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The identification, diversity and ecology of gastropod - trematode associations of economic significance in Lake Kariba, Zimbabwe

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Preface

This project took more than a full year from preparation of the field trip to writing this thesis. Along the way, many people got involved or were approached for assistance and expertise. Therefore, I would like to take this space to thank those people and acknowledge every single bit that they contributed to the finalization of this thesis.

I cannot thank one more than my mentor and co-supervisor Dr. Tine Huyse. She made it possible for me to study parasites, organisms that I find most interesting. The only available master thesis subject on parasitology last year, involved an Erasmus exchange to Perpignan and because I was already on Erasmus exchange in Paris (gaining knowledge in parasitology), I could not obtain an Erasmus grant anymore. She proposed the Zimbabwe project to me and from this particular phone call on, she has guided me intensively in all aspects of this project: obtaining a travel grant, performing field and lab work, analysing data and writing the thesis itself. I would never have devoted the amount of time and work to a project that doesn't match my interests, as this project did. I would like to thank prof. Filip Volckaert and prof. Maxwell Barson for their support, advice and guidance through the application of the travel grant and partaking the field trip. I must thank VLIR-UOS for the financial support and the University of Zimbabwe and Royal Museum for Central Africa for hosting me. The intense cooperation with fellow MSc students Kudzai Muzarabani in Kariba and Ruben Schols in Tervuren deserve a heartfelt thank you as well. Furthermore, I would like to thank Cyril Hammoud very much as for the support, assistance and advice during the (seemingly everlasting) 'failing-PCR-period' and other 'hard lab-times'. I would like to pay tribute to Zenzo Khali, Godwin Mupandawana, Elmon Dlomo, Takudzwa Madzivanzira, and all Zimbabwean students that helped with sampling in Kariba. I must thank prof. Tamuka Nhiwatiwa, Maxwell and Elmon for resolving the visa issue that threatened to end my field trip early. In Leuven I must thank Bart Hellemans and Ria Van Houdt for the advice on allozyme analysis and prof. Luc De Meester for donating the allozyme electrophoresis equipment. I would like to thank Nathalie Smitz and Kenny Meganck for their advice in the lab and during barcoding analysis. During the last weeks of analysing data, I received helpful recommendations from prof. Tom Wenseleers on my data analysis and prof. Christian Albrecht on the identification of some freshwater gastropods for which I am very grateful as well. Last but not least I would like to thank my girlfriend and fellow biology student Cara Byns for the continuous support and critical review of this work.

Content

Summary

Freshwater gastropod-borne trematode parasites can pose a great public health burden and cause major economic losses in the livestock and fish industry. Knowledge on the prevalence, diversity and ecology of both gastropods and trematodes is key to understand disease transmission dynamics and control trematodes of economic significance. Trematodiases are neglected tropical diseases that are mainly understudied. The lack of adequate tools to diagnose snail infections contributes to this problem. The primary goal of our study was to develop a reliable, cost and time efficient multiplex PCR assay that can diagnose general trematode infections in snails and identify those of economic importance, namely Schistosoma and Fasciola infections. Using these molecular tools, we found a significant underestimation of snail infection prevalence and frequent misidentification of both gastropod and trematode species using traditional methods. DNA barcoding confirmed seven gastropod species and six species of trematodes present in Kariba. We provide the first account of the invasive North-American gastropod *Pseudosuccinea columella* in Zimbabwe. Up to 60% of this snail population was infected with a *Fasciola* sp., closely related to the human and cattle infecting liver fluke *F. gigantica*. Furthermore, did we find two other introduced gastropod species i.e. *Physa acuta* and *Radix* sp. Lastly, our preliminary ecological analyses reveal correlations of gastropod species with water eutrophication and occurrence of the invasive water plant *Eicchornia crassipes*. This study therefore clearly demonstrates that man-made water reservoirs create a niche for invasive gastropods and plants which can alter the epidemiology of endemic trematodiases such as fasciolosis.

Samenvatting

Gastropoden-geassocieerde trematode parasieten vormen een aanzienlijke bedreiging voor de volksgezondheid wereldwijd en kunnen grote economische verliezen veroorzaken in de vee- en visindustrie. Kennis over de prevalentie, diversiteit en ecologie van gastropoden en de trematoden die ze verspreiden is van groot belang om de infectiedynamieken te begrijpen en trematode-epidemieën van economisch belang te beheersen. Trematodiasen zijn verwaarloosde tropische ziekten. Het gebrek aan adequate technieken om slakinfecties te diagnosticeren draagt bij aan dit probleem. Het primaire doel van onze studie was om een betrouwbare, kosten- en tijdefficiënte multiplex PCR-test te ontwikkelen die algemene trematodeninfecties bij slakken kan diagnosticeren en tegelijkertijd infecties van *Schistosoma* en *Fasciola,* beide erg economisch relevant, kan aantonen. Met behulp van deze moleculaire hulpmiddelen vonden we een significante onderschatting van de prevalentie van slakinfecties en frequente misidentificatie van zowel gastropoden als trematodensoorten met behulp van traditionele methoden. 'DNA-barcoding' bevestigde de aanwezigheid van minstens zeven soorten gastropoden en zes soorten trematoden in Kariba. Dit is de eerste waarneming van de invasieve Noord-Amerikaanse slak *Pseudosuccinea columella* in Zimbabwe. Tot 60% van deze slakkenpopulatie was besmet met een *Fasciola* sp., nauw verwant aan de runderleverbot *F. gigantica*. Verder werden twee andere geïntroduceerde gastropodensoorten voor het eerst waargenomen in het Karibameer; *Physa acuta* en *Radix* sp. Tenslotte tonen onze ecologische analyses correlaties aan van bepaalde gastropodensoorten met water-eutrofiëring en de invasieve waterplant *Eicchornia crassipes*. Deze studie toont daarom aan dat door de mens gecreëerde waterreservoirs een niche vormen voor invasieve gastropoden en planten die de epidemiologie van endemische trematodiases zoals fasciolosis kunnen veranderen.

Introduction

1. Role of freshwater gastropods in disease transmission

Some freshwater gastropod species are intermediate hosts to parasitic flat- or roundworms that have their definitive host in humans and other vertebrates. Besides forming a public health burden, gastropod-borne helminths are a problem in the aquaculture and livestock industry leading to billions of US$ù in economic losses worldwide (Giannelli *et al.,* 2016). About 50 nematode species of the superfamily Metastrongyloidea use gastropods as intermediate host but nematodes are of lesser concern in medical and veterinary parasitology, which is why we limit this thesis to the gastropod-borne trematodes (GBTs). One gastropod snail species can host an array of trematode species with different intermediate and final hosts (Giannelli *et al.,* 2016). This can have an impact on public-, veterinary and ecosystem health as illustrated in figure 1.

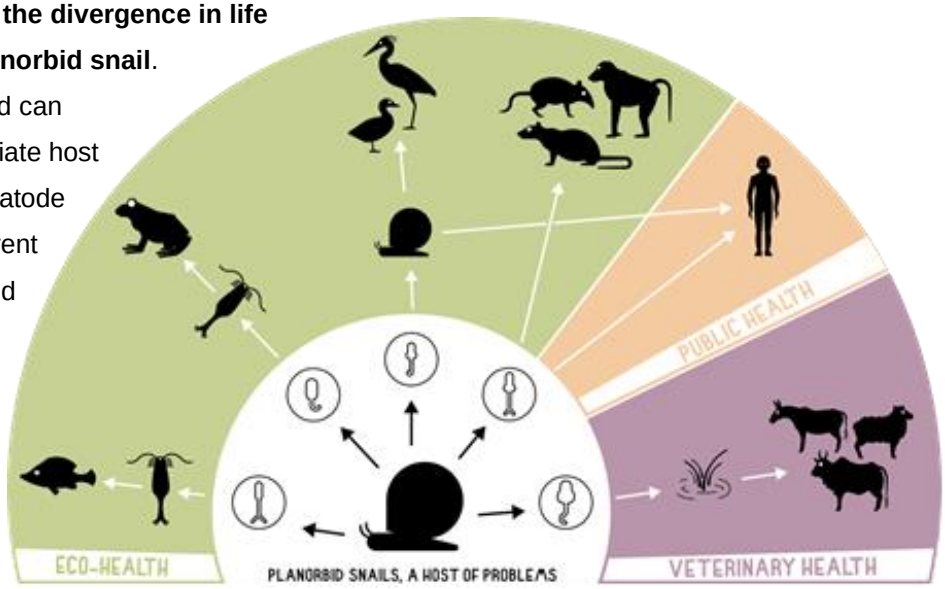


Figure 1. Illustration of the divergence in life cycles of GBTs in a planorbid snail. One species of gastropod can function as first intermediate host for an array of trematode species, each with different final and/or second intermediate hosts with an impact on eco-, human and veterinary health. (Image: courtesy of Tine Huyse).

The reality however is that all gastropod-borne diseases are neglected diseases. Gastropod-borne parasite research falls behind in the parasitic research agenda although millions of people suffer from gastropod-borne diseases. Economic losses due to infected livestock and wild or farmed fish were also found to have a tremendous economical impact. Because of the neglected status of these diseases, most research on GBTs is in its infancy when it comes to knowledge about their epidemiology, biology and ecology (Adema *et al.*, 2012; Toledo *et al.*, 2014). In this study we investigate the gastropod and trematode fauna of the understudied hydro-electric dam reservoir of Kariba, Zimbabwe.

2. African freshwater gastropods

2.1. Ecology and biology

Gastropods, commonly called snails, colonized all continents except Antarctica and are common in a wide range of terrestrial and aquatic habitats (Strong *et al.,* 2008). Freshwater gastropods mostly thrive as benthic macroinvertebrates in moist or submerged habitats such as sediment, macrophyte vegetation and decaying plant material. Most freshwater gastropods prefer specific habitats such as lakes, ponds, temporary pools, small streams or rivers, depending on their life cycle and feeding method. Almost all freshwater gastropods feed by grazing the surface of the aquatic habitat they live in with a specialized organ, called the radula, although some have been recorded as filter feeders (e.g., Bithyniidae) or active predators (e.g., Glacidorbidae) (Strong *et al.,* 2008). For freshwater gastropods, there are few exceptions to the bearing of a hard shell. The shell comes in many shapes and sizes varying from the discoidal shell of for example *Biomphalaria* sp. to the cap-shaped shell of the members of the Ancylidae family and the more common spiral, ovate shell of for example the *Bulinus* genus (Brown, 1994). Concerning ecology and life-history traits, gastropods are divided in two groups: the Caenogastropoda and the Pulmonata or heterobranchs. Whereas the Caenogastropoda possess a true ctenidium (molluscan gill), the pulmonates breathe air with a vascularized air bag or molluscan 'lung'. This makes pulmonates in general slightly more tolerant to extreme environmental factors such as drought, hypoxia and eutrophication while Caenogastropoda are rather specialists, with restricted geographic ranges. Other traits such as hermaphroditism with the ability of self-fertilization, separate the pulmonates from the strictly dioecious and sexually reproducing Caenogastropoda. Altogether, pulmonates are generally better in dispersion, colonization and less sensitive to change, both environmental and human-induced. They are also more common intermediate hosts for gastropod-borne parasites and other pests (Strong *et al.*, 2008), which will be the focus of this study.

2.2. Diversity

Gastropods contain, after bivalves, the highest number of species in the phylum of molluscs, which in turn is one of the most widespread and diverse phyla in the animal kingdom. Like many other aquatic invertebrates, the species diversity of freshwater gastropods is high in the tropics, although the highest diversity has been recorded in the Palearctic realm (Strong *et al.*, 2008). From the described 409 families of gastropods, an estimated 5%, representing 4000 species and 33 families, are freshwater gastropods (Bouchet *et al.*, 2005). Strong *et al.*, (2008) mention 366 described species from 16 families present in the Afrotropical region.

This region is, besides the Antarctic (with no representation) and the Pacific Oceanic Islands, the least represented zoogeographical region when it comes to freshwater gastropod diversity. An additional 45 newly described freshwater species per year in non-tropical regions makes the global diversity expand rapidly (Strong *et al.*, 2008). Africa is however one of the regions that recorded a lower increase in the description of new species in the most recent years although it contains some regions that were identified as diversity hotspots for freshwater gastropod fauna such as the lower Zaire basin, the lowland forests of West Africa (Guinea and Ivory Coast) and the large oligotrophic lakes of Eastern Africa: Tanganyika, Malawi and Victoria (Strong *et al.*, 2008). The interest of identifying small freshwater gastropods increased significantly in Africa only since the discovery of the role of freshwater gastropods in the transmission of human trematode parasites such as schistosomes (Brown, 1994).

2.3. Classification

The common understanding in modern classification of freshwater gastropods is that morphological features are insufficient for a sound classification and identification. Molecular tools such as DNA barcoding and molecular phylogenies of DNA and proteins are thought to be more objective (Mandahl-Barth, 1962). The concept of DNA 'barcoding' is addressing a DNA sequence or marker of a relatively short length (around 650 bp) to a certain taxonomic level (e.g. species) (Hebert *et al.*, 2003). However, examining the shell and, to a lesser extent, the soft parts of gastropods has been the standard identification technique for decades, mostly because of practical and financial reasons (Mandahl-Barth, 1962). Currently, classification and re-evaluation of the classical taxonomy is ongoing. A summary of most recent taxonomical classification of African gastropod families is listed in Strong *et al.* (2008). Although no pulmonate family is strictly endemic to Africa, the family of Planorbidae is most commonly represented by 166 described species, of which most belong to the *Bulinus* genus (Brown, 1994; Strong *et al.*, 2008). In the context of GBTs, Planoribidae is the most important African freshwater gastropod family as it includes gastropods from the *Biomphalaria* and *Bulinus* genus. Both are gastropod genera that contain species crucial in the life cycle of many trematode parasites including the schistosomes that infect humans (Brown, 1994).

3. Gastropod-Borne Trematodes

3.1. Life cycle

Most trematodes have a life cycle with multiple hosts, but all have a gastropod as first intermediate host. Life cycles with three hosts are ancestral for digenetic trematodes but

two- or four-host cycles also commonly occur (Esch *et al*, 2001). Besides intermediate and final hosts, dead-end hosts, which block the cycle (e.g. humans in the life cycle of amphistomes) and paratenic hosts, in which no major developmental processes take place (e.g. crustaceans in the life cycle of *Angiostrongylus* sp.) also commonly occur (Giannelli *et al.*, 2016). A trematode life cycle starts with an egg being deposited in a secretion of the host, commonly faeces, urine or sputum. Eggs either hatch in the (mostly humid) environment or are being consumed by a gastropod. Either way, a mobile miracidium larva emerges and penetrates the gastropod's tissue. Miracidium larvae generally migrate to a specific organ inside the gastropod; depending on the trematode species this can differ but the hepatopancreas and gonads are most common. Next, a sporocyst, which is an asexual reproductive larval phase, multiplies into many daughter sporocysts or rediae. Rediae are, in contrast to sporocysts, actively feeding larvae (i.e. they possess a mouth). The sporocysts or rediae mature into cercariae, which leave the gastropod. From this stage the immense diversity in life-cycles diverges. Most cercariae are actively swimming larvae that consist of a head and motile tail, although immobile and tailless cercarial types are also found. Free cercariae can either encyst on vegetation (then called metacerciae) waiting to be consumed by a herbivorous mammal, or they can actively seek their secondary host which they penetrate. The second intermediate host, which can vary from being a vertebrate, crustacean or another mollusc, interacts as a prey for the next host. Metacercariae encyst in the tissue of the second intermediate host and end up in the next via consumption of this infected

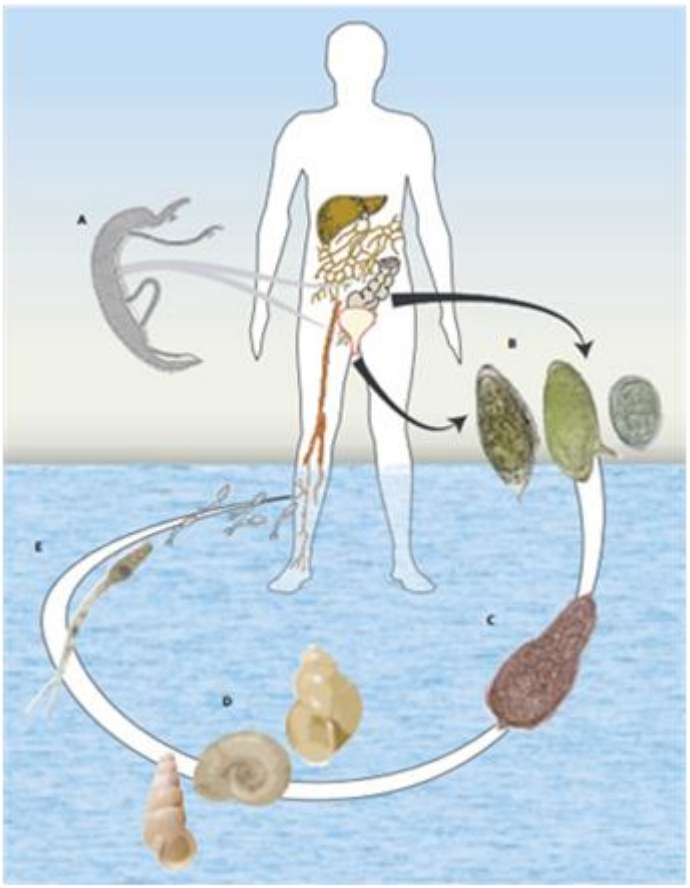


Figure 2. Illustrated life cycle of *Schistosoma* sp. A). Two adult worms (male and female) mate and deposit eggs in the mesenteric or bladder vein plexus. **B)** Schistosome eggs of f.l.t.r. *S. haematobium, S. mansoni* and *S. japonicum*. **C)** miracidium larva. **D)** Intermediate gastropod hosts f.l.t.r. *Oncomelania* sp. (host of *S. japonicum*), *Biomphalaria* sp. (host of *S. mansoni*) and *Bulinus* sp. (host of *S. haematobium*) (Gryseels *et al.* 2006).

tissue. When the (meta)cercariae finally arrive in the final host, the larvae will migrate to the appropriate tissue before developing into egg reproducing adult worms that restart the cycle (Esch *et al.,* 2001). An illustrated life cycle of the three major human infecting *Schistosoma* species is given in Figure 2.

3.2. Diversity, taxonomy and identification

The class of Trematoda (Phylum: Platyhelminthes) is divided into two subclasses: the vast group of Digenea and the smaller, four family containing Aspidogastrea. Trematodes that are most important in a medical and economical context belong to the subclass Digenea, with an estimated diversity of 18 000 species. Resolution of the evolutionary relationships of trematodes at higher taxonomic levels, has been a subject of major discussions. For the past 15 years different approaches combining morphological and life-history traits with molecular-based phylogenetic tools such as analysing 18S and 28S ribosomal RNA (rRNA) and mitochondrial genes, produced contradicting results. The molecular phylogeny of Olson *et al.* (2003) can be seen as a comprehensive phylogeny of higher taxonomic levels. The authors distinguish two groups: the order of Diplostomida, comprising six superfamilies and the order of Pagiorchiida containing 19 superfamilies from 13 suborders (Olson *et al.*, 2003). Much of the classification of Digeneans on lower taxonomic levels has been based on examination of morphological and life-cycle features. A diversity of around 18,000 species within 2,500 genera and 148 families is generally acknowledged (Toledo *et al.*, 2014). Morphological criteria for cercarial larvae identification are shape and structure of the head, tale, tegument, body suckers and excretory glands (Frandsen *et al.*, 1984). Geographical and behavioural observations can also help with the classification to respective family or sometimes genus level although species-level identification of trematodes is hard and only reliable when the life cycle is experimentally completed (Frandsen *et al.*, 1984; Moszczynska *et al.*, 2009). Species differentiation of adult trematodes is difficult or sometimes impossible for multiple reasons: the small, soft bodies have few clear characterizable traits, morphology is often similar between species (i.e. cryptic species), phenotypic plasticity is common and morphological differentiation and speciation do not occur at the same rate (Nolan *et al.*, 2005; Moszczynska *et al.*, 2009). Therefore, DNA based identification or genotyping of trematode species is highly recommended because of its sensitivity and specificity (Moszczynska *et al.*, 2009). A marker pre-eminently used for species-level identification is the cox1 gene (COI) because it is a coding, haploid inherited mitochondrial gene that evolves rapidly in the context of speciation (Hebert *et al.*, 2003). However, most studies that genotype digenean trematodes, use ribosomal DNA (rDNA) and especially the highly variable internal transcribed spacer (ITS) region as a marker. The small and large subunit

ribosomal genes 18S and 28S are other rDNA regions often used but since they are highly conserved, they are only useful to detect higher taxonomic levels (Nolan *et al.*, 2005). Identifying trematodes within infected gastropods creates constrains for developing primers. The primers have to be specific for trematode DNA but also universal for all trematodes in case there is no trematode taxon that is specifically targeted. The conserved nature of the 18S rDNA gene makes it possible to design selective, universal primers on (Routtu *et al.*, 2014). Both Moszczynska *et al.* (2009) and Routtu *et al.* (2014) propose 18S rDNA as a universal marker for acquiring basic preliminary taxonomic information of trematodes after which protein-coding genes such as *cox1* (Moszczynska *et al.*, 2009) can be sequenced to obtain higher resolution barcoding information such as species and population identity (Moszczynska *et al.*, 2009; Routtu *et al.*, 2014; Lawton *et al.*, 2015).

4. African Gastropod-Borne Trematodes of economic significance

4.1. Trematodes of medical concern

Schistosomiasis, is the most prevalent human infecting trematodiasis (Gryseels *et al.*, 2006). Schistosoma is the only genus of trematodes that contains species that almost exclusively use humans as final host (e.g. *S. haematobium*). Fever and abdominal pain due to adult worm migration is typical for acute schistosomiasis (also called Katayama fever). The main pathology originates however from migrating eggs that cause chronic infections of a variety of organs. *S. haematobium* causes urinary schistosomiasis which is the result of chronic inflammation of the bladder and ureteral wall. An associated symptom of early urinary schistosomiasis is haematuria (blood in urine) but chronic infection can lead to significant kidney damage and squamous bladder cancer. *S. mansoni* and *S. japonicum* (along with less important *S. mekongi* and *S. intercalatum*) can cause intestinal and hepatic schistosomiasis induced by migrating eggs and resulting in the inflammation of the intestinal mucosa and liver respectively. A common symptom of intestinal schistosomiasis is bloody diarrhoea. Hepatic, or hepatosplenic schistosomiasis is a more severe pathology that typically results in hepatomegaly, especially in children (Gryseels *et al.*, 2006). Concerning bird and non-human mammalian schistosomes, no serious trematode associated pathologies have been recorded in humans, although some species penetrate human skin (i.e. ‘swimmers itch’) (Toledo *et al.*, 2014). Highly endemic areas of human schistosomiasis are developing regions without adequate sanitary infrastructure (Gryseels *et al.*, 2006). A map with an overview of human schistosomiasis worldwide and details about species distribution is given in Figure 3. About 77% of all schistosomiasis occurs in Sub-Saharan Africa with the three most affected countries being Nigeria, Tanzania and Ghana (Adenowo *et al.,* 2015). The morbidity of schistosomiasis is estimated around 192 million people in

Sub-Saharan Africa, which is more than 90% of the global morbidity (207 million people) (Adenowo *et al.*, 2015). Annual mortality from schistosomiasis is highly disputable and estimates vary from 10,00 to 200,000 people worldwide (WHO, 2017a). The index of 'Disability-Adjusted Life Years or DALY is a better measure for the estimation of impact. It is calculated based on the mortality rate, prevalence and disability weight. For schistosomiasis DALY is estimated to be 1.5 million, calculated with an annual 14,000 deaths, a prevalence of 250 million people and a disability weight of 0.06 (Gryseels *et al.*, 2006). The disability weight is a disease specific value that quantifies the severity of the disease ranging from zero (no harm) to one (highly lethal) (WHO, 2017b).

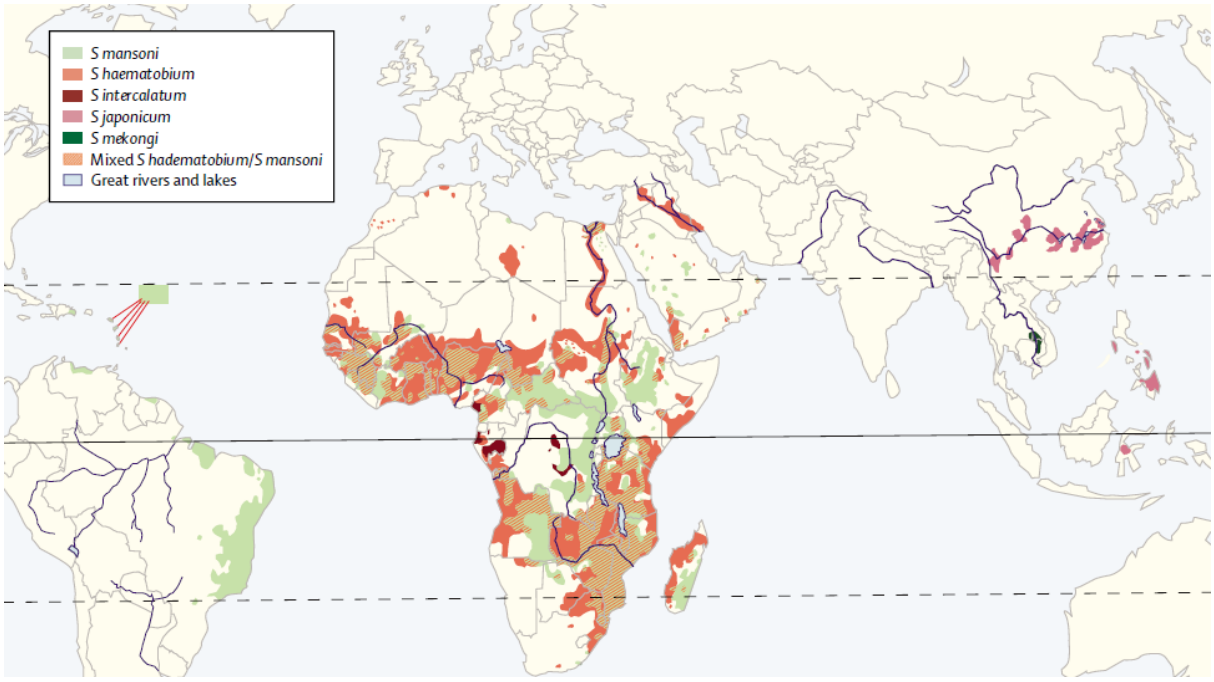


Figure 3. Distribution of the five major human infecting *Schistosoma* species. Occurrence of *S. mansoni* and *S. haematobium* (including mixed infections), is highly prevalent in Sub-saharan Africa. *S. intercalatum* is endemic to Central and West Africa. *S. japonicum* and *S. mekongi* are restricted to Asia (Gryseels *et al.* 2006).

Fasciolosis is caused by liver flukes of the Fasciola genus gallbladder (Toledo *et al.*, 2014). *Fasciola* species (family: Fasciolidae) have a two-host life cycle with one intermediate (gastropod) host and a final ruminant host (e.g. cattle, sheep and buffalo). Humans are infected by eating raw or poorly cooked liver or raw, poorly washed aquatic vegetables on which metacercariae are encysted. Two species are known to infect humans: *F. hepatica* and *F. gigantica. F. gigantica* is less prevalent but more pathogenic compared to *F. hepatica*. Acute fasciolosis results in abdominal pain, loss of appetite and fever. Chronic infections, however, can result in hepatomegaly, portal liver cirrhosis and infections of the pancreas and gallbladder (Toledo *et al.*, 2014). Global prevalence estimates of human fasciolosis vary from 2.4 million to 17 million people infected and 180 million people at risk in more than 60

countries (figure 4). *F. hepatica* has the broadest geographic range (i.e. altitude, latitude and longitude) of all human infecting trematodes, which is due to the generalist nature of both the trematode and its gastropod host, a lymnaeid (DiNardo, 2015). Human *F. hepatica* infections form a public health problem mainly in the Caribbean, Northern Africa, Western Europe and some Andean and Caspian countries. *F. gigantica* has a different geographic range and is most prevalent in Sub-Saharan Africa, Asia and Hawaii (Toledo *et al.*, 2014). There is no strong correlation between fasciolosis in livestock and humans, which makes most of the epidemiological data that assess infection rates in cattle and sheep of limited use for human fasciolosis epidemiology (Esteban *et al.,* 1999). Morbidity and mortality rates of *Fasciola* sp. are low compared to other trematodes of biomedical concern. In Africa, most reports of human fasciolosis come from the Magrheb countries (i.e. Morocco, Tunesia and Algeria) and Egypt (Ashrafi *et al.,* 2014).

Figure 4. Distribution of human fasciolosis worldwide according to WHO (2013). *Fasciola hepatica* is found in the Americas, Australia, Russia and Europe. *F. gigantica* is restricted to Asia and Africa (Ashrafi *et al.* 2014).

With the exception of schistosomiasis, the impact of most human trematodiases is commonly understudied (Harhay *et al.*, 2010). However, all trematodes combined threaten to infect more than 10% of the world population (Toledo *et al.*, 2012). More broadly, over one billion people in developing regions all over the world encounter an estimated 14 million DALY from trematode infections annually (DiNardo, 2015).

4.2. Trematodes of concern in the livestock and fish industry

Animal fasciolosis in livestock may reduce reproduction, growth rate and carcass quality as well as increase mortality in young animals, resulting in economic losses in the meat and dairy industry (Mehmood *et al.*, 2017). Both *F. hepatica* and *F. gigantica* are found in Africa where they commonly infect domestic animals such as cattle, sheep, goats, horses and donkeys (Toledo *et al.*, 2014). Furthermore, wild African ruminants have been found to be naturally infected with prevalence's up to 50% in African buffalo (*Syncerus caffer*) and some antelope species (Bindernagel, 1972). About 31 studies between 2000 and 2015 found at least eleven African countries, including Zambia and Zimbabwe, to have high prevalences of fasciolosis in cattle and sheep (Mehmood *et al.*, 2017). The highest prevalence of ovine fasciolosis was found in Ethiopia (40.2% of sheep infected). Bovine fasciolosis was found to be most prevalent in Sudan, with a 91% rate. Zambian and Zimbabwean infection rates of bovines were 48.9% and 15.4% respectively. The global economic losses due to Fasciola infections in livestock are estimated to be over 3.2 billion US$ per year. In poor, highly endemic African countries the effect on the economy is thought to be underestimated (e.g. estimated 1.94 million US$/year in Sudan) (Mehmood *et al.*, 2017).

Amphistomiasis is caused by intestinal flukes called amphistomes, a collective name for all members of the superfamily Paramphistomoidea. They represent a major economic problem infecting livestock worldwide (Toledo *et al.*, 2014). The superfamily contains twelve families of which Paramphistomidae, Gastrothylacidae and Gastrodiscidae are speciose in Africa (Laidemitt *et al.*, 2017). Amongst the most important amphistomes in Africa is *Calicophoron* (*Paramphistomum*) *microbothrium* (family: Paramphistomidae) (Pfukenyi *et al.,* 2005). These rumen flukes cause major damage to the intestine of the ruminant host due to migration of the larvae through the mucosa. Due to its high pathogenicity and prevalence, amphistomiasis tops the list of most important parasitic diseases in domesticated livestock worldwide. An estimated 500 million cattle are infected annually but the true dimension of global economic damage due to decreased reproduction and calf mortality is not clear (Toledo *et al.*, 2014). Epidemiological data on amphistomiasis and its economic impact are scarce for Sub Saharan Africa although infection rates were found to be relatively high. In two studies of faecal examination for amphistome eggs in cattle, 29.5% and 51.6% of the examined bovine found to be infected in Zimbabwe and Zambia respectively (Phiri *et al.*, 2006; Pfukenyi *et al.*, 2007).

Animal schistosomiasis is thought to infect more than 165 million bovines in Africa and Asia (Gower *et al.*, 2017). *Schistosoma bovis, S. curassoni* and *S. mattheei* infect livestock such as cattle, sheep and goats all across Sub-Saharan Africa (Toledo *et al.*, 2014). Bovine schistosomiasis forms a problem of major economical concern in the livestock industry but

epidemiological data are scarce, especially in comparison to those on human schistosomiasis (see 4.1). Hybridisation between bovine and human *Schistosoma* species has also been found and this could possibly result in strains with an altered virulence or transmission potential (Huyse *et al.*, 2009). Therefore, a One Health approach aiming at the control of both human and animal schistosomiasis ought to be the most efficient way to tackle this problem (Gower *et al.*, 2017). *S. bovis* is one of the most common schistosomes in Africa. It can be present in up to 90% of cattle, as found in Sudan where it accounted for an estimate of 7.1% in calf mortality (McCauley *et al.,* 1983). In Zimbabwe however, *S. mattheei* is the only bovine schistosome recorded, infecting up to 70% of the cattle sampled (Pfukenyi *et al.*, 2006). The pathology of bovine schistosomiasis is usually subclinical but in general, infections cause chronic diarrhoea, weight loss, reduced meat and milk quality and possibly a decreased reproductive capacity (Gower *et al.*, 2017).

Trematodes of fish are of economical concern to both the fishing industry and fish farms. Fish farms are especially vulnerable to disease because of the high-density fish populations they host. Some fish trematodiases cause mass mortalities and stunted growth rates in a wide array of commercial fish species. At least 50 families of freshwater fish trematodes can be found in African waters of which the Aporocotylidae (Sanguinicolidae) and Diplostomatidae families are most prevalent (Gebremedhn *et al.*, 2017). The family of Aporocotylidae consists of 33 genera that are also known as fish blood-flukes. The induced pathology in the final fish host can range from weight loss to acute mortality (e.g. mass mortality in juveniles of carp due to *Sanguinicola inermis*). Mass mortality is a typical result of the penetration of a large number of trematode larvae into or out of the fish gills. Species of the genus Diplostomum, or fish eye flukes, cause blindness in their host but mass mortality in juveniles has also been observed (Toledo *et al.*, 2014). Although blindness is not fatal in adult fish, it can lead to an altered foraging behaviour and a higher risk of predation (Grobbelaar *et al.*, 2014; Toledo *et al.*, 2014). Trematodes of the Uvulifer and Posthodiplostomum genera (family Diplostomatidae) cause ‘black spot disease’. No serious pathology is associated with this infection but the appearance of the black spots on the fish’ skin makes the fish less attractive to customers (Toledo *et al.*, 2014). The epidemiological context for most of the mentioned fish trematodiases is unclear, especially in Sub-Saharan Africa. Metacercariae of the fish-eye fluke *Tylodelphys* sp. (Family: Diplostomatidae) were found in some commercially important farmed fish in Kenya, including the redbelly tilapia (*Coptodon zillii*) (Otachi *et al.*, 2014). A study by Gebremedhn *et al.* (2017) found Clinostomum and Euclinostonum (Family: Clinosomatidae) to be the most prevalent trematode genera infecting commercialized fish in Ethiopia, especially African catfish (*Clarias gariepninus*) and Nile tilapia (*Oreochromus niloticus*) (Gebremedhn *et al.*, 2017).

