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The influence of snail host microbiome in trematode parasite resistance

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Supervisors:
Prof. Dr. Ellen Decaestecker
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In recent years, many of us have faced personal health challenges and mourned the loss of some remarkable individuals. These experiences underscore the importance of cherishing our health and the relationships that truly colour our lives while developing our careers.

Popularized abstract

A specific group of parasitic flatworms, also known as trematodes, causes diseases in people and animals worldwide. These parasites did not choose the “easy life” and have to subsequently infect specific animals, the so-called hosts, to survive. For trematodes, the first host is usually a freshwater snail while the definitive host is usually a vertebrate species. The illnesses caused by trematode infections range from reduced fertility and kidney failure in people, to bulging colorful eyes filled with parasites in snails or extra pairs of legs in frogs restricting their ability to jump. Which would you prefer? Probably none, yet increased transportation between continents, climate change, and environmental change increase the spread of trematodes and snails to new areas.

Controlling these parasitic diseases is complex. However, scientific breakthroughs in other systems revealed that tiny single-celled organisms, like bacteria, can reduce mosquito-borne diseases in people, the definitive host, by increasing the resistance of mosquito vectors, the first host, to these pathogens. Similarly, the bacteria of freshwater snails might influence their resistance to trematode parasites and potentially provide a new tool to combat these diseases. Before we can start adapting the bacteria of snails in the field, we first have to understand how snails, bacteria and trematodes interact and what role bacteria may play in protecting snails from these parasites.

To do so, we first study the bacteria of lab-grown snails exposed to various combinations of different parasites. This revealed that the snail’s bacteria were differently affected by each parasite species. Also, important moments of parasite development within the snail overlap with changes in the snail’s bacteria. These findings caution field-based studies investigating the bacteria of freshwater snails as these infections might obscure other biological factors under investigation.

Next, we focused on developing a protocol to manipulate the bacteria of snails. We removed all bacteria from snails with a sterilization protocol before introducing bacteria from other snail species so there would be no competition amongst the bacteria. We show, for the first time, that bacterial transplants between snails are feasible. Furthermore, snails that received bacteria from closer relatives survived better than those from distantly related snail species.

Interestingly, some snail populations in Cuba appear resistant to trematode parasites. Comparing these resistant snails with their invasive counterparts that do transmit trematodes could therefore provide valuable insights. We exchanged the bacteria between snails transmitting the parasite and those not transmitting the parasite to boost their resistance to trematode parasites. However, exchanging the bacteria between these snails did not affect the resistance of the snail host, but we did find a clear impact of parasite infection on the snail’s

bacteria. Then we went into the field in Zimbabwe to study how trematode diseases are affected by human influence on the environment. These disturbed habitats are easily overtaken by invasive snails, which in turn increase the number of parasites. Surprisingly, we found that the hippopotamus is mostly affected by these parasites. Hence a few tiny snails with their even tinier parasites, might threaten such a large enigmatic animal.

What determines why one snail transmits the parasite while another does not? Only further research can provide a conclusive answer to this question. But this thesis already demonstrated that bacteria can be exchanged between snails, that trematode parasites have a big influence on the snail's bacteria, and that invasive snails causing increased trematode transmission might threaten the enigmatic hippo yet changing the resistance of these invasive snails seems complicated.

Summary

Snail-borne diseases or trematodiases occur globally and are caused by infections with trematode parasites. Today an estimated 250 million people, over a billion livestock animals, and unknown numbers of wild animals suffer from such diseases. This leads to a reduced life expectancy and quality, enormous economic losses, and potential conservation threats. Increased globalized transport, modifications of the environment, and shifts in climatic conditions drive the spread and establishment of freshwater snails and their trematode parasites. Consequentially, transmission patterns are changing in endemic regions and spreading to new areas.

Given the serious impact of trematodiases, broad-scale control efforts have been undertaken to date. However, success has been limited due to inadequate control measures, complicating factors such as anthropogenic impacts on disease transmission, parasite hybridization events, and animal reservoirs. To better control trematodiases, drug treatments should be complemented by intervention methods targeting the snail intermediate hosts. Recently, the microbiome - all microorganisms and their molecules – of freshwater snails has been raised as a potential target for disease control by increasing resistance in wild snails to these trematode parasites. Indeed, different rates of resistance to trematode infection have been described across snail populations, ranging from almost complete susceptibility to complete resistance. Comparing the microbiome across this resistance gradient might provide valuable insights into the drivers of resistance. Moreover, the microbiome is imperative to disease vector competence in other systems whereby certain bacteria have been shown to interrupt the transmission of mosquito-borne diseases by increasing mosquito resistance to these pathogens. However, knowledge about the freshwater snail-associated microbiome is currently limited yet critical for microbiome-mediated disease control in the field. Therefore, this thesis aims to investigate to what extent the interaction between snails and the bacterial aspect of their microbiome could contribute to resistance against trematodes and how trematode infections shape the snail's bacterial profiles.

The thesis commences by investigating the bacterial communities and established parasite infections of *Biomphalaria glabrata* snails artificially exposed to various species and strain combinations of the trematode genus *Schistosoma*. Our findings reveal that parasite species, co-infection dynamics and key parasite development stages within the snail correlate with different bacterial profiles. This is important information for future studies investigating

correlative patterns in the field, as infection status and parasite development stage can shift microbial patterns, potentially obscuring other biological interactions under investigation.

Subsequently, the thesis explores the development of a transplant protocol required to delineate a potential causative effect of the snail-associated microbiome on trematode resistance. As a result, a new protocol is developed to obtain germ-free *B. glabrata* snails and the first-ever successful transplant of the bacterial aspect of the microbiome in freshwater snails is reported. Furthermore, resistance-inducing bacteria in our snail-trematode system may exist in other snail species. Therefore, the thesis incorporates a phylogenetic gradient of donor snails to assess the potential scope of microbial donors and their effect on snail fitness. Snails receiving the microbiome from a closely related host are more likely to survive, corroborating an earlier reported phylosymbiosis pattern between freshwater snails and their bacterial profiles.

Pseudosuccinea columella is a globally invasive snail, which has been linked to increased liver fluke infections in its invasive range. In contrast to the highly susceptible invasive populations, Cuba is home to six completely resistant *P. columella* populations. The *P. columella*-associated microbiome might contribute to its resistance to liver fluke infection, as vector competence can be determined by the host-associated microbiome. Therefore, the thesis describes a reciprocal transplant experiment of the bacterial aspect of the microbiome between resistant and susceptible populations of *P. columella*, followed by parasite exposure to *Fasciola hepatica*. Although transplantation was feasible, it did not shift susceptibility/resistance patterns to *F. hepatica* in *P. columella*. In contrast, parasite exposure did shape bacterial communities, which suggests a potential bacteria-mediated transgenerational effect of parasite resistance. Identifying the exact drivers of resistance will be imperative for effective disease control measures, especially given the ongoing invasions and recent introduction of *P. columella* to Southern Europe. Next, the thesis investigates the impact of habitat modification and the subsequent introduction of invasive snail species on the diversity and transmission of trematode parasites of the common hippopotamus, *Hippopotamus amphibius*. In doing so, the thesis reports on increased transmission of the liver fluke of the hippopotamus, *Fasciola nyanzae,* through the invasive snail *P. columella* in what is known as *parasite spillback*. Trematode infections combined with other stressors have been linked to declining wild herbivore populations, suggesting major consequences for hippopotamus conservation efforts.

In conclusion, while this thesis does not identify bacteria that induce resistance to trematode parasites, it does provide a novel toolset that enables future research to disentangle the role of the microbiome in the biological traits of freshwater snails. Furthermore, this thesis

significantly advances our knowledge of the interaction between parasites, snails and their bacteria while shedding more light on the potential threat of invasive snails to wildlife, livestock and human populations around the world.

Samenvatting

Parasitaire platwormen genaamd ”trematoden” komen wereldwijd voor en worden overgedragen door zoetwaterslakken. Infecties met deze parasieten veroorzaken ernstige ziektes in naar schatting 250 miljoen mensen, meer dan een miljard gedomesticeerde dieren en een onbekend aantal wilde dieren. Dit leidt tot een kortere levensverwachting, enorme economische verliezen en problemen bij de conservatie van wilde dieren. Toegenomen wereldwijd transport, aanpassingen van het milieu en verschuivingen in klimatologische omstandigheden bepalen steeds meer de verspreiding en vestiging van zoetwaterslakken en trematode parasieten. Als gevolg hiervan nemen infecties toe in endemische regio’s en verspreiden ze zich naar nieuwe gebieden.

Gezien de ernstige gevolgen van trematode infecties, zijn er tot op heden grootschalige bestrijdingspogingen ondernomen. Het succes is echter beperkt gebleven door ontoereikende bestrijdingsmaatregelen, de door de mens veroorzaakte verandering in ziekte overdracht, hybridiserende parasieten en dierlijke reservoirs waaruit de ziekte opnieuw kan opduiken. Een effectieve bestrijding van deze ziektes vereist daarom een combinatie van maatregelen die de parasiet zowel in de eindgastheer als in de slakkentussengastheer aanpakt. Het microbioom - alle micro-organismen en hun moleculen - is cruciaal voor de competentie van ziektevectoren in andere systemen. Zo zorgen microbioom manipulaties in muggen tot een verminderde overdracht van muggenoverdraagbare ziekten naar de mens. Daarom is het microbioom van zoetwaterslakken recentelijk naar voren gekomen als een potentieel doelwit voor de bestrijding van trematode parasieten. Er is echter weinig gekend over het microbioom geassocieerd met zoetwaterslakken. Dit proefschrift onderzoekt in welke mate de interactie tussen slakken en het bacteriële aspect van hun microbioom bijdraagt aan weerstand tegen trematoden.

Het proefschrift begint met het onderzoeken van de bacteriën en de infectiestatus van *Biomphalaria glabrata* slakken blootgesteld aan verschillende combinaties van *Schistosoma* soorten en stammen. Onze bevindingen onthullen dat parasietsoorten, co-infectiedynamieken, en cruciale parasietontwikkelingsstadia binnen de slak correleren met verschillende microbioom samenstellingen. Deze informatie is belangrijk voor toekomstige studies die correlatie patronen in het veld onderzoeken, aangezien infectiestatus en het ontwikkelingsstadium van de parasiet microbiële patronen kunnen doen verschuiven. Bijgevolg kunnen andere biologische interacties die onderzocht worden mogelijks verhult blijven.

Vervolgens beschrijft het proefschrift de ontwikkeling van een hulpmiddel dat helpt om een causaal verband aan te tonen tussen bacteriële gemeenschappen in slakken en hun weerstand tegen trematode infecties. Met dit protocol verkrijgen we bacterievrije *B. glabrata* slakken en wordt voor het eerst een succesvolle transplantatie van bacteriën tussen verschillende zoetwaterslakken beschreven. Bovendien is het mogelijk dat resistentie-veroorzakende bacteriën bestaan in andere slakken soorten. Daarom omvatte dit experiment donor slakken met verschillende evolutionaire afstanden tot onze ontvanger slakken. Hierdoor zagen we dat slakken die het microbioom van een nauw verwante slak ontvangen, een grotere overlevingskans hebben, wat een eerder gerapporteerd fylosymbiosepatroon tussen zoetwaterslakken en hun microbioom bevestigt.

Pseudosuccinea columella is een invasieve slak die in verband wordt gebracht met verhoogde leverbotinfecties in recent gekoloniseerde regio's. In tegenstelling tot de hoge vatbaarheid van de invasieve populaties, is Cuba de thuisbasis van zes volledig resistente *P. columella* populaties. Het aan *P. columella* geassocieerde microbioom kan een belangrijke rol spelen in zijn weerstand tegen leverbotinfectie, aangezien vectorcompetentie kan worden bepaald door het gastheer-geassocieerde microbioom. Daarom beschrijft het proefschrift een transplantatie-experiment van het bacteriële aspect van het microbioom tussen resistente en vatbare populaties van *P. columella*, gevolgd door blootstelling aan de parasiet *Fasciola hepatica*. Hoewel transplantatie mogelijk was, veranderde het de vatbaarheidspatronen van *P. columella* tegen *F. hepatica* niet. Daarentegen vormde parasietblootstelling de bacteriële gemeenschap wel, wat wijst op een potentieel transgenerationeel effect van parasietweerstand door het microbioom. Het identificeren van de exacte oorzaken van weerstand of vatbaarheid zal een cruciaal aspect vormen in effectieve ziektebestrijdingsmaatregelen, vooral gezien de aanhoudende invasies in nieuwe gebieden waaronder de recente introductie van *P. columella* in Zuid-Europa. Hierna onderzoekt het proefschrift de impact van habitatmodificatie en de daaropvolgende introductie van invasieve slakken soorten op de diversiteit en transmissie van trematode parasieten van het gewone nijlpaard, *Hippopotamus amphibius*. Hierbij rapporteert het proefschrift over verhoogde transmissie van de leverbot van het nijlpaard, *Fasciola nyanzae*, door de invasieve slak *P. columella*. Trematode-infecties gecombineerd met andere stressoren zijn eerder al in verband gebracht met afnemende populaties wilde herbivoren, wat grote gevolgen kan hebben voor de conservatie-inspanningen voor nijlpaarden.

Tot slot, hoewel dit proefschrift geen bacteriën identificeert die weerstand induceren tegen trematode parasieten in slakken, biedt het wel een nieuw protocol waarmee toekomstig onderzoek de rol van het microbioom in biologische eigenschappen van zoetwaterslakken kan achterhalen. Bovendien vergroot dit proefschrift onze kennis over de interactie tussen

parasieten, slakken en hun bacteriën. Daarnaast toont dit onderzoek de potentiële dreiging die invasieve slakken wereldwijd kunnen vormen voor wilde dieren, vee en menselijke populaties.

List of abbreviations

AIC	Akaike information criterion
ANCOVA	Analysis of covariance
ANOVA	Analysis of variance
ASV	Amplicon sequence variant
PCR	Polymerase chain reaction
RD-PCR	Rapid diagnostic PCR
qPCR	Quantitative PCR
COI	Cytochrome oxidase subunit 1
DNA	Deoxyribonucleic acid
rRNA	Ribosomal RNA
ITS	Internal transcribed spacer
GLM	Generalized linear model
RDA	Redundancy analysis
NMDS	Nonmetric multidimensional scaling
VIF	Variance inflation factor
WHO	World health organization
BLAST	Basic local alignment search tool
BOLD	Barcode of life data system
NCBI	National center for biotechnology information
Bp	Base pair
ML	Maximum likelihood
OD	Optical density
R2A	Reasoner's 2A

Use of Generative AI

I did use the generative AI assistance tool ChatGPT

ChatGPT was used for generating R code to transform code sections into functions for easy adaptation to different parameters, to optimize runtime, to debug errors, and as a prompt-based search engine.

ChatGPT and Dall-E were used to generate the background visuals of the title page of Chapter 2.

The text/code/ images in this thesis are my own (unless otherwise specified) and generative AI has only been used in accordance with the KU Leuven guidelines and appropriate references have been added. I have reviewed and edited the content as needed and I take full responsibility for the content of the thesis.

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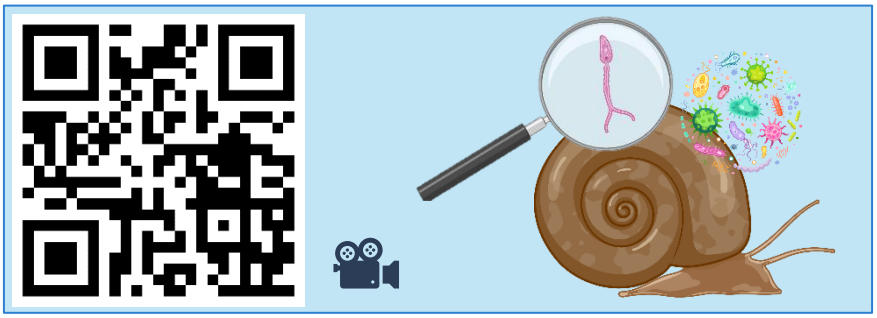
Chapter 1

General introduction

The final section of the introduction (tripartite interactions) incorporates my personal contribution to “Stock*, W., Callens*, M. *et al.* (2021) ‘Human impact on symbioses between aquatic organisms and microbes’, *Aquatic Microbial Ecology*, 87, pp. 113–138. Doi: 10.3354/ame01973” with permission from the leading authors.

*: These authors contributed equally

Introduction video:



1.1. Trematode parasites as snail-borne diseases

1.1.1. Freshwater snails

The common name ‘snails’ refers to the prosobranch and pulmonate representatives of the phylum Mollusca: class Gastropoda (Strong *et al.*, 2008). Prosobranchs are generally characterized by separate sexes, and thus, sexual reproduction; an operculum, a hard plate capable of closing the aperture or shell opening; gills for respiration; and specific habitat requirements. In contrast, pulmonates, which are hermaphroditic and can therefore self-fertilize, lack an operculum, respirate through a rudimentary lung, and have broader environmental tolerances (Strong *et al.*, 2008). Both groups, along with bivalves, are highly diverse and have colonized almost the entire world (Strong *et al.*, 2008). Over 7,000 freshwater mollusc species have been described with an estimated three to 11 thousand still awaiting discovery (Lydeard *et al.*, 2004).

Nevertheless, freshwater snails are one of the most imperilled groups in the aquatic ecosystem, with a third of the species listed as threatened and another third as data deficient (Lydeard *et al.*, 2004; Allen *et al.*, 2011; Böhm *et al.*, 2021). Almost half of the 693 recorded animal extinctions are molluscs (Lydeard *et al.*, 2004). Unsurprisingly, anthropogenic pressures lie at the source through artificial lake construction, water pollution, invasive species, and climate change (Allen *et al.*, 2011). Losing this diversity could have far-reaching consequences as snails are part of complex ecological interactions in the aquatic ecosystem through for example cascading effects of epiphyton grazing (Brönmark, 1989). Additionally, freshwater snails have a major economic impact due to their role as intermediate hosts of trematode parasites, which cause disease in human and animal populations (Allen *et al.*, 2011; Toledo and Fried, 2014).

1.1.2. Trematodes

Trematodes belong to one of the largest clade of obligate parasites, the Neodermata (Phylum: Platyhelminthes), together with cestodes and monogeneans (Littlewood *et al.*, 1999). Interestingly, the latter has recently been subject to debate as novel genomic data and analyses propose the elimination of this taxon and replacement by its daughter taxa Monopisthocotyla, which are ectoparasitic epithelial feeders, and Polyopisthocotyla, which are mostly hematophagous ectoparasites (**Figure 1**) (Brabec *et al.*, 2023). Trematodes are obligate endoparasites with a complex multi-host lifecycle, usually involving a snail first intermediate host and a vertebrate definitive host (**Figure 2**) (Toledo and Fried, 2014). The class Trematoda consists of two subclasses: the smaller group Aspidogastrea, infecting fish and turtles, and the highly diverse Digenea constituting over 99% of trematode diversity,

infecting a broad range of intermediate and definitive hosts (Gibson *et al.*, 2002; Toledo and Fried, 2014). The Aspidogastrea have a one- or two-host lifecycle, while digenean lifecycles can involve two, three or four hosts with three hosts being the most common (Shoop, 1988). Besides the number of hosts involved in the lifecycle of digeneans, further diversity arises from the different host species involved, the mode of transmission between hosts, and the reproductive modes within hosts (Shoop, 1988; Toledo and Fried, 2014).

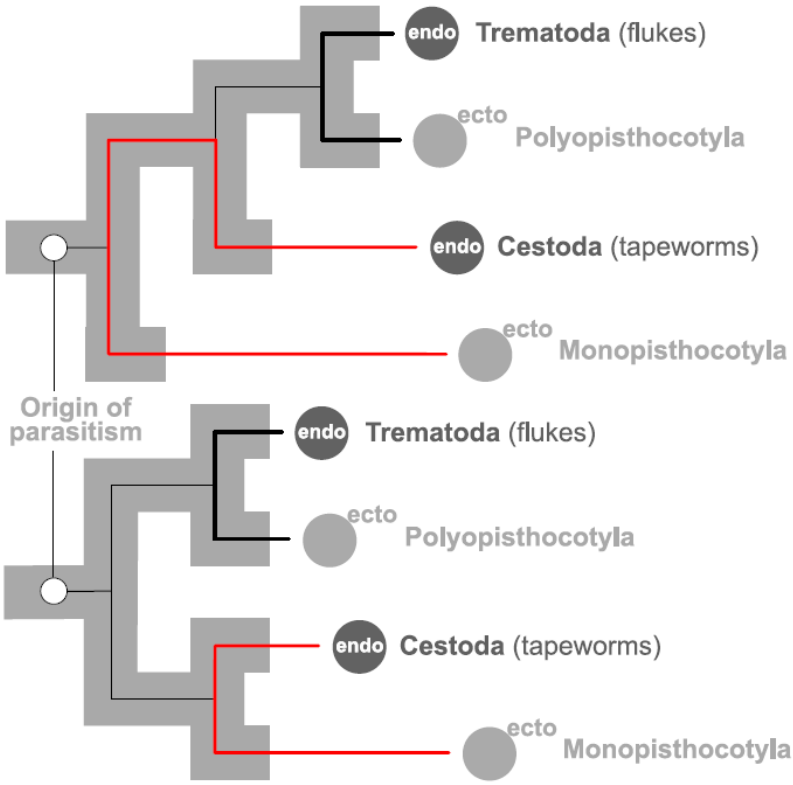


Figure 1: The four parasitic groups of the Neodermata as proposed by Brabec *et al.* (2023). Phylograms were calculated based on 255 genes whereby the top phylogram shows the maximum likelihood phylogeny and the bottom the Bayesian inferred phylogeny. The taxa Monopisthocotyla and Polyopisthocotyla were considered daughter taxa of the Monogenea prior to Brabec *et al.* (2023). “endo” refers to endoparasites, while “ecto” refers to ectoparasites. Figure reproduced from Brabec *et al.* (2023) with permission from the leading author.

Schistosoma mansoni, a model parasite recurrent throughout this thesis, is used as an example to discuss the general trematode lifecycle outline (**Figure 2**). Adult *S. mansoni* are blood parasites found in the mesenteric veins of mammalian hosts. The adults produce eggs that penetrate the vein and organ lining to eventually be released into the environment with faeces. Once fully developed and if the egg is in contact with freshwater the larva, called miracidium, emerges. This miracidium will then attempt to infect a suitable intermediate snail host. Within the snail, the miracidium initiates asexual reproduction by transforming into a mother sporocyst within the next two days. Over the next two weeks, daughter sporocysts

develop within the mother sporocyst, which then migrate to the digestive glands of the snail. Here, cercariae start to develop and are released into the environment in the following days during a process that is called *shedding*. The released cercariae swim around actively searching for a final host to infect. Once found, the cercariae rapidly lose their tail when penetrating the skin of their new host and transform into schistosomulae within hours. Next follows migration throughout the circulatory system of their definitive host until the schistosomulum reaches the liver where it transforms into juvenile worms. Roughly five weeks later the male worm grabs the female worm (see inset **Figure 2**) and carries her to its preferred reproductive site, the veins of the mesentery, restarting the lifecycle.

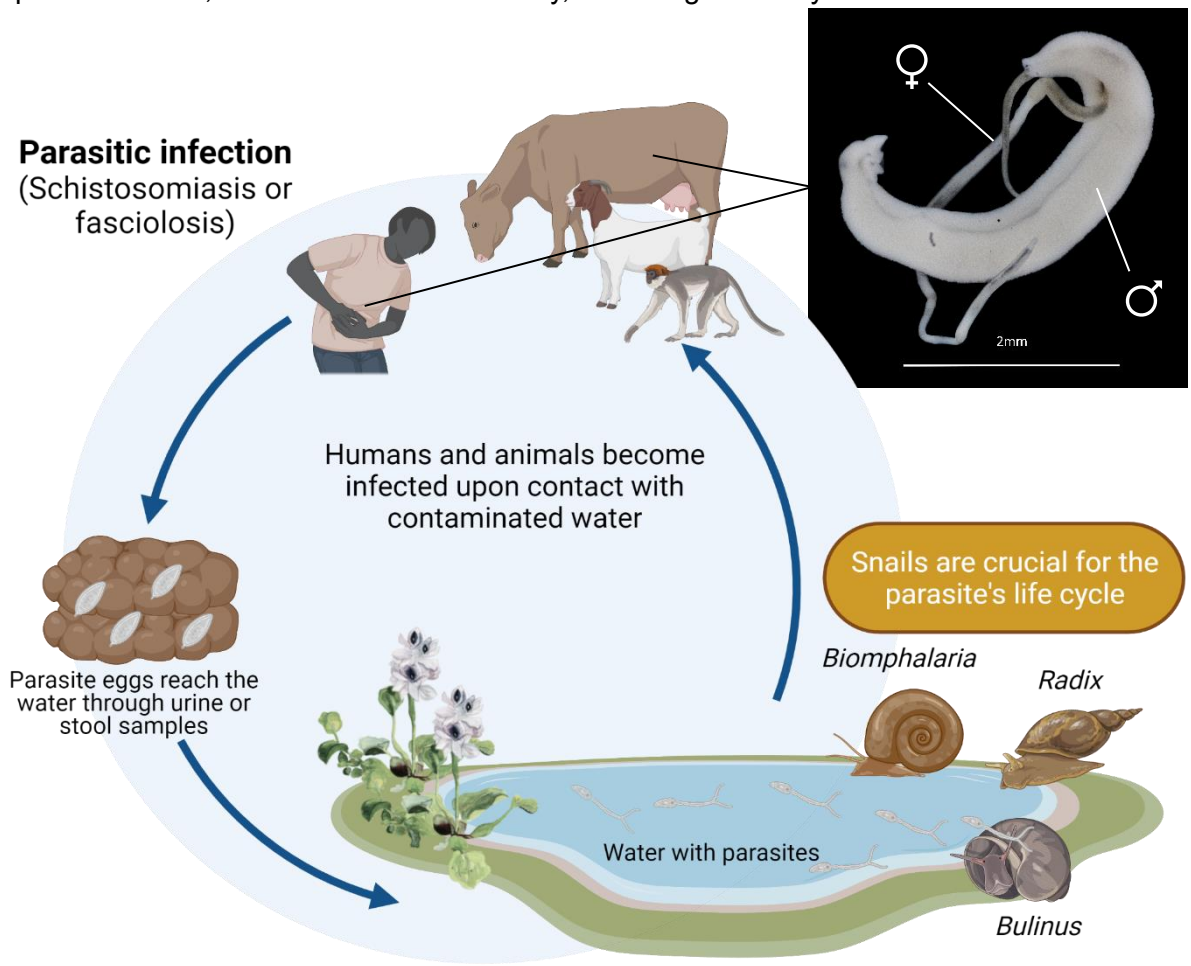


Figure 2: Generalized lifecycle for trematode parasites. Adult parasites in definitive hosts (human or animal) release eggs with excreta, from these larval stages called 'miracidia' hatch and infected snail intermediate hosts. Once infections have developed, the next larval stage 'cercariae' emerge from the snail and restart the cycle by infecting a new definitive host. Diseases as a consequence of infections with *Schistosoma* spp. or *Fasciola* spp. parasites are referred to as schistosomiasis and fasciolosis, respectively. Figure courtesy of Noelia Valderrama. Created with BioRender. Figure inset: *Schistosoma mattheei* collected by Schols R and Mudavanhu A from mesenteric veins of cattle in Zimbabwe. The larger male holds the smaller female in its gynecophoral canal.

The intricate snail-trematode interaction already starts before the intermediate host and parasite come into contact. Chemical cues released by snails can critically shape miracidial finding behavior and thereby transmission success (Magalhães *et al.*, 1997; Hertel *et al.*, 2006). Next, the schistosome must avoid destruction by the snail's immune system to successfully establish an infection (Núñez *et al.*, 1994; De Jong-Brink *et al.*, 2001). This arms-race starts the moment the parasite and snail interact when a miracidium penetrates the head-foot region and rapidly transforms into a sporocyst (Toledo and Fried, 2011). During this stage, the parasite attempts to escape the internal defence system of the snail by modulating hemocyte activity (Núñez *et al.*, 1994; De Jong-Brink *et al.*, 2001). Nevertheless, hemocytes of resistant snails enclose the recently transformed sporocyst within their tissue and kill the parasite (De Jong-Brink *et al.*, 2001). In contrast, the response in susceptible snails is not adequate and in the next two to three weeks mother sporocysts generate clonal daughter sporocysts, still located in the head-foot region (Nacif-Pimenta *et al.*, 2012). Next, daughter sporocysts migrate to the gonad-digestive gland region to further multiply asexually, inflicting significant damage in the process (Faro *et al.*, 2013; Tavalire *et al.*, 2016). This damage leads to partial or complete destruction of ovotestis tissue, typically castrating the snail host (Faro *et al.*, 2013; Tavalire *et al.*, 2016). Cercariae require arginine and glucose to mature within the daughter sporocyst thereby continuously absorbing large energy reserves of the gonad-digestive gland region during their development (Faro *et al.*, 2013). Combined, these processes reveal a direct (destruction of tissue) and indirect (snail starvation through nutrient absorption) effect on snail fitness (Faro *et al.*, 2013). However, once matured, cercariae require only a minimal supply of energy for migration and emergence which happens approximately four weeks after miracidial exposure of the snail (Faro *et al.*, 2013).

As mentioned earlier, significant variations on this lifecycle exist with usually high host specificity and different levels of complexity, giving rise to the many species seen today and over 90% still waiting to be discovered (Littlewood *et al.*, 1999; Hechinger, 2023). Nevertheless, despite allowing many speciation events, the specialized and complex lifecycles also make most trematodes especially vulnerable to extinction (Cizauskas *et al.*, 2017; Wood *et al.*, 2023). Losing trematodes could have unexpected consequences as parasites can be part of up to three-quarters of food web links, constitute a major proportion of ecosystem biomass, and regulate host populations (Lafferty *et al.*, 2006; Kuris *et al.*, 2008; Sato *et al.*, 2011). Consequentially, the idea of parasite conservation has been gaining traction (Cizauskas *et al.*, 2017; Carlson *et al.*, 2020; Kwak *et al.*, 2020; Milotic *et al.*, 2020), with the recent establishment of an IUCN SSC parasite specialist group as pinnacle (https://iucn.org/our-union/commissions/group/iucn-ssc-parasite-specialist-group). Nevertheless, certain

trematodes do impose a severe economic and public health burden (Charlier *et al.*, 2014; WHO, 2023).

1.1.3. Trematode burden and control

The most impactful of these snail-borne diseases (SBD) is schistosomiasis. It is caused by blood flukes of the genus *Schistosoma* and affects over 200 million people globally with the largest burden in Sub-Saharan Africa (WHO, 2023). Some of the consequences of a schistosome infection include an increased chance of developing bladder cancer and contracting HIV while reducing fertility and causing kidney and liver disease (Forsyth and Macdonald, 1965; King and Dangerfield-Cha, 2008; WHO, 2016). Similarly, the second most impactful SBD, fasciolosis, is caused by liver flukes of the genus *Fasciola*. It infects an estimated 2.4 - 50 million people and millions more animals across all continents except Antarctica (Mas-Coma *et al.*, 2009; Toledo and Fried, 2014; Nyindo and Lukambagire, 2015). Some consequences include severe liver disease and gastrointestinal pain in humans in addition to reduced milk production for dairy cattle (Schweizer *et al.*, 2005; Mas-Coma *et al.*, 2009). With millions of people and animals suffering from SBDs, resulting in increased mortality and billions of economic losses, concerted and broadscale control efforts have been undertaken to this date (Wright, 1972; Charlier *et al.*, 2014; Sokolow *et al.*, 2016; WHO, 2016, 2023; Mehmood *et al.*, 2017; Mutapi *et al.*, 2017).

Control measures can target any step involved in the complex life cycle of schistosomes (**Figure 3**) (Mahmoud, 2001; Sokolow *et al.*, 2016). Firstly, the availability of clean drinking water and awareness raising prevents exposure to contaminated waters and hence the infective stage, cercariae. Preliminary data indicates that soap might offer a certain degree of protection against cercariae, but further research is required before it can be used in control efforts (Lemma, 1970; Zhang *et al.*, 2022). The natural soap obtained from the endod soapberry, *Phytolacca dodecandra,* seems to be the most promising and sustainable option (Lemma, 1970; Erko *et al.*, 2002; Abebe *et al.*, 2005). Nevertheless, clean drinking water is frequently unavailable or water contact is required for food collection hence infections still occur. Currently, no effective vaccine against schistosomiasis infection exists (Molehin, 2020; WHO, 2020). Therefore, curative treatments led by the drug Praziquantel remain the golden standard to treat human populations (Mutapi *et al.*, 2017). It is a highly effective and affordable chemotherapeutic drug (Kokaliaris *et al.*, 2022), that has extended its use to 250 million tablets being distributed for mass-drug administration programmes across the disease's range (Mutapi *et al.*, 2017; WHO, 2020). Nevertheless, it does not prevent re-infection nor is it effective against juvenile worms (Chandiwana *et al.*, 1991; Pica-Mattoccia and Cioli, 2004). To avoid open defecation and prevent eggs from entering the environment, proper sanitation and

awareness are required, which are often lacking (Anyolitho *et al.*, 2022). Recently, control efforts are steering away from the typical top-down approach and are adopting bottom-up structures through citizen science, which empower communities and improve behavioural change to control the disease (Ashepet *et al.*, 2021; Anyolitho *et al.*, 2024; Tumusiime *et al.*, 2024). Simultaneously, citizen scientists can help attain broad-scale snail monitoring datasets, contributing to more efficient snail control measures which is critical for schistosomiasis control (Sokolow *et al.*, 2016).

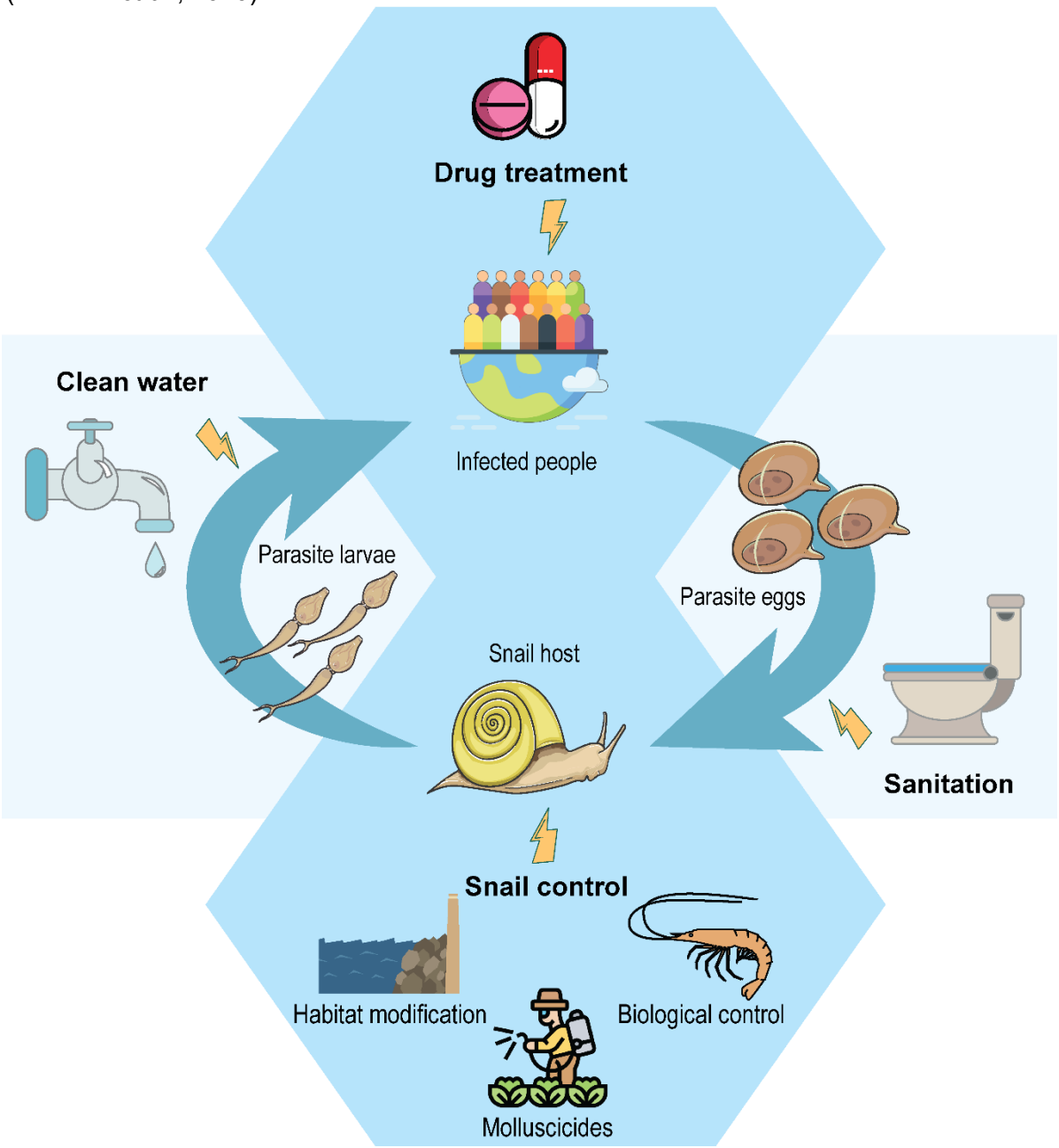


Figure 3: Current schistosomiasis control measures targeting the different schistosome life stages. Infected people can be treated with medicine such as Praziquantel. Parasite eggs can be prevented from reaching the environment with reliable sanitation. Snail control aimed at reducing snail numbers is currently conducted through habitat modification, applying molluscicides, or biological control. Contact with the infectious stage, cercariae, can be

prevented by providing reliable and clean drinking water. Figure was constructed based on the scheme shown in Sokolow *et al.* (2016).

Today snail control depends on the use of chemical molluscicides, habitat modification and biological control. These chemicals theoretically only target snails, however, in field settings frequently lead to mass mortality in other aquatic life such as fish and amphibians and cause concern for human health (Dai *et al.*, 2008; Sokolow *et al.*, 2016). Habitat modification involves the removal of aquatic vegetation, the main substrate for snails, which in turn may be used as cattle feed or as a soil fertilizer and boost crop yield (Rohr *et al.*, 2023). The water contact associated with such removal, a key transmission factor for schistosomiasis, makes protective gear imperative. Other habitat modifications such as irrigation schemes and artificial lakes can set the stage for unexpected epidemics (Picquet *et al.*, 1996; Grabner *et al.*, 2014; Sokolow *et al.*, 2017; Carolus *et al.*, 2019). Biological control has been tried through snail predators and competitors yet can have unexpected impacts on the ecosystem (Howarth, 1991; Appleton *et al.*, 2009; Ip *et al.*, 2014; Van Bocxlaer *et al.*, 2015; Sokolow *et al.*, 2016). Despite extensive control efforts to date, schistosomiasis continues to (re-)emerge across and beyond its distribution range with startling intensities (Picquet *et al.*, 1996; Boissier *et al.*, 2016; Sokolow *et al.*, 2018). In a bid to eliminate schistosomiasis as a public health concern by 2030, the WHO expressed a pressing need to improve our scientific understanding and create affordable and accurate diagnostic tools for effective disease interventions (WHO, 2020).

1.1.4. Trematodiases – complicating factors

Human impacts can manifest through globalization, climate change, pollution, and land use change. Increased connectivity through globalisation facilitates the spread of invasive species (Hulme, 2009) and presumably aided the introduction of Senegalese schistosomes in Corsica, France (Boissier *et al.*, 2016). Moreover, climate change-associated temperature rise is expected to increase distribution ranges throughout Europe of *Bulinus truncatus,* and thereby urogenital schistosomiasis since the parasite already tolerates these lower temperatures (Mulero *et al.*, 2019; De Leo *et al.*, 2020; Maes, 2024). Besides climate change, pollution and eutrophication could further increase SBD incidence. To meet food requirements intensified agriculture is required throughout developing areas (Godfray *et al.*, 2010), and consequentially environmental agrochemical concentrations are expected to increase (Snyder *et al.*, 2018; Ciceri and Allanore, 2019). Most of these agrochemicals have been linked to increased snail populations by, for example, eliminating predators or increasing the algal food source (Halstead *et al.*, 2018). Consequentially, models predict up to three times higher reproductive numbers for *S. haematobium*, leading to a much higher burden on the human population (Hoover *et al.*, 2020). The effect of agrochemicals is not limited to humans, as increased

trematode infections could in part explain amphibian decline (Rohr *et al.*, 2008). Water availability is also crucial for agriculture and combined with the need for hydropower, has been the driver for dam construction and artificial lake creation (Tortajada *et al.*, 2012; De Leo *et al.*, 2020). Nevertheless, blocking natural water flow and creating stagnant waters comes at a cost (McAllister *et al.*, 2001). SBDs have increased on many occasions due to the shift in the aquatic ecosystem, interrupted migration routes of fish and crayfish, and facilitated biological invasions associated with the creation of artificial lakes (Talla *et al.*, 1990; Havel *et al.*, 2005; Sokolow *et al.*, 2017; Carolus *et al.*, 2019).

This non-exhaustive list of anthropogenic impacts underlines the need for a *One Health* framework for SBDs whereby human and environmental health are intertwined (Degeling *et al.*, 2015; Webster *et al.*, 2016; Gower *et al.*, 2017). Moreover, it reveals how complex SBDs truly are, potentially explaining their persistence and (re-)emergence (Braks *et al.*, 2014; Toledo and Fried, 2014; Boissier *et al.*, 2016; Sokolow *et al.*, 2016; Webster *et al.*, 2016). One such complicating factor is hybridization. It is frequently reported for schistosomes and fasciolids and can result in hybrid vigour and broader host ranges (Wright and Ross, 1980; Morgan *et al.*, 2003; Hoa Le *et al.*, 2008; Huyse *et al.*, 2009; Boissier *et al.*, 2016; Nguyen *et al.*, 2018). This increased host range further complicates the already existing animal reservoirs and zoonotic tendencies of the disease (Huyse *et al.*, 2009; Toledo and Fried, 2014; Webster *et al.*, 2016; Gower *et al.*, 2017; Leger and Webster, 2017; You *et al.*, 2018; Alba *et al.*, 2021). These non-human sources of infection opened the discussion about treating animal reservoirs (Gower *et al.*, 2017), although they may increase the establishment of drug resistance (Webster *et al.*, 2016). For human populations, warnings on schistosome resistance to praziquantel have frequently emerged however remain controversial (Nuno *et al.*, 2017). Nevertheless, resistance to other drugs such as oxamniquine is widespread, revealing a cause for concern (Chevalier *et al.*, 2019). Notably, animal treatments for other helminths such as liver flukes do occur, yet targeted triclabendazole treatments against *Fasciola hepatica* are hypothesized to be partially at the basis of increased stomach fluke infections by *Calicophoron daubneyi* in European cattle by shifting intra-snail competition (Rondelaud *et al.*, 2007, 2022; Jones *et al.*, 2017). Similarly, Laidemitt *et al.* (2019) showed that within the snail *Biomphalaria pfeifferi*, the larvae of the amphistome *Calicophoron sukari* antagonize the development of *Schistosoma mansoni* yet require the latter for development within the snail host. Clearly, parasite-parasite interactions within a snail host can have a great influence on human and animal SBD epidemiology (Laidemitt *et al.*, 2019). Nevertheless, studying these parasite interactions remains challenging as species identifications are hindered due to a lack of taxonomists and reference sequences (Brant *et al.*, 2006; Schols *et al.*, 2020; Vinarski, 2020).

