 permit. Please apply in writing to MarnaL@dalrrd.gov.za;
3. All potentially infectious material utilised, collected or generated during the study are to be destroyed at the completion of the study. Records must be kept for five years for auditing purposes;

4. Liver fluke samples may only be collected from cattle carcasses at abattoirs in Limpopo, Gauteng and Mpumalanga as per the provided state veterinary letters;
5. The liver fluke samples must be preserved in 70% ethanol and may then be transported to SANBI for molecular work;
6. A separate Section 20 application must be submitted for any work to be done at UKZN;
7. Any other subsequent use or distribution of samples collected as part of this study is also subject to obtaining separate Section 20 approval;
8. All waste must be disposed of as biohazardous waste by a registered waste contractor;
9. If required, an application for an extension must be made by the responsible researcher at least one month prior to the expiry of this Section 20 approval.

Title of research/study: Mapping *Fasciola* species and their intermediate hosts in South Africa (abattoir sample phase)

Researcher: Dr Malatji

Institution: SANBI National Zoological Gardens of South Africa

Our ref Number: 12/11/1/1/18 (1866)

Your ref: P2020-13

Expiry date: 2023-03

Kind regards,

[redacted]

DR. MPHO MAJA
DIRECTOR OF ANIMAL HEALTH
Date: 2021 -05- 1 2

SUBJECT: S20 PERMISSION FOR: MAPPING *FASCIOLA* SPECIES AND THEIR INTERMEDIATE HOSTS IN SOUTH AFRICA (ABATTOIR SAMPLE PHASE) - LJVR

Appendix 2 Department of Agriculture, Land Reform and Rural Development (DALRRD section 20) ethical clearance letter.

SANBI
Biodiversity for Life
South African National Biodiversity Institute

SANBI/RES/P2020/13

04 August 2020

Dr Mokgadi Pulane Malatji

University of KwaZulu-Natal

OUTCOME OF RESUBMITTED RESEARCH PROPOSAL

This letter serves to inform you that your resubmitted research proposal titled "Mapping Fasciola species and their intermediate hosts in South Africa" was **approved** by the SANBI NZG Animal Research Ethics and Scientific Committee (RESC).

The following provisos should be taken into consideration:

1. Inform the RESC of completion or termination (with reason) of your research at the SANBI.
2. Submission of an annual progress report in November of each year. Failure to submit a progress report may result in approval to be withdrawn.
3. Submission of a written request for an extension or for any changes within the research project.
4. The SANBI should be acknowledged in all reports, scientific publications and conference contributions as follows:
   - The SANBI is acknowledged for providing samples/research platform.

The research proposal has been registered on the database as P2020/13. Please use this project number in all future correspondence.

Thank you for making use of the SANBI NZG as a research platform.

Yours sincerely

[redacted]

Prof Antoinette Kotze
Chairperson: SANBI NZG Animal Research Ethics & Scientific Committee

Appendix 3 South African National Biodiversity Institute (SANBI) ethical clearance letter.