5. Diagnostic methods for trematode infections

5.1. Diagnosis in the final host

Diagnosis of trematode infections in the final host can range from dissection of the host, examination of excreta or more advanced molecular and biochemical techniques. Direct, microscopy-based parasitological essays of stool or urine are, prior to immunological and molecular tests, most widely used in the detection of the most common GBTs in man (Keiser *et al.*, 2010). Many advanced dilution and staining techniques are applied such the Kato-Katz funnel technique (the use of a glycerol-methylene blue soaked cellophane covered tube to separate and stain the eggs) and FLOTAC, in which a solution is added to bring the eggs in flotation during centrifugation (Toledo *et al.*, 2014; Utzinger *et al.*, 2015). Immunological essays (i.e. detection of antibody against or antigens from the parasite) are commonly used to diagnose trematode infections in Western travel clinics. Several polymerase chain reaction (PCR) based techniques for parasite DNA detection have been developed in recent years. The specificity of amplifying parasite DNA to a detectable quantity is virtually 100% and it has been used for differentiating between many closely related parasite species (e.g. between the liver flukes *Opisthorchis viverrini* and *Clonorchis sinensis*) (Keiser *et al.,* 2010; Utzinger *et al.*, 2015). The fact that it can detect very early stage infections (even before serology and microscopy techniques show positive results) and hybrid species, makes it a powerfull diagnostic tool. Modifications to the traditional PCR tool such as loop-mediated isothermal amplification (LAMP), copro-DNA detection assays and real-time quantitative PCR are being studied intensively and might become important tools in the future (Johansen *et al.*, 2015). The high cost and requirement of highly trained personnel limits however the use of these techniques in poor, highly endemic countries (Utzinger *et al.*, 2015).

5.2. Diagnosis in the intermediate gastropod host

The classic method for diagnosing and quantifying trematode infections in gastropods is by either dissecting the gastropods or inducing natural emergence of cercariae (i.e. ‘shedding experiments’). By dissecting or crushing individual gastropods, germinal sacs containing sporocysts or rediae, metacercarial cysts and cercariae become detectable in a light microscope examination. However, since immature cercariae from different trematode species and types are hard to distinguish and crushing the shell results in loss of the gastropod and its possibility to shed mature cercariae after all, natural emergence is preferred (Appleton *et al.*, 2015). Natural shedding of cercariae in gastropods can be induced by placing the gastropods under direct artificial light for multiple hours. Emerging cercariae can be mounted on a microscopic slide and identification keys can be used to

identify cercarial types (a classification proposed due to the difficult determination into genera and species). Observations on its behaviour (e.g. swimming and resting position), ecological data (e.g. gastropod host species, proximity of final hosts, etc.), epidemiological knowledge (e.g. data on prevalence in the considered region) and enhanced morphological characteristics by staining the cercaria, might lead to identification of the family or sometimes genus (Frandsen *et al.*, 1984). However, shedding highly underestimates the prevalence of both single and double infections (Born-Torrijos *et al.*, 2014). Hence, assessment of true gastropod infection prefers a PCR-based technique (Born-Torrijos *et al.* 2014). There, low infection rates, immature infections and the death of the gastropod do not longer represent an obstacle to estimate the trematodal infection rate in a gastropod population (Caron *et al.,* 2008; Born-Torrijos *et al.*, 2014). PCR techniques range from single classic PCRs to multiplex PCRs (amplifying more than one specific DNA fragment using multiple primers in a single PCR reaction) and quantitative Real Time PCR. The latter is a fluorescent-based PCR technique that measures the amount of amplified DNA quantitatively based on the cleavage of a dye-labelled fluorescent probe and 5'-3' exonuclease activity of the added DNA polymerase (Caron *et al.,* 2008). In a multiplex PCR, more than one trematode taxon/species can be detected by designing multiple primer pairs hat amplify certain genetic markers, with varying lengths, in the same or different genes for different trematode taxa/species. In this way, gastropod tissue samples can be easily screened for multiple trematodes by separating the amplified DNA according to their length through agarose gel electrophoresis. Sequencing and genotyping the obtained DNA can also be used to identify multiple species in a DNA mixture (Routtu *et al.*, 2014). A selection of markers used in PCR-diagnostics for trematode infections in gastropods is given in Table 1.

Table 1. A selection of PCR assays used for the detection and discrimination of a variety of trematodes species in gastropods. The genetic marker is mentioned along with the different trematode (or gastropod) species that can be discriminated with it and the type of PCR used by the reference.

Type	Marker(s)*	Species	Reference
Single PCR	1117 bp *S. haematobium* specific region (nuclear DNA)	*S. haematobium* differentiation from *S. bovis, S. mattheei, S. curassoni, S. intercalatum* and *S. magrebowiei*	(Akinwale *et al.*, 2014)
Multiplex PCR	Microsatelites	S. *mansoni, S. haematobium* and *S. bovis*	(Van den Broeck *et al.,* 2011)
Multiplex PCR	124 bp *Fasciola* sp. specific region (nuclear DNA) and Lymnaeid ITS2 (rDNA)	*F.* hepatica and *Galba truncatula* (Lymnaeid gastropod)	(Kaplan *et al.*, 1995; Caron *et al.*, 2011)
Multiplex PCR	COI (mtDNA)	*S.haematobium* and *S. bovis*	(Webster *et al.,* 2010)

Multiplex PCR	DraI repeat	*S. haematobium, S. bovis, S. mattheei, S. curassoni, S. intercalatum* and *S. magrebowiei* and mixed infections	(Abbasi *et al.*, 2012)
Multiplex PCR	ITS1 and ITS2 (rDNA)	4 different trematode species**	(Born-Torrijos *et al.*, 2014)
LAMP	DraI and Sm1-7	*S. haematobium* and *S. mansoni*	(Hamburger *et al.*, 2013)

* If not stated otherwise (i.e. ribosomal DNA (rDNA), mitochondrial DNA (mtDNA)), all markers are nuclear. **Trematode species of no direct medical or veterinary importance in East-Africa or Zimbabwe

PCR-based techniques can be regarded as one of the most reliable, and perhaps the golden standard of diagnostic tools for detecting trematodes in their intermediate host because of their sensitivity and specificity (Caron *et al.*, 2008). Two noteworthy techniques involving PCR that are of high interest for future diagnostics are Loop-Mediated Isothermal Amplification (LAMP) and eDNA screening. LAMP is a DNA amplification technique in which DNA is amplified in an Eppendorf tube just by adding LAMP reagent under ambient temperature conditions. The amplified DNA can then be visualized with SYBR green in the same tube (or run on an agarose gel like in classical PCR). LAMP has a specificity ten times larger than classic PCR and it is a fast, easy, cheap and reliable tool for field diagnosis (Hamburger *et al.*, 2013; Gandasegui *et al.*, 2016). Environmental DNA or eDNA is DNA found in soil or water samples in which the target organism (e.g. trematode or gastropod) resides. In eDNA sample is submitted to quantitative PCR analysis of short amplicons because DNA degrades quite fast in the environment. Screening for eDNA in water samples to assess prevalence of human infecting trematodes has already successfully been done for *Schistosoma japonicum* and *Opisthorchis viveriini* (Worrell *et al.*, 2011; Hashizume *et al.*, 2017). A technique that has been abandoned since access to DNA detection with PCR-based diagnostics, is biochemical detection of differences in isoenzymes that are discriminable between different trematodes and the gastropod host. A study by Van Aken *et al.* (1987) showed that banding patterns of Glucose Phosphate Isomerase (GPI) in thin-layer starch gel electrophoresis can be used to identify infections of *F. hepatica* in a *Lymnaea truncatula* gastropod (Van Aken *et al.*, 1987). A study by Wright *et al.* (1979), used isoelectric focusing to estimate both prevalence and diversity of trematode infections in *Bulinus senegalensis* (Wright *et al.*, 1979). The authors showed that a broader range of trematode species can be detected at once using GPI or Malate Dehydrogenase (MDH) enzyme assays (Wright *et al.*, 1979). Allozyme techniques are cheap, easy in use and could be very practical in preliminary studies for infection prevalence in simple field laboratories with low budgets (Caron *et al.*, 2008).

6. Aims of the study

One of the thesis' research aims is to provide a first complete description of the gastropod – trematode associations at the Kariba lake shoreline, near the rural village of Kariba, Zimbabwe. Three goals were set to do so. The first and main goal is to develop a multiplex Rapid Diagnostic Polymerase Chain Reaction (*multiplex RD-PCR*) protocol to identify general trematode infections in snails and simultaneously check for infections of medically important *Fasciola* sp. and *Schistosoma* sp. in lymnaeid and planorbid gastropods respectively. The second goal is to identify the Kariban gastropod and trematode species found, using DNA barcoding. We do so in a One Health context, including all trematode and gastropod host species, which is unique for lake Kariba. Furthermore, we exploit and compare various other diagnostic tools and identification techniques for the assessment of gastropod-trematode diversity and infection prevalence. We compare classical techniques (morphological determination, shedding experiments) with molecular identification methods (DNA barcoding, allozyme analysis and multiplex RD-PCR) and formulate a recommendation for practical use. The third goal is to gather ecological data and to try explain differences in gastropod community structure between sites, which can be of great value for preventative consideration of economical important trematodiases.

Materials and methods

1. Ethical statement and risk assessment

Research permits were obtained by the University of Zimbabwe (Harare, Zimbabwe). Collecting and euthanizing live gastropods from the wild did not require further ethical approval. Live gastropods were euthanized by direct immersion into 90% ethanol. Although gradually increasing the ethanol concentration causes less stress, direct immersion is the standard method applied (Gilbertson *et al.,* 2016). Health risks and preventative measures taken are are described in detail in Annex I.

2. Sampling

2.1. Sampling locations

Gastropods were collected (see 2.2 Sampling methods) at 16 sites located on the banks of lake Kariba near the rural village of Kariba, Zimbabwe (see **Figure 5**). The sampling sites were adopted from Chimbari *et al.* (2003) to enable later data comparison. Each site was sampled once a month for a year by fellow master student Kudzai Muzarabani (University of Zimbabwe) as part of her master thesis project on the ecology of gastropod- and trematode populations along the Kariba shoreline. I assisted the sampling team for five weeks to collect

samples for molecular analysis (from 23/10/2017 to 05/12/2017). Additionally, three fishing camp villages (Nyaotza, Fother Gill and Tsuwa) were sampled once because of rumours on high schistosomiasis prevalence due to intensive water contact of local fishermen. All sites are described in table 1 and figure 1 of supplementary tables and figure (Annex III and IV). All in-field lab work was performed at the biology and wet chemistry laboratory of the University Lake Kariba Research Station (ULKRS), further mentioned as field lab.

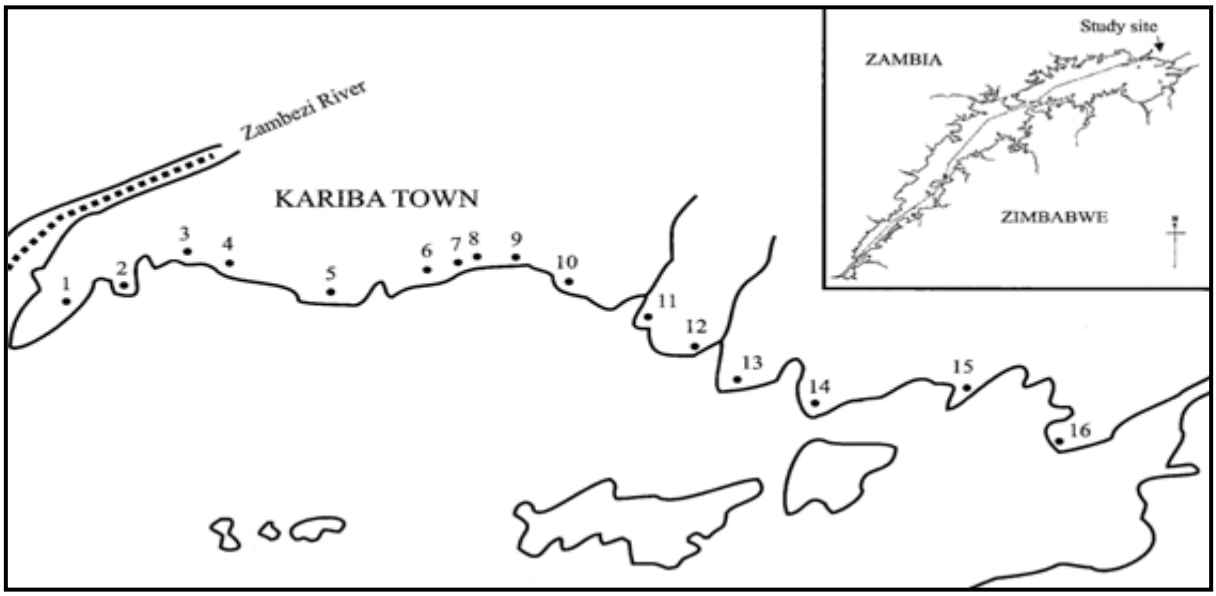


Figure 5. Scheme of the sampling sites along Kariba shoreline. Fishing camp sites are not in this figure. The names and coordinates corresponding to the site number are given in supplementary table 1 (Annex III). (Chimbari *et al.*, 2003).

2.2. Sampling methods

2.2.1. Gastropod collection and morphological identification

Gastropods were collected by examination of sediment and (floating) water plant vegetation such as water hyacinth (*Eichhornia crassipes*) using a scooping net and dredge for superficial and deep-water samples respectively. Sampling was performed by two people during 30 minutes and the collected vegetation was examined for another 30 minutes. Gastropods were stored and transported in plastic containers with lake water using a cooler box to prevent over-heating. In the field lab, gastropods were separated and counted per morphotype. The morphological identification of gastropods was based on the determination key of Brown (1994). Until the identification was confirmed by molecular analysis (see 3.1), the identified species or genera are further mentioned as 'morphotypes' (morphologically distinguishable types). After two shedding sessions (see 2.2.2), gastropods were fixated on ethanol. Uninfected gastropods were pooled per species and per site in 50 mL Falcon tubes on ethanol. Infected gastropods were individually stored in barcoded tubes filled with 2 mL

pure ethanol. At the Royal Museum for Central Africa (RMCA, Tervuren), high quality stacking photography was used to photograph each infected (shedding) gastropod specimen and at least two specimens of each gastropod morphotype for later morphometric based identification. Multiple pictures on a range of focal depths were taken and all pictures were stacked using Zerene Stacker® creating a high-resolution image of the gastropod on a calibrated scale.

2.2.2. Cercariae collection and morphological identification

Gastropod infection status was assessed by performing shedding experiments at the field lab and multiplex RD-PCR (see 3.2.2) at the RMCA. Once counted, gastropods were left to rest in darkness overnight before the first shedding protocol was performed. Gastropods were kept in foil covered plastic containers with daily refreshed lake water and fish feed for at least one week, after which shedding was repeated. For shedding, live gastropods were pooled per 5 individuals of the same morphotype in each well of a 12 or 24-well tissue culture plate filled with lake water. Next, the plates were exposed to artificial light in a self-made 'shedding box' for 1-2 hours between 8 and 10 a.m. Plates were checked every 15 minutes by a binocular microscope to identify emerged (shedded) cercariae. If positive, the snails were separated and further incubated in order to identify which gastropod was infected. Emerged cercariae were individually transferred in a drop of water to a microscopic slide using a micropipette. Cercariae were immobilized by adding a drop of pure ethanol. Based on morphology, the cercariae were catalogued to their respective type class using the determination key of Frandsen & Christensen (1984). After morphological classification, cercariae were fixated on stored in ethanol with their gastropod host (see 2.2.1).

2.2.3. Ecological parameter assessment

Every month, water samples were collected in polyethene bottles at standard depths (between 0 and 0.5m of depth) at each site. Following parameters were measured using a Portable Multimeter (HACH™, HQ40D): pH, temperature, dissolved oxygen concentration (mg/l), dissolved oxygen saturation and conductivity. The turbidity or transparency of the water was measured with a turbidity meter (Eutech Instruments™, TN100) using standard methods described by Wetzel (2001). Dissolved nutrient analysis to assess phosphate -, ammonia-, nitrate-, and Chlorophyl A concentrations was performed in the field lab. Detailed methods are described in Annex II**.** A set of discrete ecological characteristics of each site were only described once, around November 2017. Vegetation (i.e. level of abundance of most common species of floating water plant: *Eichhornia crassipes* or water hyacinth), substrate (muddy, sandy or rocky) and depth (shallow or deep) were catalogued.

3. Molecular analysis

3.1. Molecular identification of gastropod and trematode species

3.1.1. DNA extraction

The soft tissue of the preserved gastropod specimens was removed from the shell using a needle or, in case the gastropod was too small, crushing the shell with a forceps. From bigger gastropods the foot was separated from the rest of the body and discarded, for small gastropods the whole body was used for DNA extraction. Excess ethanol was removed with sterile absorbent paper. Gastropod tissue was homogenized with a scalpel on a glass plate. Cross sample contamination was prevented by sterilizing scalpel and forceps with ethanol and flame. Glass plate and gloves were rinsed with DNA AWAY™ (Thermofisher™). To extract DNA from gastropods, the proteinase *K* based E.Z.N.A. Mollusc DNA Kit (OMEGA bio-tek, Inc.) was used. This kit is a column extraction method developed for invertebrate tissue rich in mucopolysaccharides. Gastropod DNA extracts were diluted 1 in 10 to avoid PCR inhibition. For cercariae, the DNeasy Blood and Tissue Kit (Qiagen™) was used. DNA was extracted of a maximum of 4 individual cercariae per infected gastropod. Infected gastropods that did not shed cercariae but were found to be infected by the multiplex RD-PCR method (see 3.2.2) were also used to genotype both cercariae and gastropod DNA. These samples were however extracted using a different procedure i.e. Chelex® extraction (see 3.2.2.1).

3.1.2. Simplex PCR and Sanger sequencing

A simplex PCR was used for species genotyping of both gastropods and cercariae. Different markers were targeted with new primers that were designed by Tine Huyse and Cyril Hammoud (unpublished work) on an alignment including all African (and European) trematodes genera genotyped so far. The nuclear ITS rDNA marker, preferred for genotyping trematodes (Nolan *et al.*, 2005), was chosen along with COI for barcoding the cercaria and infected gastropods. Unfortunately, after multiple failed PCR and sequencing efforts of the ITS marker, this marker was abandoned (data not shown). Both Moszczynska *et al.* (2009) and Routtu *et al.* (2014) proposed 18S rDNA as a universal marker for acquiring basic preliminary taxonomic information of trematodes after which protein-coding genes such as *cox1* can be sequenced to obtain barcoding information such as species and haplotype identity (Moszczynska *et al.*, 2009; Routtu *et al.*, 2014; Lawton *et al.*, 2015). We opted for this approach. Primers used for the amplification of a cercariae-specific 18S rDNA marker of app. 1160 bp were 18S_Digenea_F (5'- CAGCTATGGTTCCTTAGATCRTA-3') and 1270_F (5'-ACTTAAAGGAATTGACGG-3') while COI was targeted using primers

COI1_dig_F (5'- CNATGATNTTNTTTTTTTTRATGCC-3') and COI1_dig_R (5'-GMASWACCAAAWTTHCGATCAAA-3') and yielded a fragment of app. 450 bp. The gastropod-specific markers were targeted using primers COI_gastropod_F (5'-TAATGTWATTGTTACAGCACATGC-3') and COI_gastropod_R (5'-GTTGRTATAAAATAGGATCACCWCC-3') for a 536 bp COI fragment while ITS was amplified using primers ITS1_gastropod_F (5'-TCGCGAGGTTCAAAGAGT-3') and ITS1_gastropod_R (5'- CTGTACTTGAGGCCACGG -3') that yielded a 393-793 bp amplicon. All PCR reactions were performed in a 15 µL volume with 1.5 µL DNA (1:10 diluted) using the Qiagen™ *Taq* DNA polymerase kit with 1.5 mM PCR buffer (Qiagen™), 0.6 mM dNTP mix (Qiagen™), 1.5 mM $MgCl_2$, 1.1 µM of each primer and 0.45 units of *Taq* Polymerase (Qiagen™). All simplex PCR protocols were performed by initial denaturation at 95°C for 5 min, 39 cycles of 94°C for 30 sec, 45°C for 30 sec and 71°C for 45 sec and a final elongation step at 71°C for 5 min in a Biometra® Tprofessional Thermal Cycler. Control of the PCR products was carried out by gel electrophoresis on a 2% agarose gel and Midori Green Direct® staining method and visualization under UV light. Samples that showed a clear amplified PCR product of the expected length were purified using an ExoSAP (Fermentas™) PCR purification protocol. Finally, 5 µL of sequencing primer (5 µM) was added to 5 µL purified PCR product and the samples were sent to Macrogen™ for Sanger sequencing using BigDye® chemistry.

3.1.3. Phylogenetic analysis

Sequences of considerable quality (HQ>50%) were processed using Geneious® software. Consensus sequences were assembled from forward and reverse sequences (if both available), all sequences were manually trimmed and ambiguities were edited. Both phylogenetic analysis and The Basic Local Alignment Search Tool (BLAST; https://blast.ncbi.nlm.nih.gov/Blast.cgi) were used as genotyping approaches (Altschul *et al.*, 1990). All parts of the phylogenetic analysis described next were computed and visualized in MEGA 7 (Kumar *et al.*, 2015). Based on the taxonomic indications from BLAST (order, superfamily, family and genus), NCBI GenBank (https://www.ncbi.nlm.nih.gov/) and BOLD (http://www.boldsystems.org/index.php/) databases were mined for closely related sequences to construct a phylogenetic tree. A selection of sequences was exported and aligned with selected sequences from this study using the MUSCLE alignment algorithm (Edgar, 2004) and trimmed homogenously for phylogenetic tree construction. The best nucleotide substitution model was calculated by Model Selection (based on the Bayesian information Criterion, BIC) and used to compute pairwise genetic distances and construct a Maximum Likelihood (ML) tree with 500 replicates for nodal support statistics (bootstrap

method). For species discrimination, genetic pairwise p-distance were calculated between COI sequences with 2 to 5% as threshold for intraspecific divergence in mitochondrial DNA (Vilas *et al.*, 2005; Lawton *et al.*, 2015).

3.2. Assessment of gastropod infection status

3.2.1. Allozyme analysis

Based on findings of Wright *et al.* (1979), we used cellulose acetate gel electrophoresis (CAGE) allozyme analysis to separate and visualize the isoforms of the protein malate dehydrogenase (MDH) from gastropods and trematode (Wright *et al.*, 1979). CAGE protocols on fresh snail tissue in the field lab were performed following Hebert *et al.* (1993). Hepatopancreas, the tissue that was reported to host most sporocyst larvae, was dissected from living gastropods and kept on ice during homogenization. Pre-made CAGE gels (Titan III®, Helena BioScience™) were cut, soaked in running buffer and loaded with protein extract of hepatopancreas homogenate. Following electrophoresis, the MDH banding patterns were visualized as described in Hebert *et al.* (1993).

3.2.2. Multiplex RD-PCR

3.2.2.1. DNA extraction and pooling design

For the assessment of infection prevalence, the Chelex® (Biorad™) DNA extraction method was used because of the reduced cost, process time and effort compared to the Phenol/Chloroform/proteinase *K* protocol, as compared by Caron *et al.* (2011). Ethanol fixated gastropod tissue was homogenized as described in 3.1.1. Tissue homogenate was incubated for one hour at 56°C and 30 min at 95°C in a 100 µL 5% Chelex® (Biorad™) solution in a spinning Thermocycler™. Samples were centrifuged for 7 minutes at 13 000 x g and supernatans extract was collected and stored at -20°C. Pure DNA extract was first used to check for infections because of the possibly low concentration of trematode DNA in the gastropod specimen. If RD-PCR was inhibited (probably due to high concentrations of inhibitors in the DNA extract), the sample was diluted 1 in 100 and the RD-PCR protocol was run again (Caron *et al.* 2011). For multiplex RD-PCR, gastropods were pooled per 4 gastropods of the same morphotype and site. A maximum of 16 gastropods was used per morphotype and per site. Sites with less than 4 gastropods successfully fixated were excluded from the analysis.

3.2.2.2. Primer design

All primers used were designed and/or tested on alignments of gastropod and trematode sequences downloaded from GenBank (NCBI) in the DNA analysis software program

Geneious 6.1.8®. Primers that proved to be suited *in silico* were custom made and ordered with Eurogentec®. Three different markers, were amplified in the multiplex RD-PCR: one internal positive control (i.e. a general gastropod marker that would confirm a successful PCR), a trematode-specific marker and a marker that targets members of a certain trematode family of interest (i.e. *Schistosoma* sp. in planorbid gastropods and *Fasciola* sp. in lymnaeid gastropods). The use of an internal control marker can be of great importance in avoiding false negative PCR results that commonly occur due to PCR inhibitors in the DNA extract (Caron *et al.* 2011). The newly designed internal control primers FW_SNAIL2 and REV_SNAIL2 target a 500-600 bp region of the conserved 18S rDNA gene. This gene was also used to target a trematode specific marker: the primers 18S_Digenea_F and 18S_Dignea_R were designed to amplify a trematode specific 392 bp fragment in the 18S rDNA region. A 124 bp *Fasciola* sp. specific repeat, first described by Kaplan *et al.* (1995), has been successfully used for *Fasciola* sp. detection in different gastropod species (Caron *et al.* 2011; Cucher *et al.* 2006; Krämer *et al.* 1998; Mostafa *et al.* 2003; Velusamy *et al.* 2004; Kaplan *et al.*, 1995). This marker is a highly repetitive sequence that makes up about 15% of the Fasciola genome (Kaplan *et al.*, 1995). The primers Kaplan_F and Kaplan_R, designed by Krämer *et al.* (1998), target the 124 bp repeat and were used in our multiplex RD-PCR for the detection of *Fasciola* sp. in lymnaeid gastropods. For the planorbid gastropod screening, we developed a two-step approach. The first step identified infections of trematodes in general (as in lymnaeids) and infections of *Schistosoma* sp. specifically (analogous to *Fasciola* sp. detection in lymnaeid gastropod). The primers FW_ITS_Schisto2 and REV_ITS_Schisto2 were designed to amplify a 369 bp *Schistosoma* sp. specific region of the ITS2 region (rDNA). If gastropods were diagnosed to be infected with *Schistosoma* sp., a second PCR was employed named: '*Schistosoma* multiplex RD-PCR'. This multiplex RD-PCR was adapted from Webster *et al.* (2010) and Van den Broeck *et al.* (2011), who designed the protocol to differentiate *Schistosma* sp. infections of particular economical interest i.e. the human infecting *S. heamatobium* and *S. mansoni* and the cattle infecting *S. bovis* and *S. curassoni* (Webster *et al.*, 2010; Van den Broeck *et al.*, 2011). A general forward primer (Asmit1) is combined with different species specific reverse primers that target regions of different lengths in the COI region, each length corresponding unique for one *Schistosoma* species. We designed a fourth, additional reverse primer for *S. mattheei,*-specific amplification (Smat.R) because this bovine schistosome is known to be prevalent in Zimbabwe (Pfukenyi et al, 2005; Pfukenyi *et al.*, 2006). All primers were chosen or designed in such a way that they could be used in the same multiplex PCR program (thus with the same melting temperature) and generate fragments of different length that can be identified

on a gel-electrophoresis run. All primers were tested both *in silico* and in simplex PCR trails. Table 2 summarizes information of all primers used.

Table 2. Primers used in multiplex RD-PCR protocols. The primer sequences and names are given along with the targeted marker, organism (target) and length of the amplicon (Len. in nt). All primes except Kaplan_F/Kaplan_R (Krämer and Schnieder, 1998) and Amsit1/Sh.R/Sb.R/Sman.R (Webster *et al.*, 2010; Van den Broeck *et al.*, 2011) were newly designed for this protocol. Fas stands for Fasciola 124 bp repeat, 18S indicates the 18S rDNA marker.

Primer name	Marker	Target	Len.	Primer sequence
General trematode multiplex RD-PCR				
18S_Digenea_F	18S	Trematoda sp.	±392	CAGCTATGGTTCCTTAGATCRTA
18S_Digenea_R				TATTTTTCGTCACTACCTCCCCGT
FW_SNAIL2	18S	Gastropoda sp.	±500	AGTATGGTTGCAAAGCTGAAACTTA
REV_SNAIL2				TACAAAGGGCAGGGACGTAAT
Kaplan_F	Fas	*Fasciola* sp.	124	ATTCACCCATTTCTGTTAGTCC
Kaplan_R				ACTAGGCTTAAAGGCGTCC
FW_ITS_Schisto2	ITS	*Schistosoma* sp.	±369	GGAAACCAATGTATGGGATTATTG
REV_ITS_Schisto2				ATTAAGCCACGACTCGAGCA
Schistosoma multiplex RD-PCR				
Asmit1	COI	*Schistosoma* sp.		TTTTTTGGTCATCCTGAGGTGTAT
Sh.R		*S haematobium*	543	TGATAATCAATGACCCTGCAATAA
Sb.R		*S. bovis*	306	CACAGGATCAGACAAACGAGTACC
Smat.R		*S. mattheei*	362	CACCAGTTACACCACCAACAGA
Sman.R		*S. mansoni*	375	TGCAGATAAAGCCACCCCTGTG

3.2.2.3. PCR protocols and analysis

Specificity and optimization tests for different primer combinations, primer concentrations, DNA polymerase types and PCR programs were performed on samples of which species identity and/or infection status were known (provided by Dr. Tine Huyse). First, all primers were tested in simplex PCR on different annealing temperatures: 43°C, 47.4°C, 55.6°C and 60°C. Next, a multiplex PCR was tested with the optimal temperature from the simplex PCR trials, using different primer combinations and both the Regular *Taq* DNA polymerase (Qiagen™) and the Multiplex *Taq* PCR kit (Qiagen™). In a third trial, concentrations of primers were adjusted and tested for sensitivity since the targets for different primers are present in different concentrations (trematode DNA in a gastropod with a low infection level is diluted compared to gastropod DNA). The fourth trial included all optimized parameters from previous tests along with a test batch of Chelex® extracted DNA samples to investigate whether the Chelex® extraction method does not interfere with high sensitivity multiplex RD-PCR. All PCR reactions were performed in a 15 µL volume with 1.5 µL gastropod DNA extract using the Qiagen™ *Taq* DNA polymerase kit containing 1.5 mM PCR buffer (Qiagen™), 0.6 mM dNTP mix (Qiagen™), 1.5 mM $MgCl_2$, 0.45 units of *Taq* Polymerase (Qiagen™) and primer mix. Primer concentrations in the final volume were: 0.2 µM

18S_Digenea_F and 18S_Digenea_R, 0.1 µM FW_SNAIL2 and REV_SNAIL2 and 0.6 µM FW_ITS_Schisto2 and REV_ITS_Schisto2 (for planorbid gastropods) or 0.6µM of Kaplan_F and Kaplan_R (for lymnaeid gastropods). The PCR reaction was performed as follows: initial denaturation at 95°C for 15 min, 39 cycles of 94°C for 30 sec, 60°C for 1 min 30 sec and 72°C for 1 min and a final elongation step at 72°C for 10 min in a Tprofessional Thermal Cycler (Biometra™). Analysis of the PCR products was carried out by gel electrophoresis (GE) on a 3% agarose gel and Midori Green direct® staining method and visualization under UV light. To separate all fragments the GE run was performed on 120 V for 1 h 45 min.

The Schistosoma multiplex RD-PCR was tested for specificity using infected gastropods or adult worm DNA extracts provided by Dr. Tine Huyse (collections of the RMCA). More extended specificity tests including more species and different DNA concentrations, were already performed by Webster *et al.* (2010). All PCR reactions were performed in a 15 µL volume with 1.5 µL gastropod DNA extract using the Qiagen™ Taq DNA polymerase kit with 1.5 mM PCR buffer (Qiagen™), 0.6 mM dNTP mix (Qiagen™), 1.5 mM $MgCl_2$, 0.45 units of *Taq* Polymerase (Qiagen™) and primer mix. Primer concentrations in the final volume were: 0.8 µM of Asmit1 and 0.3 µM of each reverse primer (Sh.R, Sb.R, Smat.R and Sman.R respectively). The PCR reaction was performed with initial denaturation at 95°C for 3 min, 39 cycles of 94°C for 30 sec, 58°C for 1 min 30 sec, 72°C for 1 min 30 sec and a final elongation step at 72°C for 7 min in a Tprofessional Thermal Cycler (Biometra™). Electrophoresis was performed as described above.

4. Multivariate ecological data analysis

The ecological data (see 2.2.3.) and gastropod count data for ten months of sampling were analysed with the statistical software program RStudio®. All R generated graphs were exported to Microsoft Powerpoint® for minor aesthetic adjustments using the *export* package created by Wenseleers (2016). Significance threshold for all statistical tests was $P \leq 0.05$. The data analysis was performed in three consecutive steps as described below.

4.1. Composition of the gastropod community

Two methods were used to visualize the relationships amongst sites based on their gastropod community structure: Nonmetric Multi-Dimensional Scaling (NMDS) and hierarchical cluster analysis. NMDS analysis was performed using the function *metaMDS* from package *vegan* (Oksanen *et al.*, 2016). The ordination was visualized in a NMDS plot. Different distance measures, suitable for community data as described in Legendre *et al.* (2013), were used to create the association matrix for NMDS ordination. The distance measure resulting in the lowest stress value was used (Legendre *et al.*, 2013). The functions

goodness and *stressplot* from package *vegan* were used to assess the goodness of fit (Oksanen *et al.*, 2016). Four hierarchical clustering strategies were tested (i.e. single linkage agglomerative clustering, complete linkage agglomerative clustering, Unweighted average linkage agglomerative clustering (UPGMA) and Ward's minimum variance method). The same distance measure used in the NMDS analysis was used for the association matrix created by *vegdist* from package *vegan* (Oksanen *et al.*, 2016). Clustering was performed using the function *hclust* from package *stats.* Diagnostics (i.e. cophenetic correlation and Gower distance) for choosing best clustering methods were based on the cophenetic distances in the dendrogram, calculated by function *cophenetic* from package *stats* (R Development Core Team, 2008). The optimal number of clusters or number of distinctive groups in the cluster dendrogram was determined by creating fusion level plots and calculating silhouette widths. The dendrogram was both plotted separate and as a projectedion on the NMDS plot using the *ordicluster* and *ordiplot* functions from package *vegan* (Oksanen *et al.*, 2016).

4.2. Physico-chemical habitat differentiation of sites and redundancy analysis

Principal component analysis (PCA) was assessed on a set of of averaged physicochemical water parameters per site to visualize the differentiation amongst sites based on these parameters. PCA was performed using function *prcomp* from package *stats* (R Development Core Team, 2011) on a scaled correlation matrix because of the differences in dimensions of the different variables. The scree plot was made by function *fviz_eig* from package *factoextra* (Kassambara *et al.*, 2017). The contribution of the different variables to the principal components were visualized using *corrplot* from package *corrplot* (Wei *et al.*, 2017) and functions *fviz_contrib* and *fviz_pca_var* from package *factoextra* (Kassambara *et al.*, 2017). The biplot was plotted with *fviz_pca_biplot* in package *factoextra* (Kassambara *et al.*, 2017). Canonical ordination was next assessed to investigate whether the variability in gastropod community composition over all sites can be explained by the physicochemical parameter profile of these sites and if this relationship was significant. Gastropod count data were Hellinger transformed as recommended for community data (Legendre *et al.*, 2001). Collinear physicochemical parameters (vif > 4) were removed from the data set in a stepwise fashion (Fox *et al.*, 2011; *Collinearity and stepwise VIF selection*, 2013). Canonical redundancy analysis (RDA) was performed as described by Borcard *et al.* (2011), using function *rda* from package *vegan* (Oksanen *et al.*, 2016). Step-back model selection based on permutation F-statistic for significance of each variable and AIC criterion of the fit was performed using the *ordistep* function from package *vegan*. The best model RDA fit was

analysed using permutation F-test statistics from *anova.cca* and plotted with function *triplot* from package *vegan* (Oksanen *et al.*, 2016).