Similarly, some snail groups remain hard to identify due to cryptic species and species complexes despite the importance of accurate snail identification in assessing snail-parasite compatibility (Mukaratirwa *et al.*, 1998; Schniebs *et al.*, 2016; Babbitt *et al.*, 2023). Notably, such compatibilities go deeper than the species level, with dramatic differences at the population level within species. Noteworthy examples exist for fasciolosis and schistosomiasis. *Pseudosuccinea columella* transmits *Fasciola* spp., causative agents of fasciolosis or liver rot in wildlife, livestock and human populations (McCully *et al.*, 1967; Toledo and Fried, 2014). It is one of the major vector snails of *F. hepatica* (Krull, 1933; Dar *et al.*, 2015; Alba *et al.*, 2019) worldwide and increases the transmission of *F. nyanzae* (Carolus *et al.*, 2019; Schols *et al.*, 2021) and *F. gigantica* (Grabner *et al.*, 2014). Interestingly, certain naturally occurring *P. columella* populations in Cuba show complete resistance to *F. hepatica* infections, contrasting the high susceptibility of the globally distributed genotype (Alba *et al.*, 2022). After the construction of the Diama dam to embank the Senegal River Basin (SRB) in 1988, an important outbreak of urinary schistosomiasis rapidly ensued (Southgate *et al.*, 2000; Meurs *et al.*, 2012). This form of the disease is caused by *S. haematobium*, which uses *Bulinus globosus* and *Bulinus truncatu*s as intermediate hosts. In the lower part of the valley, *B. globosus* acts as the main snail host for *S. haematobium*, whereas *B. truncatus* does not transmit local *S. haematobium* strains (Picquet *et al.*, 1996; Sène *et al.*, 2004). In the upper valley, however, the situation is reversed and *B. truncatus* is the main host for *S. haematobium*, whereas *B. globosus* is not involved in transmission (Southgate *et al.*, 2000; Sène *et al.*, 2004). Clearly, biological interactions and human impacts play critical roles in the transmission of debilitating SBDs.

Hosts and parasites are in an evolutionary arms race, potentially leading to frequency dependent selection (Decaestecker *et al.*, 2007; Wolinska and King, 2009; Decaestecker and King, 2019). Specifically, parasites will evolve to infect the dominant host genotype, thereby, facilitating the cohabitation of multiple host genotypes in what is called the Red Queen hypothesis (Jokela *et al.*, 2009). The snail-trematode interaction has strong reciprocal selection as snails become castrated upon successful infection or trematodes are destroyed upon failed infection (King *et al.*, 2011; Decaestecker and King, 2019). Similarly, this hypothesis explains why trematode parasites enable sexually reproducing snails to co-exist with asexually reproducing snails, despite the latter having a much higher reproductive potential (Jokela *et al.*, 2009). However, the environment shapes host-parasite interactions thereby altering the strength and direction of selection (Wolinska and King, 2009). The confounding effect of environment on host-parasite coevolution may explain why Red Queen dynamics are so difficult to detect in the wild (Wolinska and King, 2009), but not impossible (Decaestecker *et al.*, 2007; Jokela *et al.*, 2009). One such confounding factor is the microbiome, which can

mediate host-parasite coevolution (Decaestecker and King, 2019). As a result, recent works have proposed that the snail-associated microbiome might be a sustainable and specific target for SBD control (Portet *et al.*, 2021; Le Clec'h *et al.*, 2022; Sun *et al.*, 2024).

1.2. Microbiome

1.2.1. Definitions and Importance

The microbiome is defined as the combination of the microbiota – all living organisms: archaea, fungi, algae, protists, and bacteria – and all of their molecules – including metabolites and structural elements (Berg *et al.*, 2020). Phages, viruses and other mobile genetic elements are, therefore, considered part of the microbiome but not the microbiota, as they are not considered living organisms (Berg *et al.*, 2020). Nevertheless, these "non-living" elements can critically affect other microbiome components. For example, phages can shift natural selection in *Escherichia coli* in the gut of mice whereby phage domestication – maintaining the benefits of phage genes but avoiding cell lysis - leads to directional selection rather than diversifying selection (Frazão *et al.*, 2022). These domesticated phages form an excellent source of genetic diversity, with nearly 35% of the *E. coli* pangenome (genome of the entire species) originating from prophages (Bobay *et al.*, 2014). Furthermore, metabolites that are exchanged between co-occurring and successive yeast cells extend their lifespan (Correia-Melo *et al.*, 2023). More directly, the bacterial Type VI secretion system can be used to inject effector molecules in nearby cells, shifting bacterial competition, even in the mammalian gut, to gain a competitive advantage and secure a niche (Chatzidaki-Livanis *et al.*, 2016; Cianfanelli *et al.*, 2016). Moreover, the Black Queen Hypothesis states that microbes can undergo genome reduction through natural selection if the resulting metabolite is produced by other members of the community and is accessible to that individual (Morris *et al.*, 2012). Macro-ecological effects such as the effect of grazing and predation also present at the microscopic scale. Bacterial communities themselves can be top-down regulated by protist predators, leading to increased bacterial diversity which in turn stabilizes predator performance (Corno *et al.*, 2008; Saleem *et al.*, 2013; Mosca *et al.*, 2016). These studies touch upon the complexity of the microbiome, yet these effects can extend to multicellular lifeforms. Some consider the human microbiome our latest organ under study (Baquero and Nombela, 2012), with the number of bacterial cells rivalling that of human cells (Sender *et al.*, 2016). The human-associated microbiome contains an estimated 250,000 unique genes, vastly exceeding the 20,500 unique genes in the human genome (Zilber-Rosenberg and Rosenberg, 2008).

The microbiome's influence on metazoan biology has become fully established over the last decade (Margulis, 1991; Rohwer *et al.*, 2002; Theis *et al.*, 2016; Berg *et al.*, 2020; Destoumieux-Garzón *et al.*, 2024). The importance of endosymbionts in the evolution of

eukaryotic organisms was first raised by Margulis in 1991. In 2002 a new term was coined by Rohwer and colleagues – *the holobiont* – which refers to the intimate link between, and thus the unity of, a host and its microbiome, of which the latter constitutes a rapidly evolving aspect (Zilber-Rosenberg and Rosenberg, 2008; Alberdi *et al.*, 2016; Theis *et al.*, 2016; Bisschop *et al.*, 2022). The genome of the holobiont, or hologenome, consists of the genome of a host and the genomes of the host-associated microbiome (**Figure 4**) (Theis *et al.*, 2016). Notably, microbial genes in the holobiont can directly affect host phenotype, not affect host phenotype, or coevolved with host genes to affect the host phenotype. In contrast, environmental microbes are not part of the holobiont yet they can become part of it in the future (Theis *et al.*, 2016).

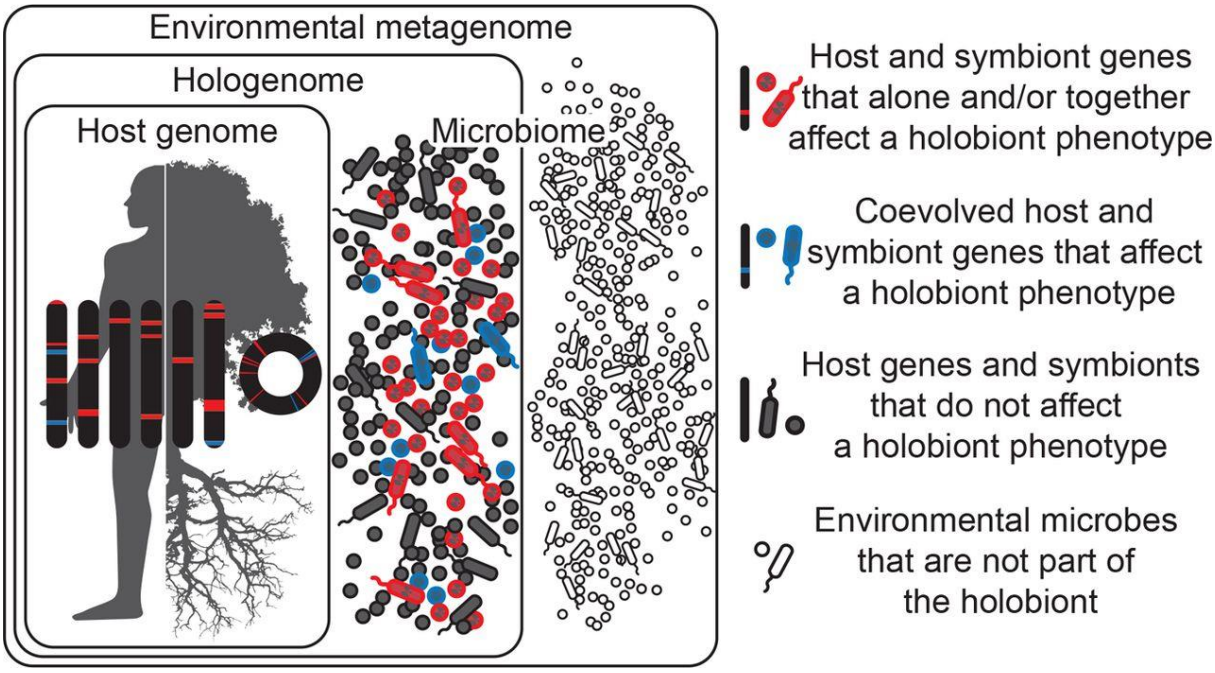


Figure 4: Schematic overview of the hologenome and environmental bacteria (white) as represented in Theis *et al.* (2016). Host and symbiont genes can either directly shape host phenotype (red), have coevolved to shape the host phenotype (blue), or do not affect the host phenotype (dark grey). The hologenome consists of the host genome and the genomes of all symbionts. The figure was reproduced from Theis *et al.* (2016).

Microbial symbiosis usually refers to a larger unicellular or multicellular organism or host colonized by a unicellular microorganism or symbiont (Stock *et al.*, 2021). These microorganisms occur at various sites of a host ranging from the epithelial surface to specialized organs called bacteriocytes (Zilber-Rosenberg and Rosenberg, 2008). The symbiotic interactions can be strong, weak, beneficial, negative or neutral and can change depending on the circumstances (Daskin and Alford, 2012). The endosymbiotic algae providing nutrients while living within corals might be the most well-known mutually beneficial symbiosis (Yellowlees *et al.*, 2008). Another frequently used example is the collaboration between squids and *Vibrio fischeri*, whereby the bacterium resides in a specialized light organ within the squid

allowing hosts to avoid casting a shadow and thus remain camouflaged (Dunlap *et al.*, 2007). Within the molluscs, deep-sea mussels have symbiotic sulphur- and methane-oxidizing bacteria which also protect them from parasitic bacteria infecting the host clam nucleus and feeding on host chromatin (Zielinski *et al.*, 2009). Plant-sap-feeding insects in general are highly dependent on their endosymbionts to obtain sufficient nutrients due to their monotonous and nutritionally simple food sources (Douglas, 2009). However, not all insects depend on symbionts as exemplified by the lack of resident gut bacteria in certain caterpillars (Hammer *et al.*, 2017). Others only depend on endosymbionts when it suits them, cereal-feeding weevils need the *Sodalis pierantonius* symbiont to be able to make a strong cuticle yet subsequently eliminate this bacterium to avoid costs associated with the symbiosis (Dell'Aglio *et al.*, 2023). In contrast to the host ending the mutually beneficial interaction, the deviation may also come from the bacteria's side. Opportunistic pathogens are perhaps the most renowned, whereby usually commensal bacteria and fungi can cause deadly infections in people with a weakened immune system (Overstreet and Lotz, 2016). The shift in pathogenicity can also originate from an environmental trigger whereby a change in, for example, temperature can drastically alter the virulence of the amphibian disease chytridiomycosis (Daskin and Alford, 2012). Mounting evidence points to a shaping effect of human-induced environmental change on the host-associated microbiome (**Figure 5**) (Stock *et al.*, 2021). For example, eutrophication leads to dysbiosis in the skin microbiota of fish (Krotman *et al.*, 2020), antibiotic use to a shift in microbial ecological interactions in water fleas (Callens *et al.*, 2018), and global warming to altering microbial metabolism in the gut of tadpoles (Fontaine and Kohl, 2020). In contrast, the microbiome itself can modify host phenotypes and thereby ultimately affect ecosystem functioning (Moreira *et al.*, 2009; Koch and Schmid-Hempel, 2012; Ridaura *et al.*, 2013; Macke *et al.*, 2017) through adaptations in for example, host physiology (Sudo *et al.*, 2004), behaviour (Desbonnet *et al.*, 2014), and disease resistance (Harris *et al.*, 2009; Lee and Mazmanian, 2010; Graça *et al.*, 2013; McLaren and Callahan, 2020). As mentioned earlier, snails are key members of the aquatic ecosystem (Brönmark, 1989), upon recognition of their importance, scientists have been expanding their efforts to better understand their microbiome.

1.2.2. Snail microbiome

The repertoire of microbiome studies in freshwater snails is limited, therefore, we will occasionally also include studies on slugs and the related class Bivalvia. Investigations on the microbiome of freshwater snails started in the mid-20th century. Chernin and colleagues pioneered the field with the limited resources of the time, developing protocols to obtain axenic (bacteria-free) snails, maintaining them in culture and exposing them to schistosome parasites (Chernin, 1957, 1960; Chernin and Schork, 1959). Some decades later, the bacterial ecology of schistosome vector snails started receiving attention. These works revealed a 10-fold

increase in bacterial cell count when snails were stressed or moribund, suggesting an interaction between the snail immune system and the microbiome, while also revealing the potential for opportunistic pathogens in this system (Ducklow *et al.*, 1979, 1981). Moreover, these earlier works already describe a limited effect of environmental bacteria on the snail microbiome, which is confirmed by recent studies using more sensitive 16S rRNA gene metabarcoding to characterize the bacterial community (Chevalier *et al.*, 2020; Huot *et al.*, 2020). However, this is not a ubiquitous pattern across molluscs as the microbiome of the invasive snail *Potamopyrgus antipodarum,* the invasive bivalve *Corbicula fluminea,* and the snail *Galba truncatula* are affected by the environment (Bankers *et al.*, 2021; Chiarello *et al.*, 2022; McCann *et al.*, 2024). Moreover, a cellulose-rich or cellulose-poor diet modifies the gut microbiome in *Planorbella trivolvis* (Hu *et al.*, 2021), which could also be seen as an environmental effect. The gut microbiome changes throughout the gut and with snail host ageing in *Pomacea canaliculata* (Li *et al.*, 2019; Chen *et al.*, 2021). In the intertidal snail *Littorina saxatilis* different gut microbiome profiles are found depending on the ecotype and the vertical gradient on cliffs (Panova *et al.*, 2023). How far these patterns extrapolate across species is difficult to assess since the snails' phylogeny and their associated microbiome are congruent (Huot *et al.*, 2020), hence patterns could be species-specific.

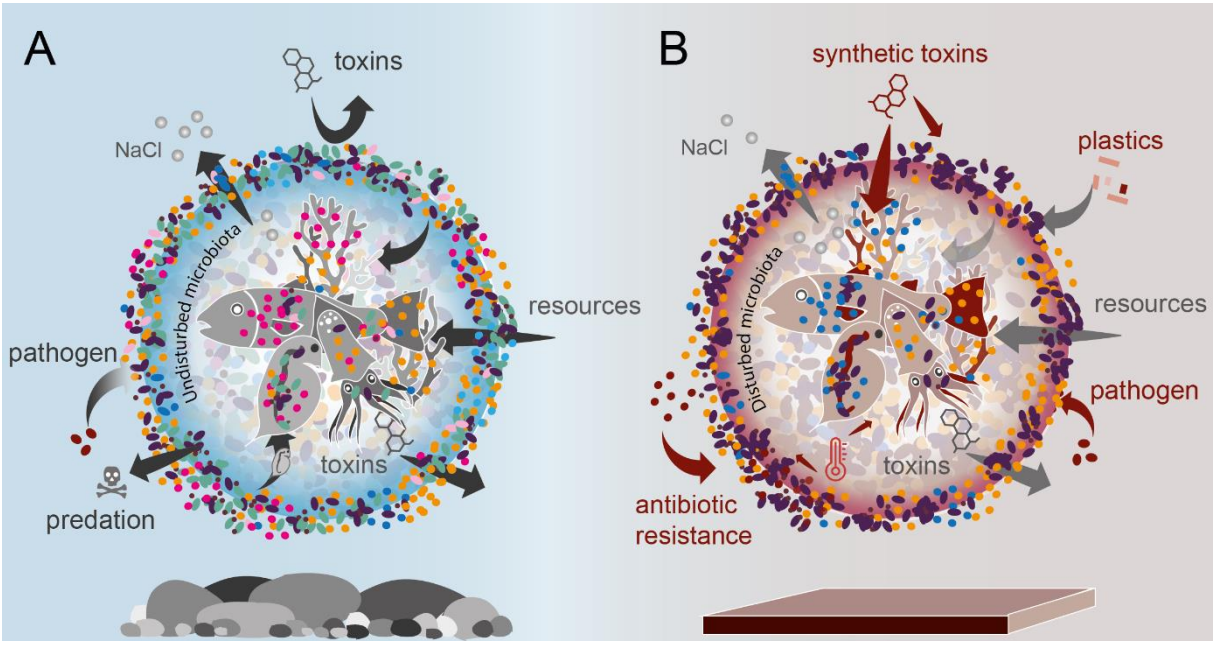


Figure 5: Overview of benefits provided to a host through microbial symbionts and how anthropogenic influences could alter these interactions. A) Beneficial services under natural conditions: Protection against toxins, pathogens and predation, osmoregulation, resource uptake, host development, providing metabolites, and the production of toxins. B) (red arrows) Human-induced disruption of the microbiome can happen through climate change, antibiotic resistance, toxins, pathogenic organisms, and microplastics. (grey arrows) Disturbed microbiota can affect the host through reduced toxin production or metabolisation, reduced

resource acquisition, reduced pathogen resistance, and ineffective osmoregulation. Figure reproduced from Stock *et al.* (2021) with courtesy of Ester M. Eckert.

Not all microbiota are beneficial or neutral for snails. Several examples exist of microbial pathogens of snails yet bivalves have had several reports of DNA-eating bacteria in deep-sea mussels (Zielinski *et al.*, 2009), viral disease in Chinese pearl mussels (Grizzle and Brunner, 2009), and larval mycosis caused by an oomycete infection in Eastern oysters (Grizzle and Brunner, 2009). Limited diseases are reported in freshwater bivalves presumably due to their highly efficient sorting of unwanted particles from their filtrate and sensitive differentiation up to cyanobacteria strain level (Grizzle and Brunner, 2009). Cole and colleagues did provide the first report of a bacterial infection in the freshwater snail *Bulinus jousseaumei* (Cole *et al.*, 1977), which, despite causing nodules in the snail tissue, did not increase mortality nor reduce fertility. More recently, however, *Paenibacillus glabratella* was isolated and described from nodules in *B. glabrata* (Duval *et al.*, 2015). This pathogen is both vertically and horizontally transmitted to other snail individuals, killing the new host within 30 days.

Microbiome transmission mostly occurs between snail individuals horizontally, but vertical transmission also occurs (Lin *et al.*, 2023) albeit not within snail eggs for *B. glabrata* (Schols *et al.*, 2023). Across snail species *Cloacibacterium* sp. and *Aeromonas* sp. have frequently been reported as members of the core microbiome, suggesting cross-generational transmission or consistent re-assimilation from the environment (Lin *et al.*, 2023). This suggests the importance of pseudo-vertical transmission - the indirect transfer of microbiota from parent to offspring through for example feces or nursing (Alberdi *et al.*, 2016)- in this system. Moreover, the reproductive mode as well as trematode resistance of *P. antipodarum* are thought to be linked to the microbial symbionts (Takacs-Vesbach *et al.*, 2016).

1.2.3. Trematode microbiome

Better understanding the microbial symbionts of parasitic organisms might be key to understanding the varying degrees of parasite virulence and transmission, as the microbiome can modify host phenotypes. Indeed, RNA viruses have been reported to increase disease pathology, parasite load, and drug resistance of infections by protozoan *Leishmania* species (Ives *et al.*, 2011). Moreover, increased parasite gene flow and hybridization events seem to stimulate the frequency of this parasite-virus symbiosis (Heeren *et al.*, 2023). Hybridization frequently occurs in both free-living and parasitic flatworms (Théron, 1989; Agatsuma *et al.*, 2000; Henrich *et al.*, 2013; Webster *et al.*, 2013; Singh *et al.*, 2020). Notably, the virome differs between free-living and parasitic flatworms, on most occasions co-diversified with the parasite, and most likely led to the emergence of some vertebrate-associated viruses (Hahn *et al.*, 2020;

Dheilly *et al.*, 2022). For now, though, it remains unclear how such viral associations might affect the outcome of flatworm infections.

Like flatworms, roundworms infect vast numbers of people and animals globally. Recently, it was revealed that the intestinal nematode *Ascaris suum* has a less diverse microbiome than its host yet the parasite's microbiome is structured by that of the host (Midha *et al.*, 2022). The intimate relationship between microbial symbionts and parasites may also serve as an innovative target for disease control to an otherwise difficult-to-manage disease. Indeed, *Wolbachia* bacteria are obligate symbionts of filarial nematodes that are needed for nematode reproduction, development and survival while also increasing parasite survival and facilitating the establishment of chronic infections (Manoj *et al.*, 2021). Targeting this *Wolbachia* symbiont of filarial nematodes through doxycycline kills both adult and juvenile stages of the parasite in humans (Manoj *et al.*, 2021). This provides a viable alternative to the problems associated with current treatments that sometimes require several years, emerging drug resistance, and adverse reactions (Manoj *et al.*, 2021).

The field of trematode microbial symbionts is in its infancy, yet some notable patterns were already discovered. The first description of bacteria closely associated with a trematode was made in 1973 by Morris, describing a bacterial species strongly attached to the tegument of the trematode *Megalodiscus temperatus*, which infects frogs (Morris, 1973). The function remains unknown to this date yet trematode tegument plays a crucial role in avoiding detection by the host's immune system (Mahmoud, 2001), suggesting a potentially relevant role for bacteria in parasite camouflage. *Neorickettsia* are non-obligate intracellular bacteria of trematodes that can jump to vertebrate hosts and cause disease (Toledo and Fried, 2014; Fischer *et al.*, 2017). These bacteria have been shown to occur throughout various tissues in their trematode hosts, which suggests the potential for vertical transmission and horizontal transmission to other trematodes through mating and as mentioned earlier to the vertebrate host (Fischer *et al.*, 2017).

Similarly, parasitic flatworms maintain parts of their microbiome (bacterial and viral component) throughout their lifecycle and various hosts but also possess microbial components unique for each stage and associated host (Hahn *et al.*, 2020; Jorge *et al.*, 2020). This indicates the potential for co-evolution between the trematode and its microbiome (Jorge *et al.*, 2020). In contrast to the phylosymbiosis pattern reported for freshwater snails (Huot *et al.*, 2020), no such pattern appears visible for trematode parasites (Salloum *et al.*, 2023b). Furthermore, trematodes co-infecting a snail host mostly have a unilateral bacterial exchange (Salloum *et al.*, 2023b). Some trematodes can cut their complex multi-host lifecycle short by asexually reproducing within an intermediate host, following that of the first intermediate snail host

(Salloum *et al.*, 2023a). Preliminary results indicate a potential yet weak link between this alternative life cycle and the relative abundance of certain taxa but not the diversity of the microbiome (Salloum *et al.*, 2023a). Similar to social bees, certain trematodes are considered social and have a caste system whereby soldiers protect the trematode against other trematodes and reproducers ensure parasite reproduction and transmission (Poulin *et al.*, 2019). Bacterial communities were shown to differ between both castes whereby the community level is potentially driving the caste classification as no single bacterial taxon was prevalent enough to be indicative of a caste (Jorge *et al.*, 2022a). Moreover, AB exposure would lead to increased proportions of reproducers compared to soldiers, suggesting a possible causative relationship between the microbiome and caste (Jorge *et al.*, 2022a). However, no direct relationship could be shown and similarly exposing the snail host to antibiotics has been indicated to cause a shift in the post-exposure trematode microbiome compared to the pre-exposure microbiome (Jorge *et al.*, 2022b). Potentially, also, a tripartite interaction could be at play with the snail host, trematode parasite and their microbiomes all interacting to determine infection outcome (Le Clec'h *et al.*, 2022), in line with disease resistance reported elsewhere (Harris *et al.*, 2009; Lee and Mazmanian, 2010; Graça *et al.*, 2013; McLaren and Callahan, 2020).

1.2.4. Tripartite interactions

The pathogen-host interaction can have major consequences for the outcome of subsequent infections. For example, in humans and lab models infections with *Schistosoma* spp. can occasionally lead to less severe subsequent infections resulting in, for example, a reduced *Fasciola* spp. worm burden and reduced gastrointestinal complaints associated with *Helicobacter pylori* infections (Abruzzi and Fried, 2011). However, in most cases, prior *Schistosoma* infections increased the severity of subsequent infections. For example, *Salmonella* infections were more persistent, more virulent and caused higher host mortality while *Staphylococcus aureus* infections were linked to higher counts of liver abscesses (Abruzzi and Fried, 2011). However, the host-associated microbiome can also influence host physiology and disease outcome (Ford and King, 2016; Brinker *et al.*, 2019; Greyson-Gaito *et al.*, 2020).

The associated microbiota of aquatic organisms often plays a crucial role in the host's resistance or tolerance to pathogen infection (Kimura and Tomaru, 2014; Dheilly *et al.*, 2015; Greenspan *et al.*, 2019; Davoodi and Foley, 2020; Schellenberg *et al.*, 2020; Portet *et al.*, 2021). For instance, Huot *et al.* (2020) reported a congruence of vector snails' phylogeny and their associated microbiome, combined with variable susceptibility between snail species and even populations of the same species, suggesting that the snail's microbiome might play a role

in parasite resistance. Microbiota-induced pathogen resistance or tolerance has three underlying mechanisms: competition for resources and space, production of antimicrobial substances and stimulation of the host's immune response (Dheilly *et al.*, 2015; Mallon *et al.*, 2015; Knutie *et al.*, 2017).

An example of colonization resistance through resource competition can be found in the associated microbiota of the coral *Acropora palmata*. In this host species, both the commensal microbiota and the white-pox-causing pathogen *Serratia marcescens* are known to employ glycosidases and Nacetyl-glucosaminidase to utilize components present in the coral mucus. Krediet *et al.* (2013) showed that several members of the *A. palmata* microbiota can inhibit the induction of these catabolic enzymes in *S. marcescens*. This inhibition gives the pathogen a competitive disadvantage when growing in coral mucus, which results in reduced virulence.

Antimicrobials produced by the associated microbiota can also play a crucial role in the pathogen resistance of the host. In the marine sponge *Erylus discophorus*, a large and diverse fraction of the associated microbiota is known to produce antimicrobial compounds (Graça *et al.*, 2013). However, sometimes the presence or absence of one specific bacterial strain producing antimicrobial substances can determine pathogen susceptibility or resistance. For example, the presence of the bacterium *Janthinobacterium lividum* in the cutaneous microbiota of the mountain yellow-legged frog *Rana muscosa* leads to a significantly reduced mortality as a consequence of infections by the lethal fungus *Batrachochytrium dendrobatidis*. This bacterial strain is capable of producing the anti-chytrid metabolite violacein, effectively reducing the chytrid pathogen burden on the mountain yellow-legged frog (Harris *et al.*, 2009).

The associated microbiome can also stimulate the host's immune response (Knutie *et al.*, 2017; Murdoch and Rawls, 2019). Knutie *et al.* (2017) showed that early life disturbance of the bacterial communities associated with Cuban tree frog *Osteopilus septentrionalis* tadpoles results in a significantly higher infection by the parasitic worm *Aplectana hamatospicula* in adulthood. In contrast, disturbance of the associated bacterial communities of adult frogs did not result in a higher parasitic burden. This study indicates that the microbiota likely plays an important role in priming the immune system of juvenile frogs, protecting the host from parasites later in life. Indeed, microbial antigens and metabolites are critical for immune system development in human infants (Donald and Finlay, 2023). In zebrafish larvae, the immune response is boosted by an increase in microbiota complexity but can also be affected by specific bacterial strains. By comparing germ-free to conventional zebrafish larvae, it became apparent that many of the immunity-related genes are microbiota-regulated (Murdoch and Rawls, 2019).

It is worth noting that the diversity of the microbiota is often positively correlated with colonization resistance, although a specific mechanistic explanation is often lacking. On the one hand, such a pattern could be caused by competition, as communities with high species richness occupy more niches and hence are more resistant to invading pathogens by leaving less space and nutrients available for the pathogen to exploit (Mallon *et al.*, 2015). On the other hand, it could be that key species providing resistance (e.g. through the production of antimicrobial substances) are often missing in less diverse communities. A positive correlation between microbiota diversity and colonization resistance was observed in the European common frog *R. temporaria*, which showed increased resistance to the emerging Ranavirus with a more diverse skin microbiome (Harrison *et al.*, 2019). When the microbiota diversity in zebrafish is reduced through exposure to the antibiotic olaquindox, they also become more susceptible to infection by the pathogen *Aeromonas hydrophila* (He *et al.*, 2017).

Combined, these examples illustrate the important role of the microbiome in disease transmission. Consequentially, the vector-associated microbiome may indirectly drive the health of definitive hosts by determining the vector's transmission potential (Hoffmann *et al.*, 2011; Pinto *et al.*, 2021). Indeed, the prevalence of mosquito-borne diseases like malaria, dengue, and chikungunya have all been reduced by releasing microbiome-altered mosquito vectors, which have mostly been targeting the *Wolbachia* endosymbiont (Moreira *et al.*, 2009; Hoffmann *et al.*, 2011; Pinto *et al.*, 2021). This reduction in transmission can occur due to various microbiome-mediated effects such as: reducing the host's lifespan (McMeniman and O'Neill, 2010), stimulating the mosquito's immune response (Bai *et al.*, 2019), producing anti-pathogen proteins (Wang *et al.*, 2017), and supporting the formation of physical barriers (Song *et al.*, 2018). However, it is not a one-shoe-fits-all system as several studies have since reported an increase in pathogen transmission associated with *Wolbachia* infections in mosquitos (Hughes *et al.*, 2012; Zélé *et al.*, 2014). These conflicting results indicate a complex tripartite interaction between vectors, their microbiome and vector-borne diseases, which can change depending on the interacting species and strains (Moreira *et al.*, 2009). Nevertheless, combined with a fundamental understanding of all the epidemiological drivers across a disease's range, microbiome-mediated vector-borne disease control provides an exciting and promising avenue for future disease control efforts.

The snail's immune response to infection is influenced by host genetic factors (Allan *et al.*, 2018) and by abiotic environmental factors like temperature (Leicht *et al.*, 2013; Knight *et al.*, 2015). Resistance can artificially be induced by stimulating the snail's hemocytes (Pila *et al.*, 2016), which indicates the potential for modulating the snail immune response. Combined with the successes in mosquito-borne diseases, the idea of microbiome-mediated schistosomiasis control got a major boost after Chernin first touched upon the concept in 1957 (Chernin, 1957;

Chevalier *et al.*, 2020; Huot *et al.*, 2020; Portet *et al.*, 2021; Le Clec'h *et al.*, 2022; Sun *et al.*, 2024). In 1960 Chernin showed that bacteriologically sterile (as determined by thioglycolate broth) *B. glabrata* were equally receptive to schistosome infection and survived better than an untreated strain of *B. glabrata*. Since then, no studies repeated Chernin's protocol and validated it with state-of-the-art and increasingly sensitive molecular tools. Moreover, once these protocols are validated it lays the foundation for microbiome transplant experiments whereby specific bacteria or bacterial communities can be actively or passively exchanged from one host or substrate to another (Greyson-Gaito *et al.*, 2020). Such transplant experiments disentangle causation from correlations and have shown that the microbiome is critical for toxic cyanobacteria tolerance in water fleas (Macke *et al.*, 2017; Houwenhuyse *et al.*, 2021), for obesity in lab mice (Ridaura *et al.*, 2013), and for parasite resistance in bumblebees (Koch and Schmid-Hempel, 2012).

Altogether, field- and lab-based studies, including lab infection experiments, field-based correlative data, microbiome transplant experiments, and microcosm studies can converge to a fundamental understanding of all the involved epidemiological drivers such as complex compatibility patterns (Sène *et al.*, 2004) and parasite-parasite interactions (Laidemitt *et al.*, 2019). As a result, sustainable disease control will hopefully be possible through a nature-based solution, ensuring public, economic and environmental health as dictated by the *One Health* framework (Webster *et al.*, 2016).

1.3. Aims of the thesis

The first aim of the thesis targeted a lab-based infection experiment. This experiment aimed to understand what changes the snail microbiome goes through during the maturation of schistosome infections. Moreover, we aimed to understand what happens to the bacterial aspect of the microbiome during key parasite development stages, the various effects different schistosome species might have on the snail bacterial profiles, and to compare these effects under single parasite infections to co-infection conditions (**Chapter 2**). The second aim of the thesis is to validate the axenic tool of Chernin (1957) and build upon its foundations to create a viable transplant protocol for the bacterial aspect of the microbiome of freshwater snails (**Chapter 3**). The third aim of the thesis was to investigate a potential link between the bacterial aspect of the microbiome of a globally invasive snail and its resistance to trematode infection through a reciprocal transplant experiment (**Chapter 4**).The final aim of the thesis steered away from the microbiome and zooms in on the anthropogenic pressures driving snail-parasite interactions in man-made lakes (**Chapter 5**).

1.4. Thesis outline

Chapter 2: Schistosome species, onset of shedding, and co-infection dynamics determine bacterial profiles in the snail *Biomphalaria glabrata*

To identify putative points for microbiome-mediated schistosomiasis control, we study in Chapter 2 the bacterial aspect of the microbiome during key parasite development stages, the various effects of different schistosome species, and compare single to co-infection conditions in experimental infection experiments on the lab model snail *Biomphalaria glabrata*. Earlier work on these samples allowed us to assess the impact of parasite exposure but also detailed infection status information through the high throughput amplicon sequencing (HTAS) workflow designed by Hammoud *et al.* (2022). The HTAS workflow and infection experiments were originally designed and performed in collaboration with colleagues from the Université Perpignan Via Domitia (UPVD, France). This Chapter is currently under review at *mbio*.

Chapter 3: Host-microbiome transplants of the schistosome snail host *Biomphalaria glabrata* reflect species-specific associations

Chapter 3 focuses on validating, optimizing and expanding the protocol of Chernin (1957) to establish a viable tool in the lab model snail *B. glabrata* to transplant the bacterial aspect of the microbiome. We start by optimizing and validating the protocol through bacterial growth assays, quantitative PCR (qPCR), and microscopic investigation. Next, we investigate what conditions permit microbiome transplantations, whether snails can acquire a new bacterial community, and how the relatedness between donor and recipient affects the transplant outcome. The *B. glabrata* strain used in the experiment originated from UPVD (France). This chapter is published in *FEMS Microbiology Ecology*.

Chapter 4: The bacterial aspect of the microbiome does not drive liver fluke resistance in some populations of the globally invasive snail *Pseudosuccinea columella*

In Chapter 4, we apply the developed protocol of Chapter 3 to investigate a causative role of the snail's bacterial profiles to trematode infection. First, the protocol to obtain germ-free snails and transplant the bacterial aspect of the microbiome was re-optimized to suit *P. columella*. Next, we show in a reciprocal transplant experiment that the bacterial aspect of the microbiome is not responsible for determining the highly contrasting susceptible/resistant phenotypes seen in the field. The experiment was conducted at UPVD (France). The manuscript is currently in preparation.

Chapter 5: Invasive snails, parasite spillback, and potential parasite spillover drive parasitic diseases of *Hippopotamus amphibius* in artificial lakes of Zimbabwe

Focusing on the effect of anthropogenic pressures on snail-parasite interactions, we investigate in Chapter 5 the snail and parasite communities of a man-made lake in Zimbabwe.

We start by collecting snails from the reservoir and adult trematodes from culled wildlife. Next, we determine infection prevalence through RD-PCRs (Carolus *et al.,* 2019 and Schols *et al.,* 2019) and study snail and parasite genetics. Eventually, we link trematode infections in invasive freshwater snails to infections in a keystone herbivore, focusing on *Fasciola nyanzae* a liver parasite of the common hippopotamus, and discuss the potential threat this imposes on wildlife conservation. The sampling was performed in collaboration with colleagues from the University of Zimbabwe and the Kariba research station. This chapter is published in *BMC Biology*.

Chapter 6: General discussion

The general discussion first discusses the high susceptibility of invasive snails and whether a resistant microbiome could bring rescue by integrating Chapter 4 and Chapter 5. Chapter 2 and Chapter 4 are then used to touch upon the effect of trematode infection on the snail-associated microbiome. Thereafter, we discuss the future for microbiome-mediated control of snail-borne diseases based on the information gathered in this thesis and what consequences it may have for a *One Health* framework. Next, we raise a few considerations before microbiome manipulations should be done in the wild. Thereafter, we delve into how an invasive snail could threaten the enigmatic hippopotamus based on Chapter 5, as invasions continue and wildlife persistently declines in the Anthropocene. Finally, we critically assess the applied methodology within the current state-of-the-art and provide possible solutions and directions for further research.

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Chapter 2

Schistosome species, onset of shedding, and co-infection dynamics determine bacterial profiles in the snail *Biomphalaria glabrata*



Chapter 2

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Schistosome species, onset of shedding, and co-infection dynamics determine bacterial profiles in the snail *Biomphalaria glabrata*

Trematode parasites and host-microbiome

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Abstract

Schistosomiasis is a snail-borne disease affecting over 200 million people worldwide. Despite dedicated control efforts and the deployment of effective diagnostic tools, schistosomiasis remains prevalent, manifesting with exceptional intensities and in unanticipated regions. Novel and sustainable control measures are urgently needed. Bacteria might offer such a solution as links between bacteria, disease resistance and transmission potential of intermediate hosts have been established in other systems. To better understand the tripartite interaction potentially driving snail-schistosome compatibility patterns, microbial communities must be investigated throughout and across various parasite exposure conditions. Therefore, we studied *Biomphalaria glabrata* snails exposed to a high- and low-shedder strain of *Schistosoma mansoni* and *Schistosoma rodhaini* in single and co-exposure experiments. Snails were sacrificed at different time points post-exposure and their bacterial communities and trematode (co-)infection status were determined through metabarcoding tools. Snails infected by low- and high-shedder *S. mansoni* strains were more likely to have bacterial community dysbiosis than those infected by *S. rodhaini* but this was also affected by miracidial load. Moreover, the single-infection hierarchical effect on the bacterial component of the microbiome is not maintained under co-infection with *S. rodhaini,* which appears to stabilize the snail's bacterial profile even after being outcompeted by high-shedder *S. mansoni*. Finally, alpha diversity only differed significantly between infected and uninfected snails around the onset period of shedding at 30 days post-miracidial exposure. The timing of this bacterial shift suggests an intricate parasite-snail interaction around key parasite development moments. Combined with earlier reports, this suggests that delayed shedding may potentially be a new target for schistosomiasis control. Furthermore, the impact of outcompeted parasites on the snail's bacterial profile serves as a warning for field-based studies investigating the snail microbiome as earlier or prepatent parasite infections may have a large influence on the detected bacterial patterns.

Author Summary

Schistosomiasis is a widespread snail-borne disease that impacts over 200 million people globally. The disease continues to persist despite extensive control efforts, indicating a need for innovative strategies. Bacteria might offer such a solution as they have been shown to influence disease resistance and transmission potential of intermediate hosts. Therefore, we studied the bacterial component of the microbiome of snails exposed to various schistosome strains and species, either in single or co-infections. We show that the bacterial community is affected differently depending on the virulence of the parasite strain and the parasite species. However, when snails are exposed to two parasite species and one parasite outcompetes the other, the “losing” parasite is still able to stabilize the effect on the bacterial community. Interestingly, 30 days after parasite exposure, when parasite larvae start to emerge from the snail to infect the next host, a significant difference arises in the bacterial diversity in infected snails compared to uninfected snails. Combined with earlier studies, this work suggests the potential for delayed parasite release as a control measure for schistosomiasis. Also, future studies should be aware of the effects outcompeted parasites could have on the snail’s microbiome when assessing links between infection status and microbial patterns.

Subject areas

Snail-borne disease, parasite development, tripartite interaction, microbiota, trematode, bacteria, schistosomiasis

1. Introduction

Schistosomiasis is a neglected tropical disease affecting more than 200 million people worldwide with over 90% of the cases situated in Africa [1]. The disease is caused by flatworm parasites of the *Schistosoma* genus, manifesting as either urinary or intestinal schistosomiasis. Most intestinal schistosomiasis cases are caused by *Schistosoma mansoni* and can lead to bloody stool, diarrhea, and anemia [1]. Freshwater snails of the genus *Biomphalaria* serve as intermediate hosts for *S. mansoni* [2]. However, not all snail strains transmit schistosomes equally well, providing an interesting landscape to better understand the compatibility landscape, as currently the driving factors often remain ill-understood [3,4]. Further confounding the compatibility between schistosomes and their snail hosts are co-infections with different strains of the same species, different schistosome species and other co-infecting trematode species in a single snail specimen (e.g. [5–9]). Such co-infections could affect disease outcome through immune priming of the snail host [10] or synergistic or antagonistic parasite-parasite interactions [7–9,11]. At the intraspecific level, co-infections by up to nine genotypes of *S. mansoni* are possible, leading to reduced individual fitness due to reproductive competition [6,12]. At the interspecific level of schistosome species, the rodent infecting *Schistosoma rodhaini* has a partially overlapping distribution and host range with *S. mansoni* potentially influencing the transmission of both schistosomes [13]. Nevertheless, *S. mansoni* and *S. rodhaini* co-infections in *Biomphalaria pfeifferi* and *Biomphalaria sudanica* did not show an altered cercarial emergence compared to single infections [13]. In contrast, co-infection experiments in *Biomphalaria glabrata* by both schistosome species did reveal a shift in the timing of cercarial emergence and a reduction in the number of released cercariae compared to single infection with and without patent co-infection [9]. At the interspecific level of trematode species, an interesting two-way interaction was reported by Laidemitt and colleagues [8] whereby the cattle amphistome *Calicophoron sukari* requires an earlier infection of *S. mansoni* in *B. pfeifferi* to establish itself but will in turn inhibit further development of *S. mansoni.* Moreover, such interactions illustrate that the control of one disease could inadvertently lead to the increase of another [8]. Therefore, including parasite-parasite interactions within snail-parasite compatibility studies becomes imperative.

Today snail control depends on the use of chemical molluscicides, habitat modification and biological control. Chemical molluscicides theoretically only target snails, however, in field settings frequently lead to mass mortality in other aquatic life such as fish and amphibians and cause concerns for human health [14,15]. Furthermore, habitat modifications, such as the removal of aquatic vegetation, sometimes have a limited effect on snail populations while also reducing aquatic biodiversity [16,17]. Biological control has been attempted through the introduction of snail predators and competitors yet can have unexpected and often negative impacts on the ecosystem [15,18–21]. Moreover, freshwater snails take part in complex ecological interactions, even contributing to nitrogen cycling [22,23]. Therefore, schistosomiasis control measures should ideally be highly specific and reduce parasite transmission without removing key factors from the aquatic environment following the *One Health* principle. Microbiome-mediated resistance to parasite infection could provide such a sustainable solution, as seen for mosquito-borne diseases whereby the release of microbiome-altered mosquitoes significantly reduced the transmission of several arboviruses and *Plasmodium* parasites [24–27].