4.3. Generalized linear models for discrete ecological predictors

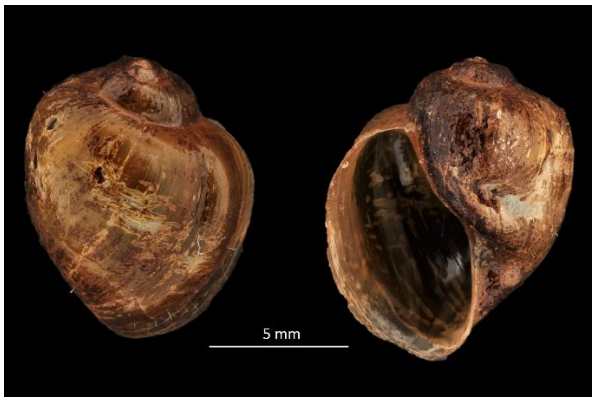
For discrete ecological parameters, Generalized Linear Models (GLM) were fitted using *glm* from package *stats* (R Development Core Team, 2011). Akaike information criterion (AIC) based model selection was performed using the function *stepAIC* in package *MASS* (Venables *et al.* 2002). Overdispersion was assessed by changing the distribution to 'quasipoisson' (instead of poisson) and checking the dispersion parameter. Systematic deviations of observed values compared to model predictions were checked for using *residualPlots* from package *car* (Fox *et al.*, 2011). All predictors that showed to be significantly influencing gastropod abundance based on a likelihood ratio tests (type III Anova with Chi square test statistics) were plotted with the function *ggplot* from the *ggplot2* package (Wilkinson, 2011).

Results

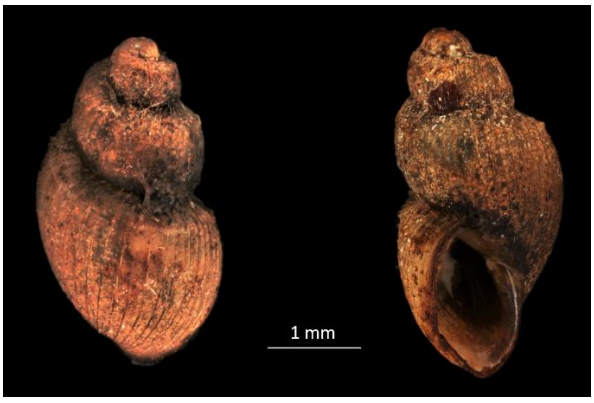
1. Gastropod and trematode diversity

1.1 Morphological vs molecular Identification of gastropods

Eight different morphotypes of gastropods were morphologically identified based on determination keys of Brown (1994) and Mandahl-Barth (1962): *Bulinus truncatus, B. forskalii, Biomphalaria pfeifferi, Lymnaea* sp., *L. natalesis, L. radix*, *Bellamya* sp. and *Physa acuta*. High quality stacked photographs of each morphotype are depicted in figure 6. Morphological characteristics of the weak body parts were included in the determination on live gastropods but not visible on the photographs since the photographed specimens were fixed on ethanol.



A



B

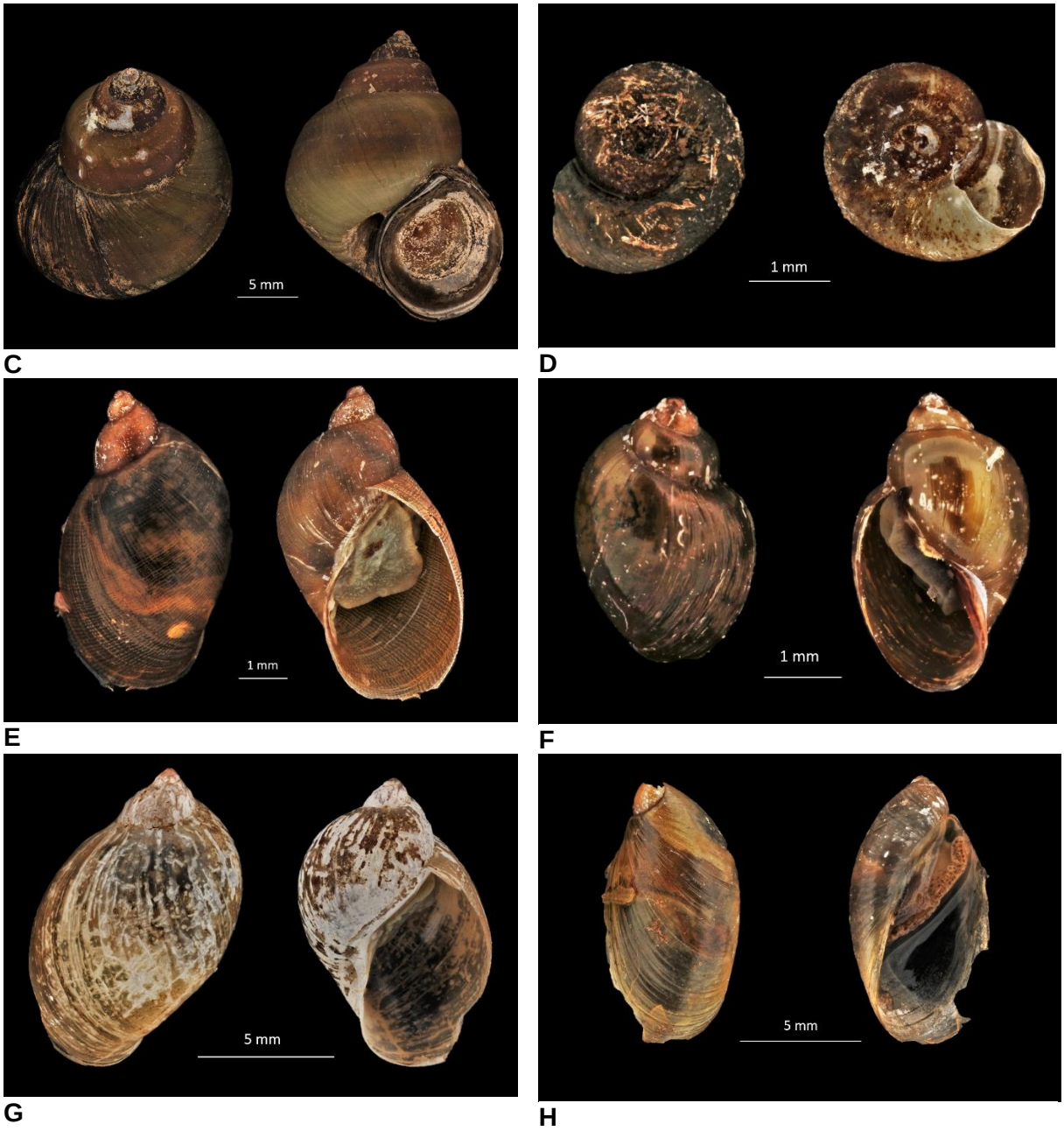


Figure 6. Photographs of all Kariban gastropod morphotypes. *Bulinus truncatus* (A)*, B. forskalii* (B)*, Bellamya* sp. (C), *Biomphalaria pfeifferi* (D)*. Lymnaea natalensis* (E)*, Physa acuta* (F), *L. radix* (G) and *Lymnaea* sp. (H). Based on molecular identification these are: *Bulinus truncatus* (A)*, B. forskalii* (B)*, Bellamya* sp. (C), *Gyraulus sp*. (D)*, Pseudosuccinea columella* (E)*, Physella (Physa) acuta* (F), *Radix* sp. (G) and *Succinea* sp. (H) respectively.

Two gastropods from each morphotype (including all specimens shown in Figure 6), all shedding gastropods and additional infected gastropods extracted with the Chelex® method were used for genotyping analysis for identification. From a total of 40 specimens tested, 28 and 24 samples showed sufficient PCR amplification of the COI and ITS marker respectively. There was no PCR amplification of the ITS marker of morphotype *L. radix* after

multiple attempts. By assembling a consensus from forward and reverse sequences, manual editing and trimming, a total of 23 COI and 20 ITS sequences of sufficient quality (HQ>50%) were obtained for analysis. COI was successfully sequenced for every gastropod morphotype but no reliable sequencing results were obtained for the ITS marker for morphotypes *L. natalensis, L. radix, Lymnaea* sp., and *Bellamya* sp. The success rate of the BLAST analysis (for ITS no BLAST results could be obtained for five out of 20 sequences using high similarity megaBLAST) and the quality after trimming of the sequences (average HQ_{COI}=69% and average HQ_{ITS}=62%) were higher for COI compared to ITS. Furthermore, did the COI marker show to be much more represented in the GenBank database for freshwater Gastropods. ITS-based genotyping showed results similar to COI-barcoding for morphotype *B. truncatus, B. forskalii* and *P. acuta* but no reliable BLAST hits were obtained for morphotype *B. pfeifferi* and *Lymnaea* sp. Because these constrains, and based on literature suggestions (Jones *et al.*, 1999; Pfenninger *et al.*, 2006; Jørgensen *et al.*, 2007; Kane *et al.*, 2008; Lawton *et al.*, 2015), the COI marker was used in further analysis to genotype the gastropod morphotypes. Based on pairwise genetic distances of all COI sequences, all representatives of the same morphotype belonged to the same species with p-distance < 0.1% (data not shown). A selection of the best (quality, length) COI sequence for each morphotype along with the length, HQ and top 3 BLAST hits is given in table 3.

Table 3. BLAST results for COI sequences with highest quality and length for each gastropod morphotype. The assigned sequence identifier or name is given (Seq.) along with its provisionally assigned morphotype name (MT), the sequencing quality (HQ), length (len) and top 3 BLAST species hits with the respective identity value (ID). Morphotype abbreviations are BFO: *B. forskalii*, BTR: *B. truncatus*; BPF: *B. pfeifferi*, LNA: *L. natalensis*, LRA: *L. radix.*, LSP: *Lymnaea* sp., PHY: *P. acuta* and BEL: *Bellamya* sp.

Seq.	MT	HQ (%)	len (bp)	BLAST 1	ID (%)	BLAST 2	ID (%)	BLAST 3	ID (%)
G	BFO	98	399	*Bulinus forskalii*	99	*Bulinus camerunensis*	95	*Bulinus* sp.	91
D	BPF	87	460	*Gyraulus* sp.	88	*Biomphalaria tenagophila*	87	*Bulinus globosus*	86
O	BTR	97	326	*Bulinus truncatus*	99	*Bulinus tropicus*	99	*Bulinus nyassanus*	98
E	LNA	96	395	*Pseudosuccinea columella*	100	*Lymnaea columella*	100	/	/
K	LRA	92	417	*Radix* sp.	96	*Radix auricularia*	92	*Lymnaea diaphana*	85
S98	LSP	92	484	*Succinea* sp.	89	*Succinea caduca*	88	*Succinea putris*	88
A	PHY	95	483	*Physella acuta*	99	*Physa heterostropha*	98	*Radix swinhoei*	98
BEL	BEL	96	450	*Bellamya monardi*	93	*Bellamya capillata*	91	*Bellamya* sp.	92

The COI-based BLAST results from morphotypes *B. forskalii*, *B. truncatus*, *L. natalensis* and *P. acuta* were sufficiently strong (BLAST identity≥99% with query cover of 100%, sequence length of +300bp and HQ≥95%) to conclude that they represent the species *B. forskalii, B. truncatus, Pseudosuccinea columella,* and *Physella (Physa) acuta* respectively. Next, a phylogeny-based barcoding approach was used to gather more taxonomic information from the morphotypes for which BLAST results were not conclusive. The gastropod with morphotype *Bellamya* sp. was excluded from this analysis because it is not a host for economically important trematode species in Africa (Toledo *et al.*, 2014) and it belongs to a different group (i.e. Caenogastropoda) compared to most trematode-hosting gastropods (Pulmonata group) (Strong *et al.*, 2008). Of each closely related genus of the gastropod family indicated by BLAST, COI sequences of representative species were downloaded from the BOLD database. Sequences were aligned and trimmed and yielded alignments of 349 bp for Lymnaeidae, 325 bp for Planorbidae and 481 bp for Succineidae. For the Succineidae alignment, *Athoracophorus bitentaculatus* was used as outgroup since its family (i.e. Athoracophoridae) is a sister taxon of the Succineidae forming the clade of Elasmognatha (Strong *et al.*, 2008). For the Planorbidae and Lymnaeidae phylogeny, *P. acuta* (family: Physidae) was used as outgroup since Physidae is a closely related taxon for both families (Strong *et al.*, 2008). For Succineidae phylogeny, the Hasegawa-Kishino-Yano model with discrete Gamma distribution ([+G] = 0.48) and invariant sites ([+I] = 30.87%) was used. For Lymnaeidae and Planorbidae the General Time Reversible (GTR) model with Gamma distribution (for Planorbidae [+G] = 0.42; for Lymnaeidae, [+G] = 0.90) and invariant sites (for Planoribdae [+I] = 27.07%; for Lymnaeidae [+I] = 51.18 %) was selected as best model for constructing the Maximum Likelihood (ML) tree (Nei and Kumar, 2000). The ML phylogenetic trees for the 3 different families are given in supplementary figures 2, 3 and 4 (Annex IV). The Planorbidae phylogeny shows that the morphotype *B. pfeifferi* does not cluster with the genus of *Biomphalaria* but is rather related to the genus *Gyraulus*. Pairwise genetic distances show that the Kariban specimen is most closely related to *Gyraulus* sp. from Turkey (KC495833.1) with a *p*-distance of 12.9% (data not shown). The phylogenetic tree of Planorbidae again confirms the identity of *B. truncatus* and *B. forskalii*. From the phylogenetic tree of Lymnaeidae we conclude that the morphotype *L. radix* belongs to the genus Radix rather than Lymnaea and it is further mentioned as *Radix* sp., since no exact species determination could be obtained. The lowest pairwise genetic distance between the Radix and those from GenBank was 3.7% with *Radix* sp. specimen from Vietnam (see supplementary table 7, Annex III). Pairwise genetic distances between COI sequences of the *P. columella* specimen from Kariba and *P. columella* specimens from different geographical regions (i.e. Egypt (GBMIN110283-17), Australia (GBMLG0711-06), Colombia (GBMPL238-

13), Spain (GBMPL1442-15) and USA (GBMPL484-13)) showed that the sequences were 100% identical (data not shown). The Succineidae phylogeny suggests that the morphotype *Lymnaea* sp. belongs to the family of Succineidae with a phylogenetic relatedness to the African genus *Oxyloma*. However, nodal support for this clustering was only 42% and pairwise genetic distances between the Kariban succineid gastropod and the closest related *Oxyloma* was lower (12.3%) compared to its relatedness to some Succinea species (e.g. 11.9% p-distance with a *Succinea* sp. from Canada (KT708734)). Therefore, we name this gastropod species *Succinea* sp.

1.2 Identification of trematodes

Both cercariae from shedding gastropods and a selection of infected gastropods (as diagnosed with the multiplex RD-PCR) were used for trematode genotyping with 18S rDNA and COI markers. COI PCR amplification was less successful compared to 18S rDNA. From the 45 samples tested, 25 gave positive PCR signals for the 18S rDNA amplicon of which 22 could be successfully sequenced (HQ>50% post trim). For COI, 50 samples were tested, resulting in 22 successful PCR amplifications and 18 successfully sequenced amplicons. The sequencing quality (HQ) post trim did not differ significantly between 18S rDNA and COI, the sequencing length however did (average $length_{COI}$=395.4 and average $length_{18S}$=572.7). The length, HQ, origin and top 3 BLAST hits of each sequence for both the 18S rDNA and COI marker can be found in supplementary table 2 (Annex III). COI sequences showed low BLAST identity matches, never exceeding 92% identity with COI sequences in the GenBank or BOLD database. 18S rDNA showed high BLAST identity matches ranging from 97% to 100%. Phylogenetic tree-based genotyping was used for more taxonomic resolution. 18 and 15 trematode sequences of the 18S rDNA and the COI marker respectively, had sufficient quality and length for tree-based genotyping. For the 18S rDNA phylogenetic analysis, the top 5 BLAST species hit results of each sequence were downloaded from GenBank, aligned with obtained sequences and trimmed, yielding a 550bp alignment representing 2 orders, 5 super families, 11 families, 22 genera and 25 species. For the 18S rDNA phylogeny the Kimura 2-parameter model with discrete Gamma distribution ([+G] = 0.23) and invariant sites ([+I] = 39%) was proposed for constructing the ML tree (Nei and Kumar, 2000). The ML phylogenetic tree for 18S rDNA is displayed in supplementary figure 5 (Annex IV). For COI-based phylogeny, BOLD and GenBank sequences were selected from one represented genus of each family and superfamily found by 18S rDNA genotyping. However, the lack of COI sequences for many families of interest (e.g. superfamily Pronocephaloidea with only 1 of 5 families represented in GenBank) constrained this effort. The trematode taxonomy of Olson *et al.* (2003) and additional

classification of amphistomes by Laidemitt *et al.* (2017) were used as guideline for family classification. Based on pairwise genetic distances of the COI alignment of all Kariban trematodes, six trematode species can be identified (supplementary table 3, Annex III). One representative COI sequence of high quality was next selected for each species (further indicated as 'type') to simplify the analysis. The alignment used for the ML building, contained 402bp long COI sequences representing 10 families with 19 genera and 22 species. The General Time Reversible model with discrete Gamma distribution ([+G] = 0.60) and invariant sites ([+I] = 19.28%) was proposed for COI evolution and used for constructing of the ML tree, displayed in figure 7 (Nei *et al.*, 2000). Pairwise genetic distances between all COI sequences included in the phylogeny are shown in supplementary table 8 (Annex III).

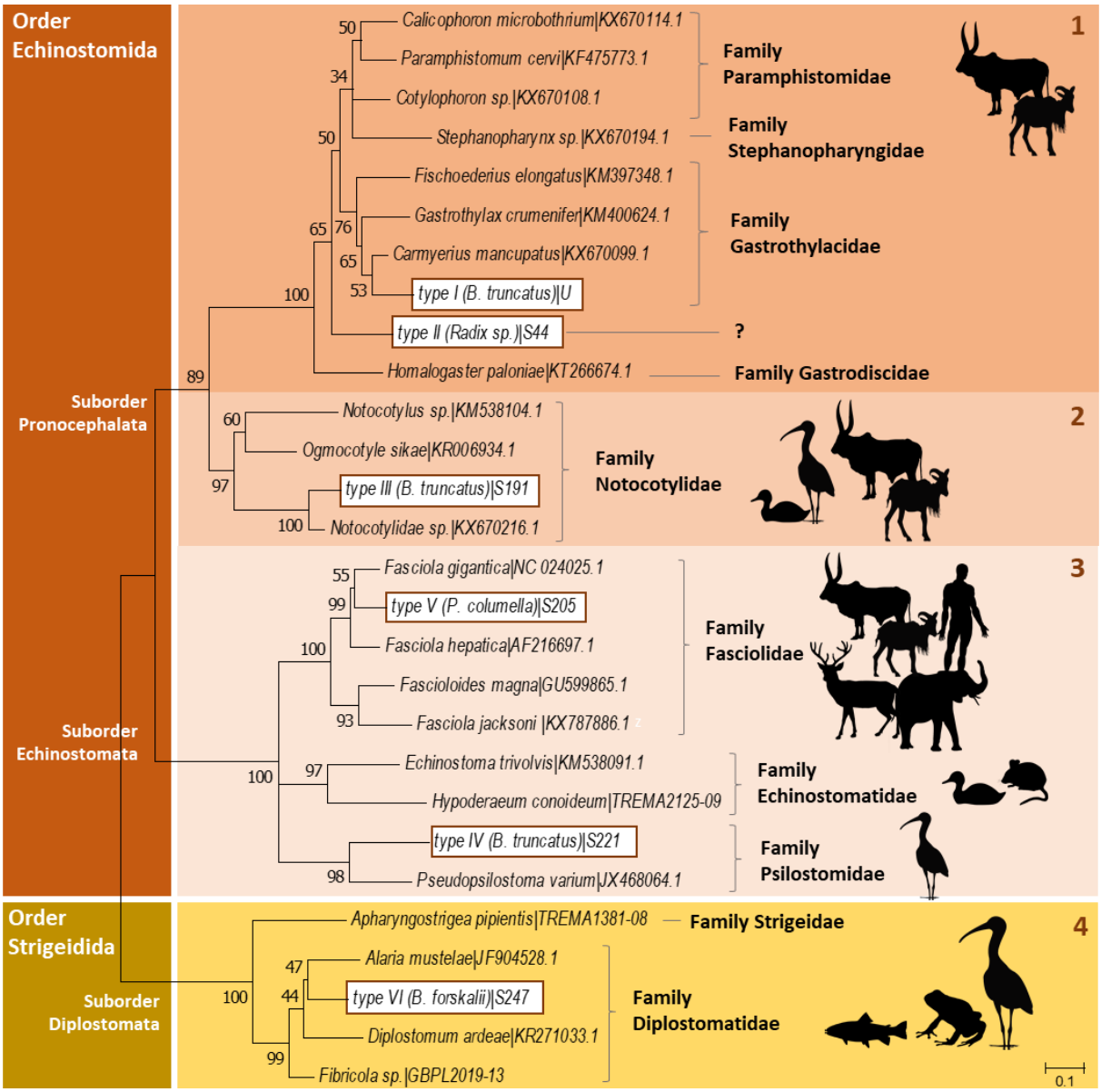


Figure 7. Molecular phylogenetic tree, based on COI alignment (402 bp). The evolutionary history was inferred using Maximum Likelihood based on the GTR+G+I model. The tree with the highest log

likelihood (Ln = -5412.94) is shown. Bootstrap values (500 replicates) are shown. Numbers 1 to 4 indicate superfamilies with 1: Paramphistomoidea, 2: Pronocephaloidea, 3: Echinostomoidea and 4: Diplostomoidea. An assembly of final hosts of the trematode species included in the phylogeny is displayed by silhouette pictograms. BOLD/GenBank accession number or sequence identifier are indicated after '|'. Each Kariban trematode type sequence is indicated by a white grid and the gastropod host is mentioned between parentheses

Phylogenetic position of the Kariban trematode species (type I to VI) in their respective order, suborder and superfamily is confirmed by both 18S rDNA and COI phylogeny. The specimens used for 18S rDNA and COI phylogeny were not all from the same gastropod origin because of failed PCR amplification for one of both markers for some samples. The site of sampling, quality (HQ), length, host origin and proposed taxonomy of every sample included in the COI and 18S based phylogeny is summarized in supplementary table 4 (Annex III). The six different trematode species were found in four different gastropod species. *P. columella, Radix* sp., and *B. forskalii* were each infected by (at least) one trematode species whereas *B. truncatus* showed to be infected with at least 3 different trematode species. One 18S rDNA sequence was obtained from an infected *Succinea* sp. but not included in the phylogenetic analysis because of its short length (280 bp; HQ=89.6%) but since it is the only amplicon for *Succinea* sp. it is worthwhile discussing. Based on 18S rDNA BLAST results (supplementary table 2, Annex III) we propose *Succinea* sp. to be infected with a species of the Leucochloridiidae family (Order Strigeida; Superfamily Brachylaimoidea) (Olson *et al.*, 2003).

2. Infection prevalence assessment

2.1 Shedding results

Shedding experiments revealed only nine infected gastropods (i.e. 0.08% infection prevalence). Eight of them were *B. truncatus* and one was a *P. columella* specimen. Six *B. truncatus* specimens from Charara site (site 16) and one from Nzou lodge (site 15) shedded amphistome-type cercariae. One *B. truncatus* (from Charara) shedded furcocercous (fork-tailed) cercariae of the longifurcate-pharyngeate distome type (Strigea cercariae). The shedding *P. columella* was infected with gymnocephalous cercariae of which *Fasciola* sp. is a type species. This specimen was found in Nzou lodge (site 15). Regarding total gastropods assessed in the shedding procedure (see 3.1: gastropod count), only 0.9% shedded with 5.4% of *B. truncatus* and 0.2% of *P. columella* being infected. All shedding gastropods were caught between August and November. No additional gastropods shedded after one week of incubation but the gastropod mortality rate during incubation was relatively high, however not quantified. Unfortunately, all pictures taken from shedding cercaria using a

microscope mounted camera were deleted from the memory card by accident at the field lab in January. Therefore morphological identification cannot be reproduced.

2.2 Allozyme analysis

We did not obtain reliable results from Cellulose Acetate Gel Electrophoreses (CAGE) based allozyme analysis. After multiple test trials and adjustments of running time, protein concentration, running voltage and other parameters, we concluded that the lack of an ultracentrifuge reaching a desired speed (13000 rpm) at the field lab lead to smothered banding patterns. The allozyme electrophoresis is therefore excluded from further analysis due to this technical constraint.

2.3 Multiplex PCR results

Representative results of the different multiplex RD-PCR trials for different infected gastropod specimens, adult trematode speciemens and controls are displayed in supplementary figure 6 (Annex IV). The general multiplex RD-PCR protocol was performed on a total of 254 gastropods of which 87 planorbids (67 *B. truncatus,* 12 *B. forskalii* and 8 *Gyraulus* sp.) and 167 lymnaeids/succinaeids (44 *Radix* sp., 24 *Succinea* sp. and 99 *P. columella*) respectively. PCR on pooled samples failed for 10 out of 64 pools (40 gastropods resp.). In those cases, samples from the pool were separately tested. Among all individual samples, 45 (from a total of 254) samples did not show clear PCR amplification. After a dilution of 1:100, nine samples remained unamplified and were discarded. Infection prevalence per gastropod species are given in Table 4. The number of gastropods tested per species was highly variable as it was depending on the number of gastropods successfully fixed, which could have been influenced by high mortality rates during the one week of incubation. The infection prevalence for all gastropod specimens tested was 32.24%. *P. columella* showed to be the most infected gastropod species (infection prevalence 62.77%) and it contributed to 75% of all infections. No infections were detected in *Gyraulus* sp. but this species accounted for only 3% of all tested gastropods specimens. 26% of all tested gastropods were *B. truncatus,* and this species contributed to 11% of all infections with an infection prevalence of 14.06%. The third most sampled gastropod species was *Radix* sp. but it contributed to a merely 4% of all infections with only 6.82% infected.

Table 4. Infection prevalence per gastropod species, based on the general trematode multiplex RD-PCR. Infection prevalence is calculated as nr of infected gastropods divided by the nr of sampled gastropods per species, excluding failed PCR results.

Species	Nr of gastropods tested	Nr. of failed PCR's	Nr of infected gastropods	Infection prevalence (%)
P. columella	99	5	59	62.77

Radix sp.	44	0	3	6.82
Succinea sp.	24	0	6	25
B. truncatus	67	3	9	14.06
B. forskalii	12	1	2	18.18
Gyraulus sp.	8	0	0	0
Total	**254**	**9**	**79**	**32.24**

The *Fasciola*-specific signal appeared in all infected *P. columella.* This indicates that this species is an important intermediate host for *Fasciola* sp. in the area. Two out of the three infected *Radix* sp. did also show the signal of *Fasciola* sp. infection and barcoding results confirmed this (see 1.2). Pairwise genetic distances between the 18S rDNA sequence of the *Radix* sp. infection was 100% identical to the *Fasciola* sp. infection in *P. columella.* However, since no COI sequences were successfully obtained for the *Fasciola* sp. infection of *Radix* sp, we cannot conclude whether this is the same species of *Fasciola* found in *P. columella.* None of the planorbid gastropods (*B. truncatus, B. forskalii* and *Gyraulus* sp. resp.) that showed a positive signal for the trematode infection (n=87), showed a *Schistosoma* sp. specific amplification. Therefore, the *Schistosoma*-specific multiplex RD-PCR was not performed on any planorbid snail specimen from Kariba. The infection prevalence per gastropod species and per site is displayed in supplementary table 5 (Annex III). The number of infected gastropods per species and per site, based on the multiplex RD-PCR results, is graphically shown in figure 8.

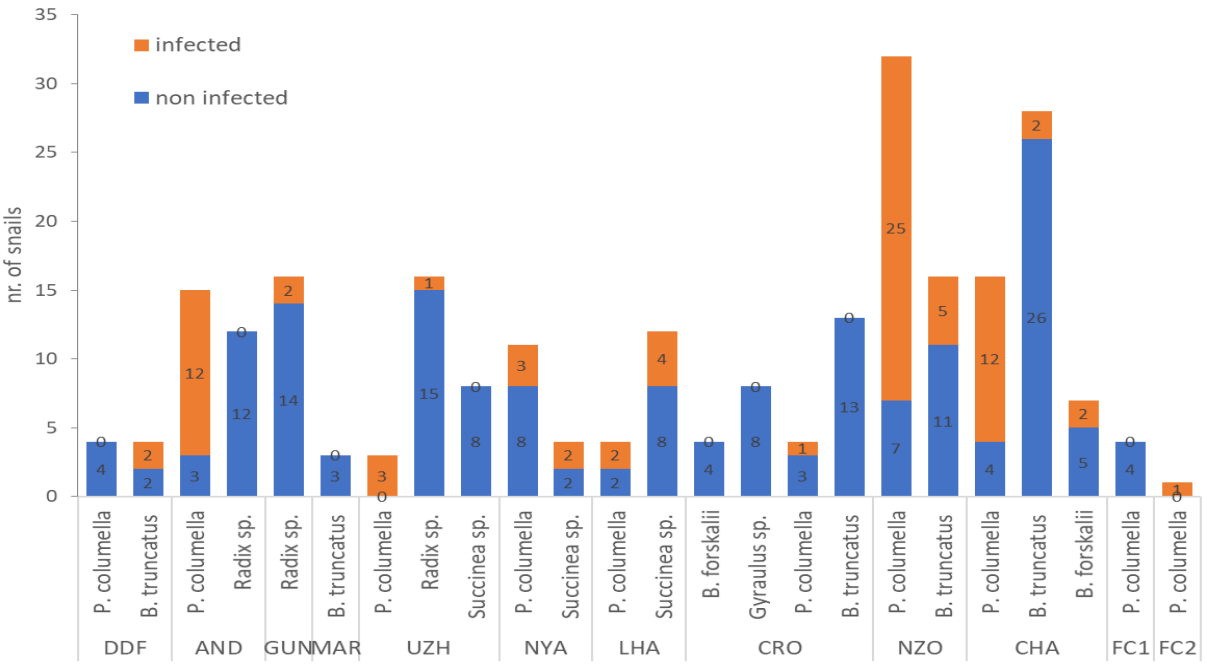


Figure 8. **Number of infected and non-infected gastropods per site and per gastropod species**. The proportion of orange and blue and the numbers plotted into these columns display the amount of

infected and non-infected gastropods respectively. The names of the sites corresponding to the site number and three letter codes are described in supplementary table 1 (Annex III).

3. Ecology of Kariban freshwater gastropod communities

3.1 Gastropod count

A total of 1024 gastropods (excluding *P. acuta* and *Bellamya* sp.) were counted from May 2017 until February 2018. The total gastropod count of each species found at each site, species richness per site and prevalence of each gastropod species over all sites are displayed in Table 5. *P. columella* was by far the most abundant gastropod species in Kariba (n=426 or 42% of all gastropods counted) and it had the highest prevalence, occurring at 14 sites. *B. forskalii* was the least abundant and least prevalent gastropod species with only 25 gastropods found in six sites. The site that harboured the highest overall gastropod count was site 3 (Andora Harbour) while site 11 (UZ harbour) had the highest species richness. Site 7, 8 and 9 were found to be very poor in gastropod count and species richness.

Table 5. Abundance, species prevalence and species richness of all sites and gastropod species, sampled between May 2017 and Feb 2018. Species taken into account are *Pseudosuccinea columella* (PCO), *Radix* sp. (RSP), *Succinea* sp. (SSP), *Gyraulus* sp. (GSP), *Bulinus* truncatus (BTR), *B*. *forskalii* (BFO). The names of the sites corresponding to the site number are described in Table 1 of supplementary tables (Annex III).

Site	PCO	RSP	SSP	GSP	BTR	BFO	Total	Richness
1	58	6	4	2	11	0	81	5
2	19	0	0	1	0	0	20	2
3	83	83	4	1	0	0	171	4
4	2	1	0	0	0	0	3	2
5	1	104	1	1	2	0	109	5
6	15	2	0	0	11	0	28	3
7	3	0	1	0	0	0	4	2
8	0	0	1	0	0	0	1	1
9	0	0	1	0	0	0	1	1
10	4	0	3	8	9	1	25	5
11	8	14	18	18	2	2	62	6
12	14	15	15	11	1	0	56	5
13	22	3	45	1	0	1	72	5
14	10	0	0	50	19	9	88	4
15	101	0	9	0	27	1	138	4
16	86	0	0	2	66	11	165	4
Total count	**426**	**228**	**102**	**95**	**148**	**25**	**1024**	**6**
Prevalence	**14**	**8**	**11**	**10**	**9**	**6**	**16**	

3.2 Gastropod community composition

Based on UPGMA (Unweighted Pair Group Method with Arithmetic mean) hierarchical clustering using a Bray-Curtis distance matrix, the 16 sites can be divided into five distinct groups. The sites within a group cluster together based on resembling gastropod community structure (gastropod counts per site). UPGMA was chosen as clustering method because of its relatively high cophenetic correlation coefficient and low Gower distance compared to other clustering methods. The Bray-Curtis distance measure was used for calculating the association matrix as it represents the relative influence of both abundant and uncommon variables well and results in a lower stress value (data not shown). In figure 9 the gastropod community composition of every site is visualized by non-metric multidimensional scaling (NMDS) along with the UPGMA clustered dendrogram (figure 9 a and b respectively). The NMDS plot displays the ordering relationship between the 16 sampled sites (displayed by numbers 1-16) based on their community composition. The plotted scores represent the sites. The relative position of the scores to each other and to the plotted variables (the species) represent their relatedness.

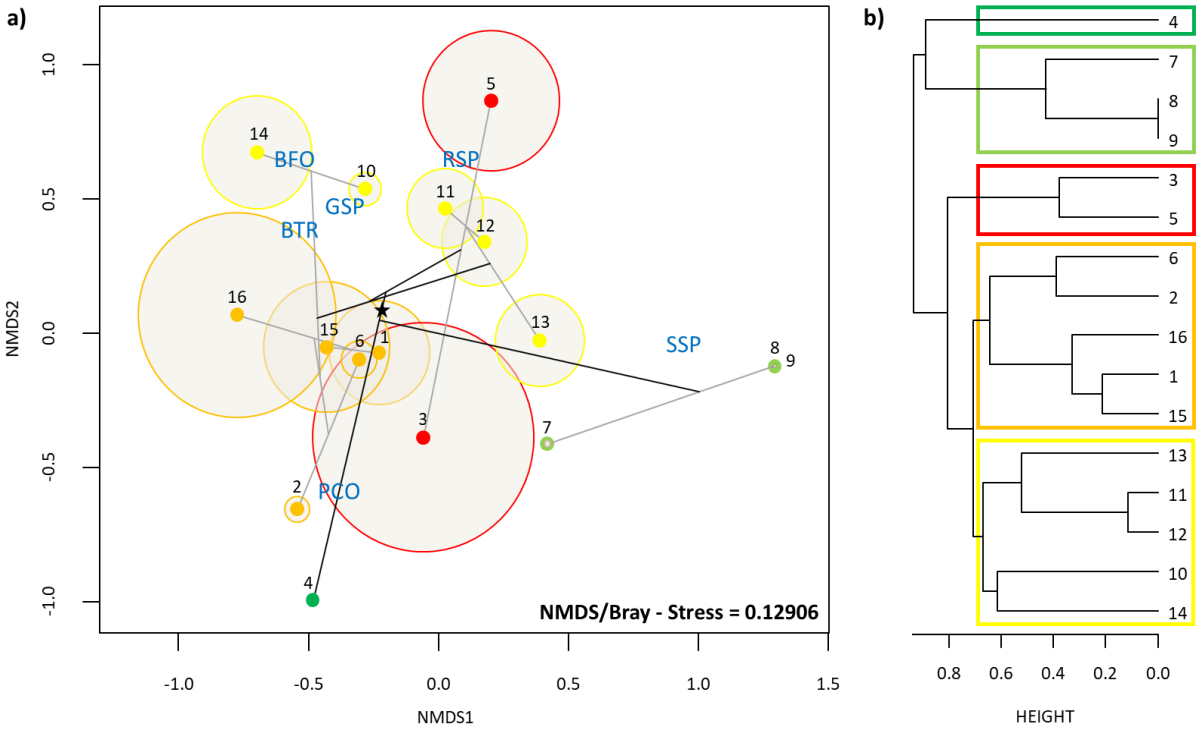


Figure 9. NMDS plot and UPGMA clustering dendrogram based on gastropod abundance per species and per site. a) NMDS plot with scores (dots with numbers) displaying the 16 different sites. Species (variables indicated by the blue species codes) displayed: *P. columella* (PCO), *Radix* sp. (RSP), *Succinea* sp. (SSP), *Gyraulus* sp. (GSP), *B. truncatus* (BTR), *B. forskalii* (BFO). The clustering dendrogram is indicated by grey lines. The star indicates the origin of the dendrogram. The size of the circles indicates the relative total number of gastropods per site. **b)** UPGMA cluster dendrogram for

different sites based on gastropod community data. 5 groups are distinguished which are displayed in different colours, also represented in a).

Sites with a high species diversity, are centred in the middle of the NMDS plot: in site 11, all gastropod species were represented with high counts of *Succinea* sp., *Gyraulus* sp. and *Radix* sp., while in site 1 all except one gastropod species could be found (no *B. forskalii*). Site 8 and 9 form a separate group along with site 7 (green cluster) because of their very low gastropod count with only 2 *Succinea* sp. found in site 8 and 9 while site 7 harboured only 3 *P columella* and 1 *Radix* sp. Site 3 and 5 form one group (red cluster) because of one common feature: the high numbers *Radix* sp. The reason both sites are separated quite far along the NMDS2 axis is the abundance of *P. columella*, which is prevalent in site 3 but not in site 5. Sites 10, 11, 12, 13 and 14 (yellow cluster) cluster together because of their shared trait: the occurrence of *Gyraulus* sp., which is absent or rare in most other sites. The fact that this group is divided into two branches can be explained by the difference in abundance of *Radix* sp. and *B. truncatus* which are dominant in site 11 and 12 and site 10 and 14 respectively, while site 13 harbours a high number of *Succinea* sp. The last group (orange cluster) forms one group because of the shared feature of high *P. columella* and *B. truncatus* counts relative to other gastropod species counts (with the exception of site 2, where only *P. columella* was dominant).

We clearly notice that planorbid gastropods group together based on community data: *B. truncatus, B. forskalii,* and *Gyraulus* sp. are most abundant in site 10, 11, 14 and 16. Except for site 16, all these sites cluster in one group (yellow cluster). Site 16 is not included in this group because it is less diverse and mainly dominated by *P. collumnella*. *B. forskalii* is a very rare gastropod species (only found in 6 of 16 sites and accounting for only 25 out of the 1024 gastropods) but it is predominantly present in site 14 and 16.

3.3 Physico-chemical differentiation among sites

The Principal Component Analysis (PCA), 88.77% of the total variance in physico-chemical profiles of all sites could be explained by the PC1 and PC2 axis of which 70.63% by the PC1 axis alone. The PCA correlation biplot of different sites (as scores) and all physico-chemical variables included (depicted as descriptor axis) is given in figure 10. The three variables with the strongest correlation with PC1 and thus the most influence on the position of scores along this axis are conductivity, phosphate concentration and ammonia concentration. For the PC2 axis, the variables dissolved oxygen concentration, oxygen saturation and temperature induced the most weight. Multiple variables showed high grades of collinearity based on VIF.

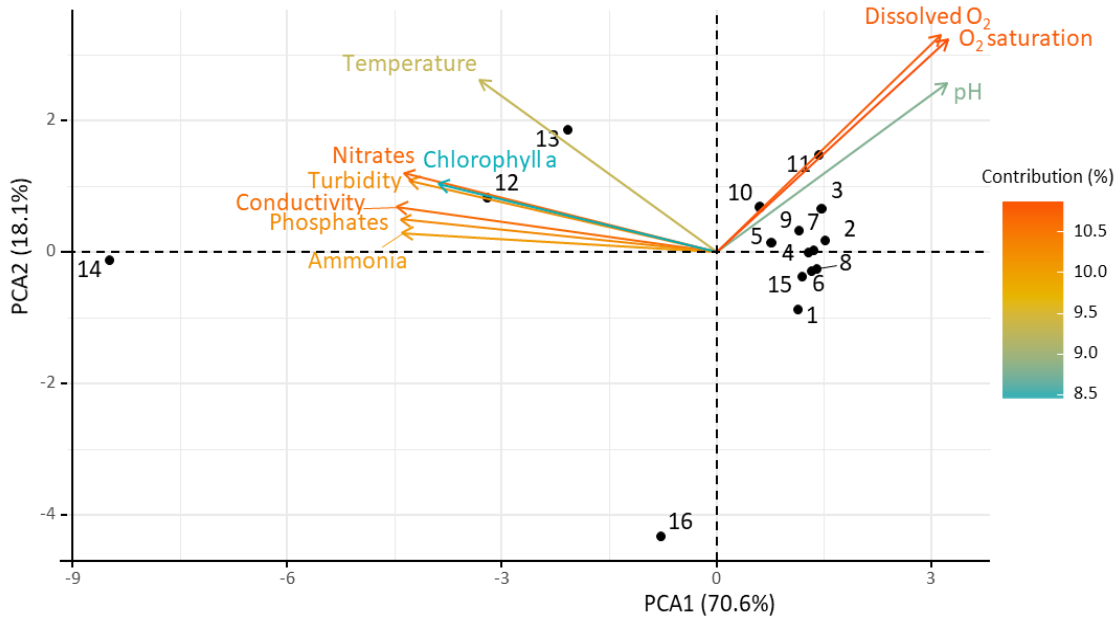


Figure 10. PCA biplot displaying correlation among sites according to physico-chemical water parameters. The scores (points) represent the 16 different sites whereas the variables are shown as arrows. Since it is a correlation type bipot (scaling = 2), the angles between the descriptor axis reflect correlations among the scores (sites) while distances between the objects do not approximate the Euclidean distance in the multidimensional space.

Overall gastropod abundance seems not to be directly correlated with physicochemical water properties since site 14, 16, 3 and 5 harboured the highest numbers although their water chemistry is totally different. The sites with lowest overall gastropod counts (site 4, 7, 8 and 9) neither show any extreme low or high physicochemical water properties but have an overall average or below average nutrient concentration. It is clear that only some sites (site 11, 12, 13, 14 and 16) differentiate from the main group in the PCA matrix. Regarding the gastropod community composition of these sites (see 3.2), many of these sites characteristically cluster together (e.g. site 10, 11, 12, 13 and 14 belong to the same group) based on specific community composition traits. Planorbid gastropod counts (*B. truncatus, B. forskalii,* and *Gyraulus* sp.) were highest in site 16 and 14, both sites have extreme physicochemical properties but in slightly different ways. Site 14 (Lake Croc) has the lowest pH and highest average nitrate-, phosphate-, ammonia- and chlorophyll a concentrations, conductivity, turbidity and temperature of all sites while the dissolved oxygen concentration and oxygen saturation is low. Site 16 has the lowest registered average temperature combined with the lowest oxygen content and low pH but the content of nutrients and the associated collinear factors chlorophyll a, turbidity and conductivity are not of an extreme kind.

3.4 Redundancy analysis

Redundancy analysis (RDA) was performed to investigate whether the variability in gastropod community composition over all sites can be explained by the physicochemical water parameters of these sites. In the RDA, the gastropod count data set represents the response matrix Y and the average physicochemical water parameter data, the explanatory variable matrix X. The hypothesis 'absence of any linear relationship between Y and X', or to put it differently 'H_0: the strength of the linear relationship, measured by the canonical R^2, is not larger than the value that would be obtained for unrelated Y and X matrices of the same sizes', was tested with the permutation *F*-test statistic (Borcard *et al.*, 2011). The variance-inflation factors (VIF) of the explanatory variables showed much collinearity between different physicochemical water parameters and all variables with a VIF larger than or equal to 5 were removed. Model selection resulted in retaining only two physicochemical parameters: pH and temperature. A distance triplot (scale = 1) of the RDA with these two variables is depicted in figure 11 and shows the relation between different sites both based on gastropod community composition, pH and temperature.

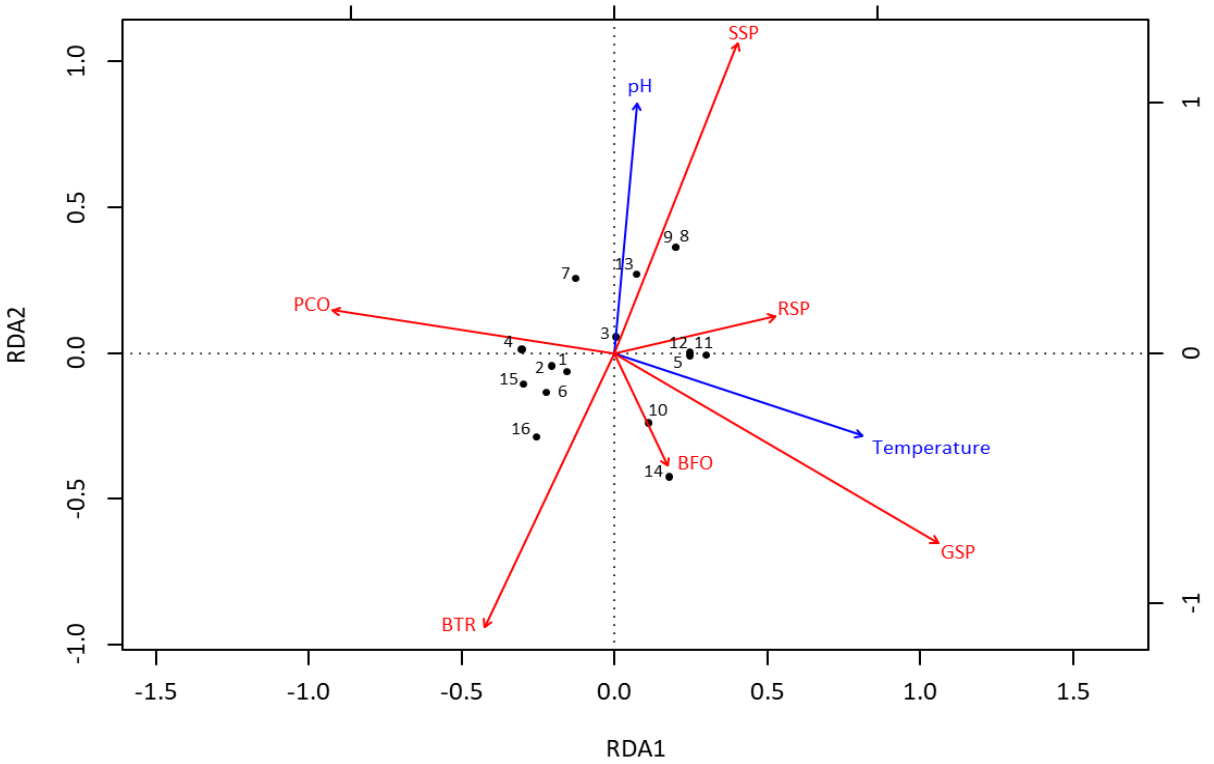


Figure 11. Distance triplot of redundancy analysis (scaling = 1). Sites (scores) are plotted in relation to the Hellinger-transformed gastropod counts per site (response variables: red arrows, scales to the bottom and left axis) and physicochemical water parameters pH and Temperature (explanatory variables: blue arrows, scaled to the top and right axis). Distances among scores approximate their Euclidean distances and angles between response and explanatory variables reflect their correlation. Abbreviations of the species are: *P. columella* (PCO), *Radix* sp. (RSP), *Succinea* sp. (SSP), *Gyraulus* sp. (GSP), *B. truncatus* (BTR), *B. forskalii* (BFO).

The *F*-test statistic was significant at Pr(>F) = 0.028. So we can assume that there is a (linear) relationship between the response and explanatory variables, expressed by the canonical R^2. The first individual RDA (RDA1) axis was significant (Pr(>F) = 0.044) but the second (RDA2) was not (Pr(>F) = 0.095). Numerical output of the redundancy analysis shows that the two canonical axes combined (RDA1 and RDA2) explain a total of 25.53% of the total variance in the model. Accumulated constrained eigenvalues show that a proportion of 55.36% and 44.63% of this variance is explained by RDA1 and RDA2 respectively. Correcting for overestimations in partition of variance with adjusted R^2 (R^2 = 0.26, adjusted R^2=0.14), the total variance explained is 7.79% by RDA1 and 6.28% by RDA2 (Borcard *et al.*, 2011). The variable pH is highly correlated with the RDA2 axis (canonical coef. = 0.64) while Temperature is correlated with the RDA1 axis (canonical coef. = 0.344). Planorbid gastropod response variables (BTR, BFO and GSP) are correlated with a low pH, which distinguishes them from lymnaeid (PCO and RSP) and succineid (SSP) gastropod species along the RDA2 axis. The abundance of *B. truncatus* (BTR) and, especially, *P. columella* (PCO) seem to negatively correlate with temperature since they are in the first and third quadrant of the triplot. *Gyraulus* sp. (GSP), *Radix* sp. (RSP) and to a lesser extent *B. forskalii* (BFO) correlate with higher temperatures.

3.5 Discrete ecological predictors

Following discrete variables (with their respective levels) were included in the analysis: *E. crassipes* (levels: high, low or none), substrate (type: muddy, sandy or rocky) and depth (level: deep or shallow). Generalized Linear Models (GLM) were built using Poisson distributed gastropod counts per species per site. AIC based backward model reduction was performed for each species-specific model fit and the fit with lowest AIC was used. Likelihood ratio tests for significance (type III Anova with Chi square test statistics) were performed. Overdispersion was detected and accounted for by using a quasi-poisson distribution. A summary of statistical output of the likelihood ratio tests (Anova type III) is given in Table 6.

Table 6. Results of likelihood ratio tests (Anova type III) for GLM of discrete environmental variables per species and total sail count. Given is the test statistic (Chisq), dispersion parameter, *p*-value and the AIC of the corresponding model fit. Asterisks indicate significance levels with '*' P<0.05; '**' P<0.01 and '***': P<0.001

species	dispersion	variable	Chisq	*p*-value
P. columella	30.96	*E.crassipes*	6.32	0.04239 *
		substrate	2.80	0.24797

Radix sp.	26.37	*E.crassipes*	0.25	0.88127
		substrate	4.12	0.12770
		depth	5.05	0.02464 *
Succinea sp.	7.06	*E.crassipes*	11.23	0.00365 **
		depth	5.28	0.02160 *
Gyrauls sp.	10.20	*E.crassipes*	2.77	0.2507
		substrate	0.92	0.6328
		depth	2.51	0.1130
B. truncatus	22.30	*E.crassipes*	4.03	0.1337
B. forskalii	3.31	*E.crassipes*	8.69	0.01298 *
all snails	27.50	*E.crassipes*	19.78	5.069e-05 ***
		depth	0.21	0.6503

Floating water plant vegetation of water hyacinth (*E. crassipes*) seemed to be a re-occurring (significant) predictor for gastropod counts. Where the quantity of water hyacinth was high, gastropod counts were generally higher compared to sites where the plant was absent or scarce. This effect seemed highly significant for the total gastropod count as well as for the species *B. forskalii, P. columella* and *Succinea* sp. separately. Depth was proven to be a significant predictor in some model fits. Generally, more gastropods were found in shallow waters compared to deep shorelines. For *Succinea* sp. however, gastropod counts were significantly higher in deeper waters. The parameter 'substrate' showed that total gastropod counts were higher for sites with a muddy substrate compared to a sandy or rocky substrate and that the few sites with a rocky substrate (site 4 and 9) harboured very few gastropods (n=3 and n=1 respectively). Although this parameter was included after model reduction for some sites it does not seemed to be a significant predictor in any of the species-specific models. Figure 12 shows the boxplots for all significant effects in the reduced models described above.

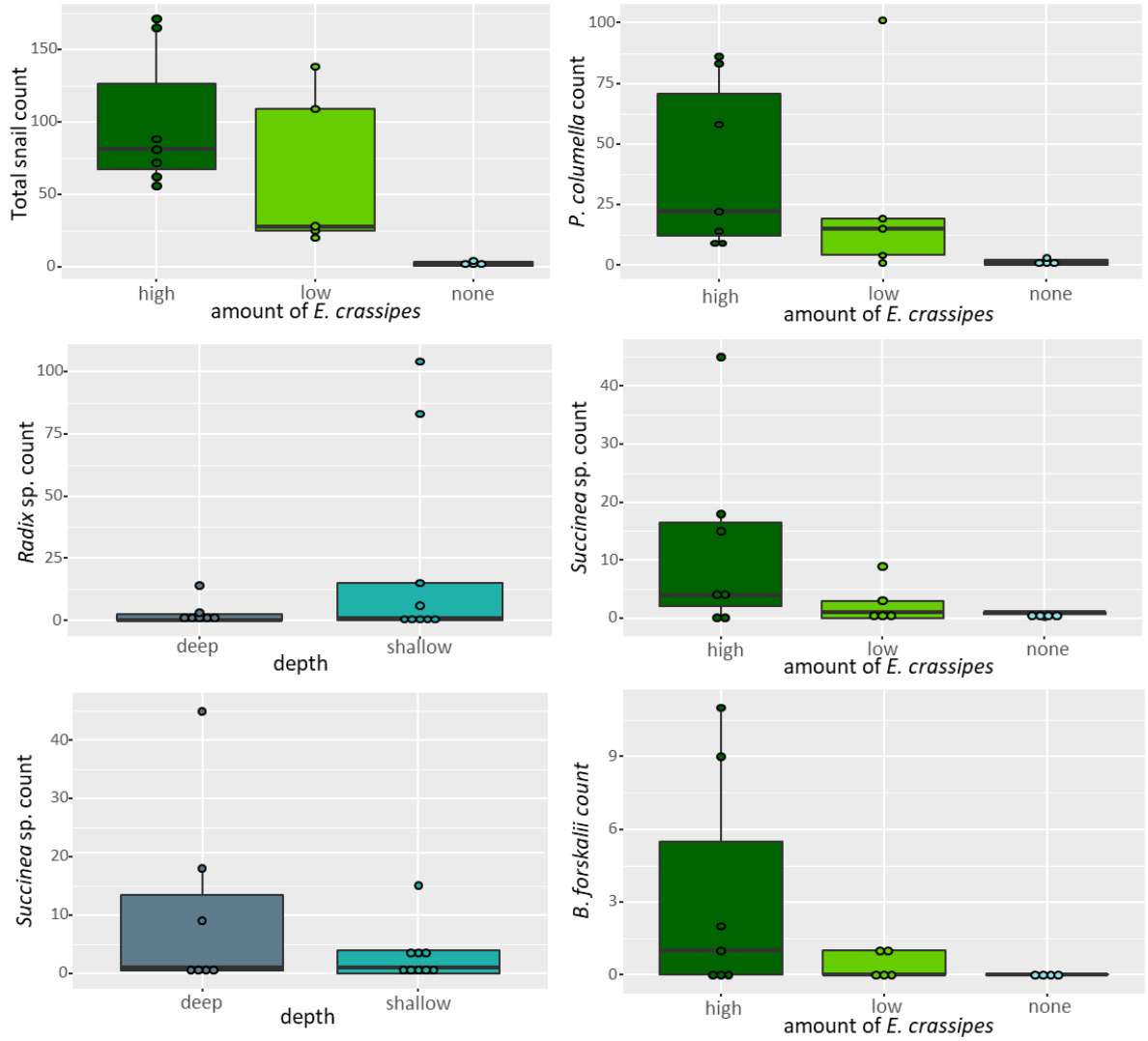


Figure 12. Gastropod counts for significant discrete ecological variables according to GLMs. Gastropod count is displayed in function of 'water hyacinth' (*E. crassipes*) with 3 levels (high, low or none) and the parameter 'depth' with two levels (shallow and deep).

Discussion

1. Freshwater gastropods of Lake Kariba

1.1 Gastropod diversity

An assessment of medical risks related to the construction of the hydro-electric dam reservoir of Kariba in the 1950' mentioned no risk of spreading gastropod-borne trematodiases near the dam wall construction area, because of its rocky soil and absence of freshwater gastropod habitat (Webster, 1960). However, it is clear by now that freshwater gastropods have colonized this area, for example from upstream rivers of the Kariba gorge and via the Zambezi river (Webster, 1960; Mungomba *et al.*, 1993). The earliest report on

freshwater gastropod diversity in lake Kariba, ten years after the flooding of the man-mad lake, mentions the presence of *Bulinus africanus*, *B. forskalii, Biomphalaria pfeifferi* and *Lymnaea natalensis* (Hira, 1969). Later on, studies proved a misidentification by Hira and colleagues and suggested *B. globosus* to be the only bulinid species present (Mubila *et al.*, 2002). All successive reports only mention and count *B. globosus* and *B. pfeifferi* because of their role in the transmission of the human schistosome species *S. haematobium* and *S. mansoni* respectively (Mungomba *et al.*, 1993; Mubila *et al.*, 2002; Chimbari *et al.*, 2003a). In all these studies, snail identification was based on morphology, which might (partly) explain the contrasting results with the present study. Our barcoding results suggest the presence of planorbid gastropods *B. forskalii, B. truncatus* and *Gyraulus* sp. but no *B. globosus* and *B. pfeifferi* were found. Based on our findings and accounts in literature (Hira, 1969; Chingwena *et al.*, 2002), we can confirm the presence of *B. forskalii* at lake Kariba. The case for *B. truncatus* and *Gyraulus* sp. is more controversial. The general shell morphology of *B. globosus* and *B. truncatus* is quite similar, especially in juveniles (Brown, 1994). Also *B. pfeifferi* and *Gyraulus* sp. can be easily misidentified especially when gastropod shells are covered by algae and microbial biofilm growth. Literature shows that identification of bullinid gastropods based on morphology is often unreliable because of the high degree of morphological variability of the shell. This phenotypical plasticity is suggested to be the consequence of slightly different environmental variables (Brown, 1994; Stothard *et al.*, 1997; Mukaratirwa *et al.*, 1998). Nevertheless, we can confirm that the genotyping of a total of nine gastropod specimens, from two different sites and labelled as 'morphotype *B. truncatus'* were indeed *B. truncatus* (98% to 100% identical for COI and ITS respectively) and not *B. globosus.* Furthermore, the group *Bulinus truncatus/tropicus,* to which *B. truncatus* but not *B. globosus* belongs to, was found to be the most widely distributed taxon in Zimbabwe (Mukaratirwa *et al.*, 1998). However, given our limited number of barcoded specimens, we cannot exclude the possibility that *B. globosus* was present but wrongly classified as *B. truncatus*. Indeed, a multitude of studies do mention *B. globosus* to be endemic to Zimbabwe and Zambia (Woolhouse *et al.*, 1990; Manning *et al.*, 1995; S Mukaratirwa *et al.*, 1996; S. Mukaratirwa *et al.*, 1996; Davies *et al.*, 1999; Pfukenyi *et al.*, 2005; Pfukenyi *et al.*, 2006). Barcoding results of 'morphotype *B. pfeifferi'* showed that this gastropod species was not *B. pfeifferi* but more related to the planorbid genus *Gyraulus.* A closer look at the shell morphology showed resemblance of our specimen (see figure 6D) to gastropods of this genus (Brown *et al.* 1969; Brown, 1994). *G. costulatus* is commonly found in Zimbabwe (Woolhouse *et al.*, 1989) and other parts of Southern Africa (Brown *et al.*, 1969). In South Africa this species was found to be a colonizer on the rise in dam reservoirs (Pretorius *et al.*, 1989). Some studies do identify *G. costulatus,* next to *B. pfeifferi* in

Zimbabwe, however no reports of this gastropod species or the *Gyraulus* genus were published so far for Lake Kariba (Chingwena *et al.*, 2002, Chingwena *et al.,* 2004). Reference sequences for the COI marker of *G. constulatus* are not available in GenBank so we cannot confirm the *G. costulatus* identity although it is possible that this species is present in Kariba. Again, one should be cautious with stating that *B. pfeifferi* is not currently present in Kariba because of the small number of samples that were barcoded successfully (n = 2). Furthermore, a multitude of records of *B. pfeifferi* at lake Kariba are found in which these gastropods shed *S. mansoni* cercariae (Mungomba *et al.*, 1993; Mubila *et al.*, 2002; Chimbari *et al.*, 2003a). Although these studies are based on morphology only, a significant prevalence of schistosomiasis has been diagnosed in children of Kariba (Chimbari *et al.*, 2003) and Siavonga (Mungomba *et al.*, 1993), a Zambian town on the banks of lake Kariba. *Gyraulus* sp. is not a known intermediate host of schistosome trematodes which increases the likelihood that *B. pfeifferi* is present in lake Kariba.

The 'morphotype *L. natalensis*' was barcoded as *P. columella.* This is the first report of this invasive gastropod species in Zimbabwe although data on lymnaeid gastropod diversity and prevalence is scarce for Zimbabwe (Hira, 1969; Chimbari *et al.*, 2003; Moyo, 2004). *P. columella* is endemic to North America but it was introduced together with agricultural aquatic crops in South Africa in the 1960's where it has become the most successful invasive freshwater gastropod (De Kock *et al.*, 1989). One of the explanations could be the capability of thriving in submerged mud when water levels retract, as this specific niche was not occupied by African molluscs in the area where *P. columella* was found (van Eeden *et al.*, 1966). Although no reference sequences are available for South African *P. columella,* our sample showed a 100% sequence similarity with isolates from Australia, Egypt, Colombia, Spain and the USA, suggesting a recent introduction of this invasive species in Zimbabwe. In South Africa, *P. columella* commonly co-occurs with *L. natalensis* in slow flowing rivers or stagnant lakes with dense vegetation and muddy substrate. But, due to its superior reproduction and semi-amphibious way of foraging, it is thought to have a competitive advantage compared to *L. natalensis* (Brown, 1994). *P. columella* is similar in shape but slightly more slender compared to the African *L. natalensis*, so misidentification based on morphological traits is plausible (Brown, 1994). Again, one has to be cautious with the assumption that there is no *L. natalensis* present in lake Kariba but the discovery of *P. columella* is at least remarkable.

The second lymnaeid gastropod morphotype, named *L. radix* could only be identified to the genus level as BLAST and phylogeny-based barcoding proposed that it belonged to the *Radix* genus, with a very close affinity to *Radix* sp. from Vietnam (von Oheimb *et al.*, 2011) with genetic p-distance of 3.7% (supplementary table 7, Annex III). This close relationship

could suggest that this *Radix* sp. was introduced form South East Asia although lack of reference sequences must be considered as well. The taxonomy of Lymnaeidae has been a subject of continuing debate. Past morphology-based species classification and genera nomenclature have been proved wrong repeatedly (Correa *et al.*, 2010). The high amount of cryptic species and the degree of morphological plasticity are the main factors that make the family of Lymnaeidae and especially the genus *Radix* hard to classify (Lawton *et al.*, 2015). Molecular-based classification and especially the use of COI as standard barcode for lymnaeid taxonomy has gained interest over recent years and although taxonomic efforts have been made, huge gaps in the reference sequence database still exist (Remigio, 2002; Pfenninger *et al.*, 2006; Correa *et al.*, 2010; Lawton *et al.*, 2015). We confirm that both lymnaeid gastropods sampled in Lake Kariba are not *R. (Lymnaea) natalensis,* the only reported lymnaeid species known as intermediate host to trematodes (i.e. *F. gigantica*) in Zimbabwe (Pfukenyi *et al.*, 2005) as the COI p-distances were > 16% (table 7 supplementary tables (Annex III)). The 'morphotype *Lymnaea* sp.' was found to be a land gastropod species from the family of Succineidae. Succineid gastropods are often found on floating water plant vegetation and included in freshwater gastropod surveys (Brown, 1994). *Succinea striata* and *Oxyloma (Succinea) patentissima* are two succineid gastropods known to be common in Southern Africa (Van Bruggen *et al.*, 1977). The position of the *Succinea* sp. from Kariba in the phylogenetic tree suggested a close relatedness to the genus *Oxyloma* but pairwise genetic distances show the closest relationship to *Succinea* sp. from Canada (p-distance=11.9%). The account of *O. petentissima* found in lake Chivero (Zimbabwe) (Brendonck *et al.*, 2003) suggests that this species could also be present in Kariba. There is no COI reference of *O. patentissima* available in GenBank but the morphological resemblance between our photograph of *Succinea* sp. and one of a South African *O. patentissima* specimen provided by Perissinotto *et al.* (2014) is striking and supports our hypothesis. Finally, also *Bellamya* sp. and *Physella (Physa) acuta* were found at lake Kariba. As previously mentioned, these gastropods are not known to host trematodes of any economic concern and are therefore excluded from our ecological and infection prevalence analysis. It must however be noted that this is the first report of the invasive *P. acuta* in lake Kariba. *P. acuta* was the most prevalent gastropod species found in this study. Like *P. columella,* it originates from North America and is likely to be introduced into Zimbabwe via South African trade in agricultural crops (Appleton, 2003).

1.2 Gastropod identification

It is clear from previous paragraphs that morphological identification can reproduce misidentifications of freshwater gastropods. Therefore, genotyping was performed with the

barcoding marker COI as it is a good marker for barcoding animals (Hebert *et al.*, 2003). Additionally, ITS was sequenced because this nuclear marker might give more insights in phylogenetic relationships between taxonomic groups (Morgan *et al.*, 2002; Kane *et al.*, 2008). Although it is recommended to use both a nuclear (e.g. ITS) and mitochondrial (e.g. COI) marker to gain better insights in evolutionary history, the COI marker fits our purpose most: quick, reproducible screening for species identification (Kane *et al.*, 2008). Also, the COI marker was the most represented marker in the GenBank database for freshwater gastropods. Based (mainly) on COI barcoding, we successfully identified four out of eight gastropod morphotypes to their species level. Both PCR and sequence success were quite low and appeared to be species-specific. For example, PCR and sequencing success were generally lower for *Gyraulus* sp. compared to *B. truncatus* and *B. forskalii* and higher for planorbid gastropods compared to lymnaeid gastropods and *Succinea* sp. specimens (data not shown). The high interspecific diversity among our gastropod species could be the reason why our universal primers did not succeed to amplify the DNA of all the species with the same success. However, one can look for reasons of consecutive failed sequencing beyond primer design. The quality and quantity of DNA in the extract may vary amongst gastropod species and have a significant influence on the sequencing result. When fixed in ethanol, gastropods can retract in their shell, providing less tissue to be penetrated by ethanol, hence resulting in DNA degradation due to this unsuccessful fixation (Galindo *et al.*, 2014). Thus, unsuccessful PCR of *Gyraulus* sp. specimens could be a result of their distinct shell morphology. Pre-treatment methods such as chemical relaxation using menthol crystals, drilling the shell, incubating the gastropod in warm water and using microwaves to remove the gastropod from its shell have been proposed to prevent this (Galindo *et al.*, 2014). Removing the intact gastropod from its shell before fixating it on ethanol also creates the opportunity to preserve the shell and thus should be considered. Besides molecular identification, other advanced biometric tools such as quantitative morphometrics of the shell might be of interest to identify gastropod species. Quantitative morphometrics use computer-derived geometric measurements of landmark distances, curves and their relationship in a statistical approach and proved to be useful in gastropod classification (Bocxlaer *et al.*, 2010).

2. Gastropod-borne trematodes of lake Kariba

2.1 Trematode diversity, possible final hosts and implications

Barcoding results showed that at least six different species of trematodes are infecting at least four different gastropod species in lake Kariba. Phylogeny reconstruction by 18S rDNA

and COI sequences provide information on the 6 respective families to which these species belong, but species-specific resolution could not be obtained (see discussion 2.2).

2.1.1 Notocotylidae

B. truncatus was found to be infected with a trematode species (type III; Fig. 7) that belongs to the family of Notocotylidae (superfamily Pronocephaloidea). Notocotylids are intestinal parasites of birds and mammals including ruminants (Toledo *et al.*, 2014; Ma *et al.*, 2016). Notocotylids have a two-host life cycle with an encysted metacercaria stage in the environment (Gonchar *et al.*, 2017). These trematodes are mostly found in Eurasia but infections in African water birds (Alexander *et al.*, 1997) and hippopotami (Sey *et al.*, 1979; Junker *et al.*, 2015) have been reported. Pronocephaloidea are closely related to paramphistomoids, together forming the suborder of Pronocephalata (Olson *et al.*, 2003). Notocotolyd flukes can, like the related paramphistomes, cause significant economic damage by infecting cattle and ovine livestock. An example is ogmocotylosis, caused by the notocotylid *Ogmocotyle* sp. This disease can lead to chronic diarrhea and hemorrhagic enteritis resulting in death (Pande *et al.*, 1960). Although *Galba (Lymnaea) truncatula* can be a host (i.e. for *Notocotylus neyrai)* (Manga-Gonzalez *et al.*, 1994), no accounts of Notocotylidae in *B. truncatus* or other African planorbid gastropods have been made so far. Since the recent discovery that some notocotylids are very closely related to Paramphistomatidae (Ma *et al.*, 2016) one could hypothesize that bullinid gastropods can function as hosts as well but further investigation is needed to conclude on this matter.

2.1.2 Psilostomatidae

The second species of trematode found in *B. truncatus* (type IV; Fig. 7), was identified to belong to the family of Psilostomidae (superfamily Echinostomoidea). Psilopostomidae are gastro-intestinal parasites of birds of no economic importance and they are found mainly in Eurasia and America (Kudlai *et al.*, 2017). African Anatidae (ducks and geese) were found to be infected as well (Alexander *et al.*, 1997). Some species of Psilostomidae (e.g. *Psilorchis hominis*) are reported to infect humans, although the route of infection and prevalence is unknown (Gakkai *et al.*, 1973; Chai *et al.*, 2009). The only genus of Psilostomidae reported in Zimbabwe is *Ribeiroia* sp*.* It has been found to infect the great egret (*Alrdea alba*) and giant kingfisher (*Megaceryle maxima*) in lake Kariba (Johnson *et al.*, 2004). *Ribeiroia* sp. is a remarkable trematode because it is known to cause severe limb deformities in amphibians (second intermediate host) that increase the chance of predation by birds (final host) and so completion of the trematode life cycle (Johnson *et al.*, 2004). This trematode has been reported to be transmitted by East African planorbid gastropods such as *Biomphalaria* sp.

(Wilson *et al.*, 2005). Phylogenetic analysis showed that despite high similarities in morphology, ecology and life cycle (Johnson *et al.*, 2004), Psilostomidae is the most divergent family within Echinostomoidea (Atopkin, 2011). Recent taxonomic efforts have produced new insights in the phylogeny of Echinostomatoidea including the displacement of *Ribeiroia* sp. into the Echinostomatidae family, thus outside Psilostomidae (Tkach *et al.*, 2016). Therefore, we conclude to have found an undescribed psilostome species and, moreover, the first account of Psilostomatidae in Zimbabwe.