A host and its microbiome are intimately linked in what is known as the 'holobiont' [28], of which the microbial component constitutes a rapidly evolving facet [29,30]. Various factors, such as environment, food, disease and age can influence the microbiome [22,31,32]. Such microbiome alterations can help the acclimatization or adaptation of a host but can also worsen the effect an environmental stressor has on a host in what is known as 'dysbiosis' [32]. Interestingly, the dysbiotic state is frequently linked to disease [32–34] and may cascade down to the immune system facilitating pathogen colonization and growth [33]. We define the dysbiotic state as an increased stochasticity in the microbial community of stressed individuals compared to healthy individuals, which is measured by an increased within-treatment Beta diversity [35].

The microbiome has previously been linked to disease resistance and thereby the transmission potential of hosts in the host-pathogen-microbiome tripartite interaction [36,37]. In line with the recent explosion of research on microbiome-mediated resistance in mosquito-borne diseases (see for example the review [38]), schistosomiasis researchers have started exploring the potential role of the microbiome in a snail's resistance to schistosomes (see the reviews of Le Clec'h *et al.* [39] and Sun *et al.* [40]).

Microbiome-mediated resistance can originate from three underlying processes: direct competition for resources and space, inhibitory products, or immune priming of the host (see for example the review of Stock *et al.* [31]). However, within the tripartite interaction framework, Chernin [41] already explored the effect of bacteria, or rather the lack thereof, on infection outcome, revealing that *B. glabrata* snails lacking viable bacteria and control snails were equally susceptible to *S. mansoni*. Nevertheless, the current knowledge on the microbiome of intermediate snail hosts of schistosomes was recently reviewed by Sun *et al.* [40]. Briefly, it is now known that the snail microbiome is specific to each species and strain, variable in time and independent of rearing conditions [42,43]. Microbiome transmission mostly occurs between snail individuals horizontally, but vertical transmission also occurs [44] albeit not within snail eggs [45]. This suggests the importance of pseudo-vertical transmission - the indirect transfer of microbiota from parent to offspring through for example feces or nursing - in this system [29]. Additionally, links between the snail microbiome and parasite resistance have been suggested, as allelic variation in a genomic region influencing resistance to schistosome infection, the Guadeloupe Resistance Complex, is correlated with the bacterial component of the microbiome [46]. Finally, the snail's bacterial profile varies with parasite exposure, sympatry of parasite-snail combinations, and primary or secondary infections [4].

To establish an infection, a miracidium (the larval stage released from schistosome eggs that is infective to snails) penetrates the head-foot region of *Biomphalaria* snails and rapidly transforms to the next developmental stage, a sporocyst [47]. During this stage, the parasite has to escape the internal defence system of the snail by modulating hemocyte activity [48,49]. Hemocytes of resistant snails enclose the recently transformed sporocyst within their tissue and attempt to kill the parasite [48]. Such a response is not adequate in susceptible snails and in the next two to three weeks mother sporocysts generate clonal daughter sporocysts, still located in the head-foot region [50]. Next, daughter sporocysts migrate to the gonad-digestive gland region to further multiply asexually, inflicting significant damage in the process [51,52]. This damage leads to partial or complete destruction of ovotestis tissue, typically castrating the snail host [51,52]. Cercariae (the larval stage infective to final hosts) require arginine and glucose to mature within the daughter sporocyst thereby continuously absorbing large energy reserve

of the gonad-digestive gland region during their development [52]. Combined, these processes reveal a direct (destruction of tissue) and indirect (snail starvation through nutrient absorption) effect on snail fitness [52]. However, once matured, cercariae require only a minimal supply of energy for migration and emergence which happens approximately four weeks after miracidial exposure of the snail [52].

The snail samples utilized for bacterial characterization in our study were sourced from the research conducted by Hammoud and colleagues [53]. Their research involved exposure experiments of *B. glabrata* to *S. mansoni* and *S. rodhaini*, of which the (co-)infection status is known using diagnostic PCRs and metabarcoding [53,54]. This created an opportunity to delve deeper into the microbial aspects of the experiment. By characterizing the bacterial component of the microbiome we aim to (1) compare bacterial community composition between infected and uninfected snails, and explore the composition throughout parasite development (Figure 1B), (2) evaluate the impact of single infections by different parasite strains and species on bacterial profiles (Figure 1C), and (3) explore the effects of co-infections on the bacterial aspect of the microbiome (Figure 1D & E). These insights will provide the necessary foundation for bacterial transplant experiments to disentangle causation from correlation and additionally contribute to the limited knowledge we have on the tripartite interaction between snails, parasites and the microbiome.

2. Material and methods

2.1 Ethics statement

All required permits and ethical permissions were obtained and reported by Hammoud and colleagues [53].

2.2 Experimental conditions

Snail samples used for bacterial characterization were obtained from the study of Hammoud and colleagues [53] which was conducted in early 2018 and therefore followed the same experimental setup (Figure 1, Suppl. Table 1). Briefly, laboratory-reared snails of *B. glabrata* strain BgGUA2 of the same age cohort from Guadeloupe, Dans Fond, were exposed to various allopatric combinations of *S.*

mansoni strain BRE (Brazil, Recife, low-shedder, from here on referred to as SmBRE), *S. mansoni* strain LE (SmLE, Brazil, Belo Horizonte, high-shedder, from here on referred to as SmLE), and *S. rodhaini* (Burundi, from here on referred to as Sr) at the Host-Pathogen-Environment Interactions laboratory of the University of Perpignan (UPVD, France). The snail and parasite strains have been in laboratory cycling for several decades [53,55,56]. Parasite strains have been maintained through their sympatric snail intermediate host and Swiss OFI mice as final hosts. Miracidia were obtained from schistosome infections in the mouse strain Swiss OFI (Figure 1A) and were allowed to infect snails for 24h. To answer the three abovementioned hypotheses, four different sub-experiments were conducted. A first sub-experiment was performed to characterize the detectability of parasites during infection development, involving 144 snails each exposed to a single SmBRE miracidium where specimens were sacrificed 2, 6, 10, 16, 20, 30 and 40 days post-parasite exposure (Figure 1B). Another sub-experiment focused on the detectability of various parasite species, involving 30 snails exposed to 10 miracidia of either of the three schistosome strains and sample collection at 40 days post-parasite exposure (Figure 1C). Finally, two last sub-experiments aimed at characterizing co-infections by SmBRE and SmLE, and SmLE and Sr, involving 96 snails exposed to 10 miracidia of each parasite strain and samples being collected at 2, 10 and 40 days post-parasite exposure (Figure 1D & E). Naïve snail specimens were collected on day 0 and day 40 of the experiment. Snails were maintained in a single aquarium per exposure experiment in 10L aquaria at 25°C under a 12h:12h light-dark regime with constant aeration. Snails were fed *ad libitum* with washed pesticide-free salad and water wat replaced once a week. Snails were killed by heat shock (70°C for 1 min) and stored in 80% ethanol.

Next, DNA was extracted from the whole soft tissue of the snail (i.e. without shell) with the E.Z.N.A. ®Mollusc DNA Kit (OMEGA Bio-tek, Norcross, GA, USA) according to the manufacturer's guidelines. The infection status (infected yes or no) was determined through the adapted infection RD-PCR of Schols and colleagues [54] whereby the PCR cycles were reduced to 25 to avoid the detection of aborted infections as determined by Hammoud and colleagues [53]. (Co-)infections were then characterized through the metabarcoding approach [53]. Sample sizes used for 16S rRNA metabarcoding ranged from two to 24 per treatment and experiment as detailed in Figure 1.

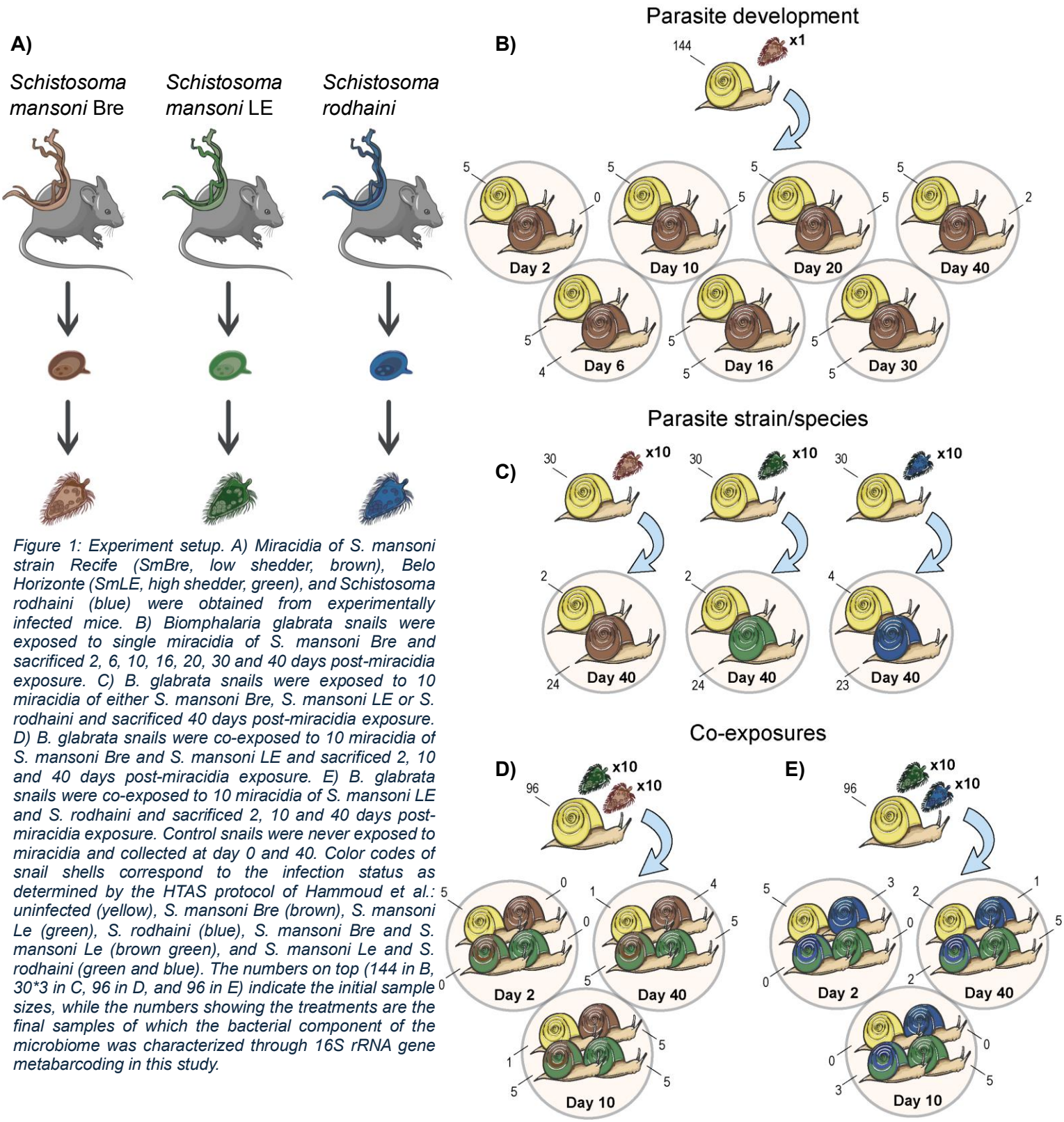


*Figure 1: Experiment setup. A) Miracidia of S. mansoni strain Recife (SmBre, low shedder, brown), Belo Horizonte (SmLE, high shedder, green), and Schistosoma rodhaini (blue) were obtained from experimentally infected mice. B) Biomphalaria glabrata snails were exposed to single miracidia of S. mansoni Bre and sacrificed 2, 6, 10, 16, 20, 30 and 40 days post-miracidia exposure. C) B. glabrata snails were exposed to 10 miracidia of either S. mansoni Bre, S. mansoni LE or S. rodhaini and sacrificed 40 days post-miracidia exposure. D) B. glabrata snails were co-exposed to 10 miracidia of S. mansoni Bre and S. mansoni LE and sacrificed 2, 10 and 40 days post-miracidia exposure. E) B. glabrata snails were co-exposed to 10 miracidia of S. mansoni LE and S. rodhaini and sacrificed 2, 10 and 40 days post-miracidia exposure. Control snails were never exposed to miracidia and collected at day 0 and 40. Color codes of snail shells correspond to the infection status as determined by the HTAS protocol of Hammoud et al.: uninfected (yellow), S. mansoni Bre (brown), S. mansoni Le (green), S. rodhaini (blue), S. mansoni Bre and S. mansoni Le (brown green), and S. mansoni Le and S. rodhaini (green and blue). The numbers on top (144 in B, 30*3 in C, 96 in D, and 96 in E) indicate the initial sample sizes, while the numbers showing the treatments are the final samples of which the bacterial component of the microbiome was characterized through 16S rRNA gene metabarcoding in this study.*

2.3 16S rRNA gene metabarcoding

A total of 212 samples (See Suppl. Table 1), four bacterial mock communities, one extraction negative and two PCR negatives were included in the 16S rRNA sequencing in a protocol adapted from Schols and colleagues [45]. Briefly, the pipeline targets the V3-V4 regions and is adapted from [57] and [42]. PCR reactions were done in triplicate in 25 µl: 12.5 µl of PCRBIO HS VeriFi Mix (PCR Biosystems), 0.75 µl of primer 341F (5'-CCTACGGGNGGCWGCAG-3'; [58]) and 0.75 µl of primer 805R (5'-GACTACHVGGGTATCTAATCC-3'; [58]) at an original concentration of 20 µM, a sample-specific volume of DNA providing 10 ng of total DNA per sample, and PCR-grade water to complete the volume. The PCR heat profile involved an initial denaturation at 98°C for 5 minutes, 25 cycles of denaturation at 98°C for 45 seconds, annealing at 60°C for 15 seconds, extension at 72°C for 10 seconds, and a final elongation at 72°C for 7 minutes. The triplicate PCR products from each sample were pooled and cleaned using magnetic beads (CleanNGS, GC Biotech) as per the manufacturer's instructions. The cleaned PCR products were then sent to Genomics Core (UZ Leuven) where, after quality control, a second PCR was performed to attach Illumina adapters and indexes. This reaction used 9 µl of the first PCR product, 0.5 µl each of the forward P7 and reverse P5 primers at 5 µM, and 10 µl of Phusion high fidelity PCR master mix (Thermo Fisher Scientific), which ran at an initial denaturation of 94°C for 30 seconds, followed by 15 cycles of denaturation at 94°C for 10 seconds, annealing at 51°C for 30 seconds, and elongation at 72°C for 30 seconds, with a final elongation at 72°C for 1 minute. Subsequent quality control confirmed proper adapter attachment and an equimolar library was prepared. Given the high number of samples, sequencing was conducted in two separate runs performed on the Illumina MiSeq v3 with a 14 pM load and aimed at 20% PhiX spike-in to improve library diversity and increase sequence quality, using the 600-cycle kit for 2 × 300 bp reads. Sample distribution per run is listed in Suppl. Table 1.

2.3.1 Mock communities

Bacterial mock communities were used as positive controls with known community composition to evaluate the success of our metabarcoding experiment, determine the appropriate filtering threshold for

the dataset, and the potential for DNA cross-contamination. The first Illumina sequencing run included a 10 Strain Even Mix Genomic Material MSA-1000™(ATCC) as reported and analyzed in [45] to ascertain a Relative Read Abundance (RRA) threshold in line with Reitmeier *et al.* [59]. Such a RRA with the help of mock communities allows for accurate filtering thresholds to improve the reproducibility of microbiome studies [59].

The second run included the ZymoBIOMICS™ Microbial Community DNA Standard I and the ZymoBIOMICS™ Microbial Community DNA Standard II (Log Distribution). Microbial Community DNA Standard I content followed by the theoretical composition of Genomic DNA (%) and 16S only (%): *Pseudomonas aeruginosa* (12, 4.2), *Escherichia coli* (12, 10.1), *Salmonella enterica* (12, 10.4), *Lactobacillus fermentum* (12, 18.4), *Enterococcus faecalis* (12, 9.9), *Staphylococcus aureus* (12, 15.5), *Listeria monocytogenes* (12, 14.1), *Bacillus subtilis* (12, 17.4), *Saccharomyces cerevisiae* (2, NA) and *Cryptococcus neoformans* (2, NA). Microbial Community DNA Standard II content followed the theoretical composition of Genomic DNA (%) and 16S only (%): *Listeria monocytogenes* (89.1, 95.9), *Pseudomonas aeruginosa* (8.9, 2.8), *Bacillus subtilis* (0.89, 1.2), *Saccharomyces cerevisiae* (0.89, NA), *Escherichia coli* (0.089, 0.069), *Salmonella enterica* (0.089, 0.07), *Lactobacillus fermentum* (0.0089, 0.012), *Enterococcus faecalis* (0.00089, 0.00067), *Cryptococcus neoformans* (0.00089, NA) and *Staphylococcus aureus* (0.000089, 0.0001).

2.3.2 Normalization of samples

2.3.2.1 qPCR

Quantitative PCRs (qPCRs) were used to evaluate the bacterial load in each sample, which subsequently allowed us to estimate the absolute abundance of the bacterial strains characterized through 16S rRNA gene metabarcoding [60]. qPCR reactions were run on the LightCycler® 480 (Roche). Each reaction consisted of 10 µl of LightCycler® 480 SYBR Green I Master (Life Science), 0.75 µl of the 16S rRNA primer 341F (5'-CCTACGGGNGGCWGCAG-3') at 20 µM, 0.75 µl of the 16S rRNA primer 805R (5'-GACTACHVGGGTATCTAATCC-3') at 20 µM and 1 µl of DNA (concentration 10 ng /µl) and 7.5 µl of sterile water to complete the volume to 20 µl. The temperature cycle was as follows,

5 minutes of initial denaturation at 95°C, followed by 50 cycles of 45 seconds at 95°C, 15 seconds at 60°C and 10 seconds at 72°C. Each sample was done in triplicate. The Femto™ Bacterial DNA Quantification Kit (Detection range of 20 fg-20 ng in seven steps) was used as a reference to quantify bacterial load through qPCR. This kit is based on a purified form of *E. coli* strain JM109. The melting and amplification curves were calculated through the LightCycler® 480 software (v. 1.5.1) and exported as reports to PDF and Excel. The Excel dataset was then used to calculate the mean and standard deviation for both the concentration and the Ct values. Bacterial load as determined by qPCR was compared across infection status through an independent t-test if normality was met, if not, comparisons were made through the Wilcoxon rank sum test both contained in the stats package (v. 4.2.2., [61]). The ratio of bacterial DNA to total DNA was then used to approximate the estimated absolute abundance (EAA) of the 16S rRNA gene metabarcoding dataset in RStudio (v. 2022.12.0) according to Zhang *et al.* [60].

2.3.2.2 RasperGade16S

16S rRNA gene copy numbers vary across bacterial taxa and provide inherent biases in sequencing-based approaches [62]. Therefore, we evaluated the use of RasperGade16S in line with [62] to correct our library for 16S rRNA gene copy numbers with or without qPCR data normalization.

2.4 Processing of sequencing data

Each Illumina MiSeq run leads to unique profiles in sequencing error. Therefore the samples for the two different MiSeq runs were processed separately until the generation of ASV tables. All steps were done according to the pipeline reported by Schols *et al.* [45] which was adapted from Janssens *et al.* [63] using the QIIME2 pipeline v2022.2 [64] on the VSC (Flemish Supercomputer Center). MultiQC was used to inspect the quality profiles of the raw reads of both runs [65]. Respectively, 37% and 12% of reads were attributed to PhiX during the first and second run. The first and second run resulted in 23,654,898 and 27,895,972 reads passing the sequencing company's first filtering step, respectively. Of these reads, 13,643,364 and 5,825,424 were attributed to the samples of this study (19,468,788 total reads). Reads of both runs were front-end trimmed (15 bp), forward reads truncated to 280 bp and reverse

reads to 240 bp with a minimal overlap of 30 bp between both reads to obtain paired-end sequences which were filtered, denoised, dereplicated and chimeras removed through the denoise function. Truncation length differed between forward and reverse reads, as reverse reads dropped faster in quality scores. Respectively, 4,021,448 (mean = 58.6%, sd = 4.7%) and 1,157,980 (mean = 45.8%, sd = 4.4%) of reads were maintained after cleaning for the first and second run.

The resulting representative sequences and data tables of both runs were then merged through the QIIME2 feature-table merge function. A total of 5,907 ASVs remained with an average length of 424 bp (sd = 11 bp). The Silva 138 SSU Ref NR 99 database [66] through a naïve Bayesian classifier was used to assign taxonomy to the ASVs. The V3-V4 region sequences of this naïve Bayesian classifier were obtained through the feature classifier extract reads function based on the 341F and 805R primers with a truncation length of 465 bp. Finally, the rooted tree was generated from the representative sequences using the phylogeny align-to-tree-mafft-fasttree function. The features, taxonomy and tree files were then exported and merged with metadata to generate a phyloseq object through the qza_to_phyloseq function of the qiime2R package [67] in RStudio (v. 2022.12.0) on a Windows machine. Mock communities were analyzed separately (see next section). Contaminant ASVs were detected and removed through the Decontam package (v. 1.18.0, [68]), using the prevalence method with a 0.1 threshold, based on two PCR negatives and one extraction negative. As a result, 25 ASVs (0.4% of the total ASVs) were classified as contaminants and removed from the dataset. Next, 179 chloroplast, mitochondria and ASVs lacking information at the phylum level were removed and taxonomic information was corrected according to Janssens *et al.* [63]. 2,444 low-abundance ASVs with less than four reads per sample were removed from the dataset (See section below 'Mock communities' for the justification and detailing of this threshold). Subsequently, a rarefaction curve was constructed through the Vegan package (v. 2.6.4, [69]). The rarefaction threshold was set at 10,403 through the phyloseq package (v. 1.42.0, [70]) using rngseed = 711 while we ensured that the plateau phase was reached. As a result, 183 ASVs were removed from the dataset as well as 14 samples including the two PCR negatives, the extraction negative and the two mock communities. The remaining nine removed samples

all originate from the second sub-experiment with various parasite infections (all originating from the second run): five Sr, three SmBRE, and two SmLE.

2.5 Statistical analyses of snail microbiome communities

Alpha diversity was assessed using Shannon and Faith's phylogenetic diversity indices, respectively, computed with the estimate_richness function from the phyloseq package and the pd function from the picante package (v. 1.8.2., [71]). To analyze the impact of treatment on alpha diversity, a generalized linear model was employed using the stats package. StepAIC from the MASS package (v. 7.3-58.1., [72]) was used to perform backward selection on the global model. The model with the lowest AIC value was used in all instances except for the second research questions "What is the effect of parasite development on the snail microbiome?" whereby the null model had a slightly lower AIC value (355 vs 363) yet we opted to investigate the interactive effect of days post-exposure and infection status. Normality was assessed with the shapiro.test function from the stats package. If normality was violated we assessed the skewness of the dataset through the moments package (v. 0.14.1., [73]) and chose either log, square root or inverse transformation depending on what was most appropriate for each dataset. Statistical significance was evaluated using a Type III ANOVA test from the car package (v. 3.1-2., [74]). Overdispersion was assessed through the check_overdispersion function of the performance package (v. 0.11.0., [75]). Outliers and influential observations were examined using the outliertest and cooks.distance functions from the car and stats packages, respectively. When applicable a pairwise comparison was made for each level through the lsmeans package with a Tukey correction for multiple testing (v. 2.30.0, [76]). Detailed statistical approaches are listed in Table 1.

Table 1:Statistical modeling. Response: lists the response variable and any required transformation. Predictors: predictor variables used as input for the models. #N: sample size. Figures: reference to the figure that show the associated output.

Question	Statistical approach	Response	Predictors	#N	Figures
Do miracidia-exposed snails have a different microbiome from naïve snails? (Figure 1)	GLM	Log Shannon	Experimental condition (1mir, 10mir, co-expSmLE-SmBRE, co-expSmLE-Sr, control)	212	Suppl. Figure 2

	GLM	Log Shannon	Sample type (naïve, exposed)	212	Suppl. Figure 2
What is the effect of parasite development on the snail microbiome? (Figure 1B)	GLM	Shannon	Days post-exposure (6, 10, 16, 20, 30, 40), infection status (yes, no), days post-exposure:infection status (interaction)	60	Suppl. Figure 3
	GLM	Faiths PD	Idem	60	Figure 2
	PERMANOVA	Bray-Curtis dissimilarity	Idem	61	Figure 3
How do different parasite species affect the snail microbiome? (Figure 1C)	GLM	Inverse Shannon	Experimental treatment (SmLE, SmBRE, Sr), parasite infection (uninfected, SmLE, SmBRE, Sr), infection status (yes, no)	79	Figure 4
	GLM	Square root Faiths PD	Experimental treatment (SmLE, SmBRE, Sr), parasite infection (uninfected, SmLE, SmBRE, Sr), infection status (yes, no)	79	Suppl. Figure 7
	PERMANOVA	Bray-Curtis dissimilarity	Idem	79	Figure 5
How do parasite species under co-infection affect the snail microbiome? (Figure 1D&E)	GLM	Log Shannon	Infection status (uninfected, SmLE, SmBRE, Sr, Sr+SmLE, SmBRE+SmLE), days post-exposure (2, 10, 40), experimental condition (co-expSmLE-SmBRE, co-expSmLE-Sr)	62	Suppl. Figure 9
	GLM	Faiths PD	Idem	62	Figure 6
	PERMANOVA	Bray-Curtis dissimilarity	Idem	62	Figure 7

Beta diversity was explored through Bray Curtis, unWeighted UniFrac and Weighted UniFrac indices, visualized using NMDS and RDA via the ordinate function from the phyloseq package and the capscale function from the vegan package. If the phylogenetic tree ended up with an edge matrix with an odd number of rows we transformed all multichotomies into a series of dichotomies using the multi2di

function of the ape package (v. 5.6-2., [77]) whereafter the tree was rerooted using the root_phyloseq_tree function of the QsRutils package (v. 0.1.5., [78]). The ordistep function from the vegan package was used for automatic stepwise model building after which the anova function was called to asses model significance (Table 1). Collinearity was assessed through the variance inflation factor obtained through the vif.acc function from the vegan package. Subsequently, the within-group Bray-Curtis dissimilarity was investigated based on an adapted version of the micrUBIfuns function (v. 0.1.0., [79]) and differences in means between levels tested with the Wilcoxon test with a Bonferroni correction for multiple testing.

In line with Lloyd-Price *et al.* [80] samples highly divergent from the reference dataset were identified based on the 90th percentile threshold. Core microbiome analysis using a detection threshold of 0.01-0.001 with a prevalence allowing up to one missing sample was done based on the microbiome package (v. 1.20.0, [81]) while adhering to the guidelines of Neu, Allen and Roy [82]. Venn diagrams were constructed based on unique ASV names through the Bioinformatics and Evolutionary Genomics website of the Ghent University (https://bioinformatics.psb.ugent.be/webtools/Venn/). Differential abundance analysis based on the negative binomial distribution was performed through the DESeq2 package (v. 1.38.3., [83]).

2.6 Mock communities

The analysis of the ATCC mock communities has been thoroughly analyzed and reported by Schols *et al.* [45], resulting in the most appropriate RRA threshold of 0.5%. The ZymoBIOMICS™ Microbial Community DNA Standard with normal distribution failed (189 reads after filtering). The log-distributed mock community was successfully sequenced and did not contain any contaminating ASV while detecting almost all expected bacterial species, with the exception of the three rarest bacterial taxa *Lactobacillus fermentum*, *Enterococcus faecalis*, and *Staphylococcus aureus* and not detecting the two fungi. The three taxa not detected in the log-distributed mock community were detected in the few reads of normal distribution mock community. *Escherichia coli* and *Salmonella enterica* (respectively, 0.089 % Genomic DNA and 0.069 % 16S only, and 0.089 % Genomic DNA and 0.07% 16S only) were the

detection limit of our pipeline with four and seven reads, respectively. Hence a RRA threshold suitable to exclude contaminants was more difficult to ascertain. Therefore, ASVs with less than four reads per sample were set to zero for that sample as it falls below the detection limit of our tool, rather than a RRA threshold for filtering. This is in line with the removal of single-, double-, and tripletons from a dataset, yet at the sample level, as they are most likely erroneous [84,85].

2.7 Data normalization

qPCR normalization of sample reads to obtain EAA proved inappropriate for our dataset as reads per sample were reduced to such an extent that the rarefaction curve would only indicate saturation of the curve at the phylum level (Suppl. Figure 1). Moreover, the inclusion or sole use of RasperGade16S to correct for 16S rRNA genome copy numbers was also deemed unsuitable for our dataset as 66.3% of the ASVs had a 16S rRNA genome copy number assigned with less than 95% probability (threshold determined by creators of the tool, [62]). Hence, neither of these normalization methods was applied to our dataset.

3. Results

3.1 Bacterial patterns

3.1.1 Comparing miracidia exposed snail samples to naïve snails

To assess whether parasite exposure affected the snail microbiome naïve and exposed snails were compared. Across the entire experiment, the Shannon diversity index (log-transformed to meet normality) did not significantly differ between snails never exposed to miracidia (naïve) and snails exposed to miracidia (sample) (LR Chi^2= 0.31, p-value = 0.58) (Suppl. Figure 2A). However, when comparing the various experimental conditions (single miracidium, various parasites at 10 miracidia, the two co-infection experiments, and naïve snails), samples separate at the start day 0 and at the end of the experiment day 40 (LR Chi^2=20.61, p-value < 0.001) (Suppl. Figure 2B). Pairwise comparison revealed a significant difference between the three experiments involving 10 miracidia exposure under single-species infections and the control snails collected at the end of the experiment (estimate = -0.20, SE = 0.06, df = 196, t-ratio = -3.38, p-value = 0.01), and between the control snails at the start of the experiment and the control snails at the end of the experiment (estimate = -0.30, SE = 0.08, df = 196, t-ratio = -3.69, p-value = 0.004). The Faith's phylogenetic diversity could not meet normality after transformation and/or outlier removal and is therefore not considered. Exploratory beta diversity analysis involving NMDS only revealed separate clustering of unexposed snails collected at 0 days post-exposure (daysPE) for the Bray-Curtis dissimilarity measure (Suppl. Figure 2C). Whether the composition among groups is similar or not could not be assessed due to heterogeneity in the dataset as tested for through permutest (F= 3.94, p-value = 0.031). No ASV-level core microbiome could be detected across all samples (n=212) at a detection threshold of 0.001 or for parasite-exposed samples alone (n=202). Notably, if up to two samples were allowed to have a detection prevalence below 0.001, we did detect a *Cloacibacterium* sp. (min= 0.0005, max.=0.74, mean=0.14, sd=0.14). The ASV-level core microbiome did consist of a *Cloacibacterium* sp. when looking at the unexposed samples alone (n=10; min= 0.03, max.=0.72, mean=0.24, sd=0.25) at a detection threshold of 0.01.

3.1.2. The temporal pattern throughout schistosome development (Figure 1B)

40 days following parasite exposure, infected and uninfected snails were compared to study the effect of parasite development on snail microbiome. When investigating the interactive effect of daysPE and infection status on the Shannon and Faith's phylogenetic diversity metrics, normality was achieved after removing the same outlier for each metric (N=1, uninfected sample at 30 daysPE). No significant effects of daysPE (Shannon: LR Chi²= 4.95, p-value = 0.42; Faith's PD: LR Chi² = 3.73, p-value = 0.59), infection status (Shannon: LR Chi²= 0.27, p-value = 0.60; Faith's PD: LR Chi² = 0.59, p-value = 0.44), or their interaction (Shannon: LR Chi²= 6.65, p-value = 0.25; Faith's PD: LR Chi² = 7.41, p-value = 0.19) were detected. However, pairwise comparisons revealed significantly higher alpha diversity (Shannon: estimate = 0.35, SE = 0.14, df = 43, t-ratio = 2.49, p-value = 0.02; Faith's PD: estimate = 0.31, SE = 0.13, df = 43, t-ratio = 2.34, p-value = 0.02) in uninfected samples compared to infected samples at 30 daysPE (Suppl. Figure 3, Figure 2). Bacterial load, measured via qPCR, was significantly lower in infected compared to uninfected samples throughout the experiment (Wilcoxon rank sum test, W= 542, p-value = 0.013). However, when performing comparisons per day, only at 16 daysPE was a significantly lower bacterial load observed in infected snails compared to uninfected snails (Suppl. Figure 4; estimate=0.67, SE=0.23, df=44, t-ratio=2.98, p-value = 0.005).

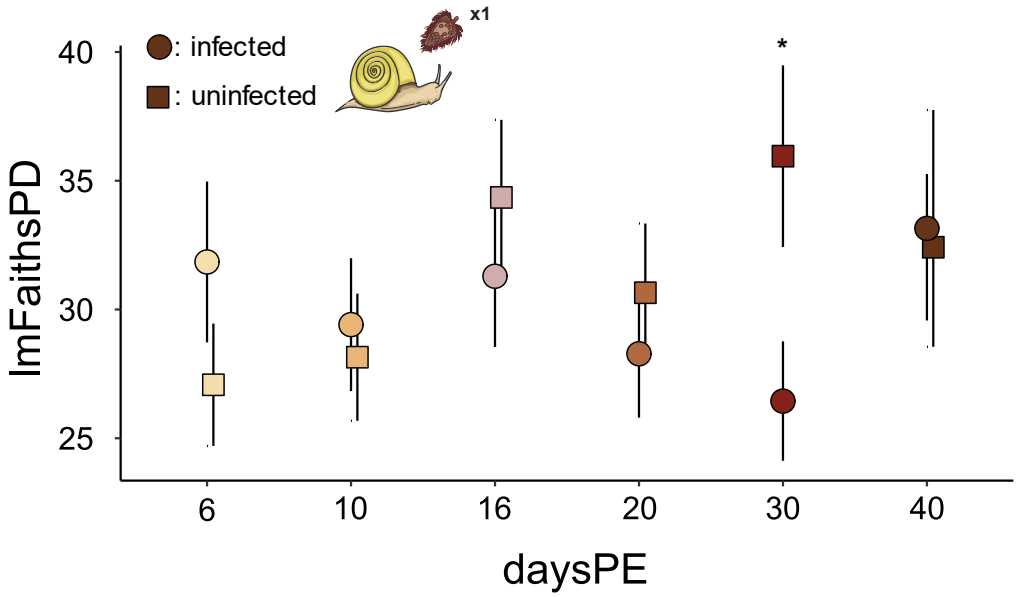


Figure 2: Faith's phylogenetic diversity metric (lmFaithsPD) for infected vs uninfected samples across various days post miracidium exposure (daysPE). Circles indicate infected samples, squares uninfected samples both indicate mean values. Error bars represent a single standard deviation. Significance: non-significant = '', <0.05 = '', <0.01 = '**', <0.001= '***'.*

Exploratory beta diversity analysis involving NMDS revealed clustering to mostly occur based on daysPE rather than the infection status for the Bray-Curtis dissimilarity and (un)weighted UniFrac measure (Suppl. Figure 5). Stepwise model selection for RDA analysis indicated that infection status did not affect the bacterial community which was therefore excluded from the model (F=1.04, p-value = 0.39). However, daysPE did have a significant effect on the bacterial community (Figure 3A; F=5.95, p-value < 0.001). The within-group Bray–Curtis dissimilarity differed significantly between infected and uninfected snails at six and ten daysPE, respectively (Figure 3B). The core microbiome at ASV-level across all samples consisted of an *Uliginosibacterium* sp. at a detection threshold of 0.001 (min= 0, max.=0.172, mean=0.040, sd=0.039). The core microbiome at ASV level across uninfected samples was further supplemented by a Comamonadaceae sp. at a detection threshold of 0.001 (min= 0.003, max.=0.175, mean=0.062, sd=0.047). The core microbiome at ASV level across infected samples consisted of nine ASVs: two *Blastopirellula* spp., two *Uliginosibacterium* sp., a *Cloacibacterium* sp., a *Mycoplasma* sp., a Phycisphaeraceae sp., a Isosphaeraceae sp., and a Comamonadaceae sp. at a detection threshold of 0.001 (min= 0.028, max.=0.676, mean=0.309, sd=0.189). At 30 daysPE ten ASVs

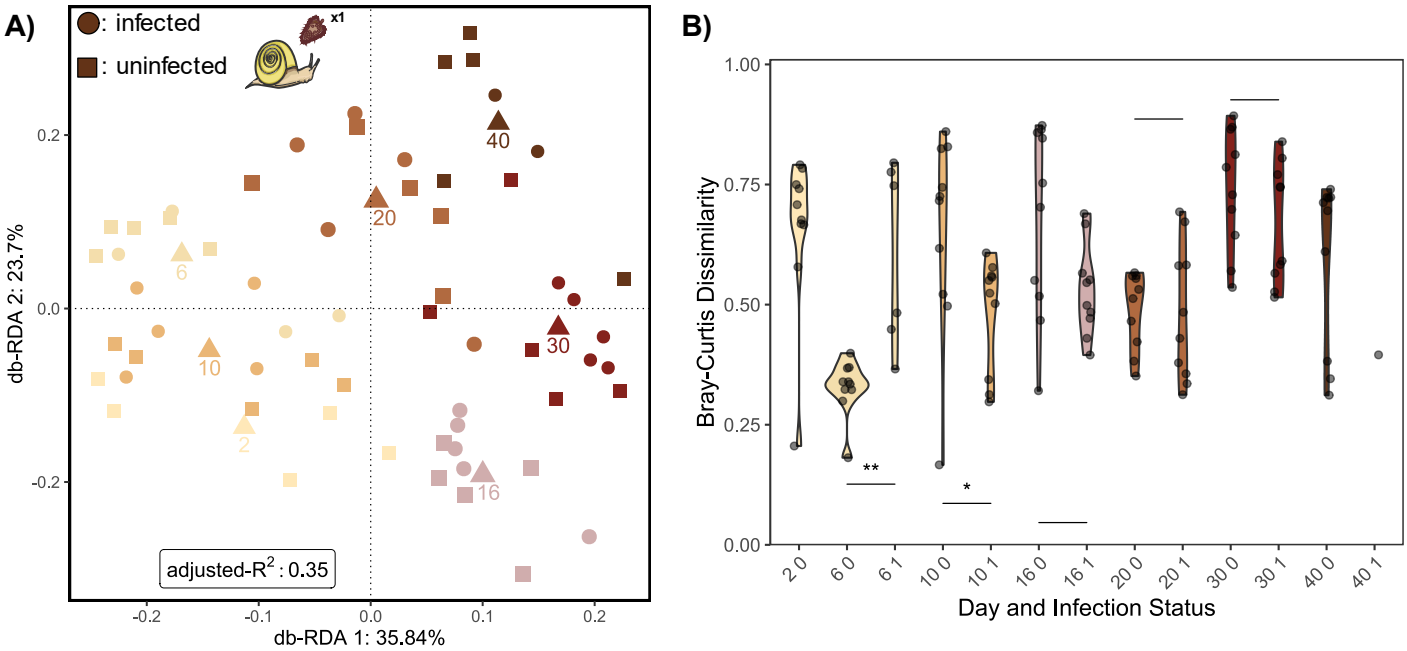


Figure 3: Beta diversity measures across days post exposure and infection status. A) Bray-Curtis dissimilarity measure as shown by RDA plot of the bacterial community adjusted for the different days post exposure. Infection status was selected out of the RDA by ordistep but is indicated for illustration purposes. The circles indicate infected samples, while the squares indicate uninfected samples. The triangles are the centroids of all samples belonging to the specific post exposure day. B) Violin plots showing the within-group Bray–Curtis dissimilarity with labels on the x-axis indicating the days post exposure (first number) followed by infection status (second number; uninfected = 0, infected = 1). Significance: non-significant = '', <0.05 = '', <0.01 = '**', <0.001= '***'.*

were present in all uninfected samples: two *Mycoplasma* spp., three Comamonadaceae spp., a *Chryseobacterium* sp., a *Pseudomonas* sp., a Chlamydiales sp., a Phycisphaeraceae sp., and a Caldilineaceae sp. (min= 0.068, max.=0.289, mean=0.183, sd=0.083). In contrast, 18 ASVs were present in all infected samples: three *Blastopirellula* spp., an *Uliginosibacterium* sp., a *Cloacibacterium* sp., two *Mycoplasma* spp., a *Fimbriiglobus* sp., a Bacilli sp., a Phycisphaeraceae sp., a Caldilineaceae sp., two *Pirellula* sp., an Isosphaeraceae sp., and four Comamonadaceae sp. (min= 0.226, max.=0.718, mean=0.489, sd=0.186). No ASVs were differentially abundant when comparing infected and uninfected samples for each daysPE nor across the entire dataset. However, at the genus level *Cerasiococcus* and *Hypomicrobium* nearly doubled (Log2fold change of 1.37 and 0.93, resp.; Padj<0.05) when comparing infected samples to uninfected samples. Further investigation of this pattern through a heatmap at ASV level did not reveal a clear pattern across infection status or daysPE (Suppl. Figure 6).