2.1.3 Amphistomes (Paramphistomoidea)

The third species of trematode we found in *B. truncatus* (type I; Fig. 7), belongs to the Gastrothylacidae family (superfamily Paramphistomoidea, commonly referred to as amphistomes). Nine out of 12 Paramphistomoidea families are known to be present in Africa (Laidemitt *et al.*, 2017). Gastrothylacidae is, together with Paramphistomidae and Gastrodiscidae, one of the most speciose ruminant infecting amphistome families in Africa, comprising 40% of all paramphistomoid species (Laidemitt *et al.*, 2017). *Carmyerius* sp. were the only Gastrotylacidae recorded so far in Zimbabwean cattle (Pfukenyi *et al.*, 2005). Different *Carmyerius* species infect African wild ruminants such as antelopes (e.g. *Redunca redunca*), African buffalo (*Syncerus caffer*) and hippopotami (Sey *et al.*, 1979). Furthermore, planorbid gastropods of the *B. tropicus/truncatus* group and wild animals such as Lechwe (*Kobus leche*) have been associated with *Carmyerius* sp. transmission in Zambia and Zimbabwe (Dinnik, 1965; Wright *et al.*, 1979). Pairwise genetic distances of COI (**supplementary table 8, Annex III**) showed a divergence of 11% between the Kariban haplotype and *C. mancepatus,* sampled in Kenyan cattle (Laidemitt *et al.*, 2017). We can therefore hypothesize that the species found in Kariba is *Carmyerius* sp. It must be noted that the Gastrothylacidae amphistome was the only shedding trematode that was successfully sequences from a shedding cercarial specimen. All other mentioned trematodes are sequenced from infected snails.

Radix sp. also showed to be infected with an amphistome (type II, Fig. 7). The family remains however unidentifiable based on the COI phylogeny. Although COI-based BLAST hits suggest affinity with the family Paramphistomidae, it does not cluster together with species from this family in the ML tree. Pairwise genetic distances (supplementary table 8, Annex III) show a relationship with the amphistome *Calicophoron microbothrium* (15.2% p distance) and amphistomes do use lymnaeid gastropod intermediate hosts (Laidemitt *et al.*, 2017). However, since there is hardly any taxonomic resolution from both the trematode and gastropod host, we can only speculate that this trematode might be of any economic concern. In general, amphistomes of livestock are of high economic importance (Laidemitt *et*

al., 2017). Amphistomes are all gastro-intestinal flukes that have a two-host life cycle with lymnaeid or planorbid gastropods as first intermediate host. Like Fasciolidae, amphistomes form metacercarial encysted stages on vegetation on which ruminants such as cattle, ovines or wild large herbivores feed. Fish, amphibians and reptiles can also host amphistomes although only rarely (Toledo *et al.*, 2014). Some amphistomes have been reported to infect humans (e.g. *Fischoederius elongatus*), but the main economic concern lies within the livestock industry. Amphistomiasis has been named the most important (neglected) infectious disease in domesticated livestock of parasitic origin. Amphistomiasis is a chronic disease that causes diarrhoea and so dehydration and exhaustion of the animal. It induces a great loss in productivity, especially in calves where lethal cases are common (Sanabria *et al.*, 2008; Toledo *et al.*, 2014). Accounts of amphistomiasis in cattle and wild ruminants of Zimbabwe and Zambia are numerous (Chingwena *et al.*, 2002; Pfukenyi *et al.*, 2005; Pfukenyi *et al.*, 2005; Phiri *et al.*, 2006; Phiri *et al.*, 2007). A review on cattle infecting trematodes of Zimbabwe showed that amphistome load and prevalence is genuinely higher than *F. gigantica* and *S. mattheeii* infections (Pfukenyi *et al.*, 2005).

2.1.4 Fasciolidae

P. columella was found to be infected with *Fasciola* sp. (type V, Fig. 7), as suggested by both genotyping and the *Fasciola*-specific marker in the multiplex RD-PCR. *P. columella* is a well-known host of *F. hepatica*, which is prevalent throughout Africa including Mozambique, Zimbabwe, Zambia, Kenya, Egypt and South Africa (Toledo *et al.*, 2014). The semi-amphibious habitat use of *P. columella* suggests its excellent capability to spread metacercariae encystments along the vegetation that can be grazed upon by large herbivores such as cattle or wild ruminants (Toledo *et al.*, 2014). Our data strongly suggest that the fasciolid found in lake Kariba is closely related to *F. gigantica* and to a lesser extent to *F. hepatica* but genetic pairwise distances of the COI alignment confirm it is none of these species since *p*-distance > 5% (supplementary table 8, Annex III). The family of Fasciolidae contains five described genera and nine species, of which only five species are available as COI reference sequenced in the GenBank database. One *Fasciola* species, endemic to Sub Saharan Africa but of which reference DNA sequences are lacking, is *F. nyanzae,* which infects hippopotami (Dinnik *et al.*, 1961). *F. nyanzae* uses lymnaeid gastropod intermediate hosts similar to other Fasciolinae (i.e. *F. gigantica*, *F. hepatica*, *F. jacksoni* and *F. magna*). Moreover, the anatomy and way of infecting its final host (i.e. inhabiting the liver rather than intestine) of *F. nyanzae* is similar to *F. gigantica* and *F. hepatica* (Lotfy *et al.*, 2008). Its cercariae and miracidia are most similar to *F. gigantica* and the adult worm shows high resemblance to *F. gigantica* and *F. hepatica* compared to other Fasciolinae such as *F.*

magna and *F. jacksoni* (Dinnik *et al.*, 1961). This advocates that *F. nyanzae* could be closely related to *F. gigantica* (Lotfy *et al.*, 2008). The Kariban trematode we have barcoded also shows most relatedness to *F. gigantica* and although no direct molecular proof can be obtained at this moment (as this would require a barcode of *F. nyanzae*) we can therefore only suggest that the Kariban fasciolid could be the species *F. nyanzae.* The fact that hippoptami are very abundant in and around lake Kariba (local source and own observation), especially when compared to cattle, goats and sheep (which are hosts to *F. gigantica* and *F. hepatica*), makes this hypothesis even more probable.

18S rDNA sequencing from one *Radix* sp. did also indicate a *Fasciola* sp. infection since the 18S rDNA sequence was identical to the specimens from *P. columella*. However, there was no successful PCR amplicon of COI obtained for this sample. Since Radix is an intermediate host of Fasciolinae (Lawton *et al.*, 2015), we can assume that both *Radix* sp. and *P. columella* are infected with the same *Fasciola* species.

2.1.5 Diplostomatidae

One furcocercous (fork-tailed) cercariae shedded from *B. truncatus* but no PCR amplification of any marker could be obtained for the specimen. Based on morphology, it was identified as a Longifurcate-pharyngeate distome (Strigea) cercaria type. These cercaria types are typically found in Diplostomatidae and Strigeidae families (superfamily Diplostomoidea). Based on barcoding, *B. forskalii* also showed to be infected with a species of Diplostomatidae (type III; Fig. 7). Diplostomatidae have three to four-host life cycles with mammals and birds as final host although the economic implications come from pathologies in their intermediate fish host. Our barcode sequence shows the highest similarity with *Alaria mustelae* with p-distance of 12.9% (**supplementary table 8, Annex III**). *Alaria* sp. are, amongst other diplostome genera (i.e. *Uvulifer, Diplostomum* and *Posthodiplostomum*), known to cause major economic problems in the fish industry (Toledo *et al.*, 2014). But no reports in the literature seem to exist from Africa. Diplostomes have been found to be the most prevalent digeneans in economically important fish species such as tilapia and other cichlids in Kenya (Otachi *et al.*, 2014) and southern Africa (Grobbelaar *et al.*, 2014). In Ethiopia, the Nile tilapia (*Oreochromis niloticus*) was discovered to be infected with the brain infecting diplostome *Tylodelphis* (Gebremedhn *et al.*, 2017). Nile tilapia is after ‘kapenta’ (*Limnothrissa miodon*) (Madamombe, 2002) and the African tigerfish (*Hydrocynus vittatus*) (Mabika *et al.*, 2016), one of the economically most important fish species in lake Kariba where it is farmed on large scale (Lake Harvest, 2017; Berg *et al.*, 1996). This emphasizes the importance of mapping the presence of diplostomes that can infect (farmed) fish in the lake. As described before (Introduction 4.2), trematodiases in (farmed) fish can have large-

scale economic complications and are hard to combat. Diplostome (strigae) cercariae have been reported in planorbid snails, mainly *B. tropicus,* in Zimbabwe (Chingwena *et al.*, 2002) and Southern Africa (Grobbelaar *et al.*, 2014). However, no account of *B. forskalii* as intermediate host for diplostomes can be found in literature from these areas. Only 12 specimens of *B. forskalii* were successfully tested for trematode infections with the multiplex RD-PCR and 18% showed to be infected. More detailed genotyping and a broader epidemiological assessment should be considered to estimate the possible impact of diplostome trematodes on the Kariban lake ecology and economy.

2.1.6 Leucochloridiidae

Of no economic importance but noteworthy to mention was the identification of a species of Leucochloridiidae found to infect the *Succinea* (*Oxyloma*) sp. land gastropod. Leucochlorids (Superfamily Brachylaimoidea; family Leucochloridiidae) are, like diplostomes, contained within the Strigei(di)da order (Olson *et al.*, 2003). These European parasites of (mainly) birds are best known for the enigmatic genus *Leucochloridium* sp. that forms pulsating sporocysts with colored bands in the ocular tentacles of their succineid gastropod hosts. Infected gastropods have been recorded to show phototactic movement towards exposed areas. This behaviour together with the pulsating tentacles are thought to have evolved to attract final avian hosts to complete the parasite life cycle (Casey *et al.*, 2003). The BLAST results could only be obtained for the 18S rDNA marker and are merely suggestive since the quality and length of the sequence was not optimal (280 bp; HQ=89.6%). Although more resolution is definitely needed, Leucochloridiidae are one of the only trematode parasites known to infect Succineidae like *Oxyloma* sp. so it is probable that these parasites have invaded the African continent as well.

2.2 Trematode identification

As described before, morphological identification of larval trematodes is highly error-prone and hard or impossible for genus or species level resolution. Therefore, molecular genotyping is indispensable. The 18S rDNA amplicon gave better sequencing results than the COI marker with 48% and 36% (resp. 22 out of 45 and 18 out of 50 amplicons) successfully sequenced. 18S rDNA sequences are much more represented in the GenBank database compared to COI. However, 18S rDNA is a more conserved marker with less interspecific variability compared to COI. This is why BLAST hits of 18S rDNA sequences showed consistently higher identity matches. Its conserved nature makes 18S rDNA useless for species-specific genotyping (Nolan *et al.*, 2005). It is however fit for ‘family genotyping’ as indicated by Olson *et al.* (2003). In contrast, is COI a good marker for barcoding animals on

species level (Hebert *et al.*, 2003) but COI barcoding of trematodes using universal primers is difficult because of nucleotide variability at the primer binding sites (Van Steenkiste *et al.*, 2015). It is thus plausible that certain trematode species were present within the infected gastropods sampled, but their DNA could not be amplified due to variability in the COI primer annealing regions. Comparing 18S rDNA and COI PCR success did not show any consistent pattern that may support this hypothesis, as failed PCR results mostly occurred in the same specimens for both 18S rDNA and COI PCR. The lack of COI reference sequences from the GenBank and BOLD database is therefore the only explanation for the low resolution of barcoding results of the Kariban trematodes. Additional genotyping with the ITS marker, combined with a broader phylogenetic analysis, can probably generate more taxonomic information about these trematodes (Nolan *et al.*, 2005; Vilas *et al.*, 2005). This is not the case for all species, since for certain species any genetic reference sequence is missing from the database (e.g. *F. nyanzae*). The complementation of the reference sequence database requires efforts that combine sampling, molecular genotyping and morphological classification of different trematode larval stages which sometimes implies the completion of the trematode life cycle (Moszczynska *et al.*, 2009). Our research group, led by Dr. Tine Huyse, is currently genotyping an adult worm specimen of *F. nyanzea* present in the museum collection of the RMCA (Tervuren). This is an example of such a taxonomic effort as it might confirm whether the *Fasciola* sp. found in Kariba is the liver fluke of hippopotamus.

2.3 Infection prevalence

Our shedding experiments yielded few infections: nine out of 1024 sampled gastropods shedded (i.e. prevalence of 0.8%), a sharp contrast with the infection rates found with multiplex RD-PCR, in which 32.24% of the gastropods were found to be infected. The number of shedding gastropods is also low compared to studies that use the traditional method conducted in other East African study areas. Studies in Zimbabwe (Chingwena *et al.*, 2002), Zambia (Phiri *et al.*, 2007) and Tanzania (Loker *et al.,* 1981) showed shedding rates in gastropods ranging from 6.6% in Zimbabwe to 13.7% and 14.9% in Zambia and Tanzania respectively (Loker *et al.,* 1981; Chingwena *et al.*, 2002; Phiri *et al.*, 2007). Of the shedding gastropods, eight were *B. truncatus,* and one was a *P. columella* gastropod. Based on the multiplex RD-PCR however, *P. columella* and not *B. truncatus* was the most infected gastropod species with 62.77% and 14.06% infected gastropods respectively. This was followed by infection rates of 25% in *Succinea* sp., 18.18% in *B. forskalii*, 6.82% in *Radix* sp. and 0% in *Gyraulus* sp. As described in result section 2.3 it must be emphasized that the difference in samples tested per species must be considered when comparing these

infection prevalences. Besides being the most infected gastropod species, P*. columella* was also the most sampled gastropod species: 39% of all sampled gastropods were *P. columella* whereas 26% were *B. truncatus,* 17% *Radix* sp., 10% *Succinea* sp. 5% *B. forskalii* and 3% *Gyraulus* sp. (see Fig. 8). Six of the nine shedding gastropods came from site 16 (Charara) and two came from site 11 (Nzou lodge) but multiplex RD-PCR showed that trematodes are present in gastropod populations of many more sites (i.e. site 1, 3, 5, 11, 12, 13, 14 and Fishing camp 1). Based on the multiplex RD-PCR results, we found 44.93% of all lymnaeid gastropods to be infected, which is similar to infection prevalence by shedding in lymnaeid gastropods of Zimbabwe (38.2% infected) and the Kafue wetlands (in the vicinity of Lake Kariba) of Zambia (42.8% infected) found by Chingwena *et al.* (2002) and Phiri *et al.* (2007) respectively. All infections of *P. columella* and two out of three infected *Radix* sp. were infected with *Fasciola* sp. as the Fasciola specific signal in the multiplex RD-PCR showed.

On the other hand, no schistosomes were found to infect the planorbid snails tested. A study by Chimbari *et al.* (2003) in Kariba, in which only planorbid gastropods were investigated by shedding, found mammalian schistosome infection rates of 3.33% and 4.76% in *B. globosus* and *B. pfeifferi* respectively (Chimbari *et al.*, 2003). It is therefore striking that no schistosomes were found in this study. Schistosomiasis is known to be endemic to Zimbabwe, and an estimated 20.8% and 9% of the Zimbabwean population is assumed to be infected with *S. haematobium* and *S. mansoni* respectively (Chimbari, 2012). However, in Kariba, a decrease in prevalence of human schistosomes was noted before and attributed to improvement of sanitary infrastructure and control campaigns (Chimbari *et al.*, 2003). We can therefore suggest that infection prevalence of *Schistosoma* sp. in Kariban gastropods has further decreased since the latest assessment. It must be noted that only 64 specimens of *B. truncatus,* the endemic host for *S. haematobium* were checked for infection (by multiplex RD-PCR). Furthermore, no *B. pfeifferi* were sampled in our assessment, while this species is known to be the endemic host for *S. mansoni* in the area (Chimbari, 2012). Therefore, we cannot draw major conclusion on the overall epidemiology of human schistosomes in the area and a more detailed epidemiological assessment would be desirable to conclude on this matter in the future.

Infection prevalence of trematodes other than *Fasciola* sp. and *Schistosoma* sp. are not assessed in our multiplex RD-PCR approach. We can therefore only confirm their presence but not their prevalence in the gastropod population. Traditional shedding methods can morphologically categorize trematode infections of each gastropod separately, which is one of the few advantages compared to RD-PCR (see 2.4). The Zimbabwean study of Chingewena *et al.* (2002) found echinostomes (38.2% of all infections) and amphistomes (37.6% of all infections) to be the most abundant, whereas the Zambian study by Phiri *et al.*

(2007) found gymnocephalous (Fasciolidae and others) and longifurcate-pharyngeate distome cercariae (typically blood-flukes of fish such as diplostomes) to be most abundant in the lymnaeid and planorbid gastropod population (Chingwena *et al.*, 2002; Phiri *et al.* 2007). Our findings do confirm amphistome and diplostome infections in planorbid gastropods and gymnocephalous (*Fasciola* sp.) and amphistome infections in lymnaeid gastropods but no quantitative estimation can be provided.

Relative infection rates of different gastropod species and different sites are hardly comparable because not all sites and species were sampled for infection in the same standardized quantity. First of all, some sites did not harbour many – or any – gastropods (e.g. site 7, 8 and 9) and every site was different in species diversity. Second, many gastropods died during the one-week incubation period (see 2.4). A third factor that complicates the comparison is the fact that 3.5% (9 out of 254) of the Chelex® DNA extractions did not yield PCR results, even after 1:100 dilution of the DNA extract (see 2.4). Because of these limitations, a detailed epidemiological assessment of the infection rates is not integrated into the ecological data analysis (see 4). It is rather the presence or absence of the gastropod host that is taken into account if we compare sites and their ecological differences for the infection risk assessment.

2.4 Methods for the assessment of infection prevalence

2.4.1 Allozyme analysis

As previously mentioned, the allozyme analysis was stopped due to technical constrains (i.e. lack of proper centrifuge system at the field lab). Although it is a technique not commonly used anymore because of the availability of more sensitive and specific molecular based diagnostic tools (e.g. PCR), it seemed to be still a promising low-cost method for on-site diagnosis in rural areas such as Kariba (Caron *et al.*, 2008; Toledo *et al.*, 2014).

2.4.2 Shedding vs multiplex RD-PCR

We cannot directly compare infection rates by shedding and multiplex RD-PCR because we did not sample the same specimens and the same quantity of specimens by both techniques. Albeit not qualitatively translatable, our results do confirm that shedding experiments, on which most trematode infection surveys of gastropods rely, highly underestimate the real epidemiological situation. Underestimations up to 66% of single infections and 80% of double infections were found when comparing PCR-based diagnostics to classical shedding (Born-Torrijos *et al.* 2014). Multiple explanations can be considered to

understand these findings: the infection may be immature, mature cercariae do not shed because sub-optimal conditions/stimuli (e.g. Temperature) and shedding of multiple species of trematodes (co-infection) rarely occurs at the same time. The latter is probably due to an antagonizing interaction or competition for gastropod resources (Dreyfuss *et al.*, 1994; Born-Torrijos *et al.*, 2014). Optimal shedding conditions vary for different trematode species using the same or different gastropod hosts. It was found that *Fasciola* sp. mainly sheds during the night and early morning in darkness whereas other trematode species of the same, lymnaeid host, shed in the morning, triggered by light (Dreyfuss *et al.*, 1994). This might explain why the intensively sampled *P. columella* did not shed more than once while more than half of the population seems to be infected in our PCR study. The workflow that we used, i.e. shedding experiments followed by fixation and multiplex RD-PCR, had several consequences for our analysis. High mortality rates during incubation were noted and could be the result of excess heat, insufficient food source, low oxygen levels or other incubation conditions. Parasite-induced host mortality is another, very important factor that has been recorded in many field and laboratory studies of gastropod-borne trematodes (Sorenson *et al.*, 2001). Decreased survivorship due to rediae or sporocyst development was shown in both lymnaeid and planorbid gastropods where prepatent as well as maturing infections commonly reduced survival by more than 50%. Increased mortality was most common in studies where gastropods were pooled (75% when grouped per species, 50% when individually kept) (Sorenson *et al.*, 2001). This thus could have had a significant influence on our results as well.

2.4.3 Limitations and possible improvements of the multiplex RD-PCR protocol

Our designed multiplex RD-PCR protocols show promising results for future in-field epidemiological use, especially because of the cheap, easy and fast Chelex® extraction procedure and pooled design that allows for high-throughput sample processing at relatively low costs (Caron *et al.*, 2008). However, a few remarks and future directions have to be mentioned to emphasize their limitations. First of all, our multiplex RD-PCR protocols were not subjected to detailed sensitivity assays. This was due to time constraints, and we based our protocol on sensitivity and specificity assays of Caron *et al.* (2011) who did perform sensitivity analysis by gradually diluting the template DNA (Caron *et al.*, 2011). It should however be considered for our protocols as well because we use different primers, primer combinations, gastropod species, target genes and pooling strategies. Although our protocols were optimized and screened for cross-reactivity and specificity, we did not test the sensitivity for the trematode marker amplification in individual and pooled gastropods using our newly designed primers. For the general trematode multiplex RD-PCR, the integration of

the internal control marker already eliminated false negative results by PCR inhibition, but the possibility of false negative results for trematode amplicons still exists and should be examined in sensitivity screens. Second, co-infections of multiple trematode species in the same gastropod were not accounted for in our general trematode multiplex RD-PCR protocol. Double (tripel or multiple) co-infections are considered to be more important than previously assumed and can be identified by amplifying two (or more) markers of different length, each specific for one trematode species (Born-Torrijos *et al.*, 2014). Since we did not target specific trematode species but aimed to identify any trematode infection, we could not integrate this approach into our multiplex RD-PCR (although we did adopt this approach in the Schistosoma multiplex RD-PCR). A third disadvantage is the phenomenon of PCR inhibition that commonly occurs in Chelex® DNA extracted PCR (Caron *et al.*, 2011). This can be prevented by integrating an internal control (like we did) and further dilution of the extract upon inhibition. By diluting however, the chance exists to over-dilute the sample which could have been the case for the failed PCR results in our study. A solution is dilution after measurement of DNA concentration in the DNA extract, for example by Qubit® Fluorometric Quantitation technology (Thermofisher™). By doing so, an equal concentration of DNA can be obtained by dilution and high throughput screens can become more standardized. It decreases the chance for PCR inhibition or over-dilution of the DNA samples. The most important disadvantage of the multiplex PCR protocols as the ones we propose, is the cost (app. PCR 0.6 EUR/sample, without costs of DNA extraction included), requirement of high-tech laboratory equipment (e.g. thermal cycler) and the possibility of false positive (contamination) and false negative results although the latter two are partly eliminated by the internal control marker and a negative control to test for contamination (Caron *et al.*, 2008). Especially when a multistep process is needed to identify the trematode species like we designed with the schistosome multiplex RD-PCR and sequencing step, the costs can exceed the available resources for epidemiological screens in endemic countries.

Subtle variations to the conventional multiplex PCR protocol are nested PCR (NPCR) and single tube nested PCR (STNPCR) which increase the sensitivity and decrease the chance of false positive results due to cross contamination (STNPCR) (Melo *et al.*, 2006). These adaptations could be implemented into de multiplex design we propose here. Other, promising PCR-based diagnostic tools such as loop-mediated isothermal amplification (LAMP) and (multiplex) quantitative PCR (qPCR) have several advantages compared to conventional (multiplex) PCR (Minetti *et al.*, 2016). The use of qPCR, also called Real-Time PCR (RT-PCR) is currently the most efficient diagnostic tool developed for high throughput diagnostic screening in both final and intermediate trematode hosts because of it is quick, very specific, it allows quantification of the infection, and there is no post-PCR manipulation

(such as gel electrophoresis) necessary. The latter feature makes it less probe to cross-contamination. qPCR has been successfully tested for human schistosome (ten Hove *et al.*, 2008; Kane *et al.*, 2013), avian schistosome (Jothikumar *et al.*, 2015) and fasciolosis (Alasaad *et al.*, 2011) diagnosis. However, qPCR still requires highly specialized laboratory equipment and personnel amongst other financial drawbacks. More low-tech alternatives are LAMP and RPA (see introduction 5.2) that have been developed for *Schistosoma* diagnostics and can be used in-field because of the isothermal reaction (no need for expensive thermal cycler) and easy detection methods applicable (Minetti *et al.*, 2016). However, RPA is still too expensive and detection limits are higher compared to PCR (Rosser *et al.*, 2015). LAMP is cheap and very user friendly but both RPA and LAMP are still to be optimized for multiplex targeted screening (Hamburger *et al.*, 2013; Rosser *et al.*, 2015; Minetti *et al.*, 2016).

3. Ecology of gastropods at lake Kariba

The ecology of freshwater gastropod community structures and dynamics can be of great importance for the optimization of targeted gastropod control strategies and the risk assessments of gastropod-borne parasites infections of animal and man (Brown, 1994; Giovanelli *et al.*, 2005). In this study, gastropod distribution data were gathered both along the spatial and temporal gradient. A detailed ecological study, including the assessment of temporal variability in the gastropod community of lake Kariba in relation to ecological variables is currently led by fellow Master student Kudzai Muzarabani (University of Zimbabwe; unpublished work). We provide preliminary data on spatial differences in gastropod community and ecological parameters among the sampled sites with remarks on the infection risks of GBTs.

3.1 Gastropod community composition and physico-chemical habitat differentiation

Since ecological data rarely meet assumptions such as normal distribution, parametric tests are not suited for analysis. Using permutation tests for testing statistical significance is an alternative that deals with this problem (Borcard *et al.*, 2011). To explore the relationship between physico-chemical water parameters and gastropod community composition, we applied redundancy analysis and we used a permutation *F*-test statistic to investigate the significance of their relationship. Our RDA results show that the variance in gastropod community composition among sites can be significantly explained by variance in water pH and Temperature.

By comparing gastropod community composition (NMDS; Figure 9) and water chemistry (PCA; Figure 10) among the 16 sites, some main trends become clear. It seems that the

planorbid gastropod *Gyraulus* sp. has a preference or at least high tolerance for highly eutrophicated sites there it is very abundant in site 14, 12 and 11 but absent from most other sites. *B. forskalii* was almost exclusively found in site 16 and 14, both sites with low oxygen content and pH. *B. truncatus* shows the same pattern but was also common in other, less eutrophicated sites such as site 15. Lymnaeid gastropods seem to be less tolerant to waters with high nutrient contents. *Radix* sp. was abundant in site 5 and 3 (with n=104 and n=83 of the total of 228 *Radix* sp.), both sites characterized by low nutrient concentrations. Counts of *P. columella* were slightly lower in highly eutrophicated sites such as site 14 and site 12. From the canonical redundancy analysis (RDA; Figure 11) we can observe parallel trends. The abundance of *B. forskalii, B. truncatus* and, to a lesser extent *Gyraulus* sp., negatively correlates with pH. Low pH levels in turn proved to co-linearize with low oxygen content, high nutrient levels, turbidity, conductivity and chlorophyll-A concentrations. In literature, comparable trends are described for planorbid gastropods. Turbidity and high nutrient concentrations were found to enhance the abundance of *B. truncatus* (Slootweg *et al.* 1993). *B. truncatus* is a meso-saprobic species and is commonly found in human-associated polluted water bodies and streams connected to sewage. *B. truncatus* can also tolerate a significant lower oxygen saturation level compared to other gastropods (Watson, 1958). Both *G. costulatus* and *B. forskalii* seem to favour a similar environment based on our findings and literature (Ndifon *et al.*, 1989), although ecological studies including these species are rare.

The correlation of eutrophication and planorbid gastropod count might be very important in prevention and control of trematodiases. The bullinid gastropods sampled in Kariba were found to be intermediate hosts of different GBT. The fact that *B. truncatus* has been found to thrive in waters polluted with human excreta (Watson, 1958) and it is the host to (humane) trematodes that are transmitted via excreta, indicates that sanitary infrastructure is very important. Although *Gyraulus* sp. was not found to be infected, this species might be of interest since it can be the intermediate host of intestinal echinostomes, which can infect multiple vertebrates including humans (Brown, 1994; Fried *et al.*, 2009). Site 14 was a highly polluted site and this can be explained because of the proximity of a waste stream from the Padenga crocodile farm. Planorbid gastropod counts were high for this site and regarding the importance of planorbids gastropod for trematode dispersal, these kind of waste streams should be managed properly to prevent GBT spread via planorbid gastropods. The lymnaeid gastropods *P. columella* and *Radix* sp., seem to favour waters that are more oxygenated and have lower concentrations of nutrients. A study from Cuba, where *P. columella* is endemic, showed that the prevalence of *P. columella* and other lymnaeids is significant negatively correlated with nutrient concentrations and ‘anthropization’ or human disturbance

(Cañete *et al.*, 2004). Similar narrow tolerances to pollution have been found for African lymnaeids like *Radix natalensis* (Van Someren, 1946). However, P. columella was found to be an extremely flexible invader as it successfully colonized a range of different South African hydro-electric dams explored in a study by Pretorius *et al.* (1989).

Conductivity was the most influential factor for the separation of sites based on water chemistry traits along the PCA1 axis (Fig. 10), while pH was shown to be best qualified to describe partitioning in variance along the RDA2 axis (Fig. 11). But actually, both conductivity and pH are rather consequences of variability in other explanatory variables: electrical conductivity is mainly influenced by salt and ion (Calcium, Magnesium,…) concentrations and pH is mainly influenced by dissolved nutrient content (Brown, 1994). Temperature was another variable that showed to be of importance in the RDA analysis. Temperature is investigated in most freshwater gastropod ecology studies and is often mentioned as the most important factor determining freshwater gastropod diversity and abundance (Salawu *et al.*, 2013). It is however a complex factor because of micro-environmental differences in the water column and its influence on multiple life history traits such as reproduction, fecundity and growth of juveniles (Brown, 1994). This can perhaps be the underlying driving factor of the significant positive correlation trends of *B. forskalli*, *Radix* sp. and *Gyraulus* sp. with warmer waters (RDA: Fig. 11). Another important aspect of temperature tolerance, but rather on wider geographic scale, is the ability of certain species to invade the habitat and even outcompete indigenous species because of superior thermotolerance. This phenomenon was recorded for the invading *P. acuta* in South Africa by Brackenbury *et al.* (1991). One account even suggests the competitive replacement of *B. forskalii* populations by *P. acuta* invaders in river systems near Kinshasa, DR Congo (De Clercq, 1987). Competitive replacement could also have accounted for the high count of *P. columella* in Kariba, where previously *R. natalensis* was the only lymnaeid gastropod found. From our RDA analysis we can conclude that the invading *P. columella* correlates with lower temperatures. Spatial variability in lake water temperature could thus have a significant influence on the ability of *P. columella* to invade (and disperse trematodes) along the Kariban shoreline. The extreme rapid spread of *P. columella* has been proved to change the epidemiology of trematodes such as *F. hepatica* in the invaded countries (Madsen *et al.*, 1989). An extreme example is the competitive replacement of a local lymnaeid gastropod by *P. columella* in New Zealand (Madsen *et al.*, 1989).

3.2 Discrete ecological predictors

GLM based data analysis proved that the discrete ecological variables (i.e. *E. crassipes,* depth and substrate) did influence gastropod abundancy of different species, whether or not

in a significant way but trends were mostly similar. The floating water plant *E. crassipes* (water hyacinth) was the most re-occurring significant predictor for gastropod abundancy for almost all species. Floating vegetation can offer protection from the sun and heat but also from visual predators such as fish and birds (Plummer, 2005). Furthermore, do water plants provide oxygen to the water, act as substrate for egg mass deposition or functions as direct (plant tissue) or indirect (plant's microflora or detritus) food source. Another beneficial effect of water plant vegetation mats is the decrease in water velocity and tidal wave action. High current velocity can be detrimental for freshwater gastropods because it can wash away food substrates, important nutrients, eggs, hatchlings and adults, and consequently reduce growth, reproduction and thus abundance (Plummer, 2005). *E. crassipes* is an invasive species from South America. It is a problematic water plant in lake Kariba where it is known to form dense, vast mats that out-compete local water plants (Adams *et al.*, 2002). The link between water hyacinth and trematode epidemiology was made before and it is also suggested that the plants can provide refuges for amphibious gastropods like *P. columella* during molluscicide treatment of waterbodies for trematodiases prevention (Grabner *et al.*, 2014). It could be usefull to consider the control of water hyacinth simultaneously with the control of GBT spread by its associated gastropod fauna (e.g. *P. columella*) in lake Kariba. Besides promoting the spread of gastropods, water hyacinth causes many more problems (e.g. in fisheries) and is often seen as the most problematic invasive water weed in the world (Adams *et al.*, 2002). More gastropods were generally found in shallow waters because they rely on light for reproduction and food supply (e.g. algal growth) (Plummer, 2005). *Succinea* sp. was however found to be significantly more prevalent in deeper sites. The fact that *Succinea* sp. is a land gastropod and does not use the water itself to forage for food can perhaps explain its indifference for the depth of the lake. Another explanation can be that deeper habitats are less vulnerable to desiccation of the floating water plant and bank vegetation on which the gastropod possibly relies for shade and moist habitat. Gastropod abundance of most species was higher on muddy substrate compared to sandy or rocky lake soil but this effect was never significant. It also must be noted that most of the sampled sites had a muddy substrate and rocky shores were rare. Muddy substrates usually contain high amounts of detritus (decaying plant material, microbial biofilm) on which most fresh water gastropods forage.

3.3 Limitations of our ecological study

Although our RDA results show that the variance in gastropod community composition among sites can be significantly explained by variance in water pH and temperature, the total variance explained by the RDA fit was quite low (i.e. 25.53%). Furthermore, the

adjusted R^2 value of the canonical ordination was low (adj. R^2 =0.14), which implies that the correlation between both data matrices is weak. Although noise in ecological data sets is common (Borcard *et al.*, 2011), much more ecological variability not included in our analysis, is probable to explain the differences in gastropod communities better and could be assessed in the future. Additional well-studied explanatory variables that were not assessed in this study but might be of importance are desiccation and ionic calcium concentrations, the latter relates to calcification of the gastropod shell. Both factors can aid in explaining distribution patterns of different freshwater gastropod species (Lodge *et al.*, 1987; Brown, 1994). It must be noted that although water chemistry and habitat traits can be decisive for freshwater gastropod distribution and diversity, colonization constrains and biotic factors such as competition and predation can definitely play major roles as well (Lodge *et al.*, 1987). Our ecological assessment thus lacks possibly very important explanatory variables and can therefore be misleading. Furthermore, is the species-specific distribution of freshwater gastropods usually not solely and directly determined by physico-chemical factors and prone to micro-environmental influences. The population dynamics of freshwater gastropods are therefore mostly dependent on a combination of multiple circumstances which are hard to assess both in the field and lab (Brown, 1994). An integrated sampling program of gastropod counts and ecological parameters (including the ones proposed but not assessed in our study), along with standardized infection prevalence screening using our multiplex RD-PCR can be of great value to monitor the trematode-gastropod associations in lake Kariba.

4. Conclusion, implactions and future perspectives

While scarce in Africa, documentation on freshwater gastropod species and their larval trematode fauna can be of great value for the epidemiology and control of trematodiases in humans, wildlife and livestock. By targeting trematode transmission hotspots (e.g. site 15 or Nzou Logde in our study) in molluscicide campaigns or mass drug administration campaigns, the general cost and effort could be reduced.