3.1.3. The effect of parasite strain and species in single infections on the bacterial aspect of the microbiome (Figure 1C)

To understand how different parasite species and strains affect the snail microbiome, snails exposed to three different parasites were studied 40 days after parasite exposure. For the alpha diversity, the Shannon diversity metric needed to be inverse transformed to meet normality. Stepwise model selection only kept parasite infection (uninfected, or established infections of SmLE, SmBRE and Sr) when fed the interaction term with experimental treatment (exposure to either SmLE, SmBRE and Sr), parasite infection and infection status (infected or uninfected). The infection status significantly influenced the

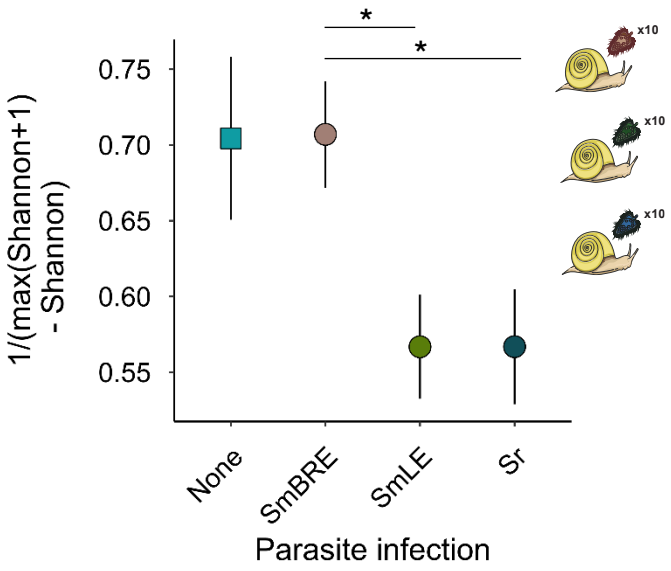


Figure 4: Inverse transformed Shannon diversity metric (alpha diversity) for snails 40 days post exposure to S. mansoni (SmBRE, low shedder), S. mansoni (SmLE, high shedder), and S. rodhaini (Sr). Uninfected samples from all exposure experiments are combined under "None" as selected for by the model. Squares indicate mean inverse Shannon diversity. Error bars indicate a single standard deviation. Significance: non-significant = '', <0.05 = '', <0.01 = '**', <0.001= '***'.*

inverse Shannon diversity (LR Chi²= 12.84, p-value = 0.005). A pairwise comparison revealed a significantly lower inverse Shannon diversity of snails infected by SmLE and Sr when compared to SmBRE (Figure 4; estimate=0.14, SE=0.05, df=66, t-ratio=2.85, p-value = 0.03; estimate=0.14, SE=0.05, df=66, t-ratio=2.71, p-value = 0.04, resp.) but not when compared to uninfected snails (estimate=0.14, SE=0.06, df=66, t-ratio=2.16, p-value = 0.15; estimate=0.14, SE=0.07, df=66, t-ratio=2.09, p-value = 0.17, resp.). Generally similar patterns were also noted for the Faith's phylogenetic diversity metric which had to be square root transformed to meet normality. Stepwise selection also only kept the parasite infection in the model (Suppl. Figure 7; LR Chi²= 11.52, p-value = 0.009). However, only SmLE-infected snails remained with a significantly higher square root transformed alpha diversity from SmBRE-infected ones but also from uninfected snails (estimate=0.13, SE=0.05, df=66, t-ratio=2.66, p-value = 0.05; estimate=0.18, SE=0.06, df=66, t-ratio=2.93, p-value = 0.02, resp.). Bacterial load, measured via qPCR, was not affected by parasite infection status (LR Chi²= 4.07, p-value = 0.25). Ordistep model selection for the beta diversity selected the additive effect of infection status (infected or uninfected) and experimental treatment (exposure to either SmLE, SmBRE and Sr) rather than the parasite infection (uninfected, or established infections of SmLE, SmBRE and Sr). The entire model was significant (Figure 5A; F=9.39, p-value < 0.001) as well as infection status (F=3.18, p-value = 0.007) and experimental condition (F=12.1, p-value < 0.001). The within-group Bray–Curtis dissimilarity differed significantly between all parasite infections and when compared to uninfected samples, except for snails infected with *S. rodhaini* which were not significantly differing from uninfected snails (Figure 5B). The core microbiome at ASV-level across all samples consisted of three ASVs: a *Uliginosibacterium* sp., a *Cloacibacterium* sp., and a Comamonadaceae sp. (min=0.014, max.=0.417, mean=0.190, sd=0.099). An additional 52 ASVs made up the core microbiome of uninfected samples (min=0.652, max.=0.783, mean=0.736, sd=0.038). The core microbiome of infected samples was limited to the same three ASVs as those across all samples (min=0.014, max.=0.417, mean=0.192, sd=0.104). However, when looking at the core microbiome of various infections (i.e., *S. rodhaini, S. mansoni* Bre, and *S. mansoni* LE) 23, 22, and 13 ASVs were respectively detected (resp., min=0.157, max.=0.717, mean=0.593, sd=0.139; min=0.035, max.=0.626, mean=0.411, sd=0.156; min=0.157, max.=0.820, mean=0.403, sd=0.147).

Supplementary Figure 8 shows the overlap of these core ASVs through a Venn diagram. Notably, one ASV is not part of the overall core microbiome because it is missing from two samples but each of these two samples originates from a different treatment. Hence, the ASV is not included in the total overview but is included in each separate one.

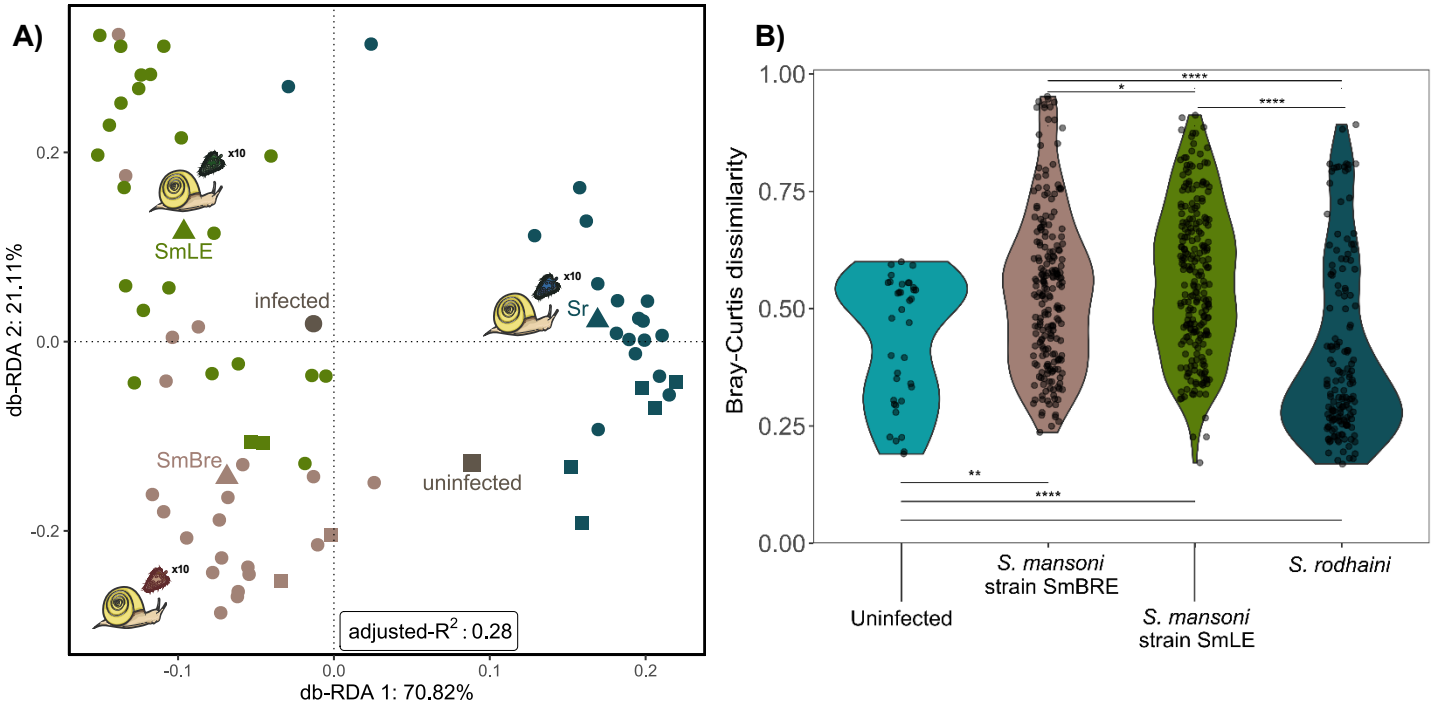


Figure 5: Beta diversity measures across the different infection experiments and infection diagnosis. A) Bray-Curtis dissimilarity measure of the bacterial community across the different experimental treatments as shown by a RDA plot with the infection status indicated by shape (circle: infected, square: uninfected) and parasite exposure by color. The RDA analysis considers experimental treatment and infection status additively. Ordistep excluded the interaction term. The triangles indicate centroid values for each parasite exposure, the large grey circle the centroid for all infected samples, and the large grey square the centroid for all uninfected samples. B) The within-group Bray–Curtis dissimilarity for the different diagnosed infections. Significance: non-significant = '', <0.05 = '', <0.01 = '**', <0.001= '***'.*

3.1.4. The effect of parasite strain and species under co-infection on the bacterial aspect of the microbiome (Figure 1D-E)

Snails of two co-exposure conditions were compared during infection development to understand how co-exposure conditions shape the snail microbiome. The additive effect of daysPE (i.e., 2, 10, and 40), experimental condition (co-exposure to either SmLE and SmBRE or SmLE and Sr) and their interaction remained after feeding the model with all the possible interactions between (co-)infection status (i.e., uninfected, SmLE, SmBRE, Sr, Sr+SmLE, and SmBRE+SmLE), daysPE, and the experimental conditions. The Shannon diversity metric required a log transformation to meet normality. No significant effect of daysPE (LR Chi²= 2.79, p-value = 0.25) and experimental condition (LR Chi²= 2.19, p-value = 0.14) alone was detected. Their interaction, however, was significant (LR Chi²= 9.15, p-value = 0.01).

Pairwise comparisons revealed a significantly higher log-transformed Shannon diversity of snails co-exposed to *S. rodhaini* and *S. mansoni* (co_infSrSm) compared to snails co-exposed to two strains of *S. mansoni* (co_infLeBr) at 40 daysPE (Suppl. Figure 9; estimate=-0.105, SE=0.049, df=55, t-ratio=-2.16, p-value = 0.04). A similar trend albeit not significant is visible at two daysPE (estimate=-0.119, SE=0.066, df=55, t-ratio=-1.81, p-value = 0.08). No significant change occurred throughout the experiment in snails co-exposed to *S. rodhaini* and *S. mansoni* (co_infSrSm) while the microbiome changed significantly when comparing two and ten, and ten and 40 post-exposure but not when comparing two and 40 daysPE (respectively, estimate=-0.169, SE=0.059, df=55, t-ratio=-2.86, p-value = 0.02; estimate=0.125, SE=0.042, df=55, t-ratio=2.99, p-value = 0.1 and estimate=-0.045, SE=0.059, df=55, t-ratio=-0.74, p-value = 0.73). A similar pattern is noted for the Faith's phylogenetic diversity metric, which did not require transformation to meet normality. Also here the interaction between daysPE and experimental condition was significant (LR Chi²= 11.65, p-value = 0.003) while the individual variables were not. Pairwise comparisons revealed a significantly higher Faith's phylogenetic diversity metric of snails co-exposed to *S. rodhaini* and *S. mansoni* (co_infSrSm) compared to snails co-exposed to two strains of *S. mansoni* (co_infLeBr) at 10 daysPE (Figure 6; estimate=-0.217, SE=0.082, df=55, t-ratio=-2.63, p-value = 0.01) while the opposite is true at 40 daysPE (Figure 6; estimate=0.175, SE=0.080, df=55, t-ratio=2.179, p-value = 0.03). No significant change occurred throughout the experiment in either co-infection experiment with the exception when comparing snails co-exposed to

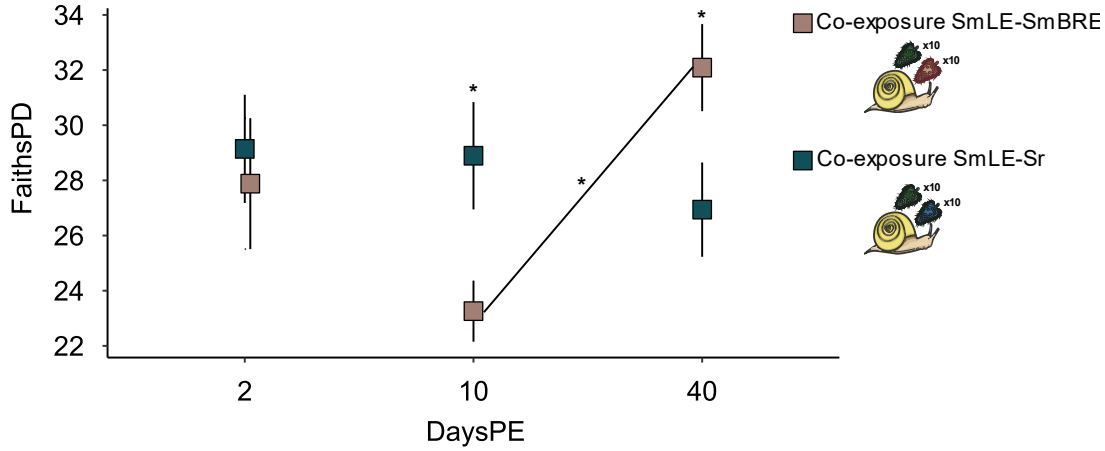


Figure 6: Faith's phylogenetic diversity metric for the two co-infection experiments across 2, 10, and 40 daysPE (irrespective of final infection outcome). Error bars indicate a single standard deviation. Significance: <0.05 = ''.*

two strains of *S. mansoni* at ten and 40 daysPE (Figure 6; estimate=-0.322, SE=0.068, df=55, t-ratio=-4.703, p-value < 0.001). RDA analysis indicated that daysPE (F=5.95, p-value < 0.001), experimental conditions (F=5.95, p-value < 0.001) and their interaction (F=5.95, p-value < 0.001) significantly affected the bacterial community (Figure 7A). The within-group Bray–Curtis dissimilarity differed significantly between two and ten, and ten and 40 daysPE for each of the experimental conditions separately but only differed significantly at day 40 post-exposure between the two experimental conditions (Figure 7B).

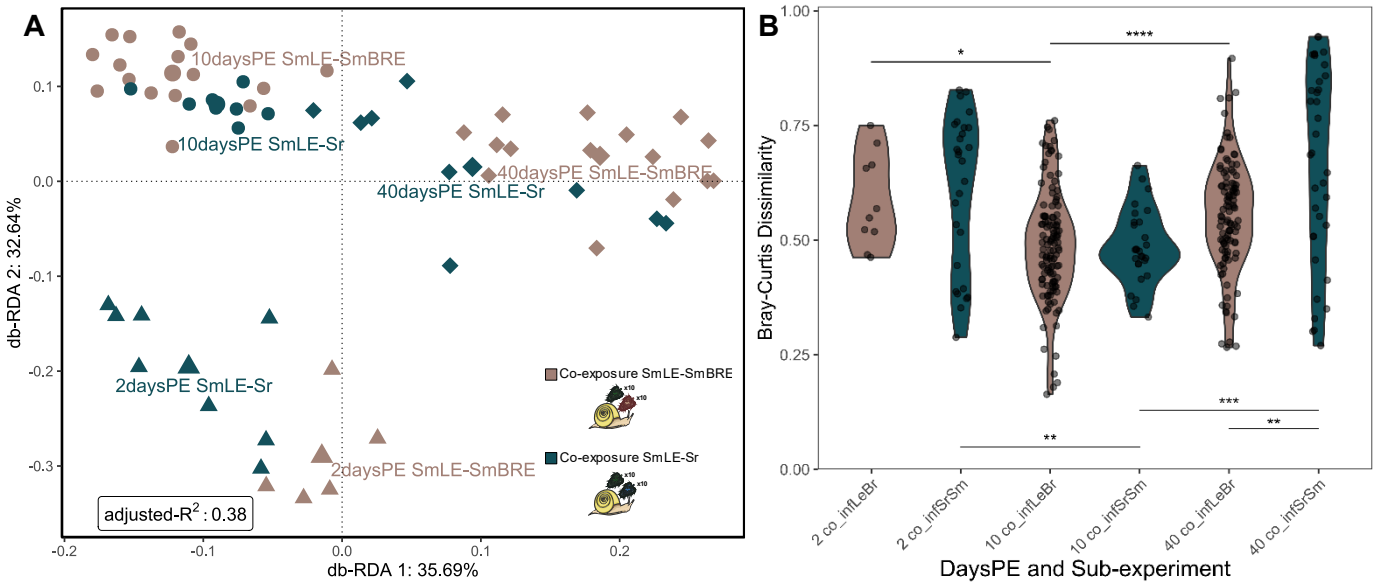


Figure 7: Beta diversity measures across the different days and co-infection experiments. A) Bray-Curtis dissimilarity measure of the bacterial community across the different days post exposure (indicated by the different shapes with the centroid indicated by the largest symbol) as shown by a RDA plot with the different co-infection experiments indicated by color. The RDA considers the co-infection experiment, days post exposure and their interaction. Ordistep excluded the infection diagnostic. B) The within-group Bray–Curtis dissimilarity with labels on the x-axis indicating the days post exposure (first number) followed by co-infection experiment (S. rodhaini and S. mansoni =co_infSrSm and two strains of S. mansoni =co_infLeBr). Significance: non-significant = '', <0.05 = '', <0.01 = '**', <0.001= '***', <0.0001= '****'.*

This pattern was not determined by the establishment of specific infections. Comparisons could not be made for the different time points of each experimental condition due to the low number of uninfected snails. The core microbiome at ASV-level across both co-infection experiments consisted of a *Cloacibacterium* sp. and a Comamonadaceae sp. at a detection threshold of 0.001 (min= 0.002, max=0.749, mean=0.169, sd=0.146). The core microbiome of the co-infection experiment with the two *S. mansoni* strains is further supplemented with an *Uliginosibacterium* sp. and an *Aquabacterium* sp. (min= 0.013, max=0.559, mean=0.206, sd=0.146) while that of the co-infection experiment with *S. rodhaini* and *S. mansoni* was supplemented by a Planctomycetes sp. (min= 0.005, max=0.739, mean=0.194, sd=0.174). Following DeSeq2 analysis, heatmap plotting indicates that the clustering per

experiment is less clear at two days PE compared to ten and 40 days PE (Figure 8). Moreover, snails exposed to two strains of *S. mansoni* are characterized by increased read abundance of the genera *Achromobacter*, *Stenotrophomonas*, *Pedospaeraceae*, and *Thermomonas* (Suppl. Figure 10). In contrast, snails exposed to *S. mansoni* and *S. rodhaini* are characterized by increased read abundance of the genus *Schlesneria*, and to a lesser extent *Mycobacterium* sp.

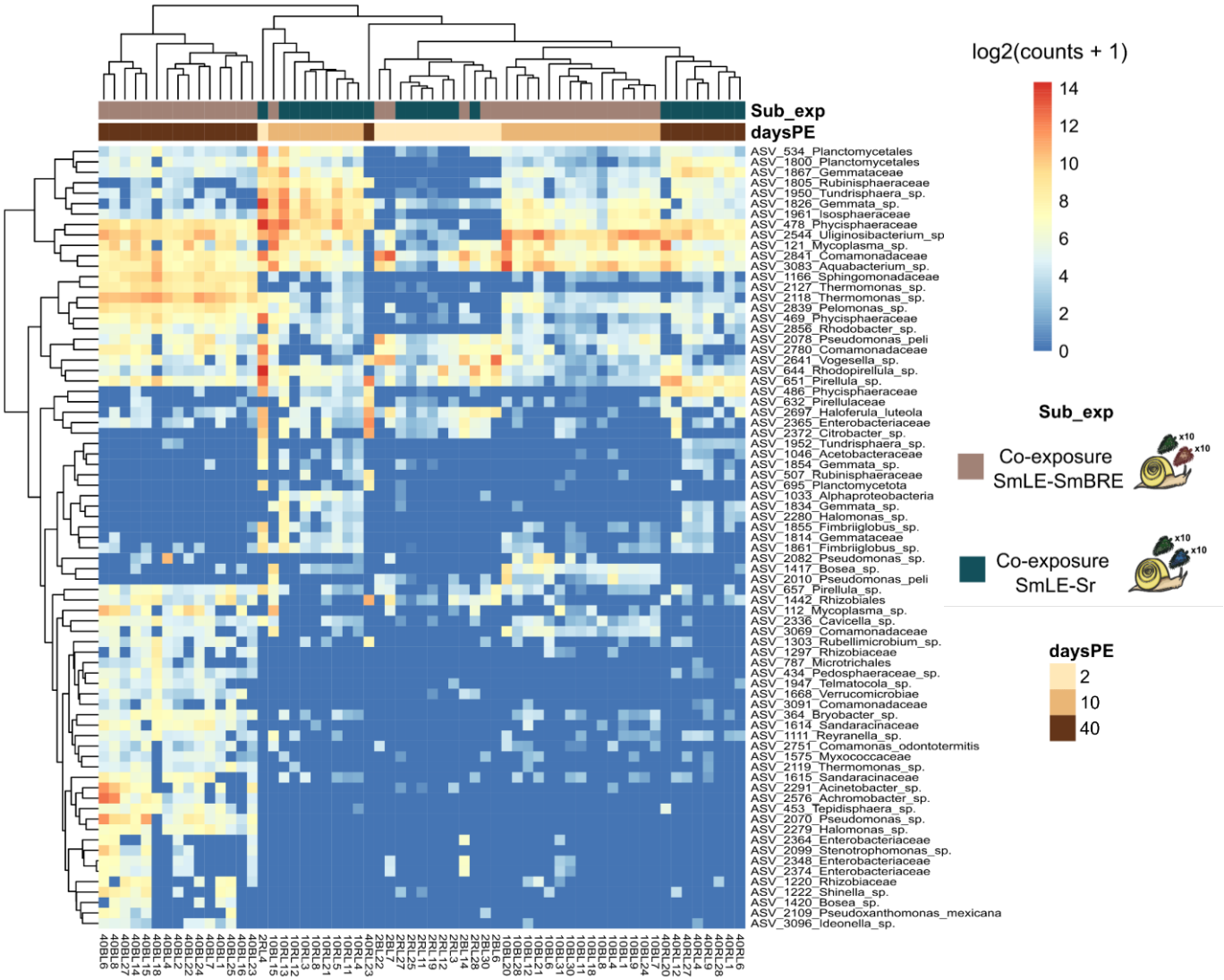


Figure 8: Heatmap showing the Log2(read counts+1) for each sample at the ASV level as selected for through DeSeq2 for significantly changing read counts with adjusted p-values <0.05. Sub_exp: co-infection experiment (S. rodhaini and S. mansoni =co_infSrSm and two strains of S. mansoni =co_infLeBr). DaysPE = days post-miracidia exposure (2, 10 and 40). Samples are listed on the X-axis while significantly changing ASVs are listed on the Y-axis. Clustering on top is based on sample similarity based on significantly different ASVs.

4. Discussion

The rise of metabarcoding tools capable of characterizing (co-)infecting parasites within snail samples (such as [53,86]) has set the stage for snail-parasitome-microbiome interaction studies. These improved infection characterization tools combined with a different experimental approach enable us to build on the valuable insights of Portet *et al.* [4]. Briefly, these authors revealed a non-reciprocal effect of snails on the bacterial profile of the water, a temporal change in the snail associated bacterial aspect of the microbiome across alpha and beta diversity metrics, a more pronounced effect of secondary vs primary infections irrespective of sympatric or allopatric snail-parasite combinations on alpha diversity metrics, and an effect through time, (re-)infection and sympatric-allopatric combinations at the community level. Despite an overlap in the general idea behind the experiment, several notable differences were exploited in our experimental setup compared to Portet *et al.* [4]. First, an increased sampling effort through time focused on parasite development during the first part of our experiment (i.e., snail collection at 2, 6, 10, 16, 20, 30 and 40 days post-exposure vs 1, 4 and 25). Second, also during this part of the experiment, snails were exposed to a single miracidium (1 vs 10), providing a higher number of uninfected snails for direct comparison as they were exposed to and reared under exactly the same conditions in the same aquarium. Third, co-infections were obtained through simultaneous not sequential parasite exposure of snails. Fourth, we worked with a different strain of *B. glabrata* (BgGUA2 vs BgGUA, BgBAR, BgBRE, and BgBS90). Fifth, we included *S. rodhaini,* a closely related schistosome to *S. mansoni*. Finally, across all experiments we determined the infection status through molecular diagnostic tools, allowing a comparison at the infection level and not only parasite exposure like Portet *et al.* [4].

4.1. The temporal pattern throughout schistosome development (Figure 1B) – the onset of shedding an intricate parasite-snail interaction

Analysis of alpha diversity metrics revealed a significant difference in alpha diversity, but not at the bacterial community level, between infected and uninfected snails at 30 days post-miracidium exposure followed by a recovery 10 days later (Figure 2 & Figure 3). Moreover, at 30 days post-exposure, infected snails show a more diverse and prominent core microbiome (18 ASVs, 48.9+-18.6%) compared to

uninfected snails (10 ASVs, 18.3+-8.3%). We hypothesize that this temporary significant difference in alpha diversity may be attributed to the onset of cercarial shedding. Using the formula from Pflüger [87] “268/(Temperature-14.2) = time until onset of shedding”, with our rearing temperature of 25°C, the estimated onset of shedding is around 25 days post-exposure. However, Theron [88] noted the onset of shedding at 33 days after miracidial exposure at 26°C. Therefore, it is likely that shedding in our experiment began around the 30-day mark. Around 30 days post-exposure, schistosome biomass [89] and read count [53] level off, potentially coinciding with the onset of shedding. In the first subsequent week, cercarial emergence peaks but during the next two weeks cercarial production significantly reduces [88], which could explain the observed recovery in alpha diversity by 40 days post-infection. Similarly, glucose concentrations dropped between 28 and 35 days post-exposure but recovered by 42 days post-exposure potentially also influencing the observed pattern [52].

For now, it remains unclear whether the drop in alpha diversity is 1) directly linked to mechanical damage and biochemical means required for the onset of shedding [48,52,90], 2) a consequence of the release of biomass and therefore reduced bacterial habitat, or 3) resulting from a relatively lower bacterial DNA template available for PCR amplification due to the increase in parasite DNA [53]. Cercariae bursting through the mantle epithelium cause severe damage to snail tissue [90,91]. Such wounds can provide an entry point for opportunistic environmental pathogens [92], and are linked to bacterial dysbiosis and an increase in potential pathogens in corrals [93,94]. Nevertheless, no signature of a dysbiotic state was seen in this part of the dataset (Figure 3B). Reduced bacterial DNA is unlikely to be the sole driver behind the observed pattern as the reduction in bacterial load in infected snails was nonsignificant at 30 days post-exposure (see section 3.1.2. & Suppl. Figure 4). Furthermore, *B. glabrata* egg count starts to drop three to four weeks after exposure to *S. mansoni* miracidia [95,96] and a similar pattern is noted for a drop in oviposition frequency of *Lymnaea stagnalis* four weeks after infection by *Trichobilharzia szidati* [97]. This suggests that the drop in alpha diversity may also coincide with the onset of snail castration and cercarial release. Moreover, infected samples at 16 days post-exposure did show a significantly lower bacterial load compared to their uninfected counterparts but did not show a reduction in any alpha diversity metric. This timing coincides with the release of daughter sporocysts from mother

sporocysts and the onset of their migration to the gonad-digestive gland region [50,98]. Combined, this data suggests an intricate snail-parasite-microbiome interaction during key points of parasite development and release.

In general, the consensus is that the initial parasite-snail interplay determines infection outcome [55,99]. Indeed, resistance to parasite infections has previously been induced in susceptible *B. glabrata* snails by artificially boosting hemocytes prior to exposure [100]. Moreover, schistosomes can modulate hemocyte activity to avoid destruction by the snail's immune system [48,49]. Notably, this change in hemocyte activity also reduces its capacity for bacterial clearance, providing a parasite-induced effect on the host's immunomodulation of the associated bacterial aspect of the microbiome [49]. Nevertheless, a later stage, when schistosome cercariae mature and emerge, is arguably equally impactful on snail physiology [48]. The data represented here suggests that microbiome-mediated schistosomiasis control in snail hosts could also attempt to target this period of parasite development. Indeed, *Wolbachia* infections in mosquitoes offer similar opportunities with severely reduced transmission to the next host by preventing infection but also by restraining the formation of the infective stage, the sporozoite [27,101]. Tavalire *et al.* [51] posit a similar perspective after observing delayed shedding of up to 30 weeks in certain inbred *B. glabrata* lines with equal resistance to infection. Moreover, snail lines capable of delaying the onset of shedding, and thus castration, also increased their reproductive period thereby underlining the viability of this target for fixation in natural populations [51]. Perhaps the microbiome was an underlying factor driving this delayed shedding which may be induced artificially through microbial manipulations. To test this hypothesis and ascertain a causative relationship between the microbiome and the onset of shedding, future experiments may attempt to artificially increase the alpha diversity around the 30-day mark and study the effect on cercarial release.

4.2. The effect of parasite strain and species in single infections on the bacterial aspect of the microbiome (Figure 1C)

High-shedder *S. mansoni* (SmLE) infections had the most profound effect on alpha diversity compared to uninfected snails while low-shedder *S. mansoni* (SmBRE) infections did not (Figure 4, Suppl. Figure

7). This difference between high- and low-shedder infections on the snail's alpha diversity, despite being the same schistosome species, could be linked to the different virulence levels of both strains. The SmBRE strain produces fewer cercariae, as is the case for *S. rodhaini*, while SmLE sheds eight times more cercariae and produces significantly more mother sporocysts [9,56,102–104], suggesting a higher impact on snail physiology and thereby the microbiome. The effect cannot be explained by reduced space for bacteria imposed by SmLE infections, as the bacterial load did not differ significantly between the established infections (see section 3.1.3. bacterial load). Both *S. mansoni* strains increased dysbiotic state of infected snails while *S. rodhaini* did not (Figure 5B), which might be explained by a different intra-molluscan development or species-specific adaptation to the intermediate snail host. Indeed, *S. mansoni* daughter sporocysts all move directly to the gonad-digestive gland region while part of the *S. rodhaini* daughter sporocysts remain in the head-foot region before joining the other daughter sporocysts that directly migrated to the gonad-digestive gland region [98]. Moreover, *S. mansoni* cercanogenous sporocysts (sporocysts producing cercariae) can transform to sporocystogenous sporocysts (sporocyst generating sporocysts) while no cercanogenous sporocysts have been detected transforming to sporocystogenous sporocysts for *S. rodhaini* [98,105]. Therefore, one hypothesis could be that these developmental variations explain why *S. mansoni* induces a dysbiotic state while *S. rodhaini* does not. Furthermore, SmLE and SmBRE infections had an *Aquabacterium* sp. as one of two core microbiome members, which was not in the core microbiome of uninfected and *S. rodhaini*-infected samples. Moreover, *Aquabacterium* spp. are described from many aquatic environments [106–108] but more importantly, do not appear to metabolize carbohydrates [108], one of the key parasite energy sources in the snail [52]. Therefore, the lack of competition with the parasite potentially explains why this bacterium became so abundant. A second hypothesis involves species-specific, and not strain-specific, snail-parasite interactions whereby SmLE and SmBRE naturally infect *B. glabrata* while Sr infects *B. pfeifferi*. Since the dysbiotic state is frequently linked to disease [32–34], this data suggests that parasites pre-adapted to the same snail species might be more virulent and increase the chance for dysbiotic state and potentially increase resource acquisition from the snail host. The different natural snail intermediate host could also explain why *S. rodhaini* infections were less successful in establishing

and persisting. Nevertheless, despite earlier passages through *B. glabrata, S. rodhaini* still produces fewer cercariae and established infections less frequently than *S. mansoni* [9] yet these authors did not describe the number of passages through the snail host and it might have, evolutionarily speaking, been a short timeframe [9]. Disturbing the microbiome might prove beneficial for the parasite as it could disrupt the host's immune response [109]. Indeed, dysbiosis is induced by nematode infection in susceptible but not resistant gastropod species [110]. However, excessive disruption might increase host mortality and thereby also the parasite's, as shown by the increased snail mortality due to SmLE infections compared to SmBRE [102]. Contrary to multi-miracidial infections, mono-miracidial infections with SmBRE do not cause dysbiosis (Figure 3B). Combined, the presented data suggests that the bacterial profile depends on parasite virulence, parasite adaptation to snails at the species level and infection load.

4.3. The effect of parasite strain and species under co-infection on the bacterial aspect of the microbiome (Figure 1 D & E)

Model selection indicated no effect of infection status (infected yes/no) and parasite infection (uninfected, SmLE, Sr, SmBRE, SmLE-Sr, or SmBRE-SmLE) on the bacterial aspect of the snail microbiome. Instead, it suggests a change through time and an effect of parasite exposure (Figure 6).

Under single infections (Section 3.1.3.), parasites with the most profound impact on the snail's bacterial profiles ranked from highest to lowest are SmLE - Sr – SmBRE at the alpha diversity level and SmLE – SmBRE – Sr at the community level. Notably, the data from the co-infection experiment suggests that this hierarchy is confounded under co-infection conditions. *S. rodhaini* under co-infection conditions negates the effect SmLE has under single infection (Figure 4, Figure 6). In contrast, co-infections with SmLE and SmBRE maintain the effect on the bacterial profiles as seen in single SmLE infections, revealing that this effect is not due to co-infection in itself but due to a direct or indirect parasite-parasite interaction. Notably, SmLE is known to outcompete SmBRE and *S. rodhaini* under co-infection [9,53,102], suggesting also a dominant effect of SmLE on the bacterial aspect of the microbiome. However, this pattern is only visible under co-infection with SmBRE but not with *S. rodhaini,* despite

outcompeting both parasites as witnessed by metabarcoded snail infections [53]. Combined, these results suggest that SmLE has a dominant effect on the microbiome due to increased competitive strength compared to SmBRE, while *S. rodhaini* appears to have a stabilizing effect on the snail's microbiome despite eventually being outcompeted by SmLE [53]. Indeed, both patent and prepatent co-infections of *S. mansoni* and *S. rodhaini* resulted in a lower cercarial output for both parasites [9], supporting an effect of outcompeted *S. rodhaini* on the *S. mansoni-B. glabrata*-microbiome interaction. Notably, the metabarcoding tool used to determine the infection status of these snail samples was specifically designed to only detect infections older than two days, in order to avoid false positives (i.e., detecting aborted infections) [53]. Hence, *S. rodhaini* infections may still have persisted as a single dormant sporocyst, that could not be picked up by our tool. Nevertheless, the data discussed here suggests that outcompeted parasite strains can affect the tripartite interaction between intermediate hosts, their microbiome, and parasites, further complicating this field of research. Indeed, uninfected but exposed snails show a similar increase in reproductive output as infected snails before the onset of castration [111], revealing an intricate effect of parasite exposure on snail biology possibly extending to the snail microbiome.

4.4. General patterns

Bacterial communities varied through time, independently of infection status (Figure 3A & 7A). Since the infection experiments were conducted with same-age snail cohorts, this result indicates a possible change in the microbiome throughout snail development. Indeed, Chen *et al.* [112] report a significantly different microbiome when comparing juvenile and adult apple snails (*Pomacea canaliculate*). Moreover, a changing microbiome during host ageing is broadly acknowledged in several organisms like humans, fish, flies and mice (see the reviews of Aleman [113] and Stock [31]). However, the bacterial communities did not follow a directional shift through time, suggesting that another factor might be at the basis of this pattern.

The genetic wealth of the microbiome frequently far exceeds that of its host [28]. To estimate the relative contribution of bacteria in the hologenome of *B. glabrata*, we can follow the same approximation of [28]

combined with the 14,423 genes described from *B. glabrata* [114]. The circa 50% of the ASVs identified from snails never exposed to parasites (n=10) in our dataset which could be classified to genus level, already results in 134 unique bacterial genera. Each bacterial species has roughly 2,500 genes of which 5% is unique, resulting in nearly 20,000 unique genes contributed by the bacterial component of the microbiome. This clear underestimation already reveals that at the bare minimum over half of the adaptation potential in the hologenome of *B. glabrata* comes from its bacteria alone. This underlines the potential importance of the bacterial aspect of snail biology as in other organisms [28].

Aeromonas and *Cloacibacterium* have been described as core members of the gut microbiome of several gastropod species, including *B. glabrata* [44]. Their relative abundance per sample ranged between 0 and 50% with means around 10% to 20% of the read count [44]. Similarly, we detected a *Cloacibacterium* sp. as a sole member of the core microbiome with a mean relative read abundance of 14% (sd=14%), indicating that the proportion of *Cloacibacterium* sp. in the gut microbiome is similar to that of the whole snail organism. However, we did not detect any *Aeromonas* sp. as core members in any of the sub-experiments nor in the entire experiment, suggesting that this taxon might be less ubiquitously present in freshwater snails than previously anticipated [44]. Uninfected samples showed less diverse core microbiomes compared to infected samples throughout most of the experiment. The established infections of each parasite strain showed a unique core microbiome, indicating a species-specific influence of trematode parasites on the microbiome [115]. Notably, part of our experiments reveal a higher impact of actual infection status rather than initial parasite exposure on the microbiome. Therefore, it becomes apparent that infection diagnostic tools are indispensable for both field and experimental settings as underlying patterns could otherwise have been missed. Therefore, we propose infection diagnostic tools to become an integral part of any future parasite exposure experiment.

4.5. Limitations

This study was characterized by some limitations. 16S rRNA gene copy numbers vary greatly with bacterial taxa [62,116]. The prospect of normalization tools to correct libraries for 16S rRNA gene copy numbers seems promising [62]. However, the majority of our dataset had a high uncertainty in the

assigned gene copy number, making RasperGade16S currently preemptive for the microbiota of freshwater snails. Nevertheless, the tool did allow us to assess this uncertainty, which already holds merits in its own right. Increased knowledge and taxonomic information on the microbiome of freshwater snails and its inclusion in RasperGade16S's database should resolve this issue and enable future works to correct sequencing biases based on 16S rRNA gene copy numbers.

As we worked with whole snail DNA extracts to monitor the entire holobiont, no information at tissue level has been obtained. Investigating the microbial patterns at tissue level could not only allow a comparative analysis at different parasite migration and development stages [52,98,105,117], but it could also increase the sensitivity of the analysis and perhaps increase the detection of rare taxa, which can be linked to important metabolic functions [118]. Although valuable insights can be drawn from the reported dataset, experimental artefacts due to single aquaria per parasite exposure condition may be a problem for the interpretation of our results [119]. Ideally, snails should be kept in individual aquaria to obtain fully independent replicates and a sufficient number of replicates per treatment should be obtained [119,120]. However, Portet *et al.* [4] showed that rearing conditions do not affect the microbiome and the larger difference between snails never exposed to miracidia compared to those in the various treatments suggests a limited effect of experimental artefacts for the current study (see section 3.1.1, Suppl. Figure 2B). Furthermore, the common garden of snails of the same treatment does allow direct comparison between infected and uninfected samples.

4.6. Conclusion

The data represented in this study revealed a complex interplay between schistosome biology and microbiome profiles in the snail *B. glabrata*. We revealed an intricate snail-parasite-microbiome interaction during key points of parasite development and a stabilizing effect of outcompeted parasites on this interaction. Most importantly, we show that exposed but uninfected snails have a distinctly different microbiome from infected snails, revealing the necessity of infection diagnostic tools in any future parasite exposure experiment studying the snail microbiome. Hopefully, studies like ours can

contribute to solving the complex tripartite interaction puzzle and eventually lead to potential avenues for sustainable schistosomiasis control.

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Author contributions

RS: Conceptualization; Data curation; Formal analysis; Funding acquisition; Investigation; Methodology; Visualization; Writing - original draft; and Writing - review & editing.

CH: Conceptualization; Data curation; Investigation; and Writing - review & editing.

KB: Investigation; Writing - review & editing

IV: Investigation; Methodology; and Writing - review & editing.

TH: Conceptualization; Data curation; Funding acquisition; Investigation; Methodology; Project administration; Resources; Supervision; Validation; and Writing - review & editing.

EDC: Conceptualization; Data curation; Funding acquisition; Investigation; Methodology; Project administration; Resources; Supervision; Validation; and Writing - review & editing.

Data availability

The metabarcoding data set generated and analyzed during the current study is freely available on the European Nucleotide Service (ENA), under the bioproject PRJEB77058.

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Chapter 3

Host-microbiome transplants of the schistosome snail host *Biomphalaria glabrata* reflect species-specific associations



Chapter 3

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Host-microbiome transplants of the schistosome snail host *Biomphalaria glabrata* reflect species-specific associations

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Abstract

Microbial symbionts can affect host phenotypes and, thereby, ecosystem functioning. The microbiome is increasingly being recognized as an important player in the tripartite interaction between parasitic flatworms, snail intermediate hosts, and the snail microbiome. In order to better understand these interactions, transplant experiments are needed, which rely on the development of a reliable and reproducible protocol to obtain microbiome-disturbed snails. Here we report on the first successful snail transplants involving the bacterial component of the microbiome, which indicate that *Biomphalaria glabrata* can accrue novel bacterial assemblies depending on the available environmental bacteria obtained from donor snails. Moreover, the phylogenetic relatedness of the donor host significantly affected recipients' survival probability , corroborating the phylosymbiosis pattern in freshwater snails. The transplant technique described here, complemented by field-based studies, could facilitate future research endeavors to investigate the role of specific bacteria or bacterial communities in parasitic flatworm resistance of *B. glabrata* and might ultimately pave the way for microbiome-mediated control of snail-borne diseases.

Introduction

Evidence is mounting that environmental factors affect the community of host-associated microorganisms and their gene functions also known as microbiomes (see for example the review of Stock *et al.* 2021 and references therein). For instance, microbiome metabolism can shift due to global warming, ecological interactions in the microbiome can alter due to antibiotic exposure, and skin microbiota can become dysbiotic due to eutrophication (Callens *et al.* 2018; Fontaine and Kohl 2020; Krotman *et al.* 2020). In turn, microbiomes can play an important role in ecosystem functioning by modifying host phenotypes (Moreira *et al.* 2009; Koch and Schmid-Hempel 2012; Ridaura *et al.* 2013; Macke *et al.* 2017). The microbiome is known to affect the host's physiology, behavior and disease resistance (Greyson-Gaito *et al.* 2020).

The latter is pertinent for the transmission potential of diseases (Ford and King 2016) and changes in the microbiome of intermediate hosts may thus indirectly affect ecosystem health (Flandroy *et al.* 2018). Intermediate hosts, parasites and microbes interact to determine infection outcome and, subsequently, the potential for disease transmission to definitive hosts (Brinker *et al.* 2019). There are precedents with mosquito-borne diseases, such as malaria, dengue and chikungunya, where the release of microbiome-altered vectors reduced disease prevalence, both at a local and at a regional scale (Moreira *et al.* 2009; Hoffmann *et al.* 2011; Pinto *et al.* 2021). It is hypothesized that microbiomes might affect the outcome of parasitic flatworm infections in snails by competing for resources, producing antimicrobials or stimulating the host's immune response (Huot *et al.* 2020; Portet *et al.* 2021; Stock *et al.* 2021; Le Clec'h *et al.* 2022).

Such findings have been stimulating schistosomiasis research to elucidate the tripartite interaction between the freshwater snail host, its microbiome (the bacterial microbiome) and its parasitic flatworm community. Schistosomiasis is a parasitic disease caused by schistosome flatworms (Digenea, Platyhelminthes). It burdens millions of people and animals globally due to inefficient control measures (Sokolow *et al.* 2018). Different snail-parasite strain combinations exhibit various reciprocity to infection, of which, the underlying pattern is often ill-understood (Sène, Southgate and Vercruysse 2004; Portet *et al.* 2021). One intermediate host, the Brazilian snail *Biomphalaria glabrata,* serves as a model system for studying schistosomiasis, placing it at the forefront of research focused on the snail-microbiome-flatworm interaction (Duval *et al.* 2015; Huot *et al.* 2020; Portet *et al.* 2021; Le Clec'h *et al.* 2022). A decades-long gap exists with earlier publications describing and manipulating the bacteria of *B. glabrata* (Chernin 1957, 1960). These works, however, provide the foundation for current efforts on host-microbiome interactions, more in particular for microbiome transplants.

Transplant experiments are manipulations whereby a number of bacterial strains or entire bacterial communities are actively or passively transplanted from a host or substrate to another host (Greyson-Gaito *et al.* 2020). These experiments help us to decipher the host-bacteria interaction and showed, for example, that the host-associated bacterial community determines bumblebee resistance to parasites (Koch and Schmid-Hempel 2012), water flea tolerance to toxic cyanobacteria (Macke *et al.* 2017) and mice's tendencies to become obese (Ridaura *et al.* 2013). Such transplant experiments, however, depend on the development of a reliable and reproducible protocol to obtain microbiome-disturbed recipients. The current study describes the optimization of the protocol designed by Chernin in 1957, its thorough validation with molecular tools, and the first successful transplants of bacterial communities across a taxonomic range of freshwater snails.

A phylosymbiosis pattern between freshwater snails and their bacterial community has been reported (Huot *et al.* 2020). Therefore, we hypothesize that exposure to a donor inoculum from a phylogenetically more distantly related snail would reduce the survivability of microbiome-disturbed snails as opposed to exposure to a donor inoculum from a conspecific or a phylogenetically more closely related snail. To test this and determine the most suitable conditions for bacterial transplants in the study system the experimental design (**Fig. 1a**) aimed to test 1) whether microbiome-disturbed snails would acquire a donor bacterial community, 2) what the optimal timing for exposure is, and 3) how the donor-recipient phylogenetic relationship affects the transplant outcome.

Material and methods

Cultivation of *Biomphalaria glabrata*

All individuals used in this study originated from *Biomphalaria glabrata* colonies in our laboratory. *Biomphalaria glabrata* (Brazil strain) has been a stock population in the Host-Pathogen-Environment Interactions lab at the University of Perpignan Via Domitia in France since its collection from Recife, Brazil in 1975. This strain has been previously studied through molecular markers and appeared to exhibit a low genetic diversity, probably due to the many cycling events in the laboratory (Dheilly *et al.* 2015). We received 30 specimens and egg clutches of this *B. glabrata* Brazil strain in June 2020, started maternal lines from individual egg clutches in October 2020, and conducted the transplant experiment described here in April 2022.

B. glabrata Brazil strain individuals were kept in 2 l and 5 l aquaria, depending on the cohort size, on a 12/12 day/night cycle at 25 °C (clear white light was generated by OSRAM FH 28W/840 HE). Each aquarium had a constant air supply provided through aeration stones. Fresh organic salad (pesticide-free) was provided three times per week at libitum. Aquaria were cleaned on a weekly basis with soap and mechanical rubbing, followed by an industrial washing machine program at 55 °C and drying in the oven. Aeration stones were cleaned by boiling in water for 10 min, overnight incubation in diluted bleach (1:3) and, finally, dried by connecting to the air line.

Donor microbiome

Microbiome-disturbed *B. glabrata* individuals (see below 'sterilization experiment') were exposed to three donor microbiomes. The donor microbiome hosts were selected according to a taxonomical gradient of relatedness to *B. glabrata*: same species but different maternal line (*B. glabrata*), same subfamily yet different tribe (*Planorbarius corneus;* collected from an artificial pond with GPS-coordinates: 50.999559, 4.955650), and the same superorder yet different family (*Stagnicola fuscus x S. palustris;* collected from an artificial pond with GPS-coordinates: 50.915337, 4.686979). All three donor snail species were molecularly identified (see suppl. Data: 'Donor snail host identification', **Fig. S1-S2**). The partial COI sequences of *B. glabrata, P. corneus* and *S. fuscus* x *S. palustris,* and the ITS2 rDNA sequence of *S. fuscus* x *S. palustris* were uploaded to GenBank (OQ954055-OQ954057, OQ955479).

Sterilization experiment

Prior to donor microbiome exposure, germ-free or microbiome-disturbed recipients had to be obtained. This was done as follows. At the start of each day, egg clutches of each maternal line

were collected and assessed for developmental status under a stereo microscope (Olympus SZX10). Late-development clutches were selected (**Fig. S3**), whereby, surrounding eggs were sliced open and debris was removed from around the egg of interest. Egg clusters were dissected for the three maternal lines. ±38 eggs were dissected to ensure that sufficient specimens were available for further manipulations. The eggs were kept separately per maternal line in autoclaved tap water (see next sentence for details) in 6-well plates to transport to the sterile biosafety cabinet. Dechlorinated tap water was autoclaved per litre, and is further referred to as “autoclaved tap water”. This water was aliquoted in tubes of 50 ml and was stored next to the snail aquaria at 25 °C. The UV light was turned on 15 min before turning on the circulation in the biosafety cabinet. The working surface and required utensils were disinfected with 70 % ethanol. Bleach was diluted with autoclaved tap water to obtain a working solution of 0.2 % Sodium hypochlorite or 0.042 % active chlorine. Snail eggs would not fit through normal 200 µl tips. Therefore, 200 µl tips were trimmed with sterilized scissors to have a larger opening. All plates required for that day were filled according to **Fig. 1b**. Briefly, the second column of the 24-well plates were filled with 1 ml of the 0.2 % bleach solution. All other wells were filled with 1ml of autoclaved tap water. Each first-column well received one dissected egg. Subsequently, eggs were exposed to 0.2 % bleach for 15 min. The timer started when the first egg entered the bleach solution. The following eggs for that plate were added at 30 sec intervals. Once all four eggs were in the bleach solution, we would swirl the plate gently for 10 s. This process was repeated for a total of six plates. Next, the first egg, now exposed to bleach for 15 min, was placed in the next well containing autoclaved tap water. We pipetted up and down 15 times in each well before transferring the egg to the next well to remove any remaining bleach. This manipulation was repeated two times until the egg entered the final well where it was allowed to hatch. This was repeated for all four eggs of that plate. Finally, the lid-tray interface was closed with parafilm.

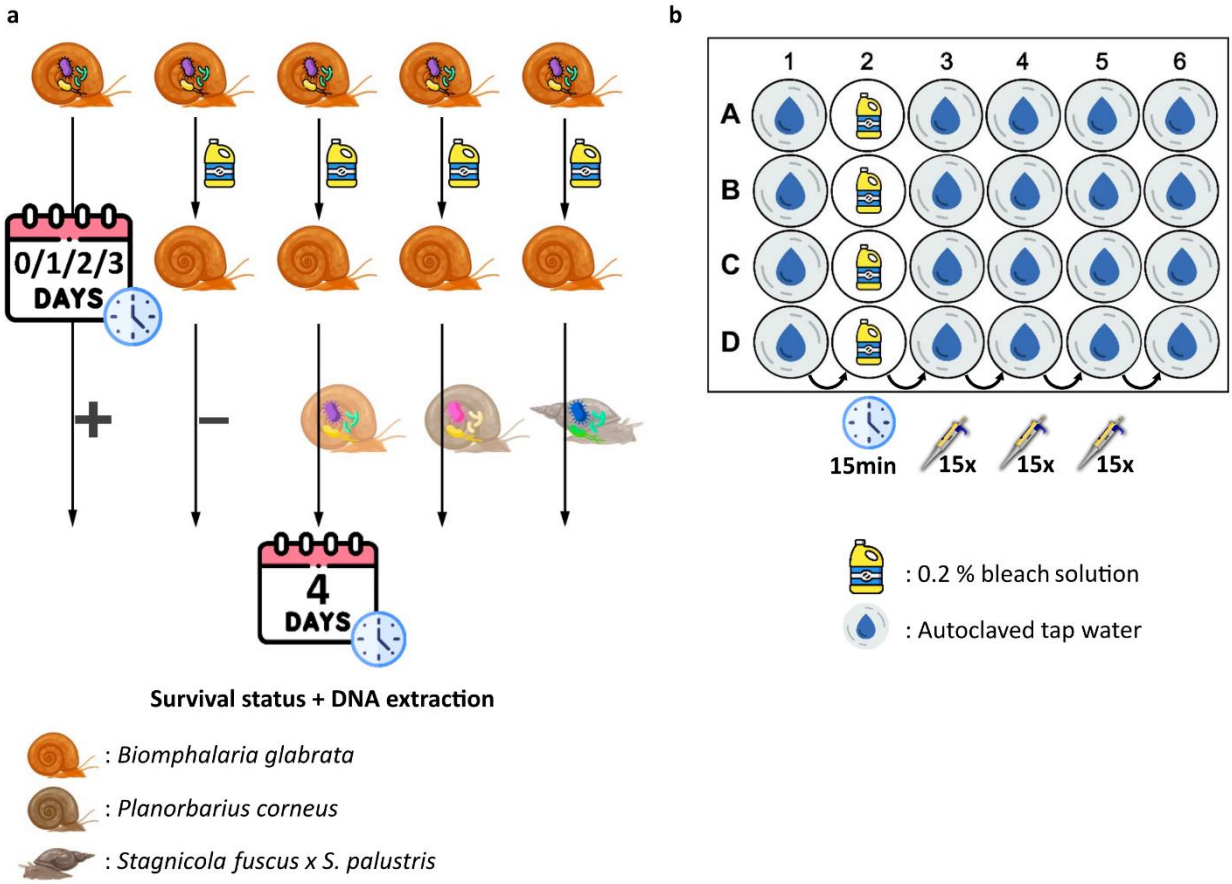


Figure 1: a) Experimental setup. Snails were sterilized on the same day as, and one day, two days and three days prior to, receiving a donor inoculum. Bacteriome-disturbed *Biomphalaria glabrata* individuals (n=8) of three maternal lines (n=3) were exposed to three donor inocula isolated from: *B. glabrata* individuals (same species but different maternal line), *Planorbarius corneus* individuals (family: Planorbidae), and *Stagnicola fuscus x S. palustris* individuals (superorder: Hygrophila). Additionally, for each factorial combination of time (n=4) and maternal line (n=3), positive (untreated samples) and negative (bleach-treated samples without donor inoculum exposure) controls were included (four technical replicates). Survival status of the egg/juvenile was noted on the day of the donor inoculum exposure, three days post donor inoculum exposure and four days post donor inoculum exposure when the specimens were sacrificed for DNA extraction. **b) The 24-well cell culture plate setup used during the sterilization protocol**. The wells of the first column receive one egg each, prior to bleach exposure. The egg from A1 is transferred to the well A2 which contains a 0.2% bleach solution and remains there for 15 min. After the 15 min bleach exposure, the egg was washed in autoclaved tap water in A3, A4 and A5 by pipetting up and down 15 times in each well before being moved to the incubation well A6. This process was done simultaneously for all four rows (A, B, C and D) for

a total of four eggs per plate. The incubation time in column six depended on the assigned condition of that sample in accordance with **Figure 1a** (0, 1, 2, or 3 days) before being exposed to a donor bacteriome. The survival status of each specimen was noted, and irrespective of whether the specimen had hatched, still in the egg, or died, was exposed to the donor inoculum in their incubation well in column six.

Transplant experiment

To test if 1) treated snails would acquire the bacterial component of a given donor microbiome, 2) what the optimal timing for exposure would be and 3) how the phylogenetic relationship between the recipient and donor host species affects the transplant outcome, we designed the following experimental setup (**Fig. 1a**). Snails were sterilized three days, two days, one day prior to and on the same day as receiving a donor inoculum. On the day of exposure donor inocula from *B. glabrata* and *P. corneus* were obtained by separately crushing three individual snails with their shell with a sterile pestle and topping the volume with autoclaved tap water until 1 ml. 300 µl of the suspension of each individual snail specimen was pooled in a 2 ml tube per donor type and topped till 1.4 ml with autoclaved tap water. Diluting the donor inocula with autoclaved tap water was required for pipet manipulations. The same protocol was used for *S. fuscus x S. palustris*, however, here only two snails were available. From the donor inocula 20 µl was each time added to the desired well. This was done for three separate maternal lines of lab-reared *B. glabrata* each time for four technical replicates (four individual snails were exposed to the same conditions). Each factorial combination (time (n=4) * maternal line (n=3)) received a donor inoculum from, either, a fourth maternal line of lab-reared *B. glabrata*, a wild-caught *P. corneus* and a wild-caught *S. fuscus x S. palustris*. Additionally, for each factorial combination of time and maternal line a positive (dissected without bleach exposure) and a negative (dissected with bleach exposure but no donor inoculum) control were included. Survival status of the egg/juvenile was noted at the day of the donor inoculum exposure, three days post donor inoculum exposure and four days post donor inoculum exposure when the specimens were sacrificed and the experiment ended. Snails were pooled by up to three specimens per factorial combination (time (n=4) * maternal line (n=3) * donor microbiome (n=3) *survival status (n=3)) in order to obtain sufficient DNA for subsequent analyses. All negative controls and the positive controls of day 0 and day three were tested for the presence of bacteria in the growth assay in combination with microscopic investigation and qPCR. These and all other samples were included for 16S rRNA gene metabarcoding.

DNA extraction

Snails were crushed with their shell with a sterile pestle in 100 µl of autoclaved tap water by up to three specimens per factorial combination in order to obtain sufficient DNA for subsequent analyses. The complete homogenate was used for recipients of donor microbiomes. For all negative controls and the positive controls at day zero and three, 90 µl of this homogenate was used for DNA extraction (5 µl was used for microscopic investigation and 5 µl for the growth assay). DNA of all samples was extracted using the E.Z.N.A.® Mollusc DNA Kit (OMEGA Bio-tek, Norcross, GA, USA) according to the manufacturer's protocol and eluted through two elution steps of 50 µL, totalling to 100 µL of DNA extract. DNA concentrations were determined using the Qubit dsDNA high-sensitivity assay (Thermo Fisher Scientific) for all DNA extracts. 3 ng of template DNA (max. 10 µl) was used for qPCR. This indicated that DNA concentrations were low for some samples (mean = 0.16 ng / µl, sd = 0.14 ng / µl). Therefore, the DNA for all samples was concentrated with the Eppendorf™ Concentrator Plus for 30 min at 30 °C, halving the total volume. Subsequently, Qubit measurements were repeated on all samples (mean = 0.35 ng / µl, sd = 0.33 ng / µl). This protocol increased the total amount of DNA per PCR reaction from 0.3 ng to 0.6 ng.

Screening for germ-free samples

We screened for germ-free samples through a growth assay, microscopic investigation and qPCR. For the growth assay, 5 µl of the homogenate was added to 4 ml of R2A medium. The R2A medium was selected as it is a medium capable of growing a broad range of bacteria (Aditi *et al.*, in prep.). The R2A medium consisted of 0.5 g/l Bacto™ Proteose peptone (Gibco), 0.5 g/l Casamino acids (Gibco), 0.5 g/l Yeast extract (OXOID), 0.5 g/l Dextrose [D-(+)-Glucose] C6H1206 – M 180, 16 g/mol (Carl Roth), 0.5 g/l Soluble starch (Merck), 0.3 g/l Dipotassium hydrogen phosphate (K2HPO4) (Merck), 0.024 g/l Magnesium sulphate (MgSO4.7H20) (Duchefa), 0.3 g/l Sodium pyruvate 100 mM (100x) (Gibco) and topped till one litre with distilled water. The resulting medium was autoclaved and stored at room temperature. The sterile 14 mL tube (Greiner Bio-one) was vortexed briefly and incubated with constant mechanical mixing at 25 °C for 72 h. Prior to OD measurements at 600 nm, the R2A medium was pipetted up and down several times to ensure adequate mixing and homogeneous OD values per sample. Measurements were done on 1 mL of medium in the Genesys 10S UV-Vis Spectrophotometer (Thermo Fisher Scientific) using the Macro-cuvette, 1 mL (Greiner Bio-one). R2A medium incubated with 5 µl of autoclaved tap water was used as a negative to obtain 0 OD values. Samples were considered germ-free if the OD measurement was 0.

To microscopically assess for bacterial presence, the LIVE/DEAD® *Bac*Light™ Bacterial Viability Kit L13152 (Thermo Fisher Scientific) was used. Each dye was dissolved in 2.5 ml of autoclaved tap water and 10 µl of a (2/3 Propidium Iodide and 1/3 SYTO9) mix to 5 µl of sample. Then it was incubated in the dark for 15 min. Subsequently, 6 µl of the sample was placed under a cover glass and the slide was visually inspected under a fluorescence microscope (Olympus BX51, U-MWB2 Blue fluorescence filter, metal halide lamp). Twenty fields of view were examined for bacterial presence at random, unless a piece of tissue/shell/matrix was seen in the field of view, then this received special attention as bacteria were notably more likely to grow on such substrates, thus increasing our chances of finding bacteria in supposedly microbiome-disturbed samples. If no bacteria were detected in these 20 fields of view, the sample was classified as germ-free.

qPCR reactions targeting bacterial 16s rRNA gene were run on the LightCycler® 480 (Roche). Each reaction consisted of 10 µl of LightCycler® 480 SYBR Green I Master (Life Science), 1 µl 16S-Fw (5'-AGACACGGTCCAGACTCCTAC-3') at 10µM, 1 µl 16S-Rv (5'-CTTGCACCCTCCGTATTACCG-3') at 10 µM, 3 ng of template DNA (n µl) and 8-n µl of sterile Milli-Q water to a final reaction volume of 20 µl, according to the protocol developed by Callens *et al.* (2018). The temperature cycle was as follows, 5min of initial denaturation at 95°C, followed by 45 cycles of 10 sec at 95°C, 20 sec at 60°C and 5 sec at 72°C. Each sample was done in triplicate. Standards were obtained by extracting DNA from a pure *Eisheria coli* culture, measuring the DNA concentration and comparing this weight to the weight of a single *E. coli* genome. Standards of the desired concentration were mixed with 4 µl of salmon sperm in a total volume of 5 µl to prevent plasmid loss caused by nonspecific binding to surfaces. The melting and amplification curves were calculated through the LightCycler® 480 software (v. 1.5.1) and exported as reports to pdf and excel. The excel dataset was then further analysed in R (v. 4.2.2, 64-bit) using RStudio (v. 2022.12.0). Samples were considered germ-free if the Cp value was not significantly different from negative controls in post-hoc pairwise comparisons calculated through the contrast and lsmeans functions with a Scheffe correction for multiple comparisons (emmeans package, v. 1.8.6, Lenth 2023).

16S rRNA gene metabarcoding

To characterize the microbiome of our samples, a 16S rRNA gene metabarcoding approach was used. The PCR protocol targeting the V3 and V4 hypervariable regions of the 16S rRNA gene (Klindworth *et al.* 2013) was adapted from (Huot *et al.* 2020). PCR reactions were conducted in triplicate for all samples in a 25 µl total volume. 12.5 µl of PCRBIO HS VeriFi™ Mix (PCR Biosystems™), 0.75 µl of the primer 341F (5'-CCTACGGGNGGCWGCAG-3') at 20 µM, 0.75 µl of

the primer 805R (5'-GACTACHVGGGTATCTAATCC-3') at 20 µM and a variable volume of DNA (0.6 ng of DNA per sample) and water (11 µl – the volume of DNA) to complete the volume to 25 µl and obtain a total DNA input of 0.6 ng. An initial denaturation at 98 °C was done for 5 min, followed by 25 cycles of denaturation at 98 °C for 45 s, annealing at 60 °C for 15 sec and extension at 72 °C for 10 sec and, finally, an elongation step at 72 °C for 7 min. The resulting triplicate PCR products were pooled per sample and subsequently purified with magnetic beads (CleanNGS, GC Biotech) following the manufacturer's protocol. The purified PCR products were sent to the Genomics core at UZ Leuven for sequencing (Illumina Miseq v3). First a quality control was performed through a fragment analyzer, and the DNA concentration was measured through Qubit. Then a second PCR reaction attached the Illumina adapters and indexes to the DNA fragments through the Genomics core tails. This PCR was done in a total volume of 20 µl and consisted of 9 µl PCR1 product (concentration 0.5-5 ng/ µl), 0.5 µl of the forward P7 primer (5 µM), 0.5 µl of the Reverse P5 primer (5 µM) and 10 µl of Phusion high fidelity PCR master mix (Thermo Fisher Scientific). PCR conditions were the following. First an initial denaturation step at 94 °C for 30 s, 15 cycles of a denaturation step at 94 °C for 10 s, an annealing step at 51 °C for 30 sec and an elongation step at 72 °C for 30 sec and, finally, an elongation step at 72 °C for 1 min. After this PCR a second quality control was done with the fragment analyzer to make sure the adapters have joined properly (i.e. fragments increased by +-60bp). Qubit analysis was performed once more and a final equimolar library was created. Finally, 14 pM was loaded with 37 % PhiX spike-in on the Illumina MiSeq v3 sequencer (2 x 300 bp with the 600-cycle kit).

Mock communities

In order to optimize and quantify any biases during our pipeline, two types of mock communities were included in the 16S rRNA gene metabarcoding run. First, the 10 Strain Even Mix Genomic Material MSA-1000™ (ATCC) is a DNA-based mock community that allowed us to exclude any potential extraction bias. It consists of 10% *Bacillus pacificus* (ATCC 10987), 10% *Bifidobacterium adolescentis* (ATCC 15703), 10% *Clostridium beijerinckii* (ATCC 35702), 10% *Deinococcus radiodurans* (ATCC BAA-816), 10% *Enterococcus faecalis* (ATCC 47077), 10% *Escherichia coli* (ATCC 700926), 10% *Lactobacillus gasseri* (ATCC 33323), 10% *Cereibacter sphaeroides* (ATCC 17029), 10% *Staphylococcus epidermidis* (ATCC 12228) and 10% *Streptococcus mutans* (ATCC 700610). Second, the 10 Strain Even Mix cell Material MSA-1000™ (ATCC) is a cell-based mock community that allowed us to assess any potential extraction bias by comparing the results to the DNA-based mock community (2x107 whole cells/vial ± 1 log). It consists of freeze-dried cell material of the same 10 strains and ratios as the MSA-1000™ mock community. The mock

communities also allow us to determine the suitable threshold below which spurious sequences should be removed as discussed by Reitmeier *et al.* (2021).

Analysis of sequencing data

The raw reads of our 16S rRNA gene metabarcoding approach were processed based on Janssens *et al.* (2022) with the QIIME2 pipeline v2022.2 (Bolyen *et al.* 2019). First, the paired-end demultiplexed sequence reads were imported and their quality scores were assessed through MultiQC (Ewels *et al.* 2016). The run resulted in 4 708 922 raw sequences (min 22 608, max 142 259), 123 622 allocated to mock communities. Using the denoise function of DADA2 within QIIME2, both forward and reverse reads were trimmed for 15 bp and truncated at 280 bp for the forward read and 240 bp for the reverse read, while demanding a 30 bp overlap between both reads. Furthermore, the paired-end sequences were filtered, denoised and dereplicated, chimeras were filtered and an amplicon sequence variants (ASVs) table was created using this denoise function. As a result, on average 59.4% of the reads were maintained per sample (sd= 9.7%). A total of 3 161 ASVs across all samples was obtained with an average length of 425.82 bp (sd= 12.27). The Silva 138 SSU Ref NR 99 database (Quast *et al.* 2013) was used to assign taxonomy to the ASVs. It contained the extracted sequences of the V3-V4 region using the forward primer (5'-CCTACGGGNGGCWGCAG-3'), reverse primer (5'-GACTACHVGGGTATCTAATCC-3') and truncation length (465 bp). The R pipeline of Callahan *et al.* (2016) was modified for this study and ran with R using RStudio on a Windows machine (Windows 11). Briefly, the resulting feature table, taxonomy, metadata and phylogenetic tree were imported in R through the phyloseq package (v. 1.42.0, McMurdie and Holmes 2013). The R package Decontam (v. 1.18.0, Davis *et al.* 2018) was used to look for contaminating DNA using the prevalence method and a 0.1 threshold. The PCR negative (n = 1) and extraction negative (n = 1) controls were used as negative samples to detect contamination. The mock community dataset did not have contamination in the 21 detected ASVs according to the Decontam package. The transplant experiment had 64 contaminating ASVs out of the total 3 156 ASVs removed from the dataset. Further manual cleaning and filtering of the dataset was performed in R based on Janssens *et al.* (2022), whereby chloroplast and mitochondrial ASVs were removed from the dataset and ASV taxonomic information was corrected. The Vegan package (v. 2.6-4, Oksanen *et al.* 2013) was used to construct a rarefaction species richness curve (Heck Jr., van Belle and Simberloff 1975). Samples were rarefied to the sampling depth of the sample with the fewest reads (i.e., 15 321) using the "rarefy_even_depth" function of the phyloseq package (rngseed = 711), while ensuring that the plateau phase was reached for all samples. Consequently, 208 ASVs were removed from the dataset. Subsequently,

low abundance taxa (cumulative relative read abundance < 0.5%, 2 046 ASVs; see end of this section for determining this threshold) were removed through the “prune_taxa” function of the phyloseq package. Subsequent analyses excluded snail specimens that were still in the egg at the end of the experiment or died prior to donor inoculum exposure. Alpha diversity measures included the Shannon (phyloseq package, function “estimate_richness”) and the Faith's phylogenetic diversity (picante package, v. 1.8.2 (Kembel *et al.* 2010); function “pd”). A generalized linear model with gamma distribution was used to model the effect of treatment on alpha diversity (stats package, v4.2.2). Significance was tested with an ANOVA type III test (car package, v3.1-2, Fox and Weisberg 2019). Outliers, influential observations and normality were assessed using the outliertest (car package), cooks.distance (stats package), and shapiro.test (stats package) functions, respectively. A Tukey pairwise post-hoc comparison was conducted through the glth function (multcomp package, v 1.4-25; Hothorn, Bretz and Westfall 2008). Beta diversity measures included the Bray Curtis and Jaccard index, visualized through NMDS (phyloseq package, function “ordinate”) and RDA (vegan package, function “capscale”). An analysis of variance model was used to model the effect of treatment on beta diversity (stats package). Significance was tested with an ANOVA type III test (car package). For model assumptions, we tested whether the ratio of maximum variance to minimum variance did not exceed five, and outliers, influential observations and normality were assessed as mentioned above. The micrUBIfuns (v. 0.1.0., Sousa 2020) function was modified to study the within group beta diversity for several diversity measures. Core microbiome analyses were conducted with the microbiome package (v. 1.20.0, Lahti and Shetty 2012) using different detection (0.01 – 0.001) and prevalence (0.85 – 0.95, value determined to allow the taxon to be absent from a maximum of one sample) values. For the analyses of the core microbiome we adhered as much as possible to the guidelines of Neu, Allen and Roy (2021) and conducted the analyses on non-rarefied, rarefied and rarefied with relative abundance thresholds of 0.25% and 0.5 %. Kaplan-Meier survival curves were created (survival package, v. 3.4-0; function “survfit”) and significance tested (p- and $\chi 2$-values, survival package, function “survdiff”, Therneau 2020) based on a subset of the dataset that excluded snail specimens still in the egg at the end of the experiment and specimens that died prior to inoculum exposure. Finally, to assess a suitable threshold below which spurious sequences should be removed (Reitmeier *et al.* 2021), a maximum likelihood (ML) tree was calculated through the IQ-tree web servers (http://iqtree.cibiv.univie.ac.at), and nodal support was assessed through 1 000 bootstrap replicates (Trifinopoulos *et al.* 2016). The resulting tree was midpoint rooted in Figtree (version 1.4.4) and tip labels were edited. The resulting tree was exported in PDF format to Adobe Acrobat Pro DC (version 2022.003.20310), in which we adjusted

the position of ill-placed nodal support values and italicized species names. Subsequently, a cumulative relative read abundance threshold of 0.5% was selected based on **Fig. S4** and **Table S1,** limiting our dataset as much as possible to the expected ASVs.

Results and discussion

The germ-free status was assigned to five out of a total of twelve bleach-treated samples (42%; **Fig. S5**, **Table S2**) and was affected by the maternal line of the recipient (**Fig. S6b**, χ^2 (2, n = 35) = 44**,** p<0.001). The timespan between sterilization and DNA extraction did not affect the germ-free status of a sample (**Fig. S6a**, χ^2 (3, n = 35) = 2.2, p=0.53). The true germ-free rate of individual snail specimens is expected to have been higher than the detected 42% because the tested samples all comprised of three individual snails. Hence, one contaminated snail is sufficient to label none of the three specimens as germ-free. Importantly, the detection of germ-free snail specimens indicates that vertical transmission within *B. glabrata* eggs is, at the minimum, not omnipresent. This does not exclude vertical transmission on the egg surface or within the gelatinous matrix of the egg clutch.

Sterilization on the same day as exposure to a donor inoculum induced a high mortality rate (**Fig. S6a**, χ^2 (3, n = 280) = 107, p<0.001). We speculate that the snail specimens might need some time (minimally 24h) to recover from bleach exposure. Perhaps, opportunistic pathogens are at play here, whereby otherwise symbiotic bacteria turn pathogenic when the opportunity arises (Brown, Cornforth and Mideo 2012). However, since the experimental setup required all specimens to be dissected and bleach-exposed per time factor, perhaps this pattern is due to a batch effect. Despite the utmost care taken during the experiment we cannot, at this point, rule out such an experimental artifact. Nevertheless, excluding the samples that were sterilized at the same day as exposure to a donor inoculum, resulted in the same pattern in survival probability across the different inoculum types for the remaining samples.

The donor inoculum affected overall survival chance of juvenile snails (**Fig. 2**, χ^2 (4, n = 280) = 26, p<0.001). Moreover, microbiome-disturbed snails that received a *P. corneus* donor inoculum survived better than snails without inoculum or with a *S. fuscus x S. palustris* donor inoculum (χ^2 (2, n = 60) = 6.79, p=0.02). A similar trend, although not significant, is notable for snails that received a *B. glabrata* donor microbiome (χ^2 (2, n = 55) = 5, p=0.07). The combination of all these results corroborate our hypothesis and the phylosymbiosis pattern reported by Huot *et al.* (2020), more in particular that exposure to a donor inoculum from a phylogenetically more distantly related snail reduces the fitness of microbiome-disturbed snails as opposed to exposure to a donor inoculum from a conspecific or a phylogenetically more closely related snail. Interestingly, survivorship was not significantly different when microbiome-disturbed snails were exposed to a donor inoculum originating from a conspecific or a species from the same family (Planorbidae). A pattern possibly explained by the wild origin of the latter species as species kept in laboratory conditions tend to rapidly loose microbial diversity (Voulgari-Kokota *et al.* 2022; Baldassarre,

Reitzel and Fraune 2023), counteracting the phylogenetic distance between the host species. However, since the alpha diversity measures between both donor types was not significantly different this seems unlikely (**Fig. S8**, p = 1). Mollusks have been reported to form novel microbial assemblies following colonization events in novel regions. Since this is often reported for invasive species (Bankers *et al.* 2021; Chiarello *et al.* 2022), it could indicate that this group has a readily adaptable microbiome if needed.

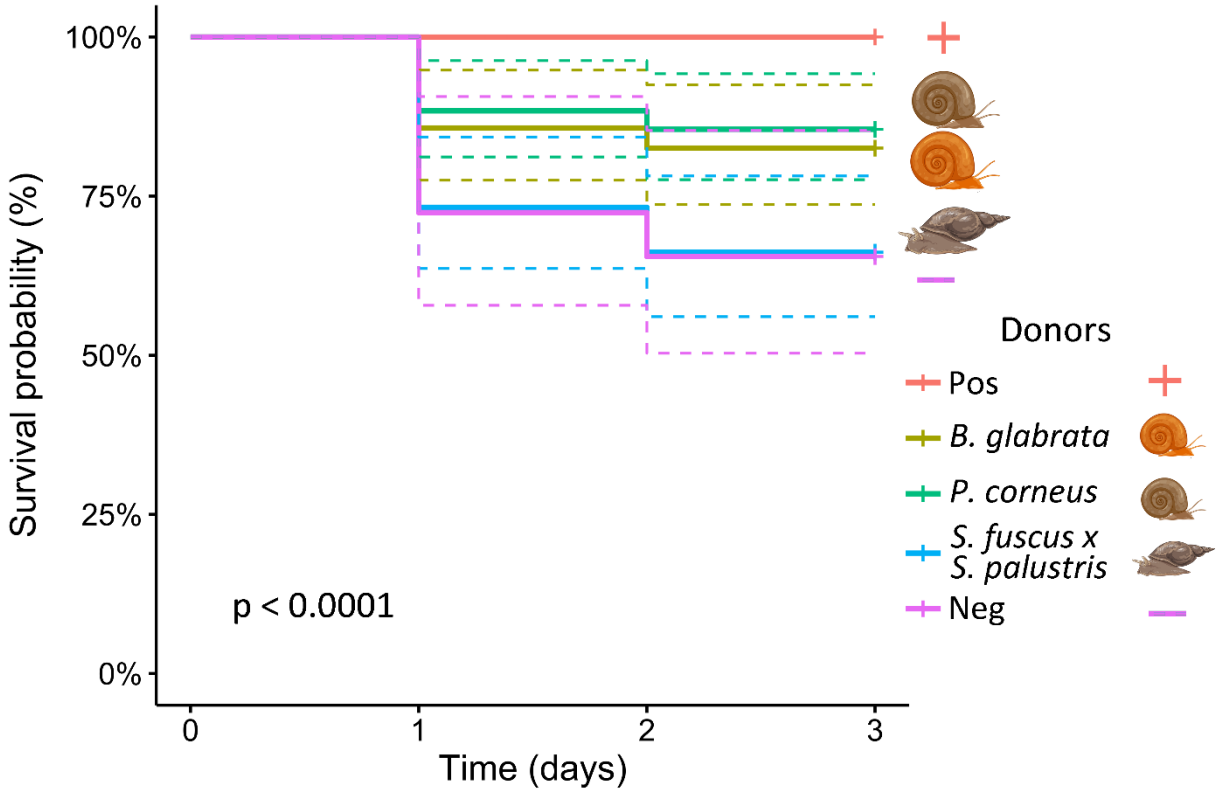


Figure 2: Kaplan-Meier survival curve across all days per donor type, excluding specimens dead at the start of the experiment or unhatched at the end of the experiment. 'Pos' refers to positive controls (untreated samples), and 'Neg' refers to negative controls (bleach-treated samples without donor inoculum exposure). Dotted lines indicate 95% CI for each respective treatment. The donor inocula were isolated from *Biomphalaria glabrata*, *Planorbarius corneus*, and *Stagnicola fuscus x S. palustris* individuals. The difference in survival probability between the different donor treatments was highly significant (p<0.0001).

The recipients of donor inocula across all three treatments shared ASVs belonging to the family Pseudomonaceae, Comamonadaceae and Flavobacteriaceae when considering a prevalence of minimally 95 % (maximum missing in one sample) and a relative read abundance of minimally 1 %. These core members made up 49 % of the reads across all samples (sd= 12 %), providing a

set of potentially ecologically relevant taxa for future research. However, the persistence of this core microbiome through time remains uncertain. ASVs belonging to the family Pirellulaceae were part of the bacterial component of the core microbiome of bleach-treated snails and occurred in all but one sample. This core member had a highly variable abundance across all negative samples (32 %, sd= 28 %). It also did not occur in the PCR negative, indicating that it is not due to post-experiment contamination.

Bleach-treated samples, negative controls and the *S. fuscus x S. palustris* donor inoculum had a lower alpha diversity compared to the other samples (**Fig. S8**, $p<0.01$). Samples that were inoculated with *B. glabrata* or *P. corneus* were not significantly different from their respective donor inocula. In contrast, samples that were exposed to the *S. fuscus x S. palustris* donor inoculum were significantly different from the donor. This discrepancy is presumed to be a consequence of a sequencing bias of the *S. fuscus x S. palustris* donor inoculum as quality checks prior to sequencing indicated failed amplification but the sample was sequenced nevertheless.

The type of donor inoculum affected the bacterial community composition of the recipients (**Fig. 3** and **Fig. S9**, $p<0.005$). Contrary to expectations, **Fig. 3a** shows each treatment type to cluster most closely to the bacterial profile of its donor inoculum yet more distant from the centroid. The previously reported phylosymbiosis pattern would suggest that a snail species assimilates more similar communities from different microbial pools, hence recipient samples were expected to be closer to the centroid. This can potentially be explained by 1) the 'Anna Karenina principle', whereby dysbiotic communities tend to be more dissimilar than healthy communities, 2) alternative stable states of bacterial communities or 3) the checkerboard pattern, whereby certain taxa tend to exclude each other from the same habitat (Levy and Borenstein 2014; Zaneveld, McMinds and Vega Thurber 2017). Our data seems to indicate that the former hypothesis is least probable, because individuals that received the same bacterial inoculum display lower dissimilarities compared to controls (**Fig. 3b, Fig. S9**) (Zaneveld, McMinds and Vega Thurber 2017). As it is unclear at this point whether the different bacterial communities have different metabolic patterns, one cannot ascertain between alternative stable states or the checkerboard pattern. To discern between the three previously listed hypotheses, future experiments should assess the stability, variability and metabolic function of the bacterial community, following a transplant event, after a small perturbation and over time (Zaneveld, McMinds and Vega Thurber 2017). The latter should also reveal how long the bacterial transplants persist over time. Notably, our dataset might have been skewed against rare taxa due the 40 PCR cycles used to create our sequencing library and the threshold value used to remove spurious sequences. Nevertheless, the inclusion of such a

threshold value is desirable as it improves reproducibility of microbiome analyses (Reitmeier *et al.* 2021).

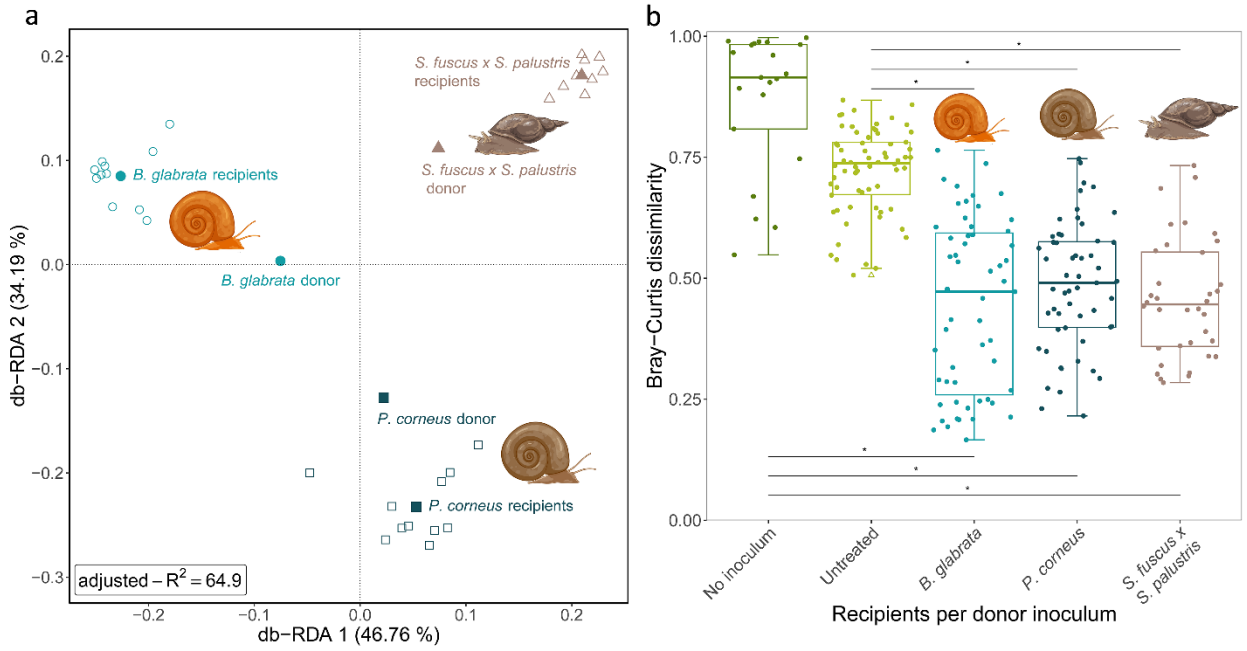


Figure 3: Beta diversity measures across the different treatments. **a** RDA plot showing the beta diversity of the samples that received a donor inoculum (Bray-Curtis dissimilarity). The model contained only the donor type and had a R^2 value of 64.9%. **b** The within-group Bray-Curtis dissimilarity (n=233). 'No inoculum' samples (negative controls) were dissected and exposed to bleach but received no donor inoculum. Untreated samples (positive controls) were dissected but were not exposed to bleach and did not receive a donor inoculum. Outliers are indicated by triangles. The lower and upper hinges correspond to the first and third quartiles (the 25th and 75th percentiles). The whiskers extend from the hinge to the largest value no further than 1.5 times the interquartile range from the hinge. '*' indicates p<0.0001.

Future studies can apply our transplant protocol to decipher how snail genotypes interact with microbiota to drive resistance to flatworm parasite infection similar to the experimental setup of Macke *et al.* (2017) for *Daphnia* genotype x bacterial community interactions with respect to toxic cyanobacteria tolerance. The described technique has been designed on an oviparous freshwater snail system and might, therefore, not necessarily be applicable to all other snail species. For example, dissecting and sterilizing eggs of ovoviviparous snails will come with a new set of challenges. Nevertheless, the technique and patterns reported here provide a foundation for future research to build upon. For example, it could help with two interesting hypotheses with respect to

the ovoviviparous freshwater snail *Potamopyrgus antipodarum* (Takacs-Vesbach *et al.* 2016). First, the data of Takacs-Vesbach et al. (2016) suggests a link between the microbiome and the reproductive mode of *P. antipodarum.* Second, the authors bring forward that snail genotype-microbiome interactions are determining snail susceptibility to *Microphallus* infections. Following optimization of our protocol, it could provide the foundation for a reciprocal transplant experiment, testing a causal link between these factors.

Another interesting pattern is the correlation between bacterial diversity and the Guadeloupe resistance complex (CRC) genomic region in *B. glabrata* (Allan *et al.* 2018). Our transplant experiment could be applied in a reciprocal transplant experiment followed by an exposure experiment to *S. mansoni* to detect a direct link between the CRC genomic region, microbiome and infected resistance of *B. glabrata* to schistosome parasites.

The microbiome can determine the transmission potential of vectors (Ford and King 2016). Previous works hypothesize this might also be the case for the tripartite interaction between parasitic flatworms, snails and their microbiome (Huot *et al.* 2020; Portet *et al.* 2021; Stock *et al.* 2021; Le Clec'h *et al.* 2022). The outcome of the tripartite interaction may have profound effects on ecosystem health as parasitic flatworm biomass is similar to that of birds or fishes in estuaries (Kuris *et al.* 2008), but is also highly relevant for human and animal health (Flandroy *et al.* 2018). The microbiome will prove an invaluable factor in better understanding the epidemiology of parasitic flatworms, especially given the wide spread of invasive and occasionally extremely permissive snail species (Lounnas *et al.* 2017; Carolus *et al.* 2019; Bankers *et al.* 2021), or the inconclusive results on snail-parasite interactions in endemic areas (Picquet *et al.* 1996; Southgate *et al.* 2000; Sène, Southgate and Vercruysse 2004; Webster *et al.* 2012).

This study reveals the potential for bacterial community transplant experiments in freshwater snails. Although such controlled laboratory experiments will undoubtedly provide vital insights into the tripartite interaction between snails, bacteria and parasitic flatworms, extrapolation of patterns and protocols to field settings might prove complicated (Greyson-Gaito *et al.* 2020). However, correlative field-based studies could fill these caveats and, likewise, transplant experiments could help discern correlation from causation in a controlled setting (Stock *et al.* 2021). Correlative studies, based on museum collections for example (Chalifour, Elder and Li 2022), could help in identifying bacterial strains involved in snail resistance. The potential of these strains should then be further examined in transplant experiments in controlled lab environments and mesocosm studies prior to manipulations in the field. We advocate in doing these contained experiments ahead of unconfined biological manipulations as the outcome can be hard to predict. Nevertheless, an improved understanding of the tripartite interaction between hosts, bacteria and

pathogens will prove paramount to fully grasp disease epidemiology and field-based correlative studies might help guide experimental efforts.

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Conflicts of interest

The authors declare no competing interests.