Although multiple studies, between 1969 and 2003, have sampled the freshwater gastropod fauna at lake Kariba, these studies focussed on planorbid gastropods that transmit schistosomiasis (Hira, 1969; Mungomba *et al.*, 1993; Mubila *et al.*, 2002; Chimbari *et al.*, 2003). This is the first time all freshwater gastropod species and their trematode parasites were taken into account and investigated in a One Health approach for this lake. We have found six different gastropod species of which *P. columella*, *Radix* sp., *Gyraulus* sp., *P. acuta* and *Succinea* sp. have not been recorded before in lake Kariba. Some of these gastropods are (cfr. *P. columella, P. acuta*) or might be (cfr. *Radix* sp., *Succinea* sp.)

invasive species. The American *P. columella* is of special interest since it was highly abundant at almost every site and it was found to be highly infected with *Fasciola* sp. We also recorded trematode infections in, probably invasive, *Radix* sp. and *Succinea* sp.

Gastropod abundance was correlated with the presence of the invasive water hyacinth *E. crassipes,* for most species but especially for *P. columella.* This cascade effect of invading plants that support invading, parasite-hosting gastropods might be an interesting topic for future research. The strong correlation of gastropods and water hyacinth implies that the control of water hyacinth should be considered in the control of GBTs. Furthermore, our ecological study showed that planorbid gastropod counts positively correlate with eutrophication. An example of this is the gastropod community rich in planorbid snails at the highly eutrophicated site 'Lake Croc' where the Padenga crocodile farm disposes its waste water. Since planorbid gastropods are important in the spread of trematodes, waste water treatment to reduce the eutrophication at this site, is an important measure to consider. Six different trematode species were identified. *B. truncatus* was found to be a host of three different trematode species from the Notocotylidae, Psilostomatidae and Gastrothylacidae (amphistomes) families. The lymnaeids *P. columella* and *Radix* sp. were found to be both infected with a *Fasciola* sp. suggested to be *F. nyanzae* (liver fluke of hippopotamus). *Radix* sp. hosted besides *Fasciola* sp. a species of amphistomes that could not be assigned to one of the described families (based on COI and 18S genotyping). *B. forskalii* was infected with a trematode from the Diplostomatidae family related to *Alaria* sp. This might imply that this trematode infects fish as intermediate host. Since the fishing and fish farming industry is the most important, and a continuously growing, economic activity at lake Kariba (Madamombe, 2002). Therefore, fish infecting parasites could be of great economic importance now and in the future and must be studied in greater detail. The presence of amphistomes, fasciolids and notocotylids indicate potential dangers for the cattle and ovine livestock industry and should be investigated in the future as well. Although no human infecting schistosomes have been found in our study, we cannot exclude the possibility that these parasites are still present. Since schistosomiasis is endemic to this area (Chimbari, 2012; Chimbari *et al.*, 2003) and we have sampled no *B. pfeifferi* and tested only 64 *B. truncatus* specimens, we cannot draw any epidemiological conclusions on this matter.

The PCR-based diagnostic protocol we have developed confirmed that classical shedding experiments highly underestimates the infection prevalence in snails. Based on our multiplex RD-PCR results, we have found trematode infection prevalence in different gastropod species ranging from 62.77% (in *P. columella*) to 0% (in *Gyraulus* sp.). But, due to our sampling design, we cannot draw any major epidemiological conclusions (see 2.3). Both the general trematode multiplex RD-PCR and schistosome multiplex RD-PCR, showed to be

promising tools for infection diagnostics. However, some remarks and constraints have to be emphasized. No epidemiological information other than the prevalence of *Fasciola* sp. and *Schistosoma* sp. can be derived from our diagnostic approach. For *Schistosoma* sp., some species discrimination via the second 'Schistosoma multiplex RD-PCR' could be obtained but no species resolution is provided for *Fasciola* sp. infections. Furthermore, does the chance of underestimating co-infections (in the general trematode multiplex RD-PCR), false negative results and PCR contamination still exist. Some future recommendations to refine this approach are mentioned in 2.4.3.

Regarding the difficulties in the species identification of both gastropods and trematodes we have provided future recommendations (see 1.1; Trematoda and 2.2; Gastropoda). Examples are optimized fixation protocols, re-designing primers and the use of quantitative morphometrics for gastropod identification. For trematodes classification, genotyping of additional markers such as ITS and 28S might broaden the phylogenetic resolution but the lack of reference sequences from the database can only be solved by increased sampling effort of intermediate and final hosts in the endemic regions.

References

Abbasi, I. *et al.* (2012) 'Differentiating *Schistosoma haematobium* from related animal schistosomes by PCR amplifying inter-repeat sequences flanking newly selected repeated sequences', *American Journal of Tropical Medicine and Hygiene*, 87(6), pp. 1059–1064. doi: 10.4269/ajtmh.2012.12-0243.

Adams, C. S. *et al.* (2002) 'The dynamics and ecology of exotic tropical species in floating plant mats: Lake Naivasha, Kenya', *Hydrobiologia*, 488, pp. 115–122. doi: 10.1023/A:1023322430005.

Adema, C. M. *et al.* (2012) 'Will all scientists working on snails and the diseases they transmit please stand up?', *PLoS Neglected Tropical Diseases*, 6(12), pp. 5–6. doi: 10.1371/journal.pntd.0001835.

Adenowo, A. F. *et al.* (2015) 'Impact of human schistosomiasis in sub-Saharan Africa', *Brazilian Journal of Infectious Diseases*. Elsevier Editora Ltda, 19(2), pp. 196–205. doi: 10.1016/j.bjid.2014.11.004.

Van Aken, D. and Brandt, J. (1987) 'Preliminary observations on the detection of the larval stages of *Fasciola hepatica* in *Lymnaea truncatula* by enzyme-electrophoresis', *Ann. Soc. belge Méd. trop.*, 67, pp. 295–298.

Akinwale, O. P. *et al.* (2014) 'Differentiating *Schistosoma haematobium* from *Schistosoma magrebowiei* and other closely related schistosomes by polymerase chain reaction amplification of a species specific mitochondrial gene.', *Tropical parasitology*, 4(1), pp. 38–42. doi: 10.4103/2229-5070.129163.

Alasaad, S. *et al.* (2011) 'A TaqMan real-time PCR-based assay for the identification of *Fasciola* spp.', *Veterinary Parasitology*, 179(1–3), pp. 266–271. doi: 10.1016/j.vetpar.2011.01.059.

Alexander, S. J. and McLaughlin, J. D. (1997) 'A checklist of helminths from the respiratory system and gastrointestinal tracts of African Anatidae', *Onderstepoort Journal of Veterinary Research*, 64(November 1996), pp. 5–16.

Altschul, S. F. *et al.* (1990) 'Basic local alignment search tool.', *Journal of molecular biology*, 215(3), pp. 403–10. doi: 10.1016/S0022-2836(05)80360-2.

Amarir, F. *et al.* (2014) '*Schistosoma haematobium* detection in snails by DraI PCR and Sh110/Sm-Sl PCR: Further evidence of the interruption of schistosomiasis transmission in Morocco', *Parasites and Vectors*, 7(1), pp. 1–8. doi: 10.1186/1756-3305-7-288.

Appleton, C. C. (2003) 'Alien and invasive freshwater gastropoda in South Africa', *African Journal of*

Aquatic Science, 28(1), pp. 69–81. doi: 10.2989/16085914.2003.9626602.
Appleton, C. C. and Madsen, H. (2012) 'Human schistosomiasis in wetlands in southern Africa', *Wetlands Ecology and Management*, 20(3), pp. 253–269. doi: 10.1007/s11273-012-9266-2.
Appleton, C. and Miranda, N. (2015) 'Locating bilharzia transmission sites in South Africa: guidelines for public health personnel', *Southern African Journal of Infectious Diseases*. Taylor & Francis, 30(3), pp. 95–102. doi: 10.1080/23120053.2015.1074438.
Ashrafi, K., Bargues, M. D. and Neill, S. O. (2014) 'Fasciolosis : A worldwide parasitic disease of importance in travel medicine', *Travel Medicine and Infectious Disease*. Elsevier Ltd, 12(6), pp. 636–649. doi: 10.1016/j.tmaid.2014.09.006.
Atopkin, D. M. (2011) 'Genetic characterization of the Psilotrema (Digenea: Psilostomatidae) genus by partial 28S ribosomal DNA sequences', *Parasitology International*. Elsevier Ireland Ltd, 60(4), pp. 541–543. doi: 10.1016/j.parint.2011.09.005.
Berg, H. *et al.* (1996) 'Managing aquaculture for sustainability in tropical Lake Kariba, Zimbabwe', *Ecological Economics*, 18(2), pp. 141–159. doi: 10.1016/0921-8009(96)00018-3.
Bindernagel, J. A. (1972) 'Liver fluke *fasciola gigantica* in african buffalo and antelopes in uganda, east africa', *Journal of Wildlife Diseases*, 8(4), pp. 315–317.
Bocxlaer, B. Van and Schultheiß, R. (2010) 'Comparison of morphometric techniques for shapes with few homologous landmarks based on machine-learning approaches to biological discrimination', *Paleobiology*, 36(03), pp. 497–515. doi: 10.1666/08068.1.
Borcard, D. *et al.* (2011) *Numerical Ecology with R*, *Springer*. Edited by R. Gentleman, K. Hornik, and G. G. Permigiani. Springer. doi: 10.1007/978-1-4419-7976-6.
Born-Torrijos, A. *et al.* (2014) 'Estimating trematode prevalence in snail hosts using a single-step duplex PCR: how badly does cercarial shedding underestimate infection rates?', *Parasites & Vectors*, 7(1), p. 243. doi: 10.1186/1756-3305-7-243.
Bouchet, P. *et al.* (2005) Classification and nomenclator of gastropod families, *Malacologia*. doi: 3-925919-72-4.
Brackenbury, T. D. and Appleton, C. c. (1991) 'Effect of controlled temperatures on gametogenesis in the gastropods *Physa acuta* (physidae) and *Bulinus tropicus* (planorbidae)', *Journal of Molluscan Studies*, 57(4), pp. 461–469. doi: 10.1093/mollus/57.4.461.
Brendonck, L. *et al.* (2003) 'The impact of water hyacinth (*Eichhornia crassipes*) in a eutrophic subtropical impoundment (Lake Chivero, Zimbabwe). II. Species diversity', *Archiv für Hydrobiologie*, 158(3), pp. 389–405. doi: 10.1127/0003-9136/2003/0158-0389.
Van den Broeck, F. *et al.* (2011) 'Optimal sample storage and extraction procotols for reliable multilocus genotyping of the human parasite *Schistosoma mansoni*', *Infection, Genetics and Evolution*. Elsevier B.V., 11(6), pp. 1413–1418. doi: 10.1016/j.meegid.2011.05.006.
Brown, D. S. (1994) Freshwater snails of Africa and their medical importance. 2nd edn, *Transactions of the Royal Society of Tropical Medicine and Hygiene*. 2nd edn. UK Taylor & Francis Ltd. doi: 10.1016/0035-9203(81)90097-3.
Brown, D. S. and van Eeden, J. A. (1969) 'The molluscan genus Gyraulus (Gastropoda: Planorbidae) in Southern Africa', pp. 305–331.
Van Bruggen, A. C. and Appleton, C. C. (1977) 'Studies on the ecology and systematics of the terrestrial molluscs of the lake Sibaya area of Zululand, South Africa.', *Zoologische verhandelingen*, 154.
Cañete, R. *et al.* (2004) 'Population dynamics of intermediate snail hosts of *Fasciola hepatica* and some environmental factors in San Juan y Martinez Municipality, Cuba', *Memorias do Instituto Oswaldo Cruz*, 99(3), pp. 257–262. doi: /S0074-02762004000300003.
Caron, Y. *et al.* (2011) 'An optimized DNA extraction and multiplex PCR for the detection of *Fasciola* sp. in lymnaeid snails', *Veterinary Parasitology*. Elsevier B.V., 178(1–2), pp. 93–99. doi: 10.1016/j.vetpar.2010.12.020.
Caron, Y., Rondelaud, D. and Losson, B. (2008) 'The detection and quantification of a digenean infection in the snail host with special emphasis on *Fasciola* sp.', *Parasitology Research*, 103(4), pp. 735–744. doi: 10.1007/s00436-008-1086-1.
Casey, S. P. *et al.* (2003) 'Use of ITS rDNA for discrimination of European green- and brown-banded sporocysts within the genus *Leucochloridium* Carus, 1835 (Digenea: Leucochloriidae)', *Systematic Parasitology*, 56(3), pp. 163–168. doi: 10.1023/B:SYPA.0000003809.15982.ca.
Chai, J. Y. *et al.* (2009) 'Foodborne intestinal flukes in Southeast Asia', *Korean Journal of Parasitology*, 47(SUPPL.), pp. 69–102. doi: 10.3347/kjp.2009.47.S.S69.
Chimbari, M. J. *et al.* (2003) 'Transmission of schistosomiasis in Kariba, Zimbabwe, and a cross-sectional comparison of schistosomiasis prevalences and intensities in the town with those in

Siavonga in Zambia', *Annals of Tropical Medicine & Parasitology*, 97(6), pp. 605–616. doi: 10.1179/000349803225001508.
Chimbari, M. J. (2012) 'Enhancing schistosomiasis control strategy for Zimbabwe: Building on past experiences', *Journal of Parasitology Research*, 2012. doi: 10.1155/2012/353768.
Chimbari, M. J. and Chirundu, D. (2003) 'Prevalence and intensity of the schistosomiasis situation along the Zimbabwean urban and peri-urban shoreline of lake Kariba.', *The Central African Journal Of Medicine*, 49(January), pp. 8–12.
Chingwena, G. *et al.* (2002) 'Larval trematode infections in freshwater snails from the highveld and lowveld areas of Zimbabwe', *Journal of Helminthology*. KU Leuven Libraries, 76(4), pp. 283–293. doi: 10.1079/JOH2002132.
Chingwena, G. *et al.* (2004) 'Population dynamics and ecology of freshwater gastropods in the highveld and lowveld regions of Zimbabwe, with emphasis on schistosome and amphistome intermediate hosts', *African Zoology*, 39(1), pp. 55–62. doi: 10.1080/15627020.2004.11407286.
De Clercq, D. (1987) 'La situation malacologique à Kinshasa et description d'un foyer autochtone de schistosomiase à *Schistosoma intercalatum*', *Annales de la Société Belge de Médecine Tropicale*, pp. 345–352.
Correa, A. C. *et al.* (2010) 'Bridging gaps in the molecular phylogeny of the Lymnaeidae (Gastropoda: Pulmonata), vectors of Fasciolosis', *BMC Evolutionary Biology*. BioMed Central Ltd, 10(1), p. 381. doi: 10.1186/1471-2148-10-381.
Cucher, M. A. *et al.* (2006) 'PCR diagnosis of *Fasciola hepatica* in field-collected *Lymnaea columella* and Lymnaea viatrix snails', *Veterinary Parasitology*, 137(1–2), pp. 74–82. doi: 10.1016/j.vetpar.2005.12.013.
Davies, C. M. *et al.* (1999) 'Host-parasite population genetics: A cross-sectional comparison of *Bulinus globosus* and *Schistosoma haematobium*', *Parasitology*, 119(3), pp. 295–302. doi: 10.1017/S0031182099004722.
DiNardo, A. (2015) *Helminth Infections and Their Impact on Global Public Health*, *Clinical Infectious Diseases*. doi: 10.1093/cid/ciu891.
Dinnik, J. A. (1965) 'The Snail Hosts of Certain Paramphistomatidae and Gastrothylacidae (Trematoda) Discovered by the late Dr. P. L. LeRoux in Africa', *Journal of Helminthology*, 39(2), pp. 141–150.
Dinnik, J. A. and Dinnik, N. N. (1961) 'On the Morphology and Life History of *Fasciola nyanzae* Leiper, 1910 from the Hippopotamus', *Journal of Helminthology*, 35(S1), pp. 53–62. doi: 10.1017/S0022149X00017570.
Dreyfuss, G. and and Rondelaud, D. (1994) '*Fasciola hepatica*: A study of the shedding of cercariae from *Lymnaea truncatula* raised under constant conditions of temperature and photoperiod.', *Parasite*, 75(11), pp. 401–404.
Edgar, R. C. (2004) 'MUSCLE: Multiple sequence alignment with high accuracy and high throughput', *Nucleic Acids Research*, 32(5), pp. 1792–1797. doi: 10.1093/nar/gkh340.
van Eeden, J. A. and Brown, D. S. (1966) 'Colonization of Fresh waters in the Rupublic of South Africa by *Lymnaea columnella* Say (Mollusca: Gastropoda)', *Nature*, 209, pp. 694–696. Available at: https://www.nature.com/articles/2101172a0.
Esch, G. W., Curtis, L. A. and Barger, M. A. (2001) 'A perspective on the ecology of trematode communities in snails', *Parasitology*, 123(07). doi: 10.1017/S0031182001007697.
Esteban, J. G. and Bargues, M. D. (1999) 'Epidemiology of human fasciolosis: a review and proposed new classification', 77(4), pp. 340–346.
Fox, J. and Weisberg, S. (2011) *No An {R} Companion to Applied Regression, Second Edition. Thousand Oaks CA : Sage*, *Sage*. Available at: http://socserv.socsci.mcmaster.ca/jfox/Books/Companion.
Frandsen, F. and Christensen, N. O. (1984) 'An introductory guide to the identification of cercariae from African freshwater snails with special reference to cercariae of trematode species of medical and veterinary importance.', *Acta tropica*, 41, pp. 181–202. doi: 10.1016/S0020-7519(03)00049-3.
Fried, B. and Toledo, R. (2009) The biology of Echinostomes - from the molecule to the community. Springer. doi: 10.1007/978-0-387-09577-6.
Gakkai, N. K. and Akao, Y. (1973) 'Description of *Psilorchis hominis* sp. nov. from man (Trematoda: Echinostomatoidea: Psilostomidae)', *Japanese Journal of Parasitology*. Elsevier, 22(3), pp. 111–15. Available at: https://www.cabdirect.org/cabdirect/abstract/19742900546 (Accessed: 12 May 2018).

Galindo, L. A. *et al.* (2014) 'Using microwaves to prepare gastropods for DNA barcoding', *Molecular Ecology Resources*, 14(4), pp. 700–705. doi: 10.1111/1755-0998.12231.
Gandasegui, J. *et al.* (2016) 'Biompha-LAMP: a new rapid loop-mediated isothermal amplification assay for detecting *Schistosoma mansoni* in *Biomphalaria glabrata* snail host', *PLoS Neglected Tropical Diseases*, 10(12). doi: 10.1371/journal.pntd.0005225.
Gebremedhn, H. G. and Tsegay, A. K. (2017) 'Review on distribution of endo-parasites of fish in Ethiopia', *Parasite Epidemiology and Control*. Elsevier, 2(4), pp. 42–47. doi: 10.1016/j.parepi.2017.10.002.
Giannelli, A. *et al.* (2016) 'Gastropod-Borne Helminths: a look at the snail-parasite interplay', *Trends in Parasitology*. Elsevier Ltd, 32(3), pp. 255–264. doi: 10.1016/j.pt.2015.12.002.
Gilbertson, C. R. and Wyatt, J. D. (2016) 'Evaluation of euthanasia techniques for an invertebrate species, land snails (*Succinea putris*).', *Journal of the American Association for Laboratory Animal Science : JAALAS*, 55(5), pp. 577–81.
Giovanelli, A. *et al.* (2005) 'Habitat preference of freshwater snails in relation to environmental factors and the presence of the competitor snail *Melanoides tuberculatus* (Müller, 1774)', *Memorias do Instituto Oswaldo Cruz*, 100(2), pp. 169–176. doi: 10.1590/S0074-02762005000200010.
Gonchar, A. and Galaktionov, K. V. (2017) 'Life cycle and biology of *Tristriata anatis* (Digenea: Notocotylidae): morphological and molecular approaches', *Parasitology Research*. Parasitology Research, 116(1), pp. 45–59. doi: 10.1007/s00436-016-5260-6.
Gower, C. M., Vince, L. and Webster, J. P. (2017) 'Should we be treating animal schistosomiasis in Africa? The need for a One Health economic evaluation of schistosomiasis control in people and their livestock', *Transactions of The Royal Society of Tropical Medicine and Hygiene*, 111(6), pp. 244–247. doi: 10.1093/trstmh/trx047.
Grabner, D. S. *et al.* (2014) 'Invasion biology meets parasitology: A case study of parasite spill-back with egyptian *Fasciola gigantica* in the invasive snail *Pseudosuccinea columella'*, *PLoS ONE*, 9(2), pp. 1–7. doi: 10.1371/journal.pone.0088537.
Grobbelaar, A. *et al.* (2014) 'Ecology of diplostomid (Trematoda: Digenea) infection in freshwater fish in southern Africa', *African Zoology*, 49(2), pp. 222–232. doi: 10.1080/15627020.2014.11407638.
Gryseels, B. *et al.* (2006) 'Human schistosomiasis', *Lancet*, 368(9541), pp. 1106–1118. doi: 10.1016/S0140-6736(06)69440-3.
Hamburger, J. *et al.* (2013) 'Evaluation of loop-mediated isothermal amplification suitable for molecular monitoring of schistosome-infected snails in field laboratories', *American Journal of Tropical Medicine and Hygiene*, 88(2), pp. 344–351. doi: 10.4269/ajtmh.2012.12-0208.
Harhay, M. O., Horton, J. and Olliaro, P. L. (2010) 'Epidemiology and control of human gastrointestinal parasites in children', *Expert Review of Anti-Infective Therapy*, 8 (2), pp. 219–234. doi: http://dx.doi.org/10.1586/14737140.8.2.219.
Hashizume, H. *et al.* (2017) 'Application of environmental DNA analysis for the detection of *Opisthorchis viverrini* DNA in water samples', *Acta Tropica*, 169, pp. 1–7. doi: 10.1016/j.actatropica.2017.01.008.
Hebert, P. D. N. *et al.* (2003) 'Biological identifications through DNA barcodes.', *Proceedings. Biological sciences / The Royal Society*, 270(1512), pp. 313–321. doi: 10.1098/rspb.2002.2218.
Hebert, P. D. N. and Beaton, M. J. (1993) 'Methodologies for allozyme analysis using cellulose acetate electrophoresis', *Zoology*, (January 1989), p. 32.
Hira, P. R. (1969) 'Transmission of schistosomiasis in lake Kariba, Zambia', *Nature*, 224(5220), pp. 177–178. doi: 10.1038/224488a0.
ten Hove, R. J. *et al.* (2008) 'Multiplex real-time PCR for the detection and quantification of *Schistosoma mansoni* and *S. haematobium* infection in stool samples collected in northern Senegal', *Transactions of the Royal Society of Tropical Medicine and Hygiene*, 102(2), pp. 179–185. doi: 10.1016/j.trstmh.2007.10.011.
Huyse, T. *et al.* (2009) 'Bidirectional introgressive hybridization between a cattle and human schistosome species.', *PLoS pathogens*. Public Library of Science, 5(9), p. e1000571. doi: 10.1371/journal.ppat.1000571.
Johansen, M. V., Lier, T. and Sithithaworn, P. (2015) 'Towards improved diagnosis of neglected zoonotic trematodes using a One Health approach', *Acta Tropica*, 141(Part B), pp. 161–169. doi: 10.1016/j.actatropica.2013.07.006.
Johnson, P. T. J. *et al.* (2004) 'Review of the trematode genus Ribeiroia (Psilostomidae): Ecology, life history and pathogenesis with special emphasis on the amphibian malformation problem',

Advances in Parasitology, 57(04), pp. 191–253. doi: 10.1016/S0065-308X(04)57003-3.
Jones, C. S. *et al.* (1999) 'Molecular identification of schistosome intermediate hosts: case studies of *Bulinus forskalii* group species (Gastropoda: Planorbidae) from Central and East Africa', *Biological Journal of the Linnean Society*, 68(1–2), pp. 215–240. doi: 10.1006/bijl.1999.0339.
Jørgensen, A. *et al.* (2007) 'Molecular phylogenetic investigations of Bulinus (Gastropoda: Planorbidae) in Lake Malawi with comments on the topological incongruence between DNA loci', *Zoologica Scripta*, 36(6), pp. 577–585. doi: 10.1111/j.1463-6409.2007.00298.x.
Jothikumar, N. *et al.* (2015) 'Real-time PCR and sequencing assays for rapid detection and identification of avian schistosomes in environmental samples', *Applied and Environmental Microbiology*, 81(12), pp. 4207–4215. doi: 10.1128/AEM.00750-15.
Junker, K., Horak, I. G. and Penzhorn, B. (2015) 'History and development of research on wildlife parasites in southern Africa, with emphasis on terrestrial mammals, especially ungulates', *International Journal for Parasitology: Parasites and Wildlife*. The Authors, 4(1), pp. 50–70. doi: 10.1016/j.ijppaw.2014.12.003.
Kane, R. A. *et al.* (2008) 'Molecular characterization of freshwater snails in the genus Bulinus: A role for barcodes?', *Parasites and Vectors*, 1(1), pp. 1–15. doi: 10.1186/1756-3305-1-15.
Kane, R. A. *et al.* (2013) 'Detection and quantification of schistosome DNA in freshwater snails using either fluorescent probes in real-time PCR or oligochromatographic dipstick assays targeting the ribosomal intergenic spacer', *Acta Tropica*. Elsevier B.V., 128(2), pp. 241–249. doi: 10.1016/j.actatropica.2011.10.019.
Kaplan, R. M. *et al.* (1995) 'A repetitive DNA probe for the sensitive detection of *Fasciola hepatica* infected snails', *International Journal for Parasitology*, 25(5), pp. 601–610. doi: 10.1016/0020-7519(94)00159-L.
Kassambara, A. and Mundt, F. (2017) 'Factoextra: extract and visualize the results of multivariate data analyses', *R package version*, 1(3), pp. 1–76. Available at: http://www.sthda.com/english/rpkgs/factoextra%0ABugReports.
Keiser, J., Duthaler, U. and Utzinger, J. (2010) 'Update on the diagnosis and treatment of food-borne trematode infections.', *Current opinion in infectious diseases*, 23(5), pp. 513–20. doi: 10.1097/QCO.0b013e32833de06a.
De Kock, K. N., Joubert, P. H. and Pretorius, S. J. (1989) 'Geographical distribution and habitat preferences of the invader freshwater snail species *Lymnaea columella* (Mollusca: Gastropoda) in South Africa.', *The Onderstepoort journal of veterinary research*, 56(4), pp. 271–5. Available at: http://www.ncbi.nlm.nih.gov/pubmed/2626264 (Accessed: 25 May 2018).
Krämer, F. and Schnieder, T. (1998) 'Sequence heterogeneity in a repetitive DNA element of Fasciola', *International Journal for Parasitology*, 28(12), pp. 1923–1929. doi: 10.1016/S0020-7519(98)00162-3.
Kudlai, O. *et al.* (2017) 'The Psilostomidae Looss, 1900 (sensu stricto) (Digenea: Echinostomatoidea): description of three new genera and a key to the genera of the family', *Systematic Parasitology*. Springer Netherlands, 94(1), pp. 21–33. doi: 10.1007/s11230-016-9681-5.
Kumar, S. *et al.* (2015) 'MEGA7: Molecular Evolutionary Genetics Analysis version 7.0.', *Molecular Biology and Evolution*, submitted(12), pp. 2725–2729. doi: 10.1093/molbev/mst197.
Laidemitt, M. R. *et al.* (2017) 'Loads of trematodes: Discovering hidden diversity of paramphistomoids in Kenyan ruminants', *Parasitology*, 144(2), pp. 131–147. doi: 10.1017/S0031182016001827.
Lake Harvest TM (no date) *Lake Harvest - fish farms - Kariba, Zimbabwe*. Available at: http://www.lakeharvest.com/fish-farms/ (Accessed: 13 May 2018).
Lawton, S. P. *et al.* (2015) 'Unravelling the riddle of Radix: DNA barcoding for species identification of freshwater snail intermediate hosts of zoonotic digeneans and estimating their inter-population evolutionary relationships', *Infection, Genetics and Evolution*. Elsevier B.V., 35, pp. 63–74. doi: 10.1016/j.meegid.2015.07.021.
Legendre, P. and De Cáceres, M. (2013) 'Beta diversity as the variance of community data: Dissimilarity coefficients and partitioning', *Ecology Letters*, 16(8), pp. 951–963. doi: 10.1111/ele.12141.
Legendre, P. and Gallagher, E. D. (2001) 'Ecologically meaningful transformations for ordination of species data', *Oecologia*, 129(2), pp. 271–280. doi: 10.1007/s004420100716.
Lodge, D. M. *et al.* (1987) 'Distribution of freshwater snails : spatial scale and the relative importance of physicochemical and biotic factors', *American Malacological Bulletin*, 5(1), pp. 73–84.
Loker, E. S., Moyo, H. G. and Gardner, S. L. (1981) 'Trematode–gastropod associations in nine non-lacustrine habitats in the Mwanza region of Tanzania', *Parasitology*, 83(02), pp. 381–399. doi: 10.1017/S0031182000085383.

Lotfy, W. M. *et al.* (2008) 'Evolutionary origins, diversification, and biogeography of liver flukes (Digenea, Fasciolidae)', *American Journal of Tropical Medicine and Hygiene*, 79(2), pp. 248–255. doi: 79/2/248

Ma, J. *et al.* (2016) 'Mitochondrial genome of *Ogmocotyle sikae* and implications for phylogenetic studies of the Notocotylidae trematodes', *Infection, Genetics and Evolution*. Elsevier B.V., 37, pp. 208–214. doi: 10.1016/j.meegid.2015.11.018.

Mabika, N. *et al.* (2016) 'Taxonomy, distribution and prevalence of parasites of tigerfish, *Hydrocynus vittatus* (Castelnau, 1861) in the Sanyati basin, Lake Kariba, Zimbabwe', *Acta Parasitologica*, 61(3), pp. 614–620. doi: 10.1515/ap-2016-0082.

Madamombe, L. (2002) The economic development of the kapenta fishery in lake Kariba. Norwegian college of fishery science, tromso. Available at: https://munin.uit.no/bitstream/handle/10037/336/thesis.pdf?sequence=1&isAllowed=y

Madsen, H. and Frandsen, F. (1989) 'The spread of freshwater snails including those of medical and veterinary importance', *Acta Tropica*, 46(3), pp. 139–146. doi: 10.1016/0001-706X(89)90030-2.

Magalhães, K. G., Jannotti Passos, L. K. and Dos Santos Carvalho, O. (2004) 'Detection of *Lymnaea columella* infection by *Fasciola hepatica* through multiplex-PCR', *Memorias do Instituto Oswaldo Cruz*, 99(4), pp. 421–424. doi: /S0074-02762004000400013.

Mandahl-Barth, G. (1962) 'Key to the identification of east and central African freshwater snails of medical and veterinary importance.', *Bulletin of the World Health Organization*, 27, pp. 135–150.

Manga-Gonzalez, Y., Gonzalez-Lanza, C. and Kanev, I. (1994) *'Lymnaea Truncatula*, Intermediate Host of some Plagiorchiidae and Notocotylidae Species in Leon, NW Spain', *Journal of Helminthology*, 68(2), pp. 135–141. doi: 10.1017/S0022149X00013663.

Manning, S. D., Woolhouse, M. E. J. and Ndamba, J. (1995) 'Geographic compatibility of the freshwater snail *Bulinus globosus* and schistosomes from the Zimbabwe highveld', *International Journal for Parasitology*, 25(1), pp. 37–42. doi: 10.1016/0020-7519(94)00097-8.

McCauley, E. H., Tayeb, A. and Majid, A. A. (1983) 'Owner survey of schitosomiasis mortality in sudanese cattle', *Tropical Animal Health and Production*, 15(4), pp. 227–233. doi: 10.1007/BF02242065.

Mehmood, K. *et al.* (2017) 'A review on epidemiology, global prevalence and economical losses of fasciolosis in ruminants', *Microbial Pathogenesis*, 109, pp. 253–262. doi: 10.1016/j.micpath.2017.06.006.

Melo, F. L. *et al.* (2006) 'Development of molecular approaches for the identification of transmission sites of schistosomiasis', *Transactions of the Royal Society of Tropical Medicine and Hygiene*, 100(11), pp. 1049–1055. doi: 10.1016/j.trstmh.2005.12.008.

Minetti, C. *et al.* (2016) 'Focusing nucleic acid-based molecular diagnostics and xenomonitoring approaches for human helminthiases amenable to preventive chemotherapy', *Parasitology Open*, 2(November), p. e16. doi: 10.1017/pao.2016.13.

Morgan, J. A. T. *et al.* (2002) 'A phylogeny of planorbid snails, with implications for the evolution of Schistosoma parasites', *Molecular Phylogenetics and Evolution*, 25(3), pp. 477–488. doi: 10.1016/S1055-7903(02)00280-4.

Mostafa, O. M. S., Taha, H. A. and Ramadan, G. (2003) 'Diagnosis of *Fasciola gigantica* in snail isung the polymerase chain reaction (PCR) assay', *J. Egypt Soc. Parasitol.*, 33(3), pp. 733–742.

Moszczynska, A. *et al.* (2009) 'Development of primers for the mitochondrial cytochrome c oxidase i gene in digenetic trematodes (Platyhelminthes) illustrates he challenge of barcoding parasitic helminths', *Molecular Ecology Resources*, 9(SUPPL. 1), pp. 75–82. doi: 10.1111/j.1755-0998.2009.02634.x.

Moyo, N. A. G. (2004) 'The feeding habits of Sargochromis Codringtonii in Lake Kariba, Zimbabwe', *Hydrobiologia*, 522(1–3), pp. 321–327. doi: 10.1023/B:HYDR.0000029966.30531.15.

Mubila, L. and Rollinson, D. (2002) 'Snail-parasite compatibility and prevalence of *Schistosoma haematobium* on the shores of Lake Kariba, Zambia', *Annals of Tropical Medicine & Parasitology*, 96(2), pp. 165–173. doi: 10.1179/000349802125000592.

Mukaratirwa, S. *et al.* (1996) 'Genetic structure and parasite compatibility of *Bulinus globosus* (Gastropoda: Planorbidae) from two areas of different endemicity of *Schistosoma haematobium* in Zimbabwe', *International Journal for Parasitology*, 26(3), pp. 269–280. doi: 10.1016/0020-7519(95)00130-1.

Mukaratirwa, S. *et al.* (1996) 'Population genetics and genetic variability of *Bulinus globosus* (Gastropoda: Planorbidae) from the two main river systems in Zimbabwe', *Journal of Heredity*,

87(4), pp. 288–294.
Mukaratirwa, S. *et al.* (1998) 'Genetic and morphological variation of populations belonging to the *Bulinus truncatus/tropicus* complex (gastropoda : Planorbidae) in south western Zimbabwe', pp. 435–446.
Mungomba, L. M., Chandiwana, S. K. and Madesen, H. (1993) 'Schistosomiasis around Siavonga, on the shores of Lake Kariba, Zambia', *Annals of Tropical Medicine and Parasitology*, 87(4), pp. 365–371. doi: 10.1080/00034983.1993.11812780.
Ndifon, G. T. and Ukoli, F. M. A. (1989) 'Ecology of freshwater snails in south-western Nigeria. I: Distribution and habitat preferences', *Hydrobiologia*, 171(3), pp. 231–253. doi: 10.1007/BF00008146.
Nei, M. and Kumar, S. (2000) *Molecular Evolutionand Phylogenetics*, *Archives of virology*. doi: 10.1046/j.1365-2540.2001.0923a.x.
Nolan, M. J. and Cribb, T. H. (2005) 'The use and implications of ribosomal DNA sequencing for the discrimination of digenean species', *Advances in Parasitology*, 60, pp. 101–163. doi: 10.1016/S0065-308X(05)60002-4.
von Oheimb, P. V. *et al.* (2011) 'Freshwater biogeography and limnological evolution of the tibetan plateau - insights from a plateau-wide distributed gastropod taxon (radix spp.)', *PLoS ONE*, 6(10). doi: 10.1371/journal.pone.0026307.
Oksanen, J. *et al.* (2016) 'Vegan: community ecology package', *R package 2.3-3*, p. Available at: https://cran.r-project.org/web/packa. doi: 10.4135/9781412971874.n145.
Olson, P. D. *et al.* (2003) 'Phylogeny and classification of the Digenea (Platyhelminthes: Trematoda)', *International Journal for Parasitology*, 33(7), pp. 733–755. doi: 10.1016/S0020-7519(03)00049-3.
Otachi, E. O. *et al.* (2014) 'Parasites of commercially important fish from Lake Naivasha, Rift Valley, Kenya', *Parasitology Research*, 113(3), pp. 1057–1067. doi: 10.1007/s00436-013-3741-4.
Pande, B. P. and Bhatia, B. B. (1960) 'On *Ogmocotyle indica* (Bhalerao, 1942) Ruiz, 1946 (Trematoda), the notocotylid monostome of Indian ovines, and its pathogenicity', *The journal of Parasitology*, 46(6), pp. 800–802. Available at: http://www.jstor.orhg/stable/3275537.
Perissinotto, R. *et al.* (2014) 'Biodiversity census of lake St Lucia, iSimangaliso Wetland Park (South Africa): Gastropod molluscs', *ZooKeys*, 43(440), pp. 1–43. doi: 10.3897/zookeys.440.7803.
Pfenninger, M., Cordellier, M. and Streit, B. (2006) 'Comparing the efficacy of morphologic and DNA-based taxonomy in the freshwater gastropod genus Radix (Basommatophora, Pulmonata)', *BMC Evolutionary Biology*, 6, pp. 1–14. doi: 10.1186/1471-2148-6-100.
Pfukenyi, D M;Mukaratirwa, S;Willingham, A L;Monrad, J. (2005) 'Epidemiological studies of amphistome infections in cattle in the highveld and lowveld communal grazing areas of Zimbabwe', *The Onderstepoort journal of veterinary research*, 72, pp. 67–86.
Pfukenyi, D. *et al.* (2006) 'Epidemiological studies of *Schistosoma mattheei* infections in cattle in the highveld and lowveld communal grazing areas of Zimbabwe.', *Onderstepoort J Vet Res*, 73(2), pp. 179–191. doi: 10.4102/ojvr.v74i2.132.
Pfukenyi, D. *et al.* (2007) 'Epidemiological studies of *Fasciola gigantica* infections in cattle in the highveld and lowveld communal grazing areas of Zimbabwe', *Onderstepoort J Vet Res*, 74(2), pp. 129–142. doi: 10.4102/ojvr.v74i2.132.
Pfukenyi, D. M., Monrad, J. and Mukaratirwa, S. (2005) 'Epidemiology and control of trematode infections in cattle in Zimbabwe: a review.', *Journal of the South African Veterinary Association*, 76(1), pp. 9–17. doi: 10.4102/jsava.v76i1.387.
Phiri, A. M. *et al.* (2007) 'Trematode infections in freshwater snails and cattle from the Kafue wetlands of Zambia during a period of highest cattle–water contact', *Journal of Helminthology*, 81(01), pp. 85–92. doi: 10.1017/S0022149X07387786.
Phiri, A. M., Phiri, I. K. and Monrad, J. (2006) 'Prevalence of amphistomiasis and its association with *Fasciola gigantica*; infections in Zambian cattle from communal grazing areas', *Journal of Helminthology*. KU Leuven Libraries, 80(1), pp. 65–68. doi: 10.1079/JOH2005313.
Plummer, M. L. (2005) 'Impact of invasive water hyacinth (*Eichhornia crassipes*) on snail hosts of schistosomiasis in Lake Victoria, East Africa', *EcoHealth*, 2(1), pp. 81–86. doi: 10.1007/s10393-004-0104-8.
Pretorius, S. J., Joubert, P. H. and Kock, K. N. De (1989) 'A review of the schistosomiasis risk in South African dams', *Water SA*, 15(2), p. 4738.
R Development Core Team, R. (2011) *R: A Language and Environment for Statistical Computing*, *R Foundation for Statistical Computing*. doi: 10.1007/978-3-540-74686-7.
R Development Core Team, R. F. F. S. C. (2008) 'R: A Language and Environment for Statistical

Computing', *Vienna Austria R Foundation for Statistical Computing*, 1(10), p. ISBN 3-900051-07-0. doi: 10.1007/978-3-540-74686-7.
Remigio, E. (2002) 'Molecular phylogenetic relationships in the aquatic snail genus Lymnaea, the intermediate host of the causative agent of fasciolosis: Insights from broader taxon sampling', *Parasitology Research*, 88(7), pp. 687–696. doi: 10.1007/s00436-002-0658-8.
Rosser, A. *et al.* (2015) 'Isothermal recombinase polymerase amplification (RPA) of *Schistosoma haematobium* DNA and oligochromatographic lateral flow detection', *Parasites and Vectors*. Parasites & Vectors, 8(1), pp. 1–5. doi: 10.1186/s13071-015-1055-3.
Routtu, J. *et al.* (2014) 'Selective and universal primers for trematode barcoding in freshwater snails', *Parasitology Research*, 113(7), pp. 2535–2540. doi: 10.1007/s00436-014-3903-z.
Salawu, O. T. and Odaibo, A. B. (2013) 'Preliminary study on ecology of *Bulinus jousseaumei* in *Schistosoma haematobium* endemic rural community of Nigeria', *African Journal of Ecology*, 51(3), pp. 441–446. doi: 10.1111/aje.12054.
Sanabria, R. and Romero, J. (2008) 'Review and update of paramphistomosis', *Helminthologia*, 45(2), pp. 64–68. doi: 10.2478/s11687-008-0012-5.
Scudder and Thayer (2005) *The Kariba Case Study*, *Social science working paper*. Pasadena, California. doi: June 2005.
Sey, O. and Graber, M. (1979) 'Examination of amphistomes (Trematoda: Paramphistomidae) of some African mammals.', *The Revue d'élevage et médecine vétérinaire des pays tropicaux*, 32(2), pp. 161–167.
Slootweg, R., Vroeg, P. A. and Wiersma, S. J. . (1993) 'Effects of molluscivorous fish, water quality and pond management on the development of schistosomiasis vector snails in aquaculture ponds', *Aquaculture Research*, 24(1), pp. 123–128. doi: 10.1111/j.1365-2109.1993.tb00835.x.
Van Someren, V. D. (1946) 'The habitats and tolerance ranges of *Lymnaea (Radix) caillaudi* , the intermediate snail host of liver fluke in East Africa', *Journal of Animal Ecology*, 15(2), pp. 170–197. Available at: url: http://www.jstor.org/stable/1557.
Sorenson, R. E. and Minchella, D. J. (2001) 'Snail-trematode life history interactions : past trends and future directions', *Parasitology*, 123(2001), pp. S3–S18. doi: 10.1017/S0031182001007843.
Van Steenkiste, N. *et al.* (2015) 'New primers for DNA barcoding of digeneans and cestodes (Platyhelminthes)', *Molecular Ecology Resources*, 15(4), pp. 945–952. doi: 10.1111/1755-0998.12358.
Stothard, J. R. *et al.* (1997) 'Observations on shell morphology, enzymes and random amplified polymorphic DNA (RAPD) in *Bulinus Africanus* group snails (Gastropoda: Planorbidae) in Zanzibar ', *Journal of Molluscan Studies* , 63(4), pp. 489–503. doi: 10.1093/mollus/63.4.489.
Strong, E. E. *et al.* (2008) 'Global diversity of gastropods (Gastropoda; Mollusca) in freshwater', *Hydrobiologia*, 595(1), pp. 149–166. doi: 10.1007/s10750-007-9012-6.
Tkach, V. V., Kudlai, O. and Kostadinova, A. (2016) 'Molecular phylogeny and systematics of the Echinostomatoidea Looss, 1899 (Platyhelminthes: Digenea)', *International Journal for Parasitology*. Australian Society for Parasitology Inc., 46(3), pp. 171–185. doi: 10.1016/j.ijpara.2015.11.001.
Toledo, R., Esteban, J. G. and Fried, B. (2012) 'Current status of food-borne trematode infections', *European Journal of Clinical Microbiology and Infectious Diseases*, 31(8), pp. 1705–1718. doi: 10.1007/s10096-011-1515-4.
Toledo, R. and Fried, B. (2014) *Digenetic Trematodes*, *Advances in Experimental Medicine and Biology*. Springer. doi: 10.1007/978-1-4939-0915-5.
Utzinger, J. *et al.* (2015) 'New diagnostic tools in schistosomiasis', *Clinical microbiology and infection*, In press(6), p. doi: 10.1016/j.cmi.2015.03.014. doi: 10.1016/j.cmi.2015.03.014.
Velusamy, R., Singh, B. P. and Raina, O. K. (2004) 'Detection of *Fasciola gigantica* infection in snails by polymerase chain reaction', *Veterinary Parasitology*, 120(1–2), pp. 85–90. doi: 10.1016/j.vetpar.2003.11.009.
Venables, W. N. and Ripley, B. D. (2002) 'MASS: modern applied statistics with S', *Issues of Accuracy and Scale*, (March), p. 868. doi: 10.1198/tech.2003.s33.
Vilas, R., Criscione, C. D. and Blouin, M. S. (2005) 'A comparison between mitochondrial DNA and the ribosomal internal transcribed regions in prospecting for cryptic species of platyhelminth parasites', *Parasitology*, 131(6), pp. 839–846. doi: 10.1017/S0031182005008437.
Watson, J. M. (1958) 'Ecology and distribution of *Bulinus truncatus* in the Middle East; with comments on the effect of some human activities in their relationship to the snail host on the incidence of bilharziasis haematobia in the Middle East and Africa.', *Bulletin of the World Health Organization*, 18(5–6), pp. 833–894.

Webster, B. L. *et al.* (2010) 'Rapid diagnostic multiplex PCR (RD-PCR) to discriminate *Schistosoma haematobium* and *S. bovis*', *Journal of Helminthology*, 84(01), p. 107. doi: 10.1017/S0022149X09990447.
Webster, M. H. (1960) 'The medical aspects of the Kariba hydro-electric scheme', *Central African Journal of Medicine*, 10, pp. 3–36. Available at: https://sci-hub.tw/https://www.ncbi.nlm.nih.gov/m/pubmed/13783665/.
Wei, T. and Simko, V. (2017) 'R package corrplot: Visualization of a Correlation Matrix (Version 0.84)'. Available at: https://github.com/taiyun/corrplot (Accessed: 17 May 2018).
Wenseleers, T. (2016) *Export: convert R graphs and statistical output to Microsoft Office / LibreOffice, HTML and Latex*. Available at: https://github.com/tomwenseleers/export.
Wetzel, R. (2001) 'Limnology, 3 E. Lake and River Ecosystems', *Academic Press, 525 B Street, Ste. 1900, San Diego, CA 92101, USA. 850*, p. 850.
WHO (2017a) *Schistosomiasis fact sheet (update of october 2017)*, *WHO Media Centre*.
WHO (2017b) *WHO | Disability weights, discounting and age weighting of DALYs*, *WHO*. World Health Organization.
Wilkinson, L. (2011) 'ggplot2: elegant graphics for data analysis by WICKHAM, H.', *Biometrics*, 67(2), pp. 678–679. doi: 10.1111/j.1541-0420.2011.01616.x.
Wilson, W. D. *et al.* (2005) 'A molecular phylogenetic study of the genus Ribeiroia (Digenea): trematodes known to cause limb malformations in amphibians.', *The Journal of parasitology*, 91(5), pp. 1040–1045. doi: 10.1645/GE-465R.1.
Woolhouse, M. E. J. and Chandiwana, S. K. (1989) 'Spatial and temporal heterogeneity in the population dynamics of *Bulinus Globosus* and *Biomphalaria pfeifferi* and in the epidemiology of their infection with schistosomes', *Parasitology*, 98(1), pp. 21–34. doi: 10.1017/S0031182000059655.
Woolhouse, M. E. J. and Chandiwana, S. K. (1990) 'Population dynamics model for *Bulinus globosus*, intermediate host for *Schistosoma haematobium*, in river habitats', *Acta Tropica*, 47(3), pp. 151–160. doi: 10.1016/0001-706X(90)90021-Q.
Worrell, C. *et al.* (2011) 'Field detection of *Schistosoma japonicum* cercariae in environmental water samples by quantitative PCR', *Applied and Environmental Microbiology*, 77(6), pp. 2192–2195. doi: 10.1128/AEM.01561-10.
Wright, C. A. and Rollinson, D. (1979) 'Analysis of enzymes in the *Bulinus africanus* group (Mollusca: Planorbidae) by isoelectric focusing', *Journal of Natural History*, 13(3), pp. 263–273. doi: 10.1080/00222937900770201.
Wright, C. A., Southgate, V. R. and Howard, G. W. (1979) 'A note on the life-cycles of some amphistome flukes in Zambia', *Journal of Helminthology*. Cambridge University Press, 53(03), p. 251. doi: 10.1017/S0022149X00006039.
Wright, C. a, Rollinson, D. and Goll, P. H. (1979) 'Parasites in *Bulinus senegalensis* (Mollusca: Planorbidae) and their detection.', *Parasitology*, pp. 95–105. doi: 10.1017/S0031182000051994.

ANNEX I: Risk assessment

The risk assessment of safety issues associated with the research performed can be divided into three distinct categories. The first includes all risks imposed by travelling and conducting field work in Zimbabwe. The second includes all risks associated with the use of hazardous reagents in the lab, both in Zimbabwe and in Belgium. The third category includes all risks linked to post-lab analysis and writing.

Prior to departure, all necessary safety precautions were considered. Financial, medical and technical aspects (e.g. insurance) were covered by lectures and an online brochure provided by the KULeuven international mobility office. Recommendations were followed, e.g. I received additional vaccinations against yellow fever and rabies, prophylactic medicine was bought (e.g. probiotics, malaria prophylaxis) and appropriate travel insurance was contracted. Some safety precautions related to the possibility of wild animal encounters had to be taken while staying in Kariba. While sampling lake shores, the shallow water was probed with long sticks and stones to check for crocodiles. Constant enclosure in group and awareness of the surroundings were daily routine since Kariba town and the sampling sites are part of a wildlife reserve and encounters of wild animals such as elephant, buffalo or hippopotamus were common. Since this study involves sampling of snails that are known to potentially harbour human infecting parasites, all safety precautions were taken to prevent direct contact with the snails and lake water. Sampling was always done wearing boots or wading suit and the picking of snails from scooping nets and vegetation was done with forceps and gloves. High index factor sun screen, a hat and lots of water prevented dehydration and sun burs in the warm and dry climate of Zimbabwe.

Safety precautions were taken while working with irritating, inflammable and carcinogenic reagents while performing allozyme analysis in Kariba and genetic lab work in Tervuren. Wearing a lab coat and gloves were standard safety measures in the lab while lab goggles were worn where necessary. Appropriate waste disposal was taken care of as indicated by the lab manager to avoid environmental contamination. The protein staining protocol in the allozyme analysis involved the use of MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) which is a chemical with potential mutagenic effects when ingested. The chemicals NAD (Nictoinamide Adenine Dinucleotide), Tris-HCl (2-Amino-2-(hydroxymethyl) propane-1,3-diol Hydro Chloride), Malic Acid and PMS (Phenazine Methosulfate) can all cause irritation to the eyes, skin and respiratory system upon contact. While performing the protein staining protocol, all safety precautions were taken, including wearing lab goggles. When performing DNA extraction procedures, care was taken when

working with volatile and flammable substances such as ethanol (70%) and isopropanol. The flame used for sterilizing the scalpel, forceps and needle was always kept within a safe distance of these chemicals and ourselfs. Since some chemicals such as chlorophorm and isopropanol are volatile and can cause dizziness, inhalation precautions were taken. Proteinase K, Chlorophorm, Chelex® resin and isopropanol, all used for DNA extraction, can cause irritation to the eyes and/or respiratory system and where therefore handled with care. PCR protocols did not include any direct safety risks although great care was taken to avoid DNA contamination of the sample which directly implies any contact with these substances. Post PCR analysis required heating of agar for casting it into an electrophoresis gel mold. The agar solution was heated up to its boiling point and handling the solution required precaution and oven mitts to avoid burns. For visualization of the DNA after PCR amplification, the staining chemical Midori Green direct (Nippon Genetics^{TM}) and UV light was used. Although Midori Green is a safer alternative for the highly carcinogenic Ethidium Bromide, the DNA intercalating chemical was handled with great care (e.g. direct lab glove replacement after use, handling only with marked zone). Visualization of the DNA with UV light was performed in an enclosed metal box with a camera and monitor since direct exposure of skin or eyes to highly concentrated UV light can have mutagenic effects. All lab work was performed under the supervision of the supervisor, a doctoral student from the lab or the lab manager. All safety precautions, waste disposal and contamination risks (in both ways) were therefore monitored in detail and help or questions linked to any of these aspects were immediately assessed.

A third and last aspect of risks linked to the writing of this thesis is the ergonomics of reading, writing and analysing data on the computer. Short pauses intervened prolonged hours of sitting behind a desk and staring at the screen to decrease ocular stress and back aches.

ANNEX II: Methods for nutrient analysis

The described methods of nutrient analysis were performed by a team of biochemistry students under the supervision of the lab technician at the University Lake Kariba Research Station (ULKRS). Nutrient concentrations were spectrophotometrically measured and calculated as described in USEPA (1983) for nitrate, phosphate and ammonia concentrations. Methods by Aminot *et al.* (2000) were followed for chlorophyll a concentrations.

a. **Sample storage**

Water samples were frozen at -20°C when analysis did not immediately followed sampling. For chlorophyll a analysis, water samples were filtered on a Whatman® filter paper and the paper was, wrapped in aluminium foil, stored in the freezer (-20°C) until multi-sample analysis was performed.

b. Nitrate analysis

Nitrate concentrations were determined as described in method 353.2 by the USEPA (1983). Nitrates were reduced to nitrites on a copper-cadmium column. Nitrite reacts with sulphanilamide in acidic conditions, forming a compound that colours red/purple when N-1-Napthylethylenediamine is added. The intensity of the colour formation is proportional to the concentration of nitrites and the absorbance at 545nm. Absorbance was measured in a Hach DR 2800™ Spectrophotometer as described by Hach (2007). The concentration of nitrates was calculated as the difference in absorbance of the sample before and after nitrate reduction.

c. Phosphate analysis

Phosphate concentrations were determined as described in method 365.2 by the USEPA (1983). Dissolved (ortho)phosphate ions react with ammonium molybdate and antimony potassium tartrate in acidic conditions forming a complex that, when reduced by ascorbic acid, colours intensively blue. The intensity of the colour formation is proportional to the concentration of phosphates and the absorbance at 882nm. Absorbance was measured in a Hach DR 2800™ Spectrophotometer as described by Hach (2007).

d. Ammonia analysis

Ammonia concentrations were determined as described in method 350.1 by the USEPA (1983). Ammonia reacts with hypochlorite in slightly alkaline conditions and catalized by sodium nittroprusside, forming monochloroamine that complexates with phenol and forms blue indophenol. Hypochlorite is added to intensify the colour. The intensity of the colour

formation is proportional to the concentration of ammonia and the absorbance at 635nm. Absorbance was measured in a Hach DR 2800™ Spectrophotometer as described by Hach (2007).

e. Chlorophyll a analysis

Chlorophyll a concentrations were determined as described by Aminot *et al.*, (2000). Chlorophyll a was extracted from the filter paper by incubation of the paper in 7ml of a 90% acetone solution for 1-3 hours while refrigerated (4°C). The sample was refrigerated for 10 minutes at 3000 revolutions per second and supernatants was collected. Since chlorophyll a is a pigment itself, the concentration can be measured at the absorbance level of the pigment. Absorbance was measured at 630nm, 647nm, 664nm and 750nm in a Hach DR 2800™ Spectrophotometer as described by Hach (2007). The chlorophyll a concentration was calculated as described by Aminot *et al.* (2000).

References

Aminot, A. and Rey, F. (2000) 'Standard procedure for the determination of chlorophyll a by spectroscopic methods', in *ICES Techniques in Marine Environmental Sciences*. Copenhagen, p. 25. Available at: https://www.researchgate.net/publication/242385183 (Accessed: 15 May 2018).

Hach Company (2007) *DR 2800 Spectrophotometer Procedures Manual*. 2nd edn. Available at: https://www.scribd.com/document/138080069/DR-2800-Spectrophotometer-Procedures-Manual (Accessed: 15 May 2018).

USEPA (1983) *Methods for Chemical Analysis of Water and Wastes*, *National Service Center for Environmental Publications (NSCEP)*. Available at: https://www.epa.gov/homeland-security-research/reference-document-methods-chemical-analysis-water-and-waste-epa6004-0.

Annex III: Supplementary tables

Table 1. Information of samples sites. Each site has a unique number and three-letter code as reference. Coordinates (longitude and latitude) and discrete ecological parameters included in the ecological data analysis are given per site: i.e. the abundance of water hyacinth (*Eicchornia crassipes*), substrate and depth.

Site nr.	Name	Code	Longitude	Latitude	*E. crassipes*	Substrate	Depth
			Sites				
1	DDF	DDF	S16.53152	E028.75891	high	sandy	shallow
2	GDI	GDI	S16.53438	E028.76714	low	sandy	shallow
3	Andora Harbor	AND	S16.53229	E028.77057	high	muddy	shallow
4	I&J	I&J	S16.53050	E028.78197	none	rocky	shallow
5	Gundamusaira	GUN	S16.53030	E028.78521	low	muddy	shallow
6	Marineland	MAR	S16.53030	E028.80208	low	muddy	deep
7	Breezes	BRE	S16.53605	E028.81407	none	sandy	deep
8	B-Line	BLI	S16.53287	E028.81571	none	sandy	deep
9	Cutty Sark	CUS	S16.53468	E028.81909	none	rocky	shallow
10	Mopani Bay	MOP	S16.53086	E028.83055	low	muddy	shallow
11	UZ Harbor	UZH	S16.52934	E028.84181	high	muddy	deep
12	Nyanyana	NYA	S16.52963	E028.84747	high	muddy	shallow
13	Lake Harvest	LHA	S16.53303	E028.85368	high	muddy	deep
14	Lake Croc	CRO	S16.54363	E028.87697	high	muddy	deep
15	Nzou Lodge	NZO	S16.56119	E028.94143	low	sandy	deep
16	Charara	CHA	S16.55410	E028.95083	high	muddy	shallow
			Fishing camps				
17	Nyaotza	FC1	S16.635086	E28.927149	NA	NA	NA
18	Fother Gill	FC2	S16.640996	E28.928856	NA	NA	NA
19	Tsuwa	FC3	S16.707669	E28.938418	NA	NA	NA

Table 2. Summary of barcoded sequences of trematodes. The top 3 BLAST hits with the respective identity cover (ID), length (len), quality (HQ) and length are shown for every sequence included in the genotyping analysis. The origin marks the snail host with PCO: *P. columella*, BTR: *B. truncatus*, BFO: *B. forskalii*, RSP: *Radix* sp. and SSP: *Succinea* sp.

seq. name	Origin	HQ (%)	len (bp)	best BLAST 1	ID (%)	best BLAST 2	ID (%)	best BLAST 3	ID (%)
18S rDNA marker									
S9	PCO	100	586	*Fasciola hepatica*	100	*Fasciola gigantica*	99	*Fasciola jacksoni*	99
S84	PCO	100	586	*Fasciola hepatica*	100	*Fasciola gigantica*	99	*Fasciola jacksoni*	99
S56	PCO	50	586	*Fasciola hepatica*	99	*Fasciola gigantica*	99	*Fasciola jacksoni*	99
S151	PCO	100	586	*Fasciola hepatica*	100	*Fasciola gigantica*	99	*Fasciola jacksoni*	99
S205	PCO	100	586	*Fasciola hepatica*	100	*Fasciola gigantica*	99	*Fasciola jacksoni*	99
S201	PCO	99.7	586	*Fasciola hepatica*	100	*Fasciola gigantica*	99	*Fasciola jacksoni*	99
S37	RSP	100	586	*Fasciola hepatica*	100	*Fasciola gigantica*	99	*Fasciola jacksoni*	99
S100	PCO	100	586	*Fasciola hepatica*	100	*Fasciola gigantica*	99	*Fasciola jacksoni*	99

S247	BFO	100	575	*Hysteromorpha triloba*	98	*Diplostomum phoxini*	97	*Strigeidida* sp.	96
S99	SSP	89.6	280	*Leucochloridium* sp.	99	*Leucochloridium holostomum*	99	*Leucochloridium paradoxum*	99
S188	BTR	99.8	575	*Nudacotyle undicola*	97	*Notocotylus pacifera*	96	*Taprobanella bicaudata*	96
S6	BTR	100	576	*Nudacotyle undicola*	97	*Notocotylus pacifera*	96	*Taprobanella bicaudata*	96
S187	BTR	94.6	614	*Nudacotyle undicola*	97	*Notocotylus pacifera*	96	*Taprobanella bicaudata*	96
S191	BTR	100	576	*Nudacotyle undicola*	97	*Notocotylus pacifera*	96	*Taprobanella bicaudata*	96
3C1	BTR (C)	100	586	*Paramphistomum cervi*	100	*Orthocoelium streptocoelium*	100	*Olveria bosi*	100
U	BTR (S)	100	586	*Paramphistomum cervi*	100	*Orthocoelium streptocoelium*	100	*Olveria bosi*	100
S	BTR (S)	99.7	587	*Paramphistomum cervi*	99	*Orthocoelium streptocoelium*	99	*Olveria bosi*	99
1A4	BTR (C)	100	586	*Paramphistomum cervi*	100	*Orthocoelium streptocoelium*	100	*Olveria bosi*	100
S44	RSP	99.7	586	*Paramphistomum cervi*	99	*Orthocoelium streptocoelium*	99	*Olveria bosi*	99
T	BTR (S)	100	586	*Paramphistomum cervi*	100	*Orthocoelium streptocoelium*	100	*Olveria bosi*	100
3C2	BTR (C)	100	586	*Paramphistomum cervi*	100	*Orthocoelium streptocoelium*	100	*Olveria bosi*	100
1A2	BTR (C)	100	586	*Paramphistomum cervi*	100	*Orthocoelium streptocoelium*	100	*Olveria bosi*	100
S222	BTR	99.1	586	*Petasiger phalacrocoracis*	99	*Paryphostomum radiatum*	97	*Isthmiophora hortensis*	97
S221	BTR	100	586	*Petasiger phalacrocoracis*	99	*Paryphostomum radiatum*	97	*Isthmiophora hortensis*	97
COI marker									
S221	BTR	98.8	419	*Apophallus* sp.	75	*Echinococcus multilocularis*	77	*Hemiuridae* sp.	76
S44	RSP	98.8	420	*Calicophoron microbothrium*	85	*Cotylophoron* sp.	85	*Calicophoron raja*	85
M	BTR (S)	100	188	*Carmyerius exoporus*	90	*Carmyerius mancupatus*	89	*Fischoederius elongatus*	87
1A2	BTR (C)	99	417	*Carmyerius mancupatus*	89	*Carmyerius exoporus*	88	*Carmyerius gregarius*	88
3C1	BTR (C)	99.5	416	*Carmyerius mancupatus*	89	*Carmyerius exoporus*	87	*Carmyerius gregarius*	87
3C2	BTR (C)	93.1	451	*Carmyerius mancupatus*	90	*Carmyerius exoporus*	88	*Carmyerius gregarius*	88
S	BTR (S)	98.8	417	*Carmyerius mancupatus*	89	*Carmyerius exoporus*	87	*Fischoederius elongatus*	86
T	BTR (S)	98.3	422	*Carmyerius mancupatus*	89	*Carmyerius exoporus*	88	*Carmyerius gregarius*	88
U	BTR (S)	99	420	*Carmyerius mancupatus*	89	*Carmyerius exoporus*	87	*Carmyerius gregarius*	87
S84	PCO	80.3	314	*Fasciola gigantica*	90	*Fasciola* sp.	90	*Fascioloides magna*	86
S9	PCO	98.8	417	*Fasciola gigantica*	92	*Fasciola hepatica*	91	*Fasciola* sp.	90
S151	PCO	98.1	416	*Fasciola gigantica*	91	*Fasciola* sp.	90	*Fasciola hepatica*	90
S201	PCO	97	304	*Fasciola gigantica*	91	*Fasciola hepatica*	91	*Fasciola* sp.	90
S205	PCO	96.9	419	*Fasciola gigantica*	91	*Fasciola* sp.	90	*Fasciola hepatica*	90
S247	BFO	98.8	419	*Fibricola* sp.	89	*Neodiplostomum americanum*	89	*Alaria marcianae*	89
S187	BTR	99	419	*Notocotylidae* sp.	91	*Ogmocotyle sikae*	81	*Ogmocotyle* sp.	79
S191	BTR	99	420	*Notocotylidae* sp.	91	*Ogmocotyle sikae*	81	*Ogmocotyle* sp.	79
S6	BTR	97.1	419	*Notocotylidae* sp.	91	*Ogmocotyle sikae*	81	*Ogmocotyle* sp.	79

Table 3. Pairwise genetic distance (p-distance) between Kariban trematode sequences of COI (410 bp).. All sequences (Seq.) with the same colour indication are assumed to belong to the same species. Colours indicate; yellow: Gastrothylacidae sp., green: Pronocepahloidea sp., blue: Fasciola sp., pink: Paramphistomoidea sp., red: Psiolostomidae sp. and grey: Diplostomidae sp.

Seq.		1	2	3	4	5	6	7	8	9	10	11	12	13
1A2	1													
3C2	2	0.002												
S	3	0.005	0.002											
S6	4	0.259	0.261	0.263										
S9	5	0.315	0.315	0.315	0.278									
S44	6	0.18	0.178	0.178	0.254	0.261								
S151	7	0.315	0.315	0.315	0.283	0.01	0.266							
S187	8	0.259	0.261	0.263	0	0.278	0.254	0.283						
S191	9	0.259	0.261	0.263	0	0.278	0.254	0.283	0					
S205	10	0.315	0.315	0.315	0.283	0.01	0.266	0	0.283	0.283				
S221	11	0.298	0.298	0.298	0.305	0.229	0.293	0.229	0.305	0.305	0.229			
S247	12	0.295	0.293	0.295	0.271	0.254	0.268	0.251	0.271	0.271	0.251	0.329		
T	13	0	0.002	0.005	0.259	0.315	0.18	0.315	0.259	0.259	0.315	0.298	0.295	
U	14	0.002	0	0.002	0.261	0.315	0.178	0.315	0.261	0.261	0.315	0.298	0.293	0.002

Table 4. Specifications of sequences included in the COI phylogeny. Given are host origin, sample site, HQ of the sequence and taxonomic resolution based on COI and 18S rDNA genotyping. (C) indicates that the specimen is a cercaria, (S) specifies that sample used was a shedding snail. No indication indicates that the snail was proved to be infected by multiplex RD-PCR. For the names corresponding to the abbreviated site codes and number: see table 1 of supplementary tables.

Seq. ID	Host origin	Site	HQ (%)	Length (bp)	Taxonomy
3C2	*B. truncatus* (C)	16	93.1	451	Family Gastrothylacidae
U	*B. truncatus* (S)	16	99	420	Family Gastrothylacidae
3C1	*B. truncatus* (C)	16	99.5	416	Family Gastrothylacidae
S	*B. truncatus* (S)	16	98.8	417	Family Gastrothylacidae
1A2	*B. truncatus* (C)	16	99	417	Family Gastrothylacidae
T	*B. truncatus* (S)	15	98.3	422	Family Gastrothylacidae
S44	*Radix* sp.	5	98.8	420	Superfamily Paramphistomoidea
S6	*B. truncatus*	1	97.1	419	Family Notocotylidae
S187	*B. truncatus*	15	99	419	Family Notocotylidae
S191	*B. truncatus*	15	99	420	Family Notocotylidae
S221	*B. truncatus*	16	98.8	419	Family Psilostomidae
S9	*P. columella*	3	98.8	417	Genus *Fasciola*
S151	*P. columella*	15	98.1	416	Genus *Fasciola*
S205	*P. columella*	16	96.9	419	Genus *Fasciola*
S247	*B. forskalii*	16	98.8	419	Family Diplostomatidae

Table 5. Total number of examined snails and the multiplex RD-PCR based infection prevalence per species and per site. The names of the sites corresponding to the numbers are listed in table 1 of supplementary figures (Annex III).

Site	Species	nr. of snails tested	nr. of failed PCR	nr. of non - infected snails	nr. of infected snails	infection prevalence (%)
1	*P. columella*	4	0	4	0	0
	B. truncatus	4	0	2	2	50
3	*P. columella*	16	1	3	12	80
	Radix sp.	12	0	12	0	0
5	*Radix* sp.	16	0	14	2	12.50
6	*B. truncatus*	3	0	3	0	0
11	*P. columella*	4	1	0	3	100
	Radix sp.	16	0	15	1	6.25
	Succinea sp.	8	0	8	0	0
12	*P. columella*	12	1	8	3	27.27
	Succinea sp.	4	0	2	2	50
13	*P. columella*	4	0	2	2	50
	Succinea sp.	12	0	8	4	33.33
14	*B. forskalii*	4	0	4	0	0
	Gyraulus sp.	8	0	8	0	0
	P. columella	4	0	3	1	25
	B. truncatus	16	3	13	0	0
15	*P. columella*	32	0	7	25	78.13
	B. truncatus	16	0	11	5	31.25
16	*P. columella*	16	0	4	12	75
	B. truncatus	28	0	26	2	7.14
	B. forskalii	8	1	5	2	28.57
FC1	*P. columella*	4	0	4	0	0
FC2	*P. columella*	3	2	0	1	100
	All	254	9	166	79	32.24

Table 6. Average values of physico-chemical water parameters measured for each site. Averages were taken from sampling of May 2017 until February 2018. Site names corresponding to the numbers and codes are listed in table 1 of supplementary tables. Units of variables are: mg/L for dissolved oxygen concentration (D.O.), % for oxygen saturation (O.S.), µS/cm for conductivity (Con.), NTU or nephelometric turbidity units for turbidity (Turb.) and µg/L for phosphate (Pho), ammonia (Amm.), nitrate (Nit.) and chlorophyll a (Chl.A) concentrations. The names of the sites corresponding to the numbers are listed in table 1 of supplementary tables.