Author contributions

RS, IV and ED conceived the study, collected the samples, and wrote the manuscript. RS and IV conducted the experiments and prepared the sequencing library. RS performed the data analysis and prepared the figures. RS, TH and ED interpreted the data. All authors revised and commented on the manuscript. All authors have read the final version of the manuscript and approved of its content.

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Data Availability Statement

The metabarcoding dataset generated and analysed during the current study is freely available on the European Nucleotide Service (ENA), under the bioproject PRJEB60803. The COI and ITS2 sequences used for the identification of the donor snail species were uploaded to GenBank and can be retrieved under accession numbers OQ954055-OQ954057, OQ955479.

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Chapter 4

The bacterial aspect of the microbiome does not drive liver fluke resistance in some populations of the globally invasive snail *Pseudosuccinea columella*



Chapter 4

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The bacterial aspect of the microbiome does not drive liver fluke resistance in some populations of the globally invasive snail *Pseudosuccinea columella*

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Abstract

Globalization has been facilitating biological invasions, including those by freshwater snails and trematode parasites. One such example is the freshwater snail *Pseudosuccinea columella* that spread globally. The invading populations are highly susceptible to infection by *Fasciola* trematodes, with major repercussions for human and animal fasciolosis or liver rot. Notably, six *P. columella* populations exist in Cuba, which are completely resistant to *F. hepatica* infection. It has been shown that resistant and susceptible populations of *P. columella* have different bacterial communities, suggesting a potential role for microbiome-mediated resistance. We adapted a sterilization and transplant protocol for the bacterial aspect of the microbiome to *P. columella*. We then applied it in a reciprocal transplant experiment involving resistant and susceptible *P. columella* snails followed by exposure to *F. hepatica* miracidia. This allowed us to investigate if resistance can be modified by the bacterial aspect of the microbiome. We studied the bacterial communities and assessed the effect of bacterial profiles on the success rate of *F. hepatica* infection. Despite high mortality, the sterilization and transplant protocols were successfully adapted to *P. columella.* Inocula drove bacterial communities of recipients, however, failed at shifting susceptibility/resistance patterns in *P. columella* to *F. hepatica* infections throughout our experiment. In contrast, parasite exposure did alter bacterial profiles at both alpha diversity and community level, suggesting the potential for microbiome-mediated transgenerational effects of parasite resistance. These findings are broadly relevant given the recent introduction of *P. columella* to Europe, whereby an ongoing invasion could set the stage for a European fasciolosis epidemic as has happened in Africa. Therefore, identifying the exact drivers of resistance will be imperative for sustainable and effective disease control measures.

Introduction

Climate change alone will increase nearly half of the currently known human pathogenic diseases, including trematodiases (Mora *et al.*, 2022). These diseases, also known as snail-borne diseases, are caused by infections with trematodes, a group of flatworm parasites with a worldwide distribution (Toledo and Fried, 2014). Trematodes and their intermediate snail hosts rapidly colonize new areas due to the globalized transport of goods and people (Appleton, 2003; Boissier *et al.*, 2016). Snails are critical to complete the lifecycle while playing a limited role in the introduction events of trematodes compared to the more mobile final hosts (Blasco-Costa and Poulin, 2013). Simultaneously with increased introduction events of organisms through globalization (Gubler, 2010), artificial impoundments increase snail habitat, facilitating biological invasions (Southgate, 1997; Carolus *et al.*, 2019). A key beneficiary of these changes is the globally invasive snail *Pseudosuccinea columella*.

This species is native to the American continent but has spread globally with ongoing invasions throughout Pacific and Caribbean islands, South America, Africa, Australia, and Europe (Pointier *et al.*, 2007; Lounnas *et al.*, 2017; Alba *et al.*, 2019b; Ngcamphalala *et al.*, 2022). *Pseudosuccinea columella* mostly self-fertilizes, which likely is a contributing factor to its invasiveness (Lounnas *et al.*, 2017; Alba *et al.*, 2019b). It is one of the major intermediate host snails of *Fasciola hepatica* (Krull, 1933; Dar *et al.*, 2015; Alba *et al.*, 2019b) worldwide and its presence has been found to increase transmission of *Fasciola nyanzae* (Carolus *et al.*, 2019; Schols *et al.*, 2021; Schols and Huyse, 2024) and *Fasciola gigantica* (Grabner *et al.*, 2014) in Africa. These *Fasciola* species are causative agents of fasciolosis or liver rot in wildlife, livestock and human populations (McCully *et al.*, 1967; Toledo and Fried, 2014). Liver fluke infections in animals drastically reduce the health, reproduction and lifespan of their host, causing hepatic fibrosis, hemorrhage, and bile duct calcification, in addition to increased abortion rate, lower productivity and higher mortality (McCully *et al.*, 1967; Toledo and Fried, 2014; Mehmood *et al.*, 2017). As a result, global economic losses are estimated to exceed 3.2 billion US dollars with over 600 million infected domestic animals (Toet *et al.*, 2014; Mehmood *et al.*, 2017). Concerning human health, over 50 million people are estimated to suffer from fasciolosis (Nyindo and Lukambagire, 2015) with continuously increasing counts (Alba *et al.*, 2021). For example, flooding of agricultural fields, an extreme weather event expected to become more common with climate change (Utaaker and Robertson, 2015), has led to fasciolosis outbreaks in Cuba (Rojas *et al.*, 2010).

Interestingly, *P. columella* presents as two different phenotypes in response to *F. hepatica* infection. Most populations, including the globally distributed ones showing the invasive genotype, are susceptible to the parasite. In contrast, Cuba is home to *P. columella* populations that are

susceptible (S) or completely resistant (R) to *F. hepatica* infections. While susceptibility is characterized by a polymorphism of compatibility, resistance to *F. hepatica* in this species is extreme and monomorphic; always 0% prevalence irrespective of parasite dose, isolate or infection scheme (Gutiérrez *et al.*, 2003; Alba *et al.*, 2018, 2020, 2022). However, the resistant phenotype has a small distribution range, occurring in merely six locations (Alba *et al.*, 2019b). This is presumably due to a fitness cost and reduced competitive ability as exemplified by reproductive constraints observed in the laboratory (Gutiérrez *et al.*, 2002; Alba *et al.*, 2019b).

The resistant phenotype is characterized by a potent immune response whereby hemocytes rapidly encapsulate invading miracidia within 24 hours *in vivo* (Gutiérrez *et al.*, 2003) and *in vitro* (Alba *et al.*, 2020). The hemocytes are potentially activated through a rapid increase in the expression of granulin, a cytokine linked to trematode resistance in snails (Pila *et al.*, 2016; Alba *et al.*, 2020). In contrast, granulin expression is downregulated in the susceptible phenotype and is potentially parasite-mediated (Alba *et al.*, 2020). These first hours are key for infection development, as also seen in other trematode systems (Núñez *et al.*, 1994; De Jong-Brink *et al.*, 2001). The resistance in *P. columella* originates from an immunological basis rather than parasite-host strain incompatibility (Alba *et al.*, 2022). Indeed, the resistant phenotype is characterized by a constitutive increase of biological processes, transcripts and proteins linked to immune and stress responses (Alba *et al.*, 2019a). Nevertheless, such resistance appears to be parasite-specific, as susceptible and resistant phenotypes are equally susceptible to *Trichobilharzia* sp. and echinostomes, suggesting, at least to some extent, parasite-driven selection (Alba *et al.*, 2022).

Host-parasite interactions can be shaped by the environment, which includes abiotic and biotic factors such as the microbiome (Wolinska and King, 2009; Macke *et al.*, 2017b). Microbiomes are suggested to be important in structuring host phenotypes with consequences up to the ecosystem level (Decaestecker *et al.*, 2024). The microbiome has been implicated in defining the immune system during host development (Knutie *et al.*, 2017) and by regulating immune-related genes (Murdoch and Rawls, 2019), and might, therefore, lay at the basis of resistance/susceptibility in *P. columella*. Indeed, the Guadeloupe Resistance Complex, a genomic region linked to schistosome resistance has been linked to specific microbiota (Allan *et al.*, 2018). Therefore, it has been suggested that the microbiome might (co-)determine a snail's resistance to trematode infection (Allan *et al.*, 2018; Huot *et al.*, 2020; Portet *et al.*, 2021; Le Clec'h *et al.*, 2022; Sun *et al.*, 2024). However, to date, a causative link between the microbiome and resistance to trematode parasites remains to be proven. This link could be investigated through a reciprocal transplant experiment,

which involves exchanging microbial communities between different hosts (Macke *et al.*, 2017a; Greyson-Gaito *et al.*, 2020; Schols *et al.*, 2023)

Preliminary results show that the resistant phenotype has distinctive indicator species within its microbiome compared to susceptible populations throughout generations, environmental shifts and even when occurring in sympatry with susceptible snails in natural or experimental conditions (Alba *et al.*, in prep.). This suggests that the selection also encompasses its microbiome as a co-evolving ecological unit. These results, combined with the recent publication of a microbiome transplant protocol for freshwater snails (Schols *et al.*, 2023) provide the ideal model system to investigate microbiome-mediated snail-parasite compatibility patterns seen elsewhere in the world. The worldwide impact, re-emergent character and increased veterinary and sanitary significance of fasciolosis (Alba *et al.*, 2021) combined with the strong invasive capacity of *P. columella* warrants further examination into the driving factors of this interaction.

Therefore, this study aimed to adapt the transplant protocol of Schols *et al.* (2023) to suit *P. columella* and assess its success. This entails: obtaining axenic and surviving snails, successfully manipulating snail microbiota, and securing a persistent new microbiome through time and snail development. Secondly, we want to assess if reciprocal transplant experiments can shift snail resistance phenotypes in *P. columella*.

Material and Methods

Experimental populations of *Pseudosuccinea columella*

Two well-established laboratory populations maintained at the Interactions Hôtes-Pathogènes-Environnements (IHPE) Molluscarium and corresponding to each *P. columella* phenotype were used: resistant population 2P-R (Segundo potrero, Cuba) and susceptible population PL-S (Port Lauragais, France). Each population also showed distinct multilocus genotypes according to microsatellites (Alba *et al.*, in prep.). All snails used in the experiment were derived from a single individual parent through self-fertilization for a maximum homogenization of within-population biological variables. Standard guidelines for lymnaeid rearing were followed, maintaining snails at 24°C in 24h light, in petri dishes containing sterilized mud and sterilized distilled water and growing algae on which they were fed *ad libitum* (Sánchez *et al.*, 1995).

Sterilization experiment and screening for germ-free samples

As Chernin (1957) noted, the sterilization protocol requires optimization for different snail species. We started from the original protocol of Chernin (1957) and Schols *et al.* (2023) optimized for *Biomphalaria glabrata* yet noted that egg isolation went easier in *P. columella*, resulting in eggs without any surrounding gel matrix. As a result, however, snail embryos lacked protection against the sterilizing agent and would all die after 15 minutes in 0.042% active chlorine. Therefore, bleach exposure was reduced to 2 minutes 45 seconds and a 0.036% active chlorine concentration (LaCroix). The rest of the sterilization protocol remained consistent with Schols *et al.* (2023).

Autoclaved algae (n=1), untreated algae (n=1), presumed axenic (n=8, 4 resistant + 4 susceptible) and untreated (n=1, 1 resistant + 1 susceptible) samples were screened for bacterial presence through a bacterial growth assay and visually screened for bacterial presence. First, samples were individually crushed using a sterile pipet tip in 50µl of molecular grade RNA and DNA-free water (Millipore). The first bacterial growth assay was conducted with LB liquid broth which was dissolved in milli-Q water, autoclaved, and stored until further use. 5µl of the abovementioned sample homogenates were incubated in 4ml of R2A medium, supplemented by triplicates of molecular grade water (n=3) and nothing (n=3), followed by incubation in an Infors HT Ecotron Incubator at 26°C for 96 hours with constant vortexing (80 rpm). Notable growth was already visible with the naked eye after 72 hours, so samples were left another 24 hours to fully allow bacterial growth in all samples. OD measurements were done in triplicate each on 1ml of medium using a Macro cuvette 1ml (Eppendorf) on 600nm through a bio photometer (Eppendorf) after

thoroughly vortexing each tube. LB medium incubated with nothing was used as a negative to obtain the 0 OD value. Samples were considered germ-free if all three measurements had a 0 OD value. The second bacterial growth assay only differed by using the R2A liquid broth medium (Himedia), an incubation time of 72 hours (since growth was already present at 48 hours), and the inclusion of two additional untreated control samples (n=2, 1 resistant + 1 susceptible). The 0 value was obtained from R2A medium incubated without adding anything.

Visual screening for bacteria was done with the LIVE/DEAD® *Bac*Light™ Bacterial Viability Kit L13152 (Thermo Fisher Scientific) by screening 20 fields of view of a slide containing 8µl of the sample-dye mixture under a fluorescence microscope (Nikon Eclipse TS100). The mixture was obtained by mixing 5µl of the sample homogenate with 10µl dye (1:1 ratio Propidium Iodide and SYTO9, each dissolved in 2 ml of autoclaved well water) and incubating for 15 minutes at room temperature in the dark. Samples were classified as germ-free if no bacteria were present in any of the fields of view. If bacteria were present, we differentiated samples with bacteria in all fields of view compared to those with occasional and low density bacterial counts.

Reciprocal transplant experiment, parasite exposure and sample collection

To evaluate the effect of the microbiota on the compatibility of *P. columella* and *F. hepatica*, we conducted two reciprocal transplant experiments using a susceptible (S) and a resistant population (R) of *P. columella* (Figure 1). The first and second reciprocal transplant experiments are from here on referred to as “transplant 1” and “transplant 2” and should not be confused with the first transplant and booster transplant, which refer to inoculation events present in both transplant 1 and transplant 2. The experiment consisted of six key milestones (Figure 2): 1) dissecting egg masses to isolate individual eggs, 2) eliminating the external native microbiota of the snail eggs through bleach sterilization, 3) transplanting a donor microbiome, 4) transplanting a booster donor microbiome, 5) experimental infection with *F. hepatica,* and 6) DNA analysis (diagnostic PCR for infection detection and 16S rRNA gene amplificon sequencing to study the bacterial component of the microbiome).

Step 1: individual eggs were dissected from egg clutches with the use of dissection needles under a dissection microscope. Several egg clutches would be dissected in a sterile petri dish containing sterile borehole water. The sterile water was replaced and the dissection needles were disinfected with 70% ethanol when changing phenotype or when the field of view would contain debris (roughly every three egg clutches). The dissected eggs were then pooled per phenotype and brought to the sterile biosafety cabinet for sterilization. Transplant 1 involved the dissection and sterilization of 235 eggs from resistant individuals and 190 eggs from sensitive individuals, while

transplant 2 involved 331 eggs from sensitive individuals and 309 eggs from resistant individuals in an attempt to increase surviving snails.

Step 2: Transplant 1 followed the sterilization setup of Schols *et al.* (2023) whereby eggs were placed in the well containing bleach, the plate swirled for 10 seconds, and then the egg was moved through three washing steps involving sterilized borehole water each time moving to the next well (hence each well only comes into contact with one egg). Snail eggs were sterilized by exposure for 2min 45sec to 0.036% active chlorine concentration (LaCroix).

In contrast, due to the higher egg count in transplant 2, individual egg-bleaching was no longer feasible. Therefore, eggs were placed per phenotype in 45 mL culture tubes, into which 6.93 ml of sterile borehole water was added. To this, 70 µL of undiluted bleach (3.6%) was added. This achieved the desired concentration of 0.036% in the tube. The eggs were left in these tubes for 2 minutes and 30 seconds to reduce mortality (15 seconds less exposure). At the end of this time, the tubes were filled up to 45 mL with sterile water to quickly dilute the bleach solution. New sterile borehole water was added, and the tube was then turned over 15 times to wash the eggs. This washing step was repeated two more times before the eggs were isolated and placed in individual wells.

Step 3: Microbiome donor inocula were obtained by crushing laboratory-reared snails from each phenotype in 5 mL sterile tubes with a sterilized pestle. Each inoculum was prepared at a ratio of 100 mg of snail/1 mL of sterilized water (w/v) as in Alba *et al.* (2015b) while ensuring sufficient volume for all wells. Once prepared, the inocula were kept on ice while aspirating 20µl of the resulting donor inocula to each well per treatment within 48 hours after sterilization. The remainder of the donor inocula were stored at -80°C. 48 hours after the transplant or 48 hours after hatching, whichever comes last, determined when the snail received autoclaved algae.

Step 4: One week after the first inoculum, a second inoculum was created and given to the recipients as a booster after the water of each well had been replaced with new sterile borehole water. These microbiome donor inocula were obtained as in step 3. For transplant two, these booster inocula were applied three days after the first inocula and into the petri dishes rather than the individual wells of the 24-well plate, as snails had to be moved to glass petri dishes to ensure survival. The inocula volume was scaled to the number of snails in a plate.

Step 5: 29 days after the booster microbiome (step 4), transplanted snails from each experimental group as well as control groups (untreated snails of both phenotypes) were individually exposed to five *F. hepatica* miracidia. Briefly, eggs laid by *F. hepatica* adult parasites collected from a single infected cow (Pyrénées strain, France) at the local abattoir at Perpignan were embryonated at 26°C, in the dark, for 15 days. Miracidia hatching was induced by exposing the eggs to light and

experimental infections of snails were carried out in 96-well plates (see Vázquez *et al.*, 2014 and Alba *et al.*, 2018 for details).

Step 6: At day 25 post-exposure (p.e.), shells were cleaned with cotton swabs soaked in ethanol and were then relaxed in 70°C-heated water for 20 sec to easily isolate the soft tissue with the aid of soft forceps. Each snail was snap-frozen individually in liquid nitrogen and stored at -80°C until DNA extraction. DNA from individual snail specimens was extracted, *F. hepatica* infection success was determined through diagnostic PCR, and the V3-V4 region of the 16S rRNA gene was amplified to study the bacterial aspect of the microbiome. Details are provided in the next two sections: “DNA extraction and parasite detection” and “16S rRNA gene metabarcoding”.

General rearing conditions: Throughout the experiment, autoclaved borehole water was used and 24-well plates and petri dishes holding snails were only opened in a sterile biosafety cabinet to avoid environmental contamination. The survival of individuals was recorded daily or every other day for as long as snail specimens were individually kept in 24-well plates. Once specimens were moved to glass petri dishes survival was only noted at milestone points as mentioned in Figure 2. Survival was determined by looking for movement or an observable heartbeat under a binocular microscope, which would correspond to surviving snails. Snails from transplant 1 were kept in individual wells in 24-wel plates until 22 days post booster transplant, being fed only autoclaved algae, after which they were moved to glass petri dishes and fed normal untreated algae. Due to the high mortality in both transplant experiments, samples of transplant 2 were moved to glass petri dishes and fed normal untreated algae three days after the first transplant had been administered.

DNA extraction and parasite detection

To avoid manipulating the snails and potentially affecting bacterial communities during dissection, infection was only determined through PCR at the end of the experiment. Moreover, snail samples tested for infection were collected 25 days after exposure to ensure that aborted infections were not detected. First, DNA from sampled snails collected throughout the experiment was extracted using the Blood and Tissue DNA kit (Qiagen) following the manufacturer’s guidelines and stored at -80°C. To determine infection, 2 µL of template DNA was tested in a multiplex PCR (GoTaq® Hot Start) targeting the ITS2 rDNA region (240 bp) of *F. hepatica* while co-amplifying part of the 18S rDNA (450 bp) of *P. columella* as internal PCR control (Alba *et al.*, 2015a). Positive control samples involved artificially spiking the DNA of a single non-exposed laboratory-reared *P. columella* with the DNA from a single *F. hepatica* miracidium. Negative controls consisted of 2 µL of DNA- and RNA-free water.

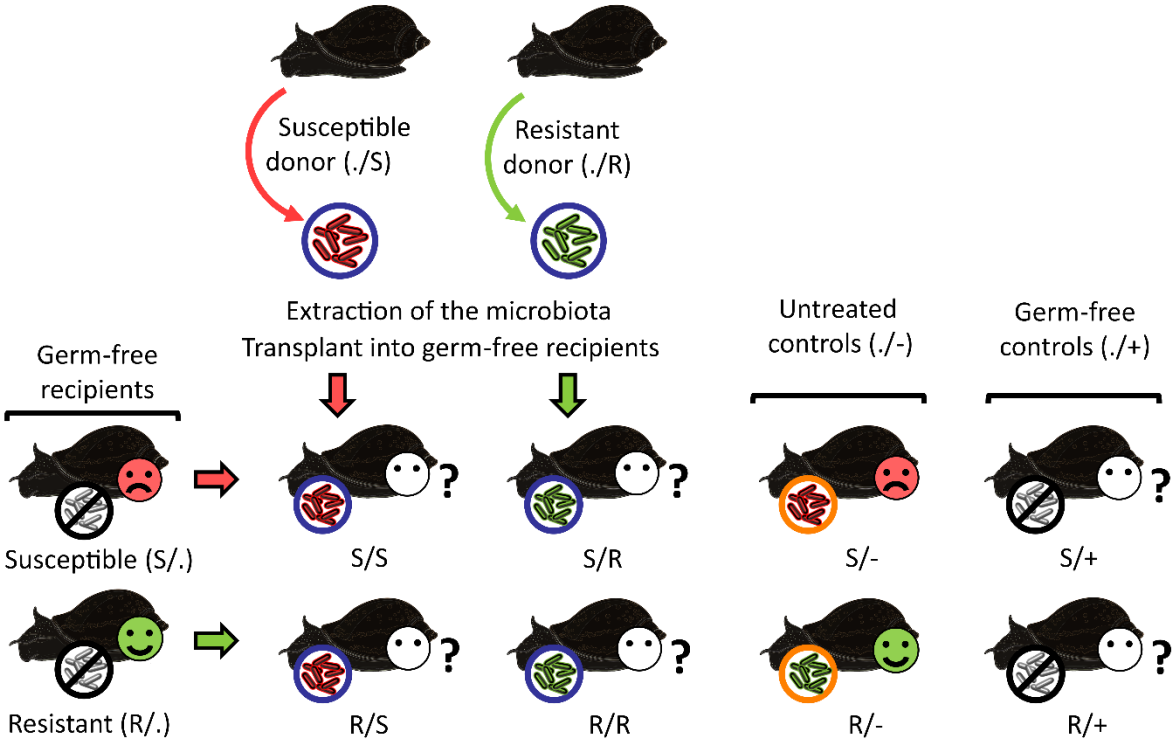


Figure 1: The reciprocal transplant setup followed for the experiment. Germ-free recipient genotypes were obtained for a resistant (R/.) and susceptible (S/.) genotype and exposed to a microbial inoculum obtained from resistant (./R) and susceptible (./S) genotype snails. This resulted in susceptible genotype individuals with susceptible genotype microbiota (S/S), susceptible genotype individuals with resistant genotype microbiota (S/R), resistant genotype individuals with susceptible genotype microbiota (R/S), and resistant genotype individuals with resistant genotype microbiota (R/R). Control samples encompassed untreated resistant (R/-) and susceptible (S/-) genotype snail specimens and germ-free resistant (R/+) and susceptible (S/+) genotype snail specimens. Sample counts for each transplant at the first exposure to donor inocula were: Transplant 1: 39 R/R, 41 R/S, 99 S/S, 98 S/R, 12 R/-, 12 S/-, 3 R/+, and 7 S/+; Transplant 2: 96 R/R, 105 R/S, 109 S/S, 114 S/R, 47 R/-, 52 S/-, 47 R/+, and 41 S/+.

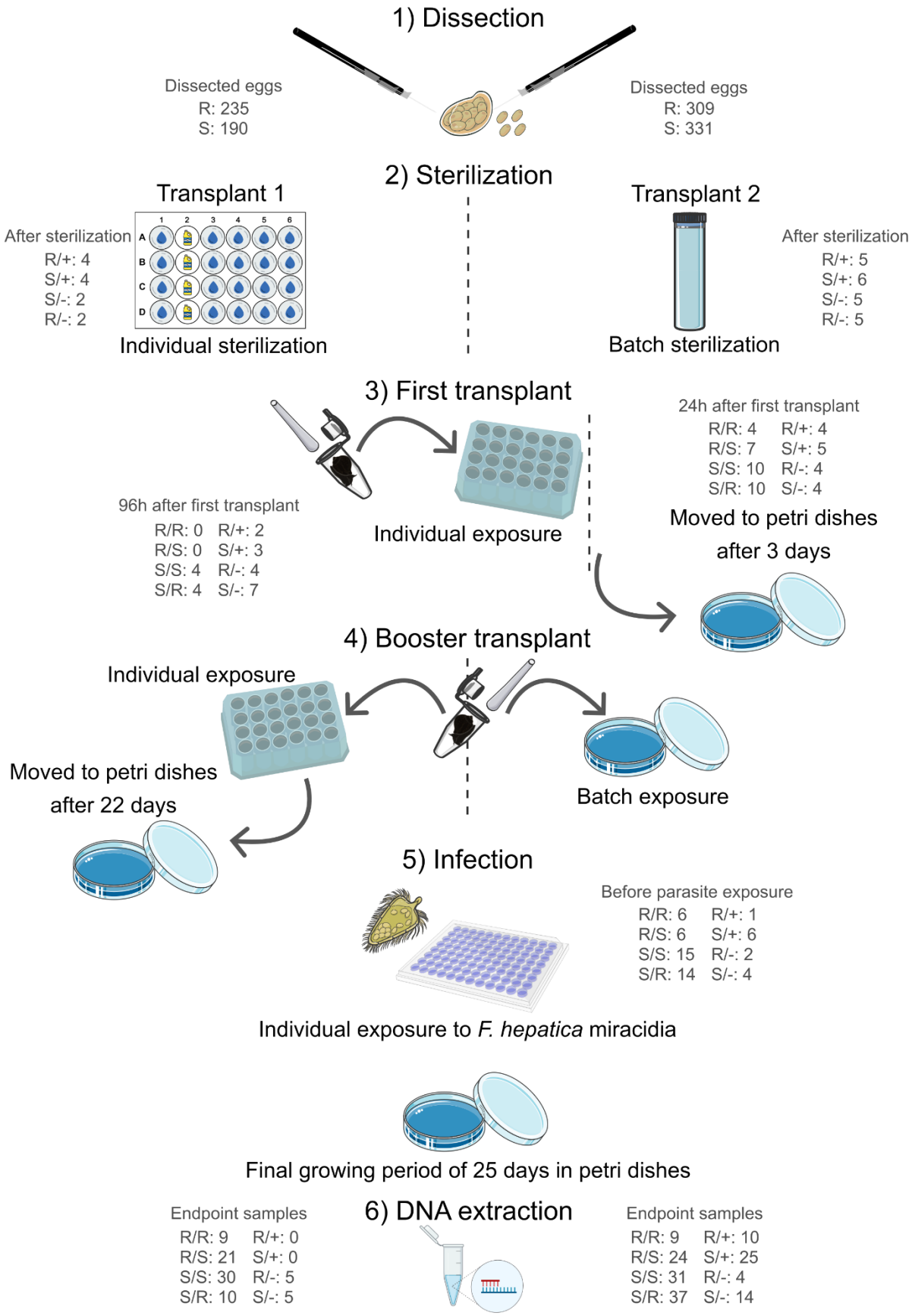

1) Dissection
Dissected eggs
R: 235
S: 190
Dissected eggs
R: 309
S: 331
2) Sterilization
Transplant 1
After sterilization
R/+: 4
S/+: 4
S/-: 2
R/-: 2
Individual sterilization
Transplant 2
Batch sterilization
After sterilization
R/+: 5
S/+: 6
S/-: 5
R/-: 5
3) First transplant
96h after first transplant
R/R: 0 R/+: 2
R/S: 0 S/+: 3
S/S: 4 R/-: 4
S/R: 4 S/-: 7
Individual exposure
24h after first transplant
R/R: 4 R/+: 4
R/S: 7 S/+: 5
S/S: 10 R/-: 4
S/R: 10 S/-: 4
Moved to petri dishes
after 3 days
4) Booster transplant
Individual exposure
Moved to petri dishes
after 22 days
Batch exposure
5) Infection
Before parasite exposure
R/R: 6 R/+: 1
R/S: 6 S/+: 6
S/S: 15 R/-: 2
S/R: 14 S/-: 4
Individual exposure to F. hepatica miracidia
Final growing period of 25 days in petri dishes
6) DNA extraction
Endpoint samples
R/R: 9 R/+: 0
R/S: 21 S/+: 0
S/S: 30 R/-: 5
S/R: 10 S/-: 5
Endpoint samples
R/R: 9 R/+: 10
R/S: 24 S/+: 25
S/S: 31 R/-: 4
S/R: 37 S/-: 14

Figure 2: The six key milestones during the reciprocal transplant experiment with parasite exposure. Dotted lines indicate differences in the manipulations of transplant 1 and transplant 2 as explained in the text. The light grey text indicates the final number of samples in the microbiome dataset taken at each time point. Sample conditions are listed as explained in **Figure 1**. Sample counts on the left of the axis indicate samples from transplant 1, samples on the right from transplant 2. **1) Dissection**: dissecting *P. columella* egg masses to isolate individual eggs. **2) Sterilization**: eliminating the external native microbiota of the snail eggs through bleach exposure. Transplant 1 followed the individual sterilization setup of Schols *et al.* (2023). Transplant 2 followed a batch bleaching procedure whereby all eggs of the same genotype were bleached simultaneously. **3) First transplant**: transplanting a donor microbiome. After the first transplant, snails of transplant 1 remained in their respective well of the 24-well plate, while snails of transplant 2 were pooled in batches in petri dishes after three days. **4) Booster transplant**: transplanting a booster donor microbiome. The booster transplant was done identically to the first transplant for transplant 1, while the batches of transplant 2 were exposed in their respective petri dish. Snails of transplant 1 were moved to petri dishes 22 days after this booster transplant. **5) Infection**: 29 days after the booster transplant specimens were experimental infected with miracidia of *F. hepatica*. Each snail was individually put in a well of a 96-well plate and exposed to 5 miracidia. **6) DNA extraction**: DNA extraction followed by diagnostic PCR for infection detection and 16S rRNA amplification to study the bacterial component of the microbiome.

16S rRNA gene metabarcoding

For the amplification of bacterial DNA, individual NGS libraries based on the 16S rRNA gene (V3–V4 regions; Klindworth *et al.*, 2013) were prepared following the Illumina two-step PCR protocol using PCR primers 341F (5'- CCTACGGGNGGCWGCAG-3') and 805R (3'-GACTACHVGGGTATCTAATCC-5'). The PCR mix consisted of the Q5® High-Fidelity DNA Polymerase Master Mix (15 µl), 1 µM of each primer, 2 µL of DNA template, and sterile DNA- and RNA-free water topped to a total volume of 30 µl. The PCR program involved an initial denaturation step at 98°C for 5 min, followed by 25 cycles of denaturation at 98°C for 45 s, annealing at 60°C for 15 sec and extension at 72°C for 10 sec and, finally, an elongation step at 72°C for 7 min. Samples were then checked on a 2% agarose gel to confirm amplification. If no amplification could be detected, the PCR was repeated for 30 cycles. This had to be done for 9 samples in transplant 1 (SS:3, SR:1, RS:1, RR:2, and unexposed bleached R:2) and 29 in transplant 2 (exposed snails: SS:1, SR:7, RS:6, RR:0, untreated R:2, and bleached R:1; unexposed snails: SS:1, SR:3, RS:2, RR:2, untreated R:1, and bleached R:3).

Amplification was individually checked by 2% agarose electrophoresis and the resulting PCR products were individually indexed using a second PCR step consisting of eight cycles using the Nextera™ XT Index (Illumina, San Diego, USA) following the manufacturer's instructions. Finally, the 435 libraries were normalized using SequalPrep™ plates (Thermo Fischer Scientific, USA) before pooling. Library 1 contained 4 PCR negatives, 2 extraction negatives, 2 mock communities, 126 samples from transplant 1, and 145 samples from transplant 2. Library 2 contained 1 PCR negative, 1 extraction negative, 3 mock communities, no samples from transplant 1, and 151 samples from transplant 2. The pooled libraries were then purified following the JetSeq™ Clean protocol (Bioline, UK), quality checked on a Bioanalyzer High Sensitivity DNA kit (Agilent, USA) and quantified using a Qubit fluorometric quantification (Thermo Fisher Scientific, USA). Paired-end sequencing (2 × 250 cycles) was performed with a MiSeq Reagent Kit v2 on an Illumina MiSeq™ instrument at the BioEnvironnement platform (University of Perpignan Via Domitia, France).

Processing of sequencing data

The error rate of every Illumina sequencing experiment varies (Stoler and Nekrutenko, 2021). Therefore, both runs were processed separately until the generation of ASV tables similar to Chapter 2. The rest of the QIIME2 pipeline v2022.2 (Bolyen *et al.*, 2019) on the VSC (Flemish Supercomputer Center) followed (Schols *et al.*, 2023) which was adapted from (Janssens *et al.*, 2022). The first and second run resulted in 10,385,805 and 7,490,544 reads, respectively. Reads of both runs were front-end trimmed (12 bp) with a minimal overlap of 12 bp between both reads to obtain paired-end sequences which were filtered, denoised, dereplicated and chimeras removed. Respectively, 6,283,074 (mean = 58%, sd = 12%) and 4,627,891 (mean = 61%, sd = 9%) reads were maintained after cleaning for the first and second run.

A total of 14,218 ASVs remained with an average length of 412 bp (sd = 59 bp). The Silva 138 SSU Ref NR 99 database (Quast *et al.*, 2013) and a naïve Bayesian classifier were used to assign taxonomy to the ASVs. The V3-V4 region sequences of this naïve Bayesian classifier were obtained through the feature classifier extract reads function based on the 341F and 805R primers with a truncation length of 464 bp.

The resulting files were then merged with the metadata to generate a phyloseq object through the qza_to_phyloseq function of the qiime2R package (Bisanz, 2018) in RStudio (v. 2022.12.0) on a Windows machine. No contaminant ASVs were detected in the dataset using the Decontam package (v. 1.18.0; Davis *et al.*, 2018), using the prevalence method with a 0.1 threshold, based on five PCR negatives and three extraction negatives. Next, 2,688 chloroplast, mitochondria and

ASVs lacking information at the phylum level were removed and taxonomic information was corrected according to Schols *et al.* (2023). 1,285 singleton and doubleton ASVs were removed from the dataset as they were most likely sequencing errors (Auer *et al.*, 2017; Zaiko *et al.*, 2022). 12 duplicated samples with the lowest read count were removed from the dataset, resulting in the removal of 99 ASVs. Subsequently, a rarefaction curve was constructed through the Vegan package (v. 2.6.4; Oksanen *et al.*, 2013). The rarefaction threshold was set at 9,267 reads per sample through the phyloseq package (v. 1.42.0; McMurdie and Holmes, 2013) using rngseed = 711 while ensuring that the plateau phase was reached. As a result, 439 ASVs were removed from the dataset as well as 11 samples. The samples lost were: the blank sequencing sample, a mock community, two R/S samples from transplant 1, from transplant 2 two R/S samples, one S/R, two S/S and two R/+. As a result, the final dataset contained 411 samples and 9,707 ASVs.

Statistical analyses

OD measurements were analysed to study the success of sterilization. The effect of treatment and genotype on triplicate OD measurements per sample were analysed through a mixed effect model with gaussian distribution using sample as a random factor. Normality was obtained after the removal of two outlier observations.

Potential differences in survival for different genotypes or treatments throughout the experiment were studied. The data associated with daily and every other day monitoring of survival were used to calculate Kaplan-Meier survival curves (survival package, v. 3.4-0; function “survfit”) and significant differences tested (survival package, function “survdiff”; Therneau, 2020).

A potential effect of genotype and microbiome on the infection probability was studied. A glm model with logit binomial distribution was used to test the interaction effect of microbial inoculum (resistant vs susceptible) and genotype (resistant vs susceptible) on the probability of infection across both experiments. A glm model with logit binomial distribution was used to test the interaction effect of treatment (untreated vs bleached) and genotype (resistant vs susceptible) on the probability of infection for transplant 2. This distribution does not assume normality and was tested for overdispersion. For transplant 1 this effect of genotype and treatment could not be tested due to the absence of bleached snails exposed to parasites.

Alpha diversity in the bacterial component of the microbiome was assessed for the Shannon, Faith’s PD, and Simpson diversity indices. The difference between donor inocula and recipients four days after microbiome transplantation was first investigated, while also considering the effect of recipient genotype and donor microbiome. The effect of treatment (untreated vs bleached), genotype (resistant vs susceptible), and their interaction effect was tested on the alpha diversity

of transplant 1 through a glm model with log gamma distribution. For transplant 2, a glmer model with log gamma distribution was used to test the effect of treatment (untreated vs bleached), genotype (resistant vs susceptible), and their interaction on alpha diversity with aquarium number as a random factor. This distribution does not assume normality and was tested for overdispersion. Underdispersion is reported as is, while overdispersion was not detected. Next, the infection parameter was included for transplant 2 to assess the effect of infection too. However, an interaction term was constructed involving all genotype*treatment*infection interactions as none of the resistant genotypes were found infected otherwise leading to multicollinearity in the model. No infection effect could be tested for transplant 1 since bleached snails without donor inoculum were not exposed to parasites. Similarly, an interaction term was constructed involving all genotype*microbiome*infection interactions, to assess the effect of genotype (resistant vs susceptible), microbiome (resistant vs susceptible), and parasite infection (infected and uninfected) on both transplant experiments. Finally, an interaction term was constructed involving all genotype*microbiome*(infection*exposure) interactions, to assess the effect of genotype (resistant vs susceptible), microbiome (resistant vs susceptible), and parasite infection and exposure (unexposed, infected and uninfected) on transplant 2. No effect of parasite exposure could be tested for transplant 1 since no unexposed snails were collected at the end of the experiment.

Beta diversity was explored through Bray Curtis, Jaccard, unWeighted UniFrac and Weighted UniFrac indices, and visualized using NMDS via the ordinate function from the phyloseq package. The function envfit (vegan package) was used to fit environmental variables on the NMDS ordination. An ANOSIM (Analysis of Similarities) was used to assess the effect of the relevant factors on beta diversity (vegan package). Specific observed patterns were then further investigated while focusing on the Bray-Curtis dissimilarity matrix with a k-means clustering with 3 to 4 centers. From the formed clusters the two major clusters were selected for subsequent analysis through a differential abundance analysis based on the negative binomial distribution with the DESeq2 package (v. 1.38.3.; Love *et al.*, 2014), targeting ASVs with a p value below 0.05 once corrected for a false discovery rate. Beta diversity was further explored through Bray Curtis dissimilarity, visualized using RDA via the capscale function from the vegan package. The ordistep function from the vegan package was used for automatic stepwise model building after which the anova function was called to assess model and factor significance. Collinearity was assessed through the variance inflation factor obtained through the vif.acc function from the vegan package.

Results

Sterility

To assess the remaining surviving bacteria after bleach treating *P. columella* eggs a bacterial growth assay was conducted. Bacterial growth was highest for the untreated algae. Bacterial growth (OD>0) was detected in all untreated samples (n=6) and part of the bleached samples (n=6/16; Figure 3A). There was a significant difference in bacterial growth across treatments (Figure 3B; bleached vs untreated; Chi^2=6.80, p=0.009) but no significant difference was detected across genotypes (resistant vs susceptible; Chi^2=1.85, p=0.17).

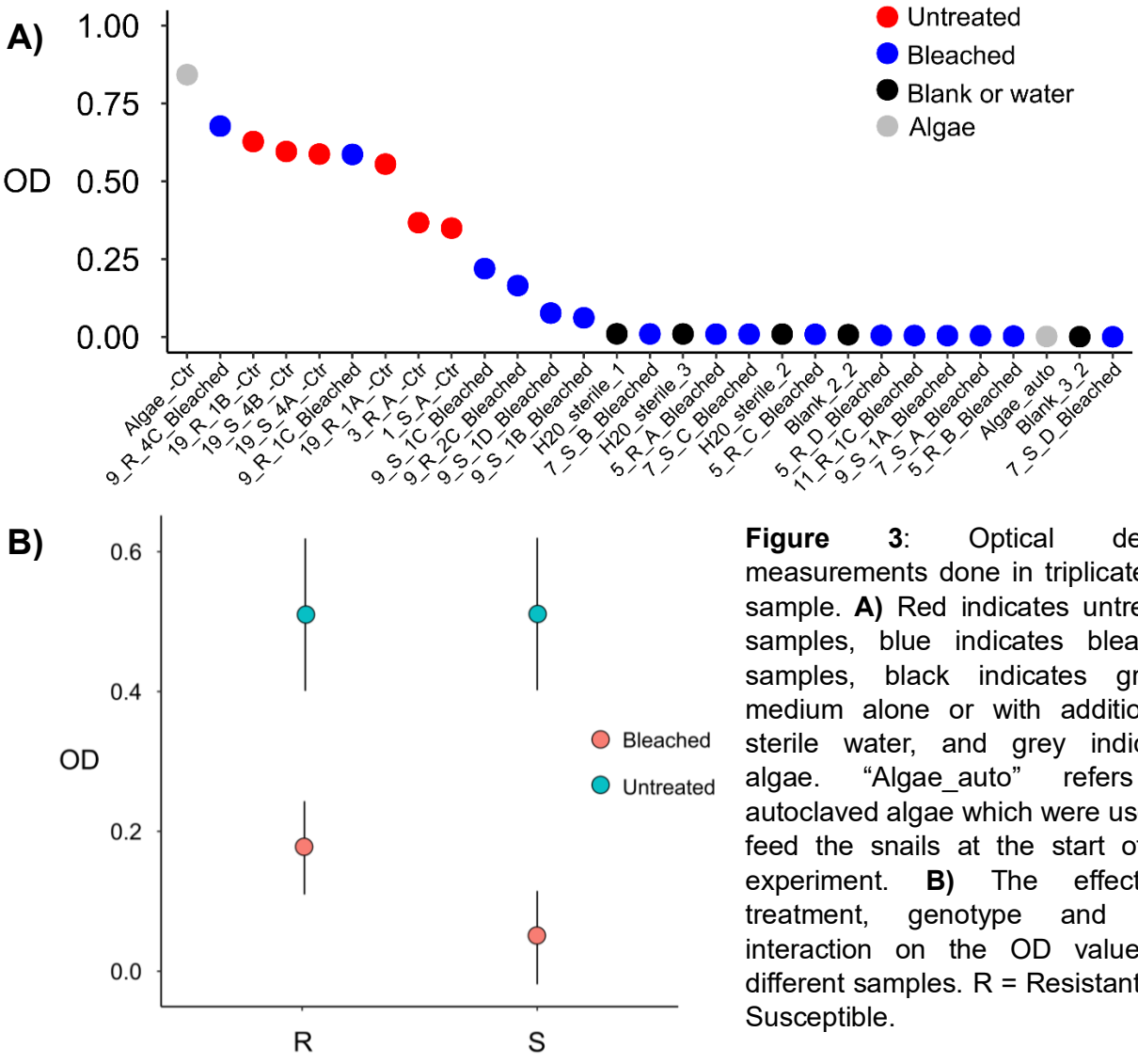


Figure 3: Optical density measurements done in triplicate per sample. **A)** Red indicates untreated samples, blue indicates bleached samples, black indicates growth medium alone or with addition of sterile water, and grey indicates algae. "Algae_auto" refers to autoclaved algae which were used to feed the snails at the start of the experiment. **B)** The effect of treatment, genotype and their interaction on the OD values of different samples. R = Resistant, S = Susceptible.