Site	pH	Temp	D.O.	O.S.	Con.	Turb.	Phos.	Amm.	Nit.	Chl.A
1	8.3	27.0	7.5	99.5	103.0	2.5	17.0	72.3	12.4	4.0
2	8.7	27.4	7.6	101.6	93.5	2.0	11.3	70.7	8.8	4.8
3	8.8	27.8	7.8	105.2	99.0	3.3	10.7	39.6	8.6	6.6
4	8.6	27.3	7.5	101.3	94.6	3.8	9.4	88.7	13.6	6.6
5	8.5	28.3	7.6	103.6	98.2	1.8	11.7	47.5	17.1	5.0
6	8.7	27.1	7.5	99.2	97.2	7.7	11.0	51.2	8.2	5.5
7	8.8	27.4	7.6	98.6	93.2	2.8	14.0	47.7	20.1	4.9
8	8.8	27.2	7.4	98.5	97.1	2.6	9.4	46.7	10.5	9.0
9	8.8	27.8	7.6	101.3	102.7	11.0	11.8	49.9	9.6	4.4
10	8.8	28.5	7.7	104.3	99.5	13.6	11.7	60.7	12.3	10.2

11	9.2	28.0	8.4	112.9	103.2	8.4	15.0	101.4	15.2	12.3
12	8.5	29.0	6.6	91.0	177.1	17.1	401.8	434.3	88.5	9.4
13	8.6	28.6	7.9	93.7	149.2	33.2	100.6	132.8	89.7	36.5
14	7.8	29.8	4.6	62.6	245.5	51.8	576.9	1345.2	127.5	68.4
15	8.7	27.4	7.3	97.3	96.3	4.6	29.5	47.8	14.4	6.4
16	8.0	26.6	4.2	54.6	106.4	7.1	20.9	76.8	13.1	9.0
average	8.6	27.8	7.2	95.3	116.0	10.8	78.9	169.6	29.3	12.7

Table 7. Pairwise genetic distance (p-distance) between *Radix* sequences of COI (349 bp). The number of nucleotide differences per site between sequences are shown. Both the Kariban morphotypes (MT) *L. natalensis* and *L. radix* are included.

Seq.		1	2	3	4	5	6	7	8	9	10	11	12	13
Pseudosuccinea columella	**1**													
Radix. ampla	**2**	0.138												
Radix auricularia	**3**	0.178	0.163											
Radix balthica	**4**	0.169	0.092	0.163										
Radix dolgini	**5**	0.149	0.115	0.181	0.126									
Radix labiata	**6**	0.163	0.109	0.175	0.129	0.097								
MT *L. natalensis*	**7**	0	0.138	0.178	0.169	0.149	0.163							
MT *L. radix*	**8**	0.178	0.16	0.158	0.175	0.158	0.169	0.178						
Radix lagotis	**9**	0.152	0.072	0.169	0.1	0.103	0.097	0.152	0.166					
Radix natalensis	**10**	0.175	0.143	0.16	0.135	0.126	0.152	0.175	0.166	0.138				
Radix ovata	**11**	0.169	0.077	0.169	0.014	0.123	0.126	0.169	0.169	0.092	0.135			
Radix rubiginosa	**12**	0.169	0.132	0.129	0.158	0.14	0.152	0.169	0.158	0.152	0.143	0.155		
***Radix* sp.**	**13**	0.169	0.166	0.158	0.178	0.163	0.169	0.169	0.037	0.16	0.166	0.172	0.146	
Radix zazurnensis	**14**	0.178	0.097	0.172	0.109	0.112	0.12	0.178	0.175	0.077	0.14	0.1	0.166	0.178

Table 8. Pairwise genetic distance (p-distance) between trematode sequences of COI (402 bp) in the COI phylogeny (figure 8).

The number of nucleotide differences per site between sequences are shown.

Seq		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27
Calicophoron microbothrium	**1**																											
Carmyerius mancupatus	**2**	0.142																										
Cotylophoron sp.	**3**	0.137	0.127																									
Fasciola jacksoni	**4**	0.318	0.313	0.303																								
Fascioloides magna	**5**	0.291	0.299	0.276	0.129																							
Fischoederius elongatus	**6**	0.157	0.139	0.179	0.306	0.294																						
Gastrothylax crumenifer	**7**	0.184	0.124	0.159	0.318	0.301	0.152																					
Homalogaster paloniae	**8**	0.182	0.184	0.179	0.308	0.291	0.192	0.216																				
Notocotylus sp.	**9**	0.279	0.254	0.271	0.286	0.279	0.259	0.244	0.279																			
Ogmocotyle sikae	**10**	0.254	0.254	0.259	0.286	0.274	0.249	0.249	0.241	0.192																		
Paramphistomum cervi	**11**	0.119	0.134	0.129	0.303	0.291	0.149	0.157	0.187	0.284	0.269																	
Pseudopsilostoma varium	**12**	0.343	0.326	0.333	0.261	0.236	0.328	0.318	0.308	0.299	0.301	0.338																
Stephanopharynx sp.	**13**	0.184	0.177	0.162	0.308	0.294	0.179	0.197	0.209	0.276	0.281	0.152	0.331															
Hypoderaeum conoideum	**14**	0.353	0.358	0.373	0.259	0.241	0.346	0.351	0.366	0.281	0.296	0.358	0.254	0.328														
Apharyngostrigea pipientis	**15**	0.301	0.279	0.279	0.313	0.286	0.291	0.291	0.291	0.291	0.284	0.294	0.321	0.301	0.331													
Diplostomum ardeae	**16**	0.296	0.291	0.306	0.279	0.269	0.289	0.291	0.311	0.301	0.296	0.296	0.351	0.294	0.323	0.197												
Fibricola sp.	**17**	0.281	0.269	0.279	0.276	0.256	0.279	0.274	0.286	0.276	0.286	0.279	0.326	0.276	0.323	0.187	0.137											
type II (*Radix* sp.)	**18**	0.152	0.157	0.157	0.306	0.286	0.174	0.172	0.189	0.249	0.231	0.159	0.301	0.197	0.331	0.286	0.291	0.264										
type III (*B. truncatus*)	**19**	0.266	0.254	0.251	0.286	0.279	0.259	0.249	0.266	0.229	0.201	0.234	0.336	0.266	0.308	0.289	0.303	0.286	0.259									
type V (*P. columella*)	**20**	0.296	0.306	0.296	0.164	0.144	0.303	0.303	0.313	0.276	0.276	0.289	0.239	0.291	0.226	0.301	0.279	0.264	0.274	0.291								
type IV (*B. truncatus*)	**21**	0.311	0.308	0.318	0.239	0.236	0.308	0.311	0.341	0.303	0.291	0.333	0.192	0.306	0.269	0.338	0.331	0.313	0.303	0.318	0.244							
type VI (*B. forskalii*)	**22**	0.296	0.276	0.291	0.291	0.299	0.308	0.291	0.313	0.294	0.303	0.281	0.328	0.301	0.338	0.184	0.134	0.114	0.281	0.281	0.259	0.343						
type I (*B. truncatus*)	**23**	0.179	0.112	0.157	0.308	0.299	0.142	0.139	0.224	0.256	0.261	0.172	0.328	0.211	0.331	0.294	0.301	0.291	0.184	0.269	0.321	0.311	0.303					
Alaria mustelae	**24**	0.316	0.308	0.316	0.281	0.284	0.318	0.316	0.321	0.316	0.311	0.301	0.343	0.311	0.323	0.199	0.137	0.134	0.321	0.301	0.281	0.356	0.129	0.313				
Notocotylidae sp.	**25**	0.276	0.254	0.261	0.286	0.289	0.266	0.241	0.264	0.211	0.194	0.259	0.321	0.276	0.308	0.301	0.289	0.279	0.261	0.097	0.286	0.318	0.284	0.259	0.308			
Echinostoma trivolvis	**26**	0.306	0.316	0.318	0.259	0.236	0.328	0.311	0.301	0.296	0.296	0.306	0.244	0.321	0.211	0.311	0.296	0.316	0.296	0.306	0.229	0.274	0.301	0.346	0.316	0.326		
Fasciola hepatica	**27**	0.289	0.284	0.284	0.152	0.152	0.291	0.301	0.286	0.279	0.261	0.289	0.234	0.289	0.241	0.306	0.264	0.261	0.256	0.281	0.104	0.239	0.264	0.313	0.274	0.289	0.201	
Fasciola gigantica	**28**	0.276	0.291	0.289	0.152	0.142	0.294	0.289	0.281	0.281	0.266	0.281	0.226	0.303	0.226	0.311	0.269	0.259	0.271	0.296	0.09	0.219	0.261	0.303	0.276	0.291	0.214	0.087

Annex IV: supplementary figures

(continued on next page)

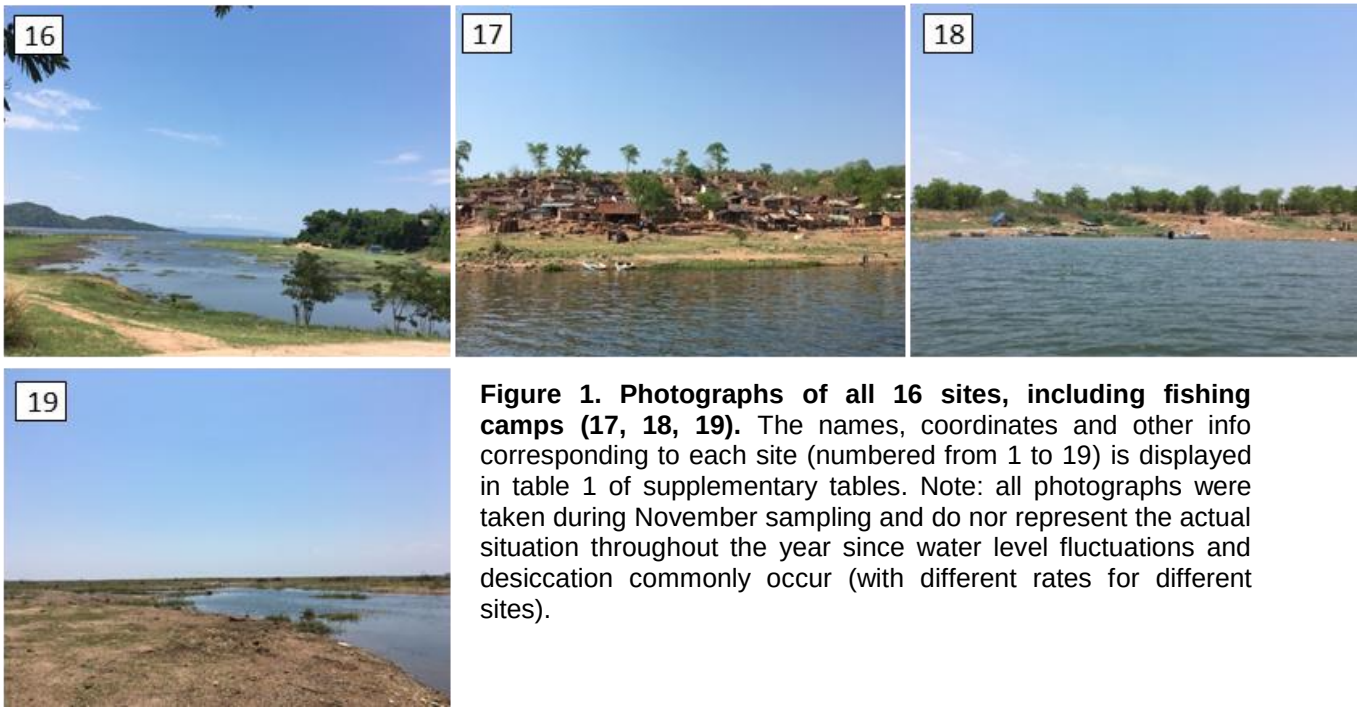


Figure 1. Photographs of all 16 sites, including fishing camps (17, 18, 19). The names, coordinates and other info corresponding to each site (numbered from 1 to 19) is displayed in table 1 of supplementary tables. Note: all photographs were taken during November sampling and do nor represent the actual situation throughout the year since water level fluctuations and desiccation commonly occur (with different rates for different sites).

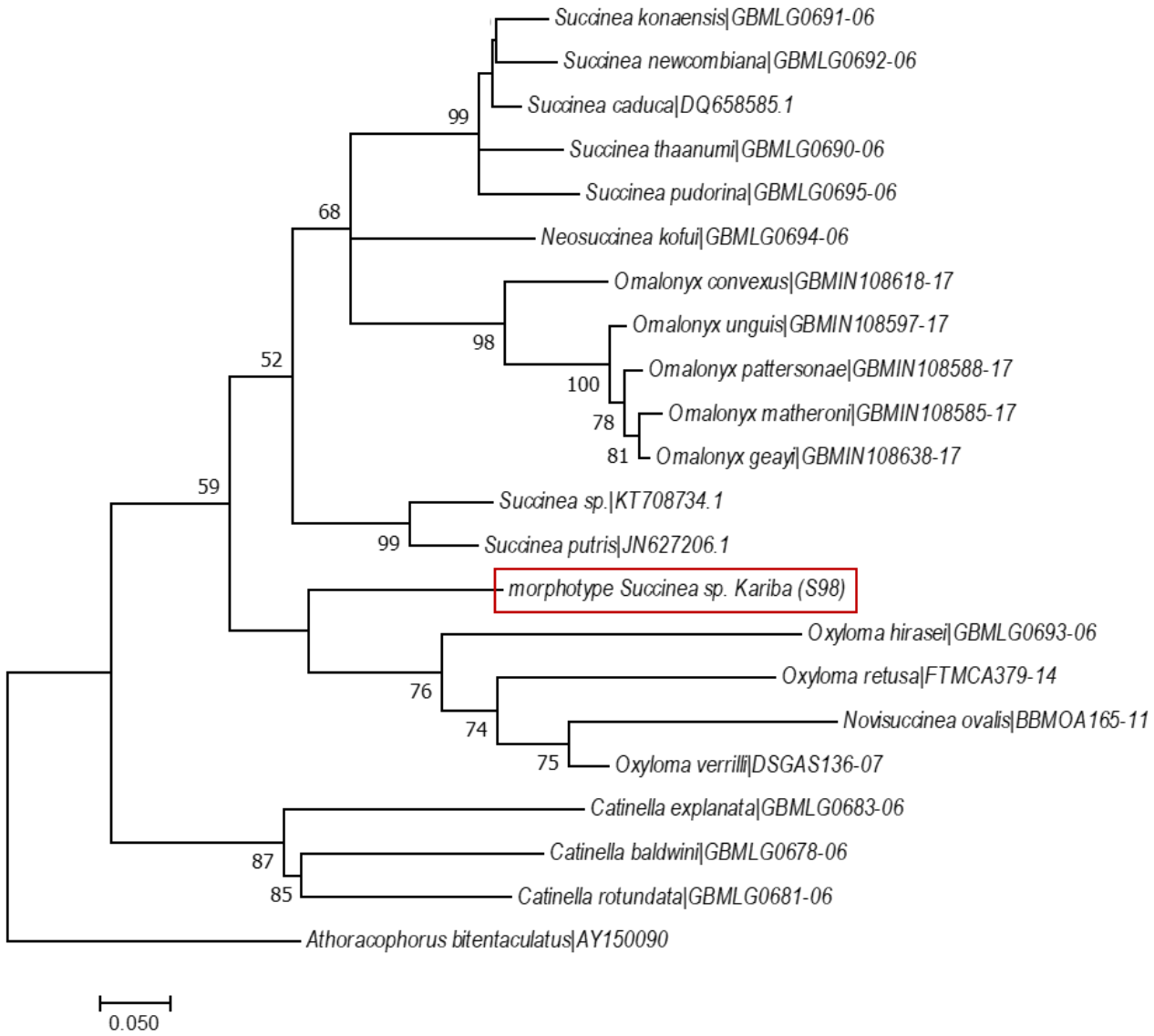


Figure 2. ML phylogenetic tree for the snail species belonging to the Succineidae family, using COI sequences (481bp) and the Hasegawa-Kishino-Yano model. The tree with the highest log likelihood (Ln = -3387.08) is shown. Bootstrap values (500 replicates) that are above or equal to 50 are shown next to the branches. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The BOLD or GenBank accession number of each sequence used is displayed after the | separator. The Kariban morphotype is boxed in red.

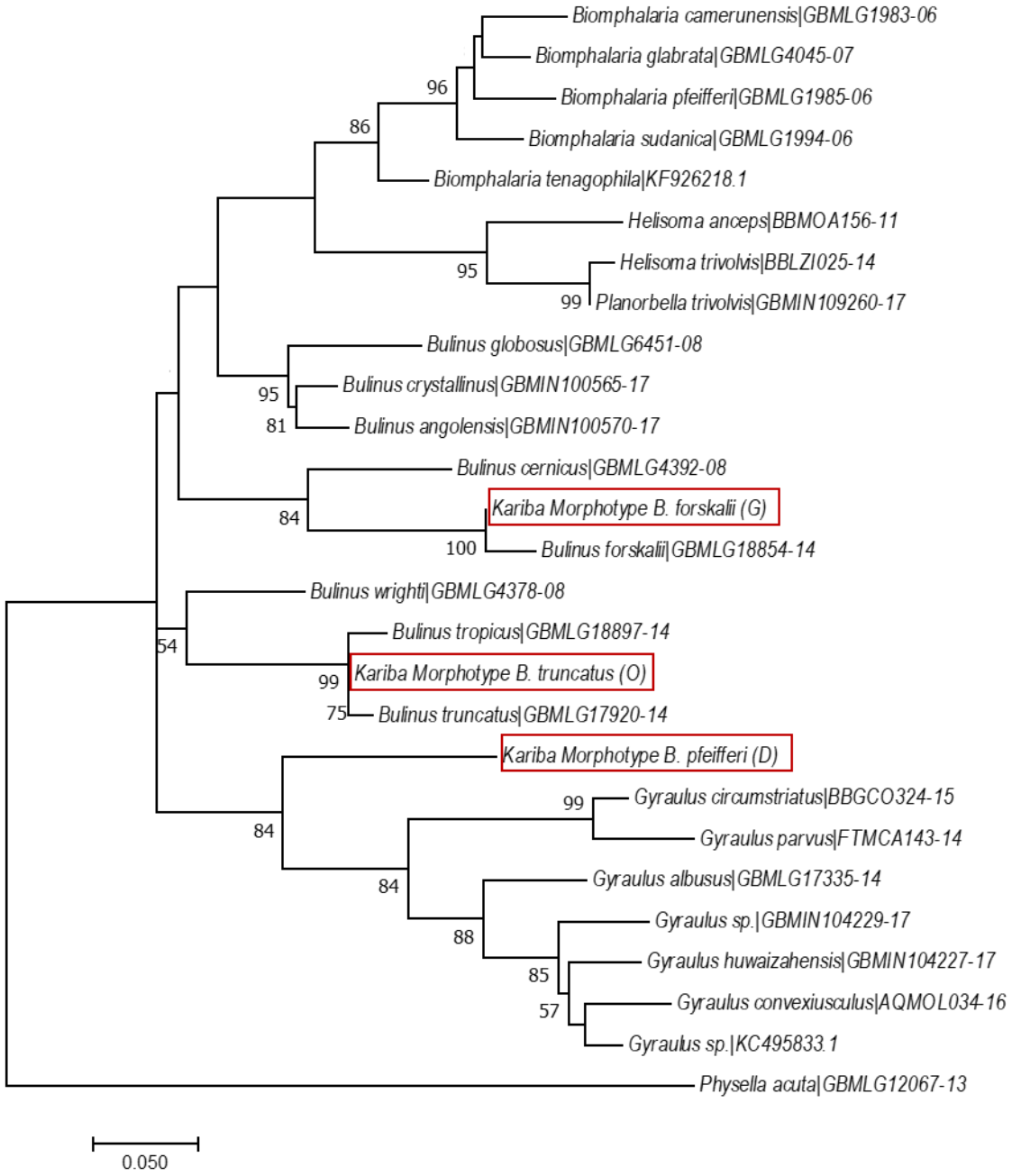


Figure 3. ML phylogenetic tree for the snail species belonging to the Planorbidae family, using COI sequences (325bp) and the General Time Reversible model. The tree with the highest log likelihood (Ln = -2549.89) is shown. Bootstrap values (500 replicates) that are above or equal to 50 are shown next to the branches. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The BOLD or GenBank accession number of each sequence used is displayed after the | separator. Kariban morphotypes are indicated by a red box.

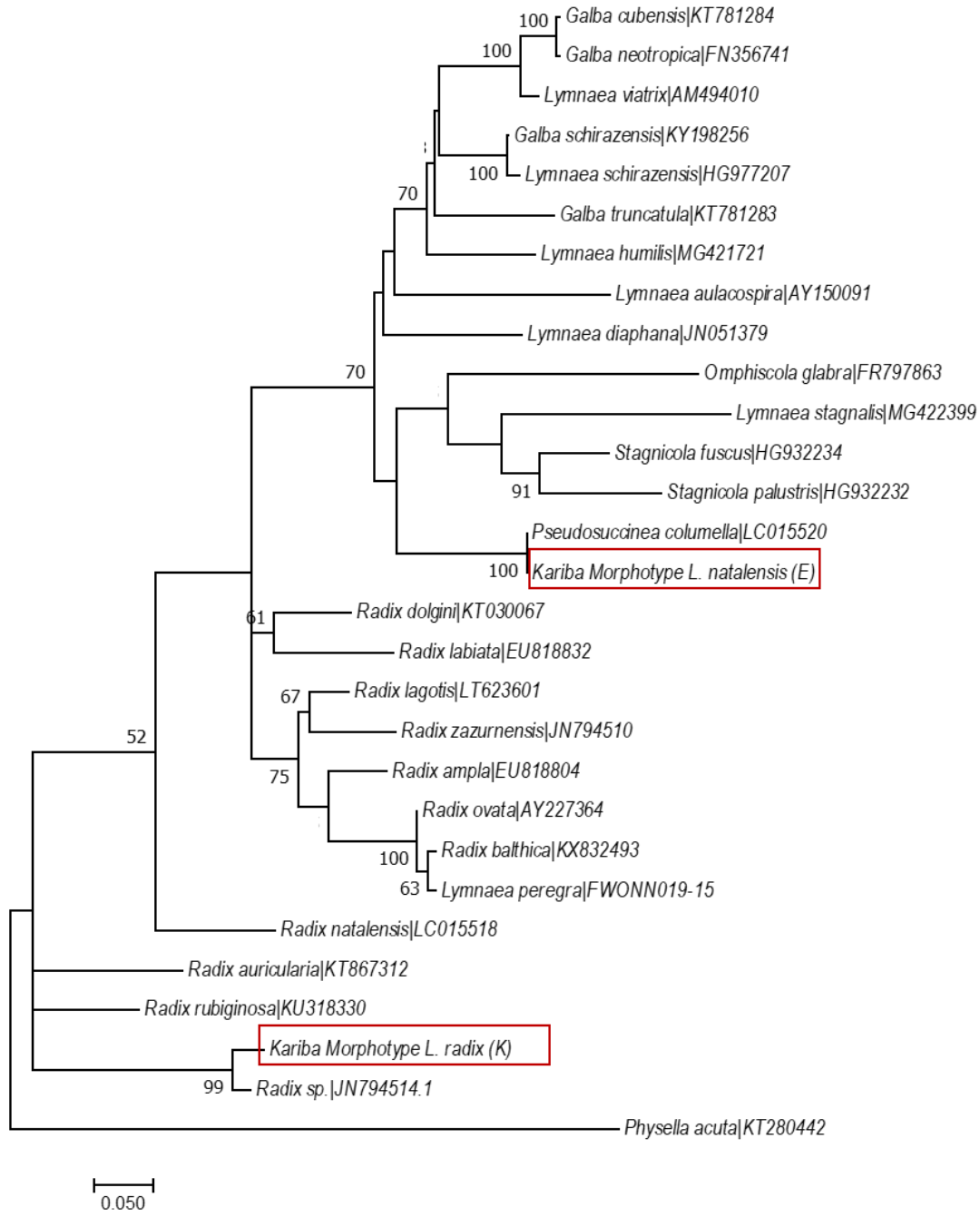


Figure 4. ML phylogenetic tree for the snail species belonging to the Lymnaeidae family, using COI sequences (349bp) and the General Time Reversible model. The tree with the highest log likelihood (Ln = -3104.02) is shown. Bootstrap values (500 replicates) are shown next to the branches. Only branch frequencies above or equal to 50 are displayed. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The BOLD or GenBank accession number of each sequence used is displayed after the | separator. Kariban morphotypes are indicated by a red box.

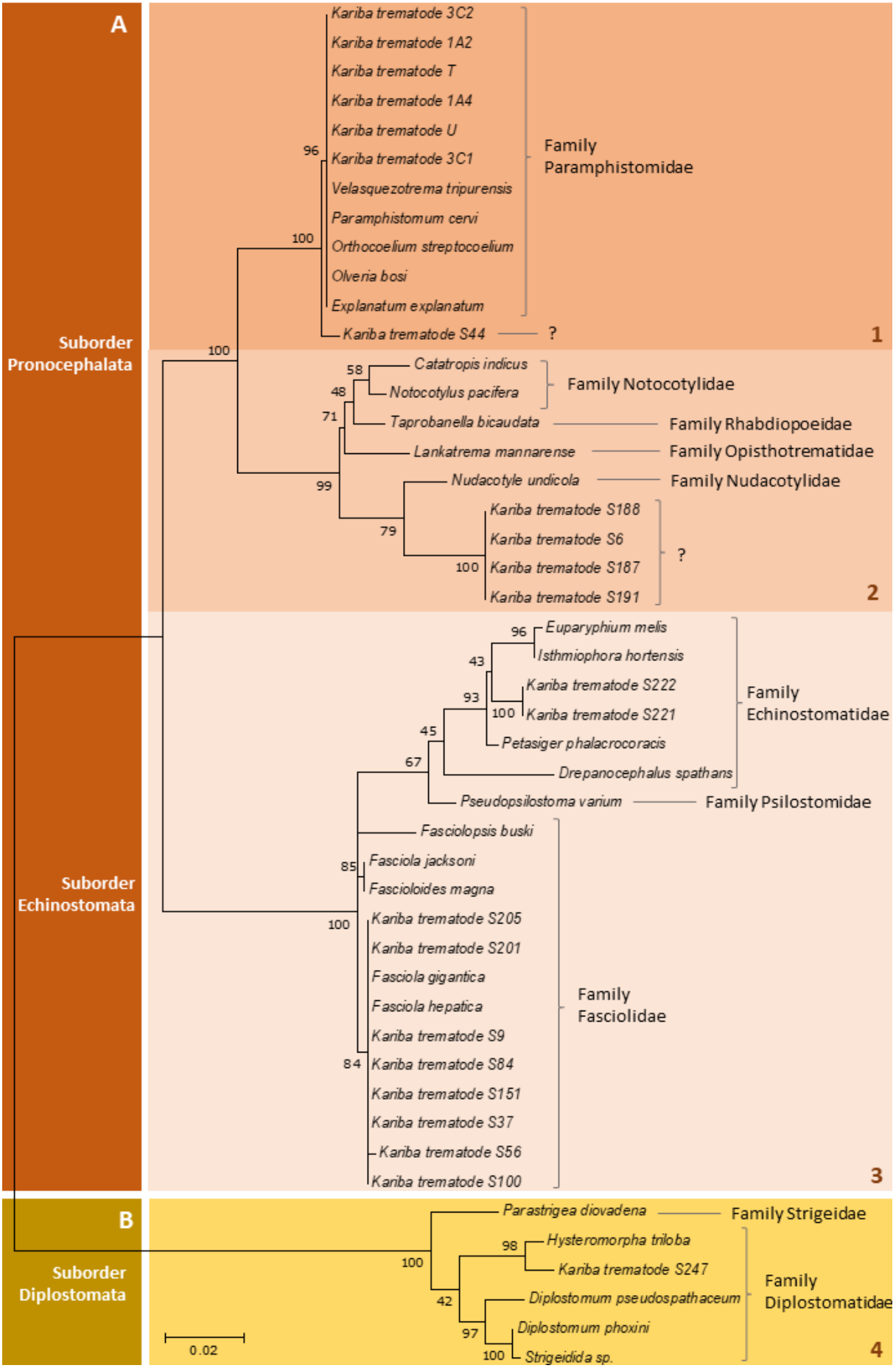
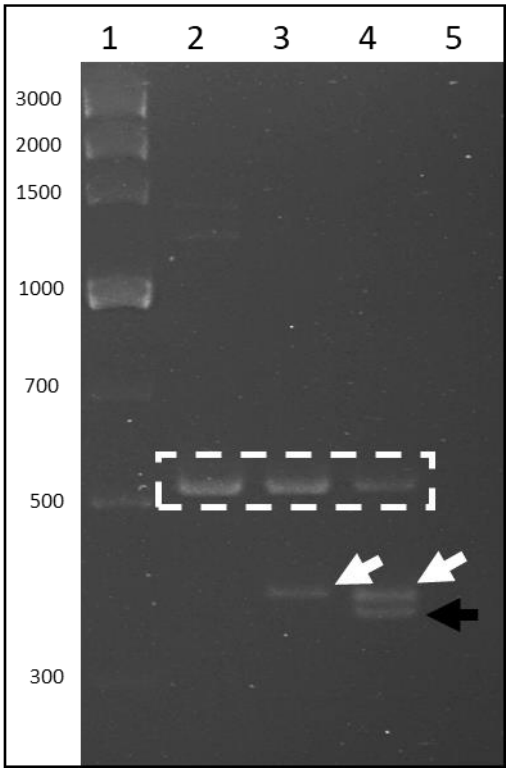
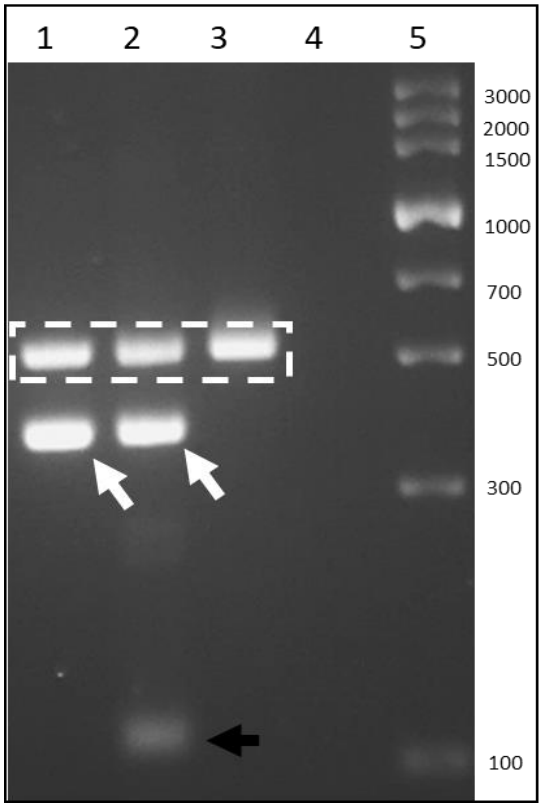

A
Suborder Pronocephalata
Kariba trematode 3C2
Kariba trematode 1A2
Kariba trematode T
Kariba trematode 1A4
Kariba trematode U
Kariba trematode 3C1
Velasquezotrema tripurensis
Paramphistomum cervi
Orthocoelium streptocoelium
Olveria bosi
Explanatum explanatum
Kariba trematode S44
Family Paramphistomidae
?
1
Catatropis indicus
Notocotylus pacifera
Family Notocotylidae
Taprobanella bicaudata
Family Rhabdiopoeidae
Lankatrema mannarense
Family Opisthotrematidae
Nudacotyle undicola
Family Nudacotylidae
Kariba trematode S188
Kariba trematode S6
Kariba trematode S187
Kariba trematode S191
?
2
Suborder Echinostomata
Euparyphium melis
Isthmiophora hortensis
Kariba trematode S222
Kariba trematode S221
Petasiger phalacrocoracis
Drepanocephalus spathans
Family Echinostomatidae
Pseudopsilostoma varium
Family Psilostomidae
Fasciolopsis buski
Fasciola jacksoni
Fascioloides magna
Kariba trematode S205
Kariba trematode S201
Fasciola gigantica
Fasciola hepatica
Kariba trematode S9
Kariba trematode S84
Kariba trematode S151
Kariba trematode S37
Kariba trematode S56
Kariba trematode S100
Family Fasciolidae
3
B
Suborder Diplostomata
Parastrigea diovadena
Family Strigeidae
Hysteromorpha triloba
Kariba trematode S247
Diplostomum pseudospathaceum
Diplostomum phoxini
Strigeidida sp.
Family Diplostomatidae
4
0.02

Figure 5. ML phylogenetic tree of trematodes, using 18S rDNA (550 bp) and the K2+G+I model. The tree with the highest log likelihood (Ln = -2026.32) is shown. Bootstrap values (500 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. Numbers 1 to 4 and corresponding coloured blocks indicate superfamilies with 1: Paramphistomoidea, 2: Pronocephaloidea, 3: Echinostomoidea and 4: Diplostomoidea. Orders are represented by dark grids with A: Echinostomida and B: Strigeidida

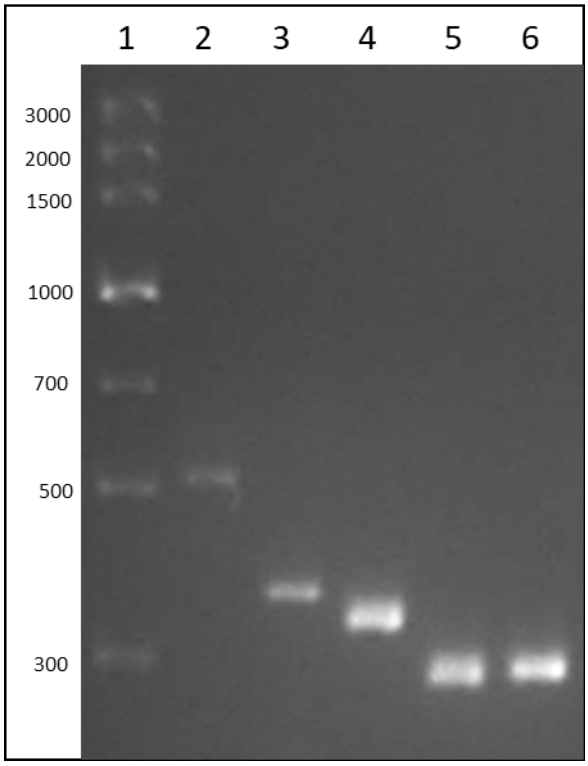
Figure 6. Representative gel electrophoresis results of the general trematode and schistosome multiplex RD-PCR that show discriminative banding patterns of the different markers targeted.



A. General trematode multiplex RD-PCR on planorbid snails. Lane 2, 3 and 4 show the internal control marker of approximately 500bp. (indicated by the white dotted frame). Lane 2 shows an uninfected snail while lane 3 and 4 show the general trematode infection marker (391bp and indicated by white arrows). Lane 4 shows the *Schistosoma* sp. specific marker indicated by the black arrow (app. 369bp). Lane 1 contains the DNA ladder and lane 5 contains a negative (no DNA). Lane 2, 3 and 4 are *B. africanus* snail samples from South Africa, provided by Ruben Schols. Barcoding showed that the snails in lane 4 is infected with *S. mattheei.*



B. General trematode multiplex RD-PCR on lymnaeid snails. Lane 1, 2 and 3 all show the internal control marker of approximately 500bp. (indicated by the white dotted frame). Lane 3 shows an uninfected snail while lane 1 and 2 show the general trematode infection marker (391bp and indicated by white arrows). Lane 4 shows the *Fasciola* sp. specific marker indicated by the black arrow (124bp). Lane 4 contains a negative (no DNA) and lane 5 the DNA ladder. Lane 1, 2 and 3 are Kariban Lymnaeid snail samples. Barcoding results show that *Radix* sp. in line 1 is infected with a Paramphistomoidea sp. while the *P. columella* in 2 is infected with *Fasciola* sp. Lane 3 contains an uninfected *P. columella.*



C. Schistosoma Multiplex RD-PCR. Lane 2, 3, 4, 5 and 6 show the *S. haematobium* (543 bp), *S. mansoni* (375 bp), *S. S. mattheei* (362 bp)*, S. bovis* and *S. curassoni* (both 306 bp) markers respectively. The sample in lane 4 was an infected *B. globosus* from South Africa, provided by Ruben Schols (barcoding confirmed *S. mattheei*). All other samples were adult worm extracts provided by Tine Huyse. Lane 1 shows the DNA ladder.

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