Survival

Kaplan-Meier survival curves were constructed to assess the effect of genetic background and microbial inocula on survival rate. Transplant 1: Snails with the resistant genotype were significantly more likely to survive (Chi^2= 11.3, $p<0.001$) while recipients of the susceptible microbiome were significantly more likely to survive (Chi^2= 17.2, $p<0.001$) (Figure 4). However, mortality seized completely after snails were placed in glass petri dishes and fed unautoclaved algae. Mortality was even higher in the first two days following microbial inoculation in transplant 2, whereby only 30% of recipients were still alive. In response specimens were placed in glass petri dishes with unautoclaved algae whereafter little to no mortality occurred. In these first days, there was a significant difference in survival across the different recipients (Chi^2= 32, $p<0.001$) (Suppl. Figure 1). Resistant genotype snails with a susceptible microbiome were more likely to survive than their resistant microbiome counterparts. Susceptible genotype snails did not differ in their survival but survived better than resistant snails.

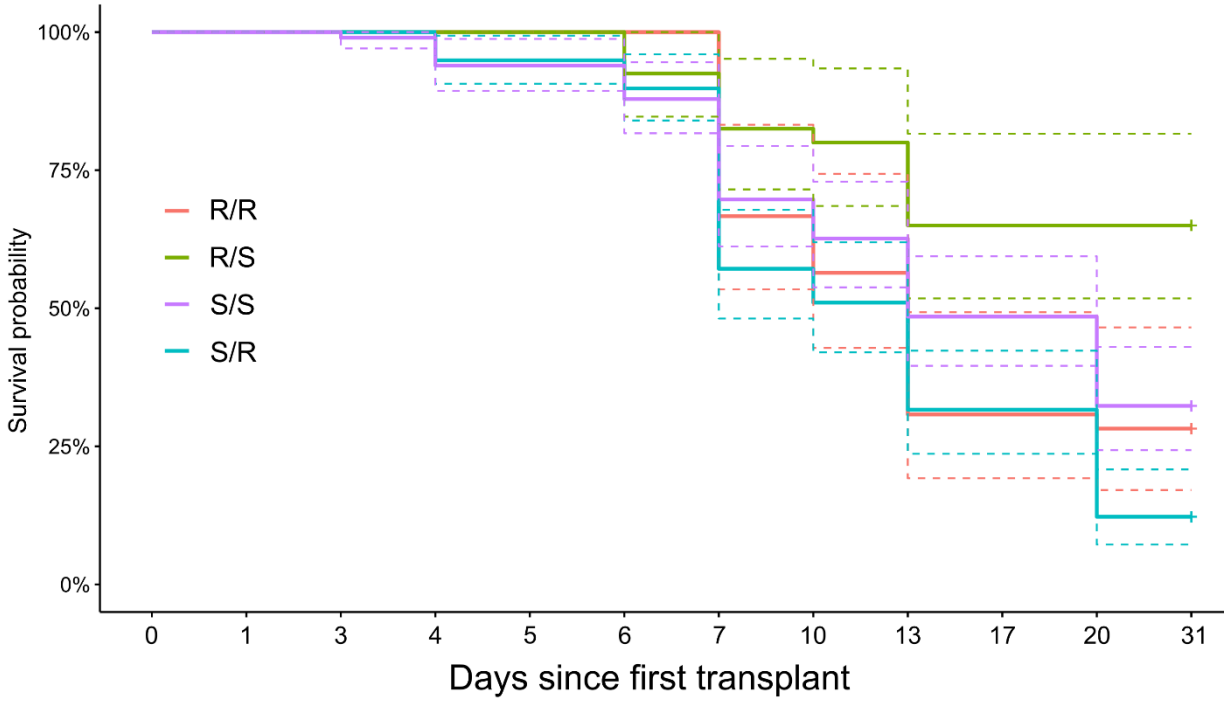


Figure 4: Kaplan-Meier curve showing the survival probability (%) for each day survival was checked in transplant 1. Each tick on the X-axis indicates a moment when survival was assessed, while the value corresponds to the number of days since the first transplant occurred. Colors indicate different genotype/microbiome combinations as explained in Figure 1. The dotted lines indicate 95% confidence intervals for each combination.

Probability of infection

Across both transplant experiments the snail's genotype purely determined the probably of infection (Chi^2= 67.53, df=1, p<0.001), there was no effect of the microbial inoculum across both transplant experiments (Transplant 1: R/R/uninfected= 9, R/S/uninfected=21, S/S/uninfected=3, S/R/infected=10 and S/S/infected=27; Transplant 2: R/R/uninfected= 9, S/R/uninfected= 3, R/S/uninfected=24, S/S/uninfected=2, S/R/infected=34 and S/S/infected=29). The mean probability of infection for resistant genotype samples was 0.03 (+-0.03) and for susceptible genotype samples 0.92 (+-0.03) whereby resistant genotype samples were never found infected. For transplant 2: When testing the effect of genotype and control on infection status, only genotype had a significant effect (Chi^2= 25.5, df=1, p<0.001) (R/+/uninfected= 10, S/+/uninfected= 9, R/-/uninfected= 4, S/-/uninfected= 2, R/+/infected= 0, S/+/infected= 16, R/-/infected= 0, S/-/infected= 12). Bleached samples of the susceptible genotype appeared infected at lower rates; however, this was not significant (estimate = -1.06, SE = 0.80, df = 49, t-ratio = -1.32, p = 0.56). We could not assess this effect for transplant 1 as there were no bleached snails (./+) surviving until parasite exposure.

Alpha diversity

Effect of genotype and bleaching

For transplant 1, two outliers had to be removed to meet normality (R/+=2, S/+=2, R/-=7, S/-=10). There was a significantly lower Shannon diversity for the susceptible genotype (Chi^2= 10.53, df=1, p=0.001) and for bleached samples (Chi^2= 10.91, df=1, p<0.001). There was no genotype-specific effect of treatment (Chi^2= 1.31, df=1, p=0.25). No effects were noted on the Faiths PD. Simpson diversity revealed a significant effect of genotype (Chi^2= 15.19, df=1, p<0.001), treatment (Chi^2= 7.85, df=1, p=0.005) and their interaction (Chi^2= 4.72, df=1, p=0.03) whereby bleaching did not reduce the Simpson diversity in the resistant genotype (Figure 5).

There was no effect of genotype, treatment nor their interaction across any alpha diversity metrics right after sterilization, one week later, and on the day of parasite exposure for transplant 2 nor was there a difference in these samples throughout those three timepoints.

Effect of genotype, bleaching and infection

For transplant 2, there was no significant effect of infection, genotype, treatment or their interactions on the Shannon diversity of control samples (R/+/uninfected= 10, S/+/uninfected= 9, R/untreated/uninfected= 4, S/untreated/uninfected= 2, R/+/infected= 0, S/+/infected= 16, R/untreated/infected= 0, S/untreated/infected= 12). However, the model did suggest a trend for

reduced Shannon diversity in infected samples (Chi^2=3.19, df=1, p=0.07). In contrast, the FaithsPD revealed a significantly lower diversity in resistant compared to susceptible genotypes (Chi^2=4.19, df=1, p=0.04) but no significant effect of infection (Chi^2=2.02, df=1, p=0.16). No significant effect of genotype (Chi^2=2.49, df=1, p=0.11) and infection (Chi^2=2.17, df=1, p=0.14) were visible for the Simpson diversity index although the general trend for a lower diversity in resistant and in infected samples remained. Underdispersion was detected in all models. This analysis was not possible for transplant 1 as bleached samples without donor microbiome all died at earlier moments.

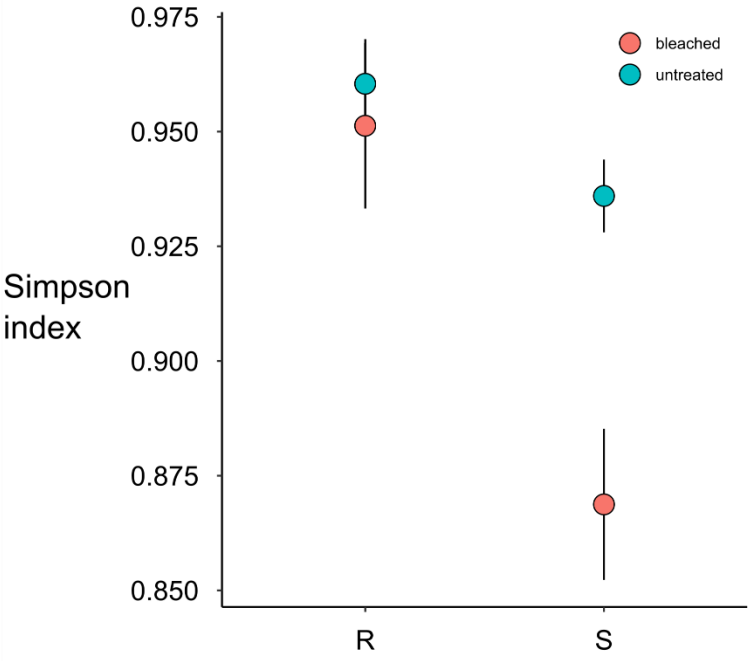


Figure 5: The Simpson diversity of bleached (sterilized without microbial inoculum) and untreated samples for the resistant (R) and susceptible (S) genotype of transplant 1, 4 days after sterilization.

Comparison donors and recipients

Donor inocula had a significantly higher alpha diversity compared to susceptible recipients in transplant 1 across all diversity metrics (p<0.001) except for the Simpson index (Chi^2= 3.28, p=0.07). No resistant samples were available for this time point to assess the effect of genotype. Transplant 2 showed no difference between donor inocula and recipients, nor the recipient genotype or microbiome inoculation (p>0.3).

Effect of genotype, microbial transplant and infection

An interaction factor had to be created combining genotype, microbiome and infection for transplant 1 and transplant 2 because all resistant genotypes were uninfected, hence highly correlated factors would otherwise confound model analysis (R/R/uninfected= 9, R/S/uninfected=

21, S/S/uninfected= 3, S/R/infected= 10, S/S/infected= 27). Alpha diversity was significantly different across the interaction factor for all diversity metrices ($p<0.001$), which originated solely from a higher diversity in infected samples of the susceptible genotype with a resistant donor microbiome (S/R/infected). The other conditions were not significantly different amongst each other. For transplant 2 no significant differences were found (R/R/uninfected= 9, S/R/uninfected= 3, R/S/uninfected= 24, S/S/uninfected= 2, S/R/infected= 34, S/S/infected= 29).

The effect of genotype, microbial transplant and exposure-infection status

Transplant 2 (analysis not available for transplant 1): (unexposed= 41, infected= 63, uninfected= 38). There was a significantly higher FaithsPD ($Chi^2=7.39$, df=2, $p=0.02$) in exposed but uninfected snails compared to infected and unexposed snails, which were not significantly different from each other. No such pattern was visible for the Shannon and Simpson diversity. Only the Shannon diversity revealed a marginal effect of the microbiome inoculum on the alpha diversity ($Chi^2=2.96$, df=1, $p=0.09$). Alpha diversity across all metrics did not differ amongst and between donor inocula and samples before parasite exposure (all $p>0.35$). Moreover, inspecting the effect plots only suggested a trend for a higher FaithsPD for the resistant donor inocula and susceptible genotype receiving susceptible microbiome ($p>0.22$).

Beta diversity

Beta diversity patterns based on NMDS

A combined NMDS plot providing an overview across both experiments of the Bray-Curtis dissimilarity. It does not reveal separation of the two transplant experiments, except for one cluster mostly dominated by samples from transplant 2 (Figure 6). This cluster is also visible in the Jaccard analysis but not in the unweighted and weighted Unifrac analysis. See below for a closer investigation of both transplant 1 and transplant 2. In the latter, we delve deeper into each cluster.

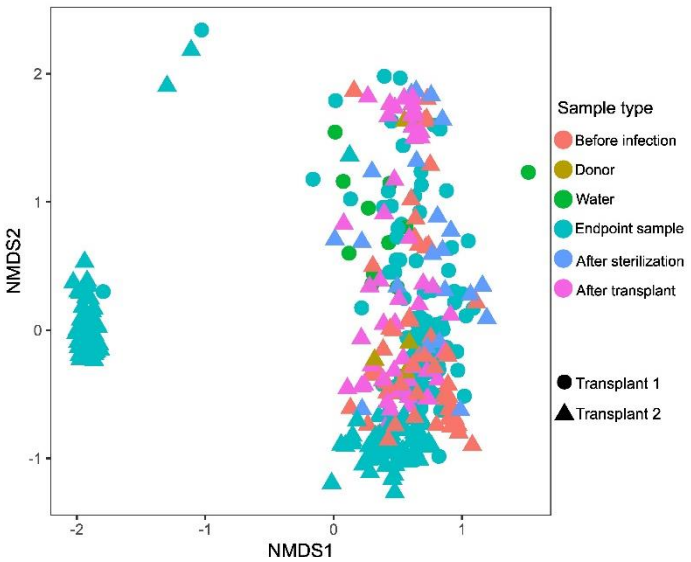


Figure 6: NMDS plot of the Bray-Curtis dissimilarity across both transplant 1 and 2. Shaped indicate different sample types.

NMDS plots of the Bray-Curtis dissimilarity revealed a separation between samples receiving a conspecific microbiome (i.e. R/R and S/S) with those receiving a heterospecific microbiome and untreated control samples (i.e. R/S, S/R, S/- and R/-) (Figure 7A&B) whereby the latter two groups form a tight cluster with high similarity (ANOSIM: R=0.12, p=0.02). Moreover, it revealed a separation between samples exposed to miracidia and those not exposed to miracidia for transplant 1 (Figure 7A). Furthermore, it revealed there was no separation of the donor inocula and, therefore, also no directional shift of samples towards their respective donor microbiome (Figure 7A; Suppl. Figure 2). This pattern was also visible for the Jaccard, weighted Unifrac and unweighted Unifrac dissimilarity measures. Differential abundance analysis revealed that 44 ASVs significantly differed between cluster one and cluster two with a Benjamini-Hochberg correction for false discovery rate (cluster two contained two subcluster, which were combined for the DESEQ2 analysis). Of these, 11 ASVs were more abundant in cluster one, which are mostly Proteobacteria spp.

NMDS plots of the Bray-Curtis dissimilarity of transplant 2 revealed no clustering based on genotype, microbiome or infection but it did reveal separation between samples exposed to miracidia and those not exposed to miracidia (Figure 8A). Bray-Curtis dissimilarity, Jaccard and Unweighted Unifrac all revealed two clusters in samples exposed to miracidia. None of the variables (MiSeq run, PCR cycle nr, microbiome, genotype, infection, batch number, or their interaction) correlate with either of the two clusters (Suppl. Figure 3). The Weighted Unifrac does not detect these two clusters nor do any of the other beta diversity measures at taxonomic levels beyond ASV (i.e. species, genus, etc.). To investigate bacteria associated with either of the two clusters, DESEQ2 analysis was conducted. Cluster 3 and 4 were omitted in the differential abundance analysis as they only contained one and two samples, respectively. Cluster one contained 62 samples and cluster two 89, whereby 178 ASVs significantly differed between both clusters with a Benjamini-Hochberg correction for false discovery rate (Figure 8B). Of these, 17 ASVs were more abundant in cluster two, while the rest was more abundant in cluster one.

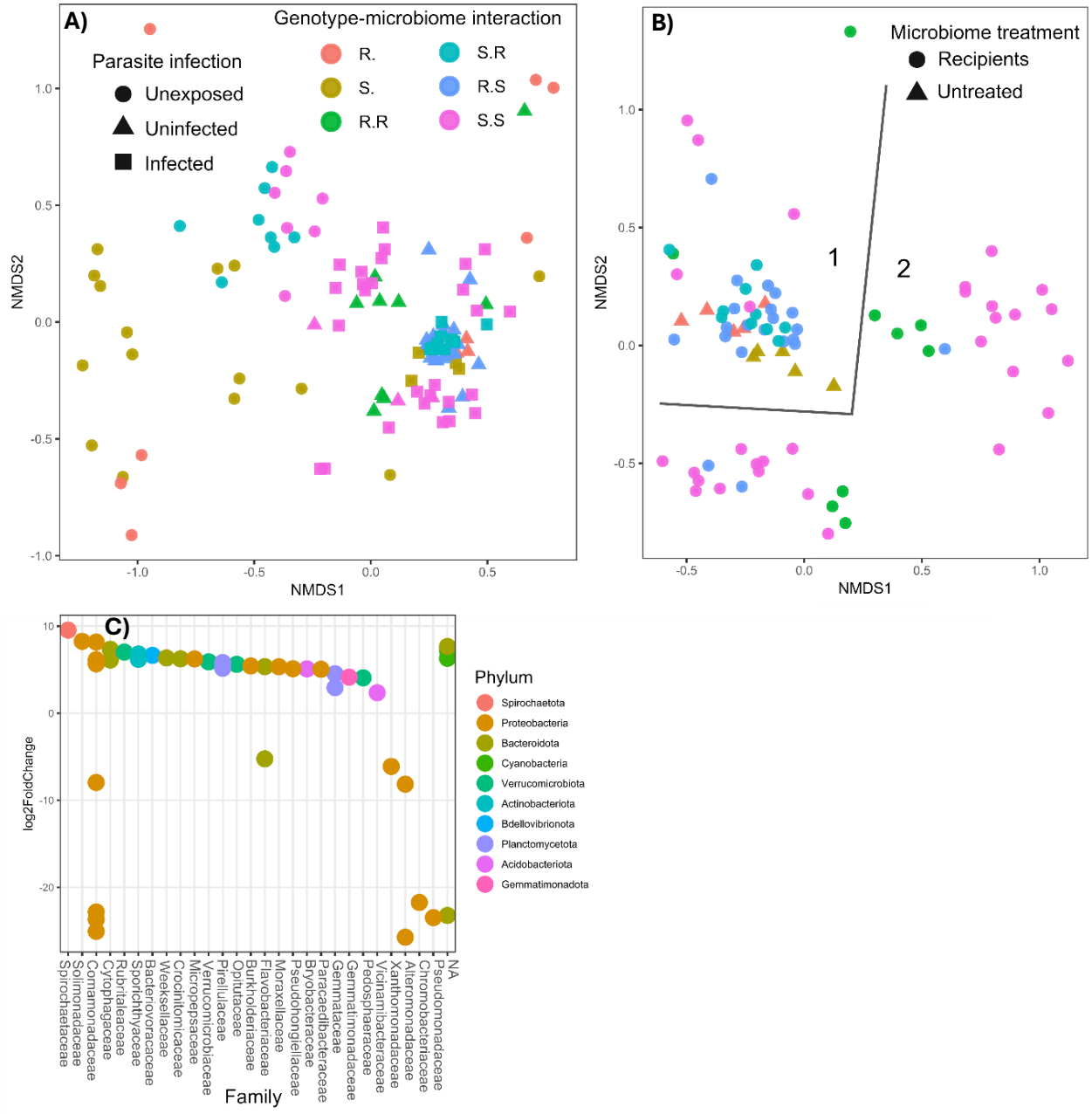


Figure 7: Beta diversity analysis of the first transplant experiment. A) NMDS plot based on the Bray-Curtis dissimilarity with parasite infection indicated by shape and the genotype-microbiome interaction as color. B) NMDS plot based on the Bray-Curtis dissimilarity focused only on the exposed samples, whereby two clusters are formed (cluster one n=45 and cluster two n=35). Cluster two was obtained by combining two separate clusters, which is required for DESEQ2. C) Differential abundance analysis revealed that 44 ASVs significantly differ between cluster one and cluster two. X-axis indicates bacterial families, Y-axis the Log2 Fold change. Positive values indicate higher abundance in cluster two compared to cluster one.

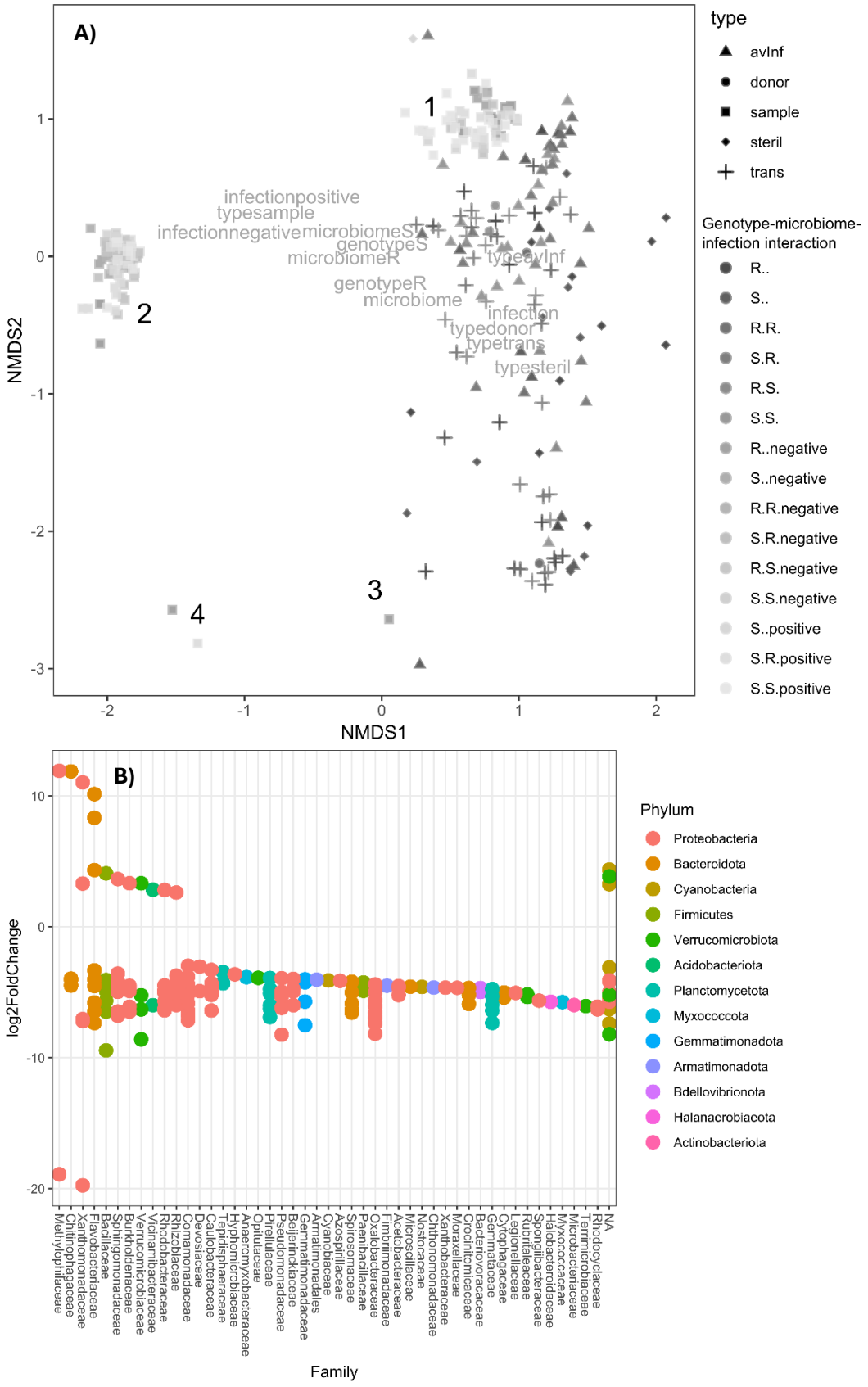


Figure 8: Beta diversity analysis of transplant 2. A) NMDS plot based on the Bray-Curtis

dissimilarity with sample types indicated by shape and the genotype, microbiome and infection status interaction as color. All variables indicated by color and shape were included in the envfit analysis and fit on the NMDS ordination. Four clusters are indicated for the sample subtype (1, 2, 3 and 4), which were obtained based on an NMDS plot based on the Bray-Curtis dissimilarity focused on the sample subtype (Suppl. Figure 4). Cluster 3 and 4 constitute only of one and two samples, respectively, and are therefore excluded from the subsequent analyses (cluster one n=62 and cluster two n=89). B) Differential abundance analysis revealed that 178 ASVs significantly differ between cluster one and cluster two. X-axis indicates bacterial families, Y-axis the Log2 Fold change. Positive values indicate higher abundance in cluster two compared to cluster one.

Beta diversity patterns investigated through RDA

For transplant 1, RDA analysis targeting donors and recipients post-transplant but pre-parasite exposure (only involving the susceptible genotype) revealed a significant difference between recipients and donors ($F=6.55$, $p=0.02$), and between recipients of either a resistant or a susceptible microbiome ($F=2.94$, $p=0.008$) of which the model combining both factors additively was significant ($F=5.54$, $p=0.003$, adj-$R^2=0.53$; Figure 9A). When looking at samples collected at the end of the experiment (Figure 9B), there was a significant effect of microbiome ($F=3.33$, $p<0.001$), genotype ($F=3.95$, $p<0.001$) and their interaction ($F=7.41$, $p<0.001$) on the Bray-Curtis dissimilarity model ($F=5.21$, $p<0.001$; Adj-$R^2=0.15$). There was no effect of infection status ($F=0.99$, $p=0.41$). The plot also reveals a separation between samples receiving a conspecific microbiome (R/R and S/S) with those receiving a heterospecific microbiome (R/S and S/R), while both heterospecific combinations also cluster separately.

Samples collected in transplant 2 after the transplantation but before infection had no signal of microbiome treatment nor genotype in their microbial communities ($F=0.89$, $p=0.67$). When looking at samples collected at the end of transplant 2 (Figure 10), there was a significant effect of microbiome ($F=5.50$, $p<0.001$), infection ($F=13.47$, $p<0.001$) and their interaction ($F=2.16$, $p=0.006$) on the Bray-Curtis dissimilarity model ($F=7.64$, $p<0.001$; Adj-$R^2=0.19$). There was no effect of genotype ($F=1.34$, $p=0.15$). The plot also revealed a separation between samples never exposed to parasites and those exposed to parasites, the latter formed two separate clusters.

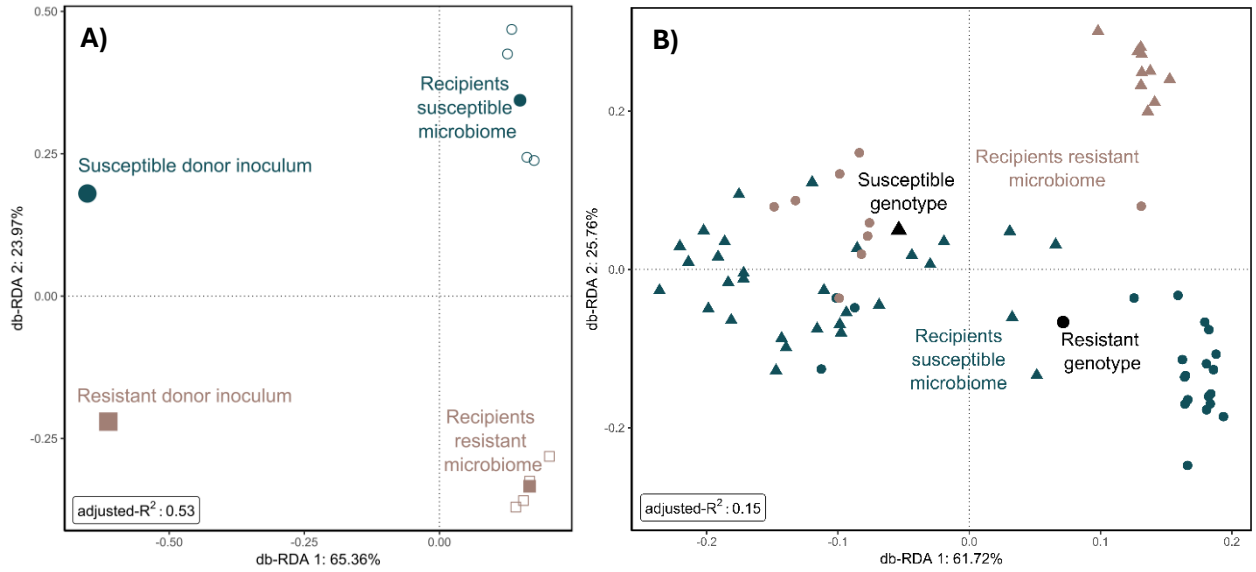


Figure 9: Beta diversity measures of transplant 1 across samples, donors, genotype and microbiome inoculum. A) Bray-Curtis dissimilarity measure of the bacterial community across the different microbial inocula for recipients and donors collected four days after inoculation as shown by RDA plot. The RDA considers the interaction of sample type and microbial inocula. B) Bray-Curtis dissimilarity measure of the bacterial community across the different recipients (microbial inoculum and recipient genotype) collected at the end of the experiment as shown by RDA plot. The RDA considers the genotype, the microbial inocula and their interaction. The circles indicate the resistant genotype, while the triangles indicate the susceptible genotype. Color represents microbial inoculum.

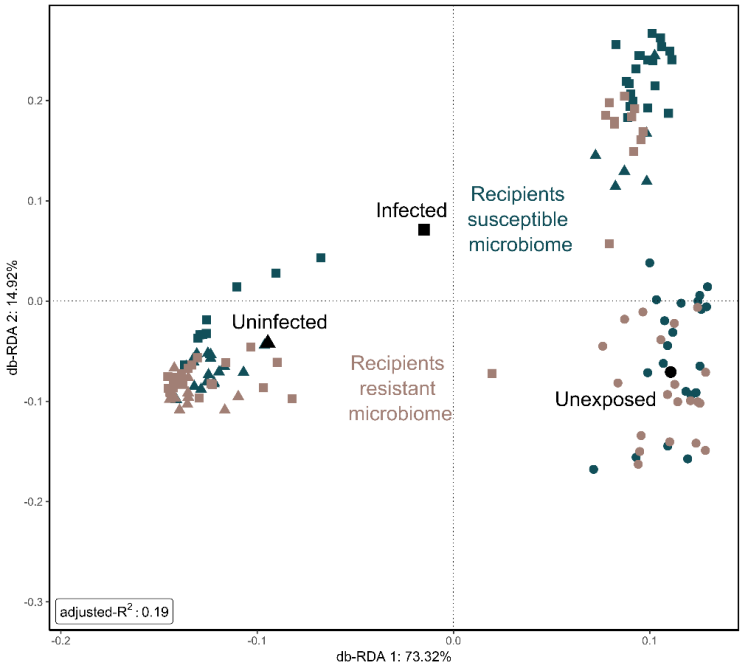


Figure 10: Beta diversity measures of transplant 2 across samples, infection status and microbiome inoculum. Bray-Curtis dissimilarity measure of the bacterial community across the different recipients (microbial inoculum and infection status) collected at the end of the experiment as shown by RDA plot. The RDA considers microbial inoculum, infection status and their interaction. Ordistep excluded genotype. The circles indicate unexposed samples, the triangles indicate uninfected samples, and the squares infected samples. Color represents microbial inoculum.

Discussion

Transplant experiments go beyond correlations and reveal the presence or absence of causative links by actively or passively introducing bacterial strains or communities to recipients (Greyson-Gaito *et al.*, 2020). In doing so, earlier works have revealed that bacterial communities are at the root cause of parasite resistance in bumblebees (Koch and Schmid-Hempel, 2012) and cyanobacteria tolerance in water fleas (Macke *et al.*, 2017a). We here attempted two such transplant experiments in the globally invasive snail *P. columella* to decipher potential microbiome-related drivers of resistance to trematode parasites (Figure 1 and Figure 2).

Transplant experiments pivot on reproducible sterilization protocols to obtain germ-free recipient hosts. No such protocol existed for *P. columella,* however, recently a protocol originally designed by Chernin (1957) was adapted and validated by Schols *et al.* (2023) on the snail *B. glabrata*. Moreover, the latter managed to passively transplant the bacterial component of the microbiome across various taxonomic levels into *B. glabrata* recipients, revealing a major impact of microbial inocula on recipient microbial communities (R^2=0.65). Nevertheless, Schols *et al.* (2023) ended the experiment four days after transplanting the microbiome, so the persistence of the newly introduced microbiome through time remained an open question. Moreover, the protocol had to be adapted to suit *P. columella* since Chernin (1957) was unable to apply it on lymnaeid snails (same family as *P. columella*). Nevertheless, we successfully obtained germ-free snails in 10 out of 16 tested samples with acceptable survival rates after reducing the bleach concentration and exposure time, revealing that bleach sterilization can be adapted to lymnaeid snails (Figure 3). Bleaching consistently reduced alpha diversity in both resistant and susceptible genotype snails (Figure 5). This holds promise for adapting the protocol to other snail groups, as initially suggested by Schols *et al.* (2023).

Similarly to Schols *et al.* (2023) a majority of the variation in the bacterial community could be explained by the donor inoculum of susceptible snails of transplant 1 in an additive model with donor samples (R^2=0.53, Figure 9A). However, despite driving the community (i.e. clear separation of recipients exposed to resistant and susceptible inocula), recipients did not cluster closely with donor inocula four days after the transplant as in Schols *et al.* (2023). Three potential and non-excluding hypotheses can be put forward: 1) a bias between the administered and the “analyzed” inoculum, 2) differences between planorbids and lymnaeids concerning microbiota acquisition from the environment, and 3) donor microbiomes from susceptible and resistant snails were too similar. First, microbial inocula were aspirated to reduce debris (shell pieces and tissue) as it would decompose, making the water unsuitable for snail survival (Maes *et al.*, 2024). However, DNA extractions of the donor inocula included this debris, which might have driven the observed

difference between donors and recipients. One potential solution for future experimentation might be filtering out this debris through a 10µm filter. Second, immersing bleached *B. glabrata* snails for four days in water containing a crude extract of other snails was sufficient for driving a directional shift in the microbiome toward the donor inocula (Schols *et al.*, 2023). However, the biological and ecological differences between *B. glabrata* and *P. columella* suggest that a customized inoculation step might be required. This can be complicated for now, as little is known about the factors that shape the snail microbiome (Panova *et al.*, 2023). Third, the susceptible and resistant population under study showed different bacterial communities in an earlier study with specimens collected one year before this experiment (Alba *et al.*, in prep.). However, the differences between both were quite limited in this experiment, complicating the observation of microbial inoculation throughout the experiment. This might be resolved with higher-resolution sequencing such as a metagenomics approach.

Susceptible genotype snails and snails exposed to the microbiome of resistant snails showed higher mortality compared to other treatments (Figure 4). This contributed to the lack of sufficient biological replicates, which is a frequent problem in experiments studying snail ecology as discussed by Maes *et al.* (2021). Nevertheless, other lab populations of both resistant and susceptible phenotypes were not breeding sufficiently at the time of study precluding their inclusion in the experiment, as we were at risk of losing the populations. We tried to compensate for this partially by having two separate experiments, yet we were unable to test genotype-specific responses per phenotype. Part of the mortality of the susceptible population appeared to be linked to increased amphibious tendencies as these snail specimens were frequently observed outside the water on lids, potentially leading to increased desiccation rates. This behavioral change might be in response to unsuitable water conditions. However, behavioral differences between resistant and susceptible *P. columella* snails have not yet been studied, leaving this observation open for future investigation. Indeed, behavioral responses to unfavorable conditions are widespread in snails and can vary intraspecifically (Rundle and Brönmark, 2001; Poznańska *et al.*, 2015), supporting the hypothesis that susceptible snails may respond differently to water quality with increased amphibious behavior as a result. The higher mortality of specimens exposed to the resistant microbiome would suggest a cost associated with this microbiome. Perhaps a potent immune response is required to keep this microbiome under control. Indeed, snail hemocytes are critical to identifying and clearing unwanted bacteria (Núñez *et al.*, 1994). However, a higher bacterial load in the resistant inoculum might have exceeded tolerance limits of recipients, which aligns with the findings of Ducklow *et al.* (1981) whereby moribund *B. glabrata* showed a 4- to 10-fold increase in bacterial load. Further examination of these samples with quantitative methods

such as qPCR or ddPCR could resolve this possibility. Irrespective of the potentially underlying pattern, the environment drove the observed mortality, as mortality seized nearly completely after switching from small plastic to larger glass containers and providing unautoclaved algae. Indeed, micro and nano plastics have been shown to impair development, induce dysbiosis, cause oxidative stress, and induce behavioral change in snails (Rodrigues *et al.*, 2023), while larger containers are less prone to oxygen depletion (Maes *et al.*, 2024). In contrast to heat killing the entire diet through autoclaving, formalin killing at least part of the diet significantly increased the growth of germ-free *B. glabrata* (Chernin and Schork, 1959). Combined, these works indicate improvements for future reciprocal transplant experiments involving snails.

When examining the bacterial community, two distinct clusters were detected after parasite exposure (Figure 8A, Suppl. Figure 4), which did not correlate with any experimental condition (MiSeq run, PCR cycle nr, microbiome, genotype, infection, batch number, or any interaction). Methodologically our work deviated from standard practice by conducting single PCR reactions and not triplicate PCR reactions which are then pooled. However, with the advancements in sequencing technology, this is deemed obsolete nowadays (Marotz *et al.*, 2019). Other biases could have been introduced into the dataset by various PCR cycles required to obtain sufficient amplification for each sample for sequencing or by splitting the samples across two MiSeq runs. Nevertheless, no significant clustering or effects were noticeable when investigating these factors, revealing that they did not introduce problematic biases into our dataset. Moreover, this pattern was only visible at the ASV level but not at higher taxonomic levels. This suggests that parasite exposure induces a shift in one of two directions according to alternative stable states or the checkerboard pattern, whereby bacteria with closely related ASVs conduct the same biological functions yet might produce slightly different metabolic products on which other species depend (Levy and Borenstein, 2014). In other words, one of the bacteria quickly occupies an ecological niche and subsequently excludes its ecologically similar, close relative (Staubach *et al.*, 2013), cascading down the bacterial community resulting in one of two communities.

When assessing the persistence of microbial inocula through time, correlation values did decrease throughout the experiment but remained a significant influence (genotype*microbiome model, R^2=0.15, Figure 9B; infection exposure status*microbiome model, R^2=0.19 Figure 10). The reduced correlation is not surprising since microbial communities are known to change through time and development (Chapter 2; Chevalier *et al.*, 2020; Huot *et al.*, 2020), while also being affected by stochastic effects and environmental contamination such as food (Staubach *et al.*, 2013). The effect of microbial inocula in transplant 2 is less clear than in transplant 1, which can potentially be explained by experimental artefacts as eggs were sterilized in batches. Indeed batch

sterilization has been shown to be less successful (Chernin, 1957; Schols *et al.*, 2023) and snails were fed unsterilized algae sooner, increasing environmental contamination (Staubach *et al.*, 2013). Nevertheless, microbial inocula drove microbial communities of recipients throughout the experiment while failing to shift susceptibility/resistance patterns in *P. columella* to *F. hepatica* infections as measured by diagnostic PCR. We did not monitor redial growth or the shedding of cercariae, hence, there is still a slight possibility that susceptible snails exposed to a resistant microbiome would have a reduced parasite intensity, which could not be measured with the applied diagnostic PCR. Parasite exposure shifted alpha and beta diversity measures in the microbiome, mostly decreasing diversity in infected snails and shifting the bacterial community after parasite exposure. Combined, these results suggest that for our study system, the microbiome may not be a driver of snail resistance to trematode parasites but rather a consequence of it. Furthermore, it revealed that random microbiome perturbations of the host will not necessarily affect the phenotype of infection by its trematode parasite. Similarly, under bacteriologically sterile conditions susceptibility patterns to *Schistosoma mansoni* in *B. glabrata* did not shift either (Chernin, 1960).

The differences in diversity are most likely not related to age or development since infected and unexposed specimens did not differ significantly from one another. Since our transplant happened within 48 hours of hatching, our experimental manipulations cover three possible routes of microbiome-mediated resistance to trematode infection (i.e. resource competition, inhibitory products, and immune priming of the host; see Stock *et al.*, 2021). However, we did not cover transgenerational effects (screen offspring of transplanted individuals) nor pre-adaptation (extract inoculum from exposed but uninfected snails in line with Macke *et al.*, 2017a). The transgenerational effect might be interesting to further investigate since immune effectors associated with the resistant phenotype in *P. columella* are produced in the albumen gland (Alba *et al.*, 2020, 2022), which also produces vital elements for embryo nourishment. Moreover, increasing evidence supports a close link between the snail's immune response and its microbiome (Núñez *et al.*, 1994; Allan *et al.*, 2018). Combined, the immune system influences both embryo nourishment and the microbiome, suggesting that a transgenerational signal in the microbiome could exist for freshwater snails. Microbiome-mediated adaptation, on the other hand, is unlikely as the microbiome shifts are most likely the consequence of the snail's immune response. Moreover, the immune response of resistant snails rapidly immobilizes the parasite (within 24 hours). Nevertheless, parental immune protection mediated by protein transfer has previously been shown to protect *B. glabrata* offspring against oomycete infection (Baron *et al.*, 2013). Furthermore, recent work on a nematode model system revealed that host genetics and

the host-associated microbiome jointly interact to determine the adaptation potential to a novel environment (Petersen *et al.*, 2023), while genetically depleted spider mites mainly adapt to new environments through their microbiome (Bisschop *et al.*, 2022). Combined, this suggests, albeit unlikely, that parasite-exposed and naïve donors might have a different effect on the recipient's phenotype.

Host-parasite interactions shift depending on the environmental conditions (Wolinska and King, 2009), which includes the microbiome. Nevertheless, the results presented here indicate that shifting the *P. columella*-associated microbiome does not necessarily influence the resistance to *F. hepatica.* Future studies focusing on specific bacteria taxa significantly associated with one or the other phenotype might shed light on the role of the microbiota and the different microbiome signatures found. This, in conjunction with investigations on the metabolome and immunome, including hemocyte subpopulation profiling and counting combined with transcriptome profiling should allow to disentangle what exactly drives resistance in *P. columella* and how it drives the biological patterns associated with this phenotype. Although we failed to demonstrate direct causality here, the microbiome might still be an important driver of snail resistance to trematode infection.

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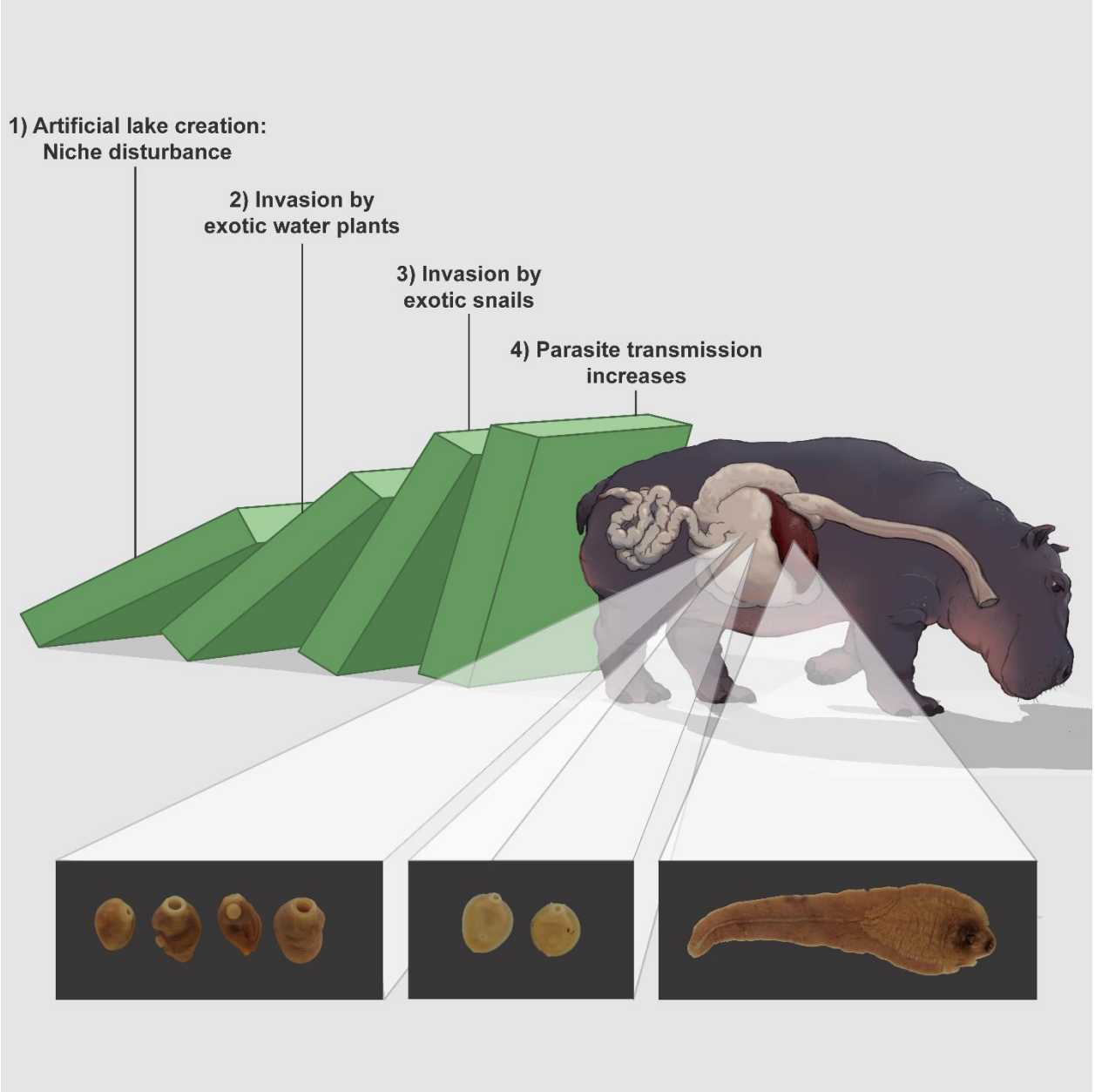
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Chapter 5

Invasive snails, parasite spillback, and potential parasite spillover drive parasitic diseases of *Hippopotamus amphibius* in artificial lakes of Zimbabwe



Chapter 5

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Invasive snails, parasite spillback and potential parasite spillover drive parasitic diseases of *Hippopotamus amphibius* in artificial lakes of Zimbabwe

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Abstract

Background

Humans impose a significant pressure on large herbivore populations, such as hippopotami, through hunting, poaching and habitat destruction. Anthropogenic pressures can also occur indirectly, such as artificial lake creation, which disrupts the local environment, facilitating the subsequent colonization of invasive species that alter the ecosystem. These events can lead to drastic changes in parasite diversity and transmission, but generally receive little scientific attention.

Results

In order to document and identify trematode parasites of the common hippopotamus (*Hippopotamus amphibius*) in artificial water systems of Zimbabwe, we applied an integrative taxonomic approach, combining molecular diagnostics and morphometrics on archived and new samples. In doing so, we provide DNA reference sequences of the hippopotamus liver fluke *Fasciola nyanzae,* enabling us to construct the first complete *Fasciola* phylogeny. We describe parasite spillback of *F. nyanzae* by the invasive freshwater snail *Pseudosuccinea columella,* as a consequence of a cascade of biological invasions in Lake Kariba, one of the biggest artificial lakes in the world. Additionally, we report an unknown stomach fluke of the hippopotamus transmitted by the non-endemic snail *Radix* aff. *plicatula*, an Asian snail species that has not been found in Africa before, and the stomach fluke *Carmyerius cruciformis* transmitted by the native snail *Bulinus truncatus*. Finally, *Biomphalaria pfeifferi* and two *Bulinus* species were found as new snail hosts for the poorly documented hippopotamus blood fluke *Schistosoma edwardiense*.

Conclusions

Our findings indicate that artificial lakes are breeding grounds for endemic and non-endemic snails that transmit trematode parasites of the common hippopotamus. This has important implications, as existing research links trematode parasite infections combined with other stressors to declining wild herbivore populations. Therefore, we argue that monitoring the anthropogenic impact on parasite transmission should become an integral part of wildlife conservation efforts.

Background

Large artificial lakes can be a major driver of the socio-economic development of a region by improving water accessibility, stimulating agricultural irrigation and generating renewable energy [1, 2]. Globally, 3,700 medium to large size artificial lakes (those with a capacity larger than 1 MW) have been built or are under construction [2, 3]. Nevertheless, dam construction also has tremendous repercussions on the surrounding environment as it can lead to the dislodgement and extinction of local aquatic communities, altered migration of aquatic biota, changes in aquatic biochemistry and physiology, disturbances of flood dynamics, introduction of exotic and invasive species, and the creation of breeding grounds for (non-)endemic vectors and intermediate hosts of (non-)endemic parasite species [4-11]. Additionally, the subsequent intensification of agriculture, animal husbandry and aquaculture near artificial lakes can lead to increased parasite transmission to humans, wildlife and domesticated animals [6, 12-15].

Snails play a key role as the intermediate host in the life cycle of trematode parasites and are a prime example of intermediate hosts that thrive in artificial lakes, as shown by multiple studies [5, 7, 10, 11, 16]. Consequently, the health burden on the definitive host of these parasites increases, as illustrated by the construction of the Diama dam in Northern Senegal, which caused drastic changes in the aquatic chemistry (e.g. reduced salinity and increased pH) that favored the colonization of planorbid snails and the subsequent establishment of one of the world's biggest foci of human intestinal schistosomiasis [17]. Another study suggests that dams block migratory river prawns that predate on planorbid snails, and thereby increase the risk of schistosomiasis for millions of people world-wide [16]. The use of agrochemicals such as fertilizers and pesticides on irrigated land can further stimulate snail - and thus trematode - prevalence by reducing snail predator populations and increasing algal growth, the main food source of freshwater snails [12, 15]. Besides favoring the colonization and proliferation of endemic snail species, artificial lakes can also facilitate the establishment of non-endemic snails that have the potential to drastically alter the dynamics of trematode transmission [5, 18, 19]. Invasive non-endemic snails can affect parasite transmission in three distinct ways: 1) by introducing non-endemic parasites, defined as "spillover", 2) by successfully transmitting endemic parasites with an overall increased transmission as a result, defined as "spillback", or 3) by transmitting endemic parasites but with a lower transmission potential, resulting in a "dilution" effect [20-22].

Recently, we found strong indications that in Lake Kariba, the world's biggest artificial lake by volume, the proliferation of the invasive water hyacinth (*Eichhornia crassipes*) has facilitated the establishment of the invasive North American snail *Pseudosuccinea columella*, which in turn supports an extremely high prevalence (65%) of an unknown fasciolid trematode

species [5]. The impressive success of *P. columella* snails in colonizing Lake Kariba, as well as the high trematode infection prevalence, suggest a significant health burden for the affected definitive host(s). The high parasite transmission by this invasive snail can be designated as either a spillback or a spillover phenomenon [22]. To discriminate between both scenarios, the identity of the parasite and final host are key. Carolus and colleagues [5] showed that the trematode transmitted by *P. columella* is phylogenetically closely related to *Fasciola gigantica* and *Fasciola hepatica.* However, the high genetic distances between this unknown species and other *Fasciola* species (based on mitochondrial cytochrome c oxidase subunit I (*COI*) sequences) suggested it to be 1) a new species, 2) a species not represented in current molecular databases, or 3) a hybrid species, as fasciolids are well-known for their hybridization potential [23-25]. Therefore, the aim of this study was to revisit Lake Kariba in order to collect more information on this parasite and its intermediate and definitive hosts. This subsequent sampling campaign involved the collection of snails and adult liver and stomach flukes from a culled hippopotamus, a prime suspect as definitive host [5]. An integrative taxonomic approach [26], combining nuclear and mitochondrial markers, was applied to reveal the identity of the unknown *Fasciola* sp. and other trematodes. In addition, we investigated whether similar phenomena of biological invasions coupled with parasite transmission are occurring in other artificial water systems in Zimbabwe.

Results

Trematode identification from the dissected hippopotamus

Morphological analysis

Two morphologically distinct liver flukes (hereafter referred to as 'Hippo liver fluke(s)') were collected from the liver bile ducts of the culled hippopotamus. In addition, several hundred bright red stomach flukes (hereafter referred to as 'Hippo stomach fluke type 1') and two small yellowish stomach flukes (hereafter referred to as 'Hippo stomach fluke type 2') were collected from the stomach wall of the hippopotamus. A high-resolution photograph of each adult fluke morphotype is shown in **Figure 1**. Scanning Electron Microscope (SEM) imaging was only possible for Hippo stomach fluke type 1 due to the low sample size of the other morphotypes (**Additional file 1: Figure S1**). Specimens of Hippo stomach fluke type 1 had a cylindrical shape that tapered towards the anterior end and showed a marked color change from bright red in fresh specimens to brown upon fixation. The Hippo stomach fluke type 2 had a discoid body shape and one specimen appeared to have two small caudal appendages (**Figure 1c**, right specimen). A comparative morphometric analysis (**Table 1**) of the fixed Hippo liver flukes described above and fixed museum specimens of *F. hepatica, F. gigantica* and *F. nyanzae* (all shown in **Additional file 1: Figure S2**), combined with the

descriptions in literature [27-29], indicates that the hitherto unknown *Fasciola* sp. present in Lake Kariba and Mwenje reservoir is morphologically most similar to *F. nyanzae.* The body width at 2 mm from the posterior end, the testis location, the testis to body length ratio and the vitellaria morphology differentiate *F. nyanzae* from *F. hepatica* and *F. gigantica*. This, together with the fact that the definitive host is the common hippopotamus, supports the morphological identification of the Hippo liver fluke(s) as *F. nyanzae*. Additionally, measurements (**Table 1**) of the metacercariae (**Additional file 1: Figure S3**) that were released from two *P. columella* snails collected in Lake Kariba, fall within the range reported in literature for metacercariae of *F. hepatica, F. gigantica* and *F. nyanzae* [27, 30, 31]. Notably, the observed shedding time between 9 and 11pm (see below 'Trematode infection prevalence in snails'), coincides with increased foraging activity of hippos at night [32].



Figure 1: The collected flukes from the male hippopotamus at Lake Kariba. A) Hippo liver flukes 1 & 2 (resp. left and right); B) Hippo stomach fluke type 1; and C) Hippo stomach fluke type 2. Scale bars represent 5 mm. Pictures were cropped and pasted to an artificial black background for contour visibility.

Table 1: Morphometric analysis of *Fasciola* species. Three reference measurements from literature are provided for *Fasciola nyanzae* collected from *Hippopotamus amphibius* [27-29]. All measurements are provided in mm, except measurements of metacercariae which are displayed in µm. Metacercarial measurements for Hippo liver fluke 1 and 2 were taken from two shedding *Pseudosuccinea columella* snails from site 3 in Kariba (**Additional file 1: Figure S3**). Metacercarial cyst measurements for *Fasciola gigantica* and *Fasciola hepatica* were taken from Alicata [30] and Vareille-Morel *et al*. [31] respectively. Variables that differentiate between the three *Fasciola* species are underlined. Measurements were taken with ImageJ software on 70% EtOH fixed samples with exception of the reports from literature. Measurement methodology is shown in **Additional file 1: Figure S2**. Abbreviations: body length to body width ratio (BL/BW), body length (BL), body width at widest point (BW), posterior width at 2 mm from the posterior end (BW2), cephalic cone length until shoulders (CL), ventral sucker anteroposterior diameter (VS), testis location in body length (Testis loc.), testis to body length ratio, vitellaria location in body length, outer diameter metacercaria (OD cyst), inner diameter metacercaria (without fibrous layer, ID cyst).

Species	Source	Host	BL/ BW	BL	BW	BW 2	CL	VS	Testis loc.	Testis /BL	Vitellaria	OD cysts (µm)	ID cysts (µm)
F. nyanzae	Leiper (1910)	*H. amphibius*	~7/ 1	69	~9	3	3	1.25	/	/	25	/	/
F. nyanzae	Jackson (1921)	*?*	3.6/ 1- 7.8/ 1	35- 59	4.5- 13.5	/	/	/	Anterior third	/	/	/	/
F. nyanzae	Dinnik and Dinnik (1961)	*H. amphibius*	~7/ 1	49- 91	~9	/	/	1.46 - 1.97	Anterior third	25% - 40%	/	242- 272	212- 228
Hippo liver fluke 1	This study	*H. amphibius*	4.9/ 1	52. 4	10.6	3.1	/	1.47	Anterior third	24%	34.1	261	221
Hippo liver fluke 2	This study	*H. amphibius*	3.5/ 1	46. 9	13.3	3.2	2.2	1.72	Anterior third	29%	27.1	259	228
F. nyanzae RMCA	This study	*H. amphibius*	4.2/ 1	41. 2	9.8	3	2.0 4	1.35	Anterior third	25%	25.4	/	/
F. gigantica RMCA	This study	Bovine	3.6/ 1	35. 3	9.9	5.1	3.1	0.83	Anterior two third	41%	10.8	238- 268	180- 206

F. hepatica RMCA	This study	*Bos taurus*	3.1/1	26.8	8.6	4.6	1.67	1.47	Anterior two third	42%	10.16	205-256	/

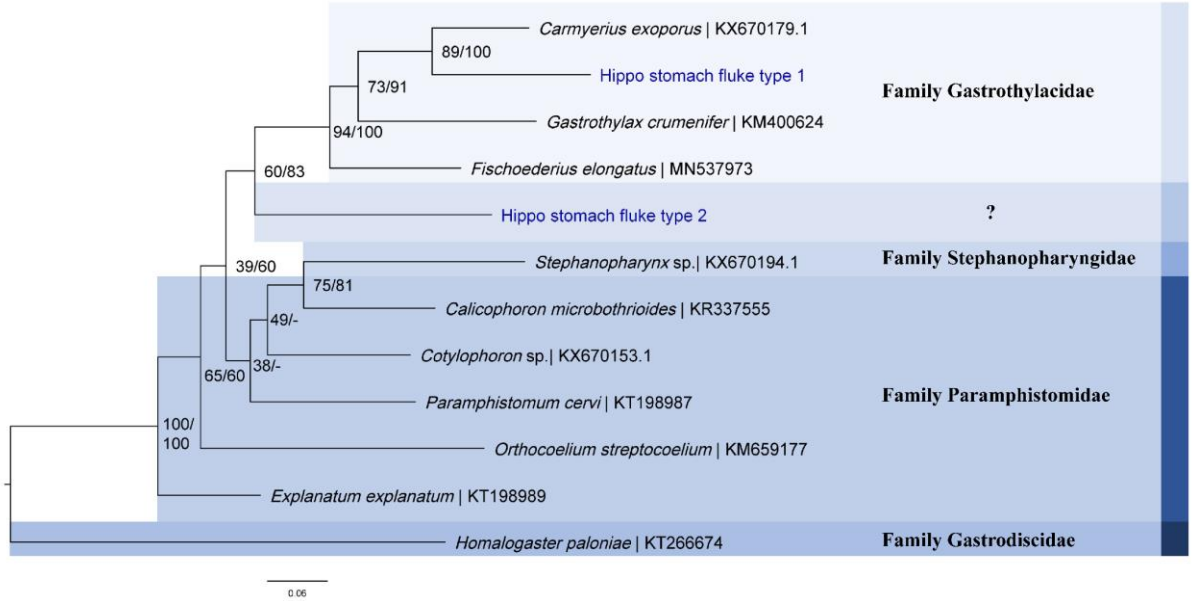


Figure 2: Maximum Likelihood analysis of the available sequences of the Superfamily Paramphistomoidea using the GTR + G (= 0.80) + I (= 0.47) model on 758 bp of the *COI* marker. *Homalogaster paloniae* ("GenBank: KT266674"), from the family Gastrodiscidae, was used as an outgroup. Nodal support is indicated as bootstrap percentages (1,000 bootstraps) and posterior probabilities, respectively before and after the "/" separator. Specimens from this study are indicated in blue. GenBank accession numbers are provided after the '|' separator.

Molecular analysis

The obtained *COI* sequences for Hippo stomach fluke type 1 and 2 (871 base pairs [bp] and 675 bp, "GenBank: MT909560 and MT909561" resp.) did not closely match any of the available sequences on the GenBank or BOLD databases. The closest BLAST hits were *Gastrothylax crumenifer* (89% identity score) and *Explanatum explanatum* (87% identity score), respectively. Our phylogenetic analyses show that Hippo stomach fluke type 1 has close affinity with the *Carmyerius* genus (**Figure 2**). Additionally, the sequence proved 100% identical (*COI*, 376 bp) to an amphistome infection isolated from *Bulinus truncatus* in 2017 in Lake Kariba ("GenBank: MT013349 and MT013355" resp. for the snail and the amphistome infection; preprint, [33]). As no deeper taxonomic resolution could be obtained from the molecular data, we further refer to this species as '*Carmyerius* sp.'. Based on *COI* sequencing, we can conclude that the Hippo stomach fluke type 2 belongs to the superfamily Paramphistomoidea, but it could not be identified to a lower taxonomic level in our phylogenetic analysis (**Figure 2**). The *COI* sequence was however 100% identical (420 bp)

to an amphistome infection in *Radix* sp. ("GenBank: MT013350"; preprint, [33]), here identified as *Radix* aff*. plicatula* (see below 'Snail identification')*,* from Lake Kariba [5; preprint, 33]. All pairwise distances with *COI* reference sequences on GenBank exceed 12% and thereby surpass the 10% divergence of congeneric trematodes [34]. Therefore, we further refer to this species as 'unidentified amphistome species'. DNA sequencing of Hippo liver fluke 1 and 2 resulted in *COI* fragments of 815 and 862 bp ("GenBank: MT909542 and MT909543" resp.) and rDNA fragments of 3,083 and 2,987 bp ("GenBank: MT909821 and MT909820"), respectively. The rDNA region, which was identical between the two flukes, covered almost the entire *18S* rDNA (1,829 bp, see methods section and **Additional file 1: Figure S4**), internal transcribed spacer 1 (*ITS1*, 454 bp), *5.8S* rDNA (160 bp), internal transcribed spacer 2 (*ITS2*, 360 bp) and partial *28S* rDNA (281 and 185 bp resp. for Hippo liver fluke 1 and 2) regions. The *COI* sequences differed 2.2% over 814 nucleotides. The *COI* and rDNA sequences showed the highest similarity (twice 99.3% for COI ["GenBank: MK330628 - MK330630"] and 99.9% for rDNA ["GenBank: MK330623 - MK330625"]) to the *Fasciola* sp. infections in *P. columella* and *Radix* sp. (here identified as *R.* aff*. plicatula,* see the following section 'Snail identification') from Lake Kariba, reported by Carolus *et al.* [5]. The *COI* pairwise distances of Hippo liver fluke 1 and 2 with *F. gigantica* (10.6% and 14.4% resp.) and *F. hepatica* (16.4% and 18% resp.) exceed the 5% threshold for species differentiation based on the *COI* barcoding marker [34, 35]. The rDNA pairwise distances (rDNA sequences were identical between Hippo liver fluke 1 and 2) to *F. gigantica* and *F. hepatica* were less pronounced compared to *COI* distances (0.4% and 0.2% resp.) (**Additional file 1: Table S1** and **S2**). Phylogenetic analysis based on *COI* sequences place the Hippo liver fluke as a sister taxon to *F. gigantica* within the *Fasciola* genus (**Figure 3a** and **Additional file 1: Table S1**). The placement in the *Fasciola* genus is confirmed by the rDNA-based phylogeny (**Figure 3c** and **Additional file 1: Table S2**). No DNA sequences could be obtained from the museum samples, probably due to DNA degradation or PCR inhibition as a result of long-term preservation, first in formaldehyde and later in 70% ethanol. This prevented the inclusion of the museum samples (shown in **Additional file 1: Figure S2**) in our phylogenetic analysis. None of the tested datasets showed conclusive evidence of substitution saturation (see supplementary file: "Raw_data" for the Steel's and Xia's method outputs per dataset).

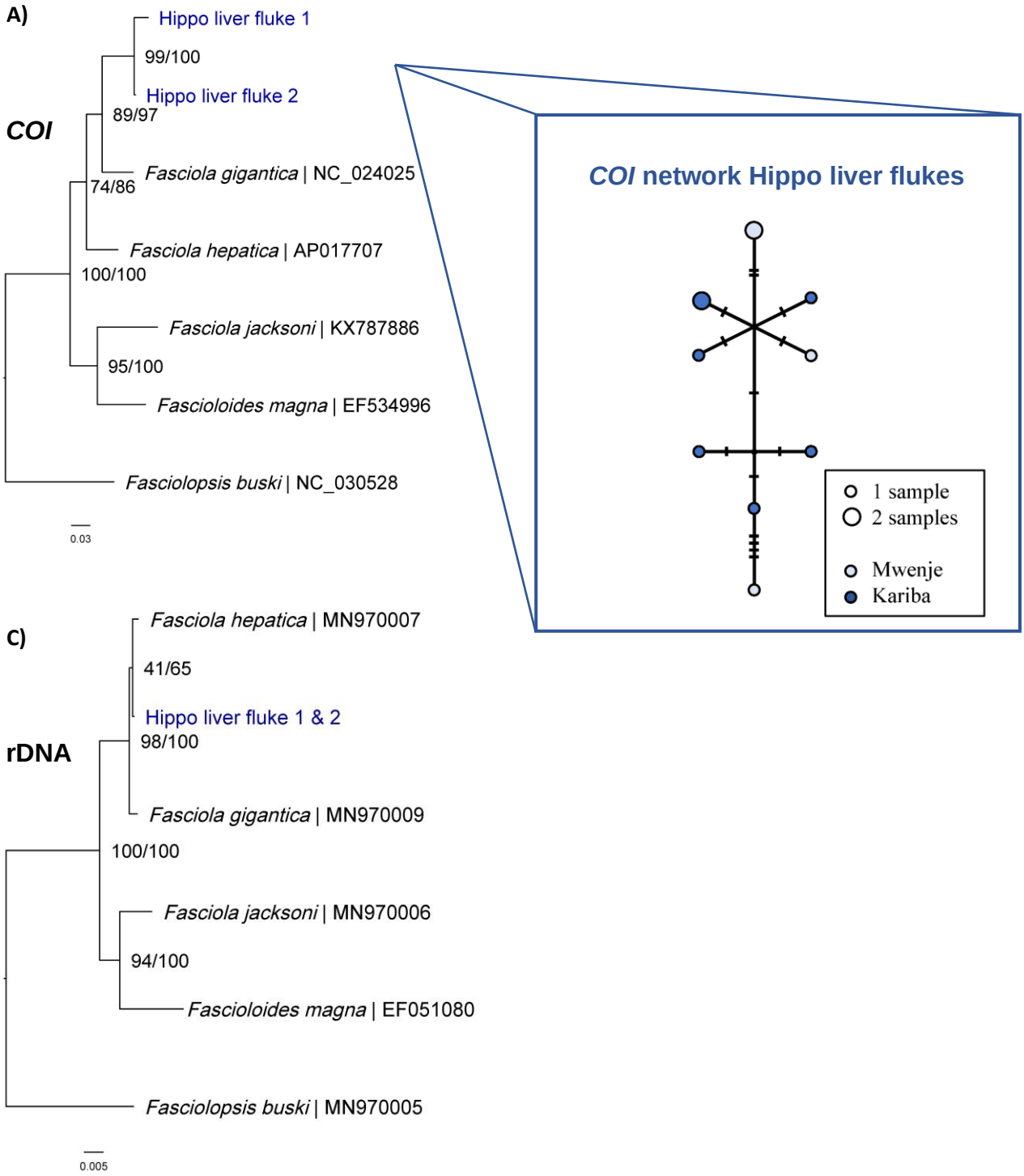


Figure 3: A) Maximum Likelihood analysis of the available sequences of the subfamily Fasciolinae using the GTR + G (= 0.25) model on 814 bp of the *COI* marker. *Fasciolopsis buski* ("GenBank: NC_030528"), from the subfamily Fasciolopsinae, was used as an outgroup. B) The *Fasciola nyanzae COI* haplotype network of 11 sequences based on 412 positions, visualized using the TCS model in PopArt®. One hatch mark represents one mutation. The legend indicates the used color-code and haplotype abundance through circle size. C) Maximum Likelihood analysis of the available sequences of the subfamily Fasciolinae using the GTR + G (= 0.05) model on 2,771 bp of the rDNA region. *Fasciolopsis*

buski (“GenBank: MN970005”), from the subfamily Fasciolopsinae, was used as an outgroup. Nodal support is indicated as bootstrap percentages (1,000 bootstraps) and posterior probabilities, respectively before and after the “/” separator. Specimens from this study are indicated in blue. GenBank accession numbers are provided after the ‘|’ separator.

Snail identification

Six different snail species were sampled in this study: *Pseudosuccinea columella* and *Radix* aff*. plicatula* at Lake Kariba and *Radix natalensis, Biomphalaria pfeifferi, Bulinus globosus* and *Bulinus* sp. at Mwenje reservoir (**Figure 4**). Out of 16 sampled sites in Lake Kariba, only site 3 and site 16 harbored snails (see **Table 2** for the number of snails per site and **Figure 4** for information on sampling locations). High-resolution photographs of one fixed specimen per species are depicted in **Figure 5**.

COI sequences of *P. columella* were 100% identical (463 & 433 bp, “GenBank: MT888842”) to the invasive *P. columella* haplotype reported across Africa, Australia and Northern America (i.e. Egypt [“BOLD: GBMIN110283-17”], Australia [“BOLD: GBMLG0711-06”] and USA [“BOLD: GBMPL484-13”]). We confirm the presence of this invasive haplotype in Lake Kariba, as published before [5] (see “GenBank: MK333465”). Carolus and colleagues [5] reported an unidentified *Radix* sp., presumably originating from Asia, but the authors were not able to identify the species, as closely related reference sequences were missing at that time. The *COI* sequences from the *Radix* species sampled in this study (“GenBank: MT888847”) show only one mutation in the 350 bp overlapping *COI* sequence compared to the *Radix* sp. sequenced by Carolus *et al.* [5] (see “GenBank: MK333466”). In our phylogenetic analyses, the *Radix* sp. shows well supported clustering with a recently generated reference sequence of *Radix plicatula* from China [36] (**Additional file 1: Figure S5 and Table S3**). However, many radicine snail species are absent from online genetic databases, and shell morphology differs from the description in Vinarski *et al*. [37]. Therefore, and in combination with the pairwise distance of 3.6% between the *Radix* sp. from Kariba and the reference sequence of *R. plicatula* on a 463 bp *COI* alignment, we identify our current specimen and the previously reported *Radix* sp. from Lake Kariba as the non-endemic *R. affinis plicatula* (**Additional file 1: Table S3**). The qualifier *affinis* is utilized in accordance with the open nomenclature guidelines of Sigovini and colleagues [38], as our specimens show clear molecular affinity to *R. plicatula,* but are not identical to it.

The identity of *R. natalensis, B. pfeifferi* and *B. globosus* collected in Mwenje (446 bp, 446 bp and 463 bp resp.; “GenBank: MT888844-MT888846”) was confirmed based on phylogenetic clustering and pairwise distances below 5%, compared to *COI* reference sequences in the BOLD and NCBI databases (**Additional file 1: Figure S5, S6** and **Table S3, S4 and S5)**. A second *Bulinus* species from Mwenje (446 bp, “GenBank: MT888843”) could not be identified

to species level based on *COI* barcoding but appears to be part of the *Bulinus truncatus/tropicus* species complex as indicated by the firm clustering within this group in our phylogenetic analysis (**Additional file 1: Figure S6a and Table S5**). Further taxonomic identification was not possible as the species group contains 14 species that are morphologically nearly indistinguishable, many of which still lack reliable reference genetic material [39, 40].

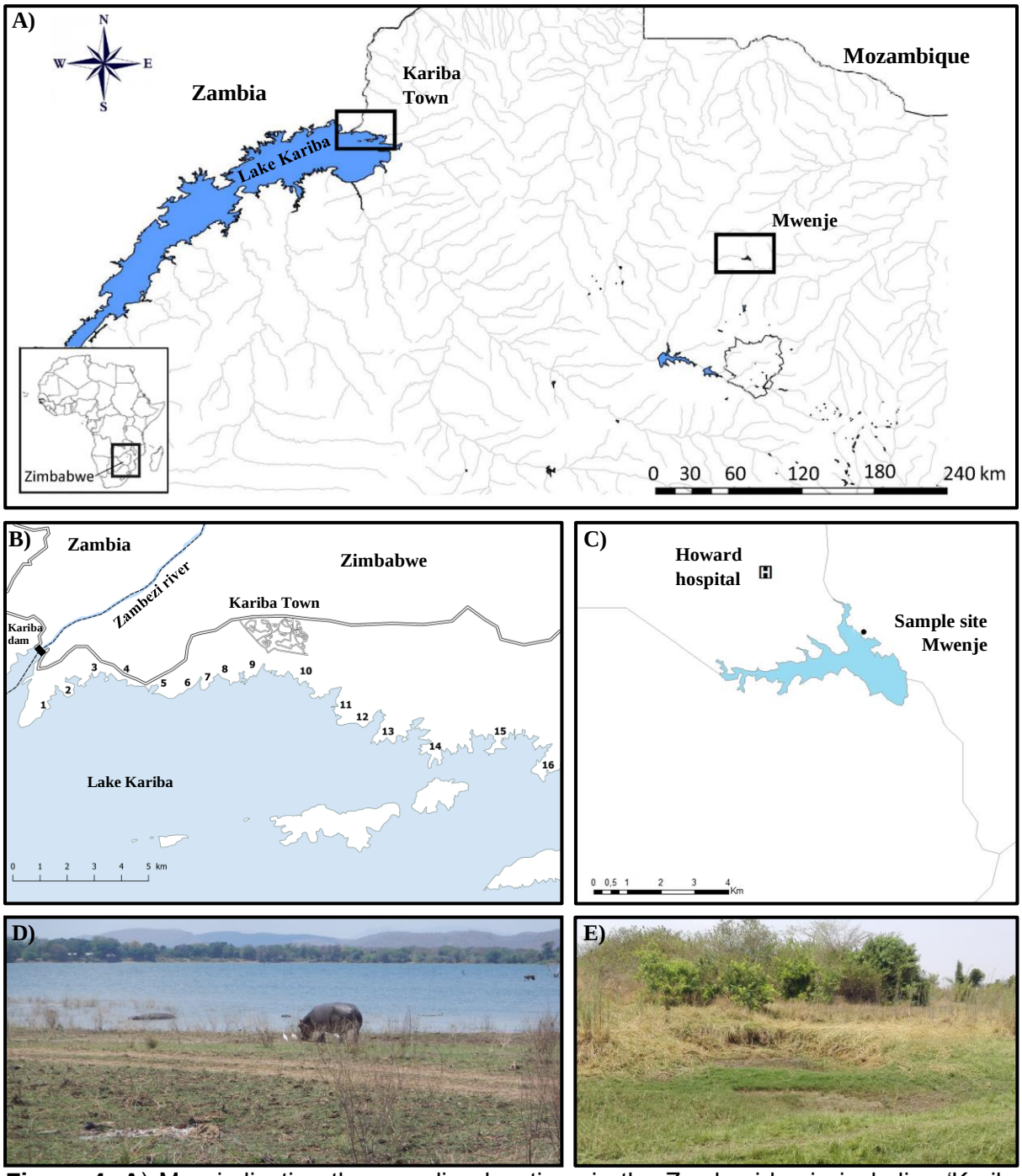


Figure 4: **A**) Map indicating the sampling locations in the Zambezi basin including 'Kariba Town' at Lake Kariba and 'Mwenje' at Mwenje reservoir in Zimbabwe. **B**) Sampling sites at Lake Kariba; figure adapted from Carolus *et al.* [5]. **C**) Sampling site at Mwenje, a temporary

puddle adjacent to the main reservoir located at S 17°14'47.9" E 31°01'07.7"; figure adapted from Schols *et al.* [39]. **D**) Sampling site 13 at Lake Kariba. **E**) Sampling site at Mwenje reservoir.

Table 2: Snail abundance and trematode infections per site. All snail species are listed per site along with the number of specimens collected (Sampled), the number of snails that shed cercariae in the shedding experiment (Shedding), the number of snails for which DNA was extracted for diagnostic multiplex PCRs (Tested), the number of samples that tested positive for a trematode infection (Trematoda inf.), and how many of those trematode infections had a *Fasciola nyanzae* (*F. nyanzae* inf.), *Schistosoma edwardiense* (*S. edwardiense* inf.) or *Schistosoma haematobium* infection (*S. haematobium* inf.). For Kariba sites, the number between brackets refers to exact sample location adopted from Carolus *et al.* [5]. High–resolution pictures of each snail morphotype are shown in **Figure 5**.

Site	Species	Sampled	Shedding	Tested	Trematoda inf.	*F. nyanzae* inf.	*S. edwardiense* inf.	*S. haematobium* inf.
Kariba (3)	*P. columella*	60	6	24	21	21	0	0
Kariba (3)	*R.* aff. *plicatula*	12	0	12	3	1	0	0
Kariba (16)	*P. columella*	43	0	24	24	24	0	0
Mwenje	*R. natalensis*	17	0	17	13	11	0	0
Mwenje	*B. pfeifferi*	6	0	6	6	0	3*	0
Mwenje	*Bulinus* sp.	1	0	1	1	0	1	0
Mwenje	*B. globosus*	9	0	9	9	0	6	1

* One more *B. pfeifferi* showed infection signals indicating a *Schistosoma* sp. infection but no sequences could be obtained, inhibiting species identification.

Trematode infection prevalence in snails

Cercarial shedding (i.e. release of trematode larvae) was exclusively observed between 9 pm and 11 pm in six out of 103 examined *P. columella* snails (5.8%) collected in Kariba. No other snail species was found to release cercariae during shedding experiments. When screening for infections through diagnostic PCRs, more infected snails were detected. **Table**

2 shows the number of snails tested, the number of trematode infections detected and the number of samples where a *Schistosoma* sp. or *Fasciola* sp. infection was detected in the multiplex PCR assay. In summary, 77 out of 93 samples (82.8%) had (pre)patent trematode infections. Of all detected infections, a total of 57 out of the 61 (93.4%) infected lymnaeid snails (*P. columella* and *R.* aff. *plicatula* from Kariba and *R. natalensis* from Mwenje) were infected with a *Fasciola* sp., while 12 out of the 16 (75%) infected planorbid snails (*B. pfeifferi, B. globosus* and *Bulinus* sp. from Mwenje) were infected with a *Schistosoma* species.

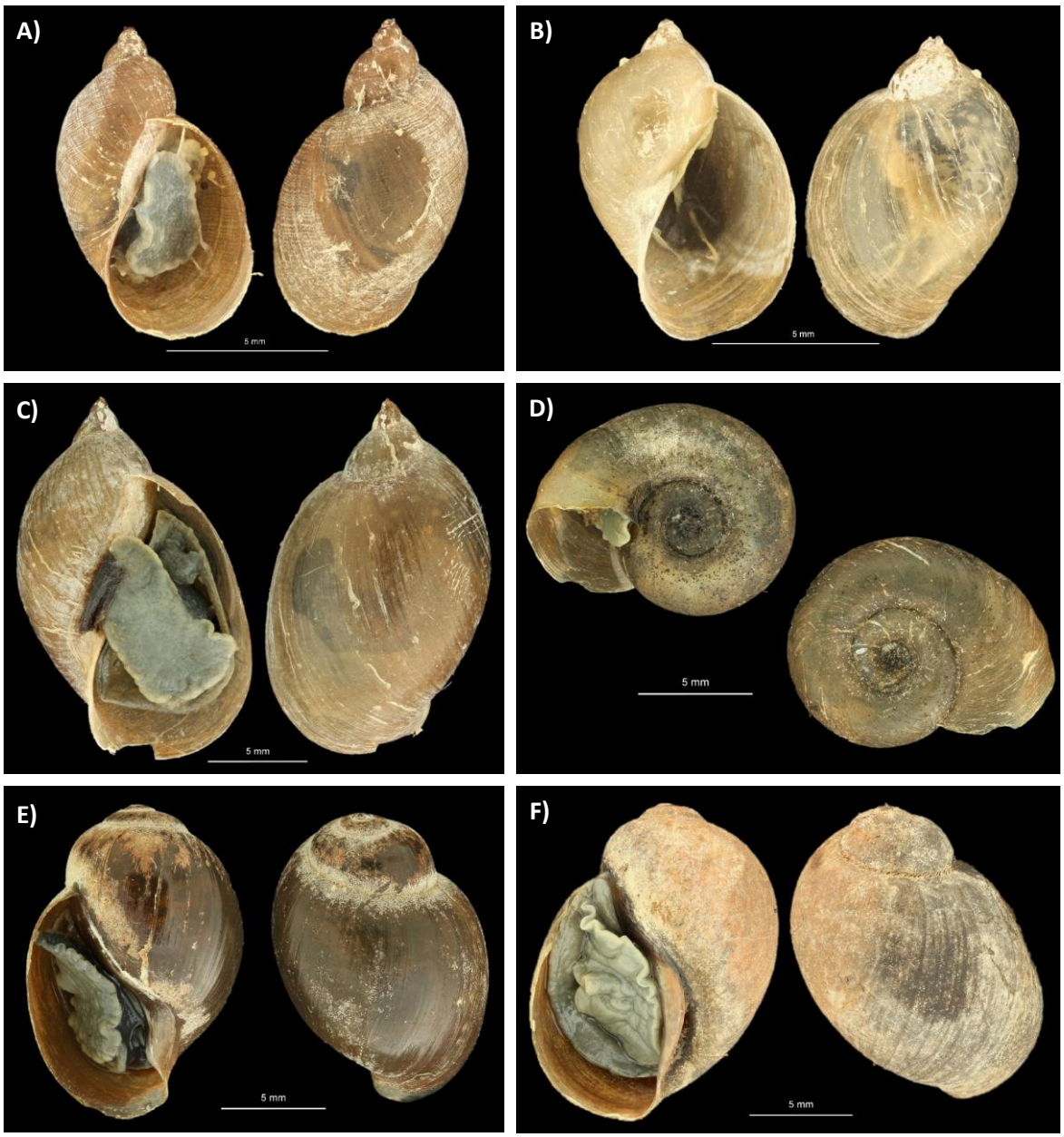


Figure 5: Pictures of snail morphotypes collected in Mwenje and Kariba. A) *Pseudosuccinea columella*, B) *Radix* sp. molecularly identified as *Radix* aff. *plicatula*, C) *Radix natalensis*, D) *Biomphalaria pfeifferi*, E) *Bulinus globosus* and F) *Bulinus* sp.. Scale bars represent 5 mm. Pictures were cropped and pasted to an artificial black background for contour visibility.

Trematode identification from snail infections

Tissue of one *P. columella* specimen and one *R.* aff*. plicatula* specimen from Lake Kariba with a positive *Fasciola* sp. PCR signal was used for trematode *COI* barcoding (425 bp and 482 bp resp., "GenBank: MT909545 and MT909549" resp.). Both *COI* sequences showed a pairwise distance of less than 0.5% compared to each other and less than 0.8% compared to the two adult *F. nyanzae* specimens from the culled hippopotamus (based on 408 bp). Eleven specimens of *Radix natalensis* from Mwenje with a positive *Fasciola* sp. PCR signal were used for *COI* and *ITS1-5.8S-ITS2* sequencing. Five *COI* sequences (~400 bp) were ~99% identical to a *Diplostomidae* sp. collected from the adjacent reservoir by Schols *et al*. [41] ("GenBank: MT994279"), two did not provide sequencing results of sufficient quality and the remaining four amplicons (424 bp - 428 bp, "GenBank: MT909546-MT909548 and MT909550") showed a pairwise distance below 2% to the two adult *F. nyanzae* specimens from Kariba (results not shown, 408 bp). The *ITS1-5.8S-ITS2* amplification and sequencing was successful for 10 out of the 11 *Fasciola* sp. positive samples (464 bp, "GenBank: MT893586-MT893595"). All 10 sequences were 100% identical to each other, to the two adult *F. nyanzae* and to the S151 haplotype from Carolus *et al.* [5] ("GenBank: MK330624").

Using the two-step diagnostic PCR assay of Schols *et al.* [42] (see methods section, 'detection of infected snails'), we identified *S. haematobium* in a *B. globosus* snail*.* This was confirmed by sequencing part of the *COI* marker (335 bp, "GenBank: MT886703") and rDNA region (985 bp, "GenBank: MT884914") in this specimen*.* The remaining *Schistosoma* sp. infections in *B. pfeifferi* (4)*, B. globosus* (6) and *Bulinus* sp. (1) gave a *Schistosoma*-specific amplicon in the first multiplex PCR but did not yield a species-specific amplicon in the second multiplex PCR, indicating it was not *Schistosoma haematobium, Schistosoma mansoni, Schistosoma mattheei, Schistosoma bovis, Schistosoma curassoni* or *Schistosoma guineensis* [42]. We, therefore, sequenced the *COI* marker and the *Schistosoma*-specific *5.8S-ITS2* amplicon generated in the first multiplex PCR. The *COI* marker (335 bp, "GenBank: MT886702") was successfully amplified for only one *B. pfeifferi* infection and showed a pairwise distance of 0.2% to the preliminary identified *S. edwardiense* isolated from *Biomphalaria sudanica* in Uganda ("GenBank: AY197347"). The *5.8S-ITS2* (~340 bp, "GenBank: MT884915-MT884924") marker was successfully sequenced for ten out of the eleven remaining samples and all sequences were 100% identical to each other and to the *S. edwardiense* reference sequence ("GenBank: AY197344"). Based on these results, we identify all ten remaining *Schistosoma* sp. infections in *B. pfeifferi* (3)*, B. globosus* (6) and *Bulinus* sp. (1) as *S. edwardiense.*

Haplotype network of *Fasciola nyanzae*

The genetic diversity of *F. nyanzae* in Lake Kariba and Mwenje reservoir was assessed using all available *COI* sequences of *F. nyanzae.* To build the haplotype network, we included both adult liver flukes collected from the hippopotamus, *F. nyanzae* sequences isolated from four infected *P. columella* from Lake Kariba (of which one from this study and three from Carolus *et al*. [5]: “GenBank: MK330623-MK330625”), one sequence from an infected *R.* aff*. plicatula* from Lake Kariba and *F. nyanzae* sequences from four infected *R. natalensis* from Mwenje reservoir. The resulting alignment (412 bp) consisted of 11 sequences, of which nine were unique, and the associated haplotype diversity (Hd) was 0.96. The highest pairwise distance between two haplotypes was 1.7%. The haplotype network shows no spatial genetic structure (**Figure 3b**).

All GenBank identifiers generated by this study, and those of Carolus *et al.* [5] and Muzarabani *et al*. (preprint, [33]) involved in the analyses, are listed together with the sample type, marker, taxonomic identification and region of origin in **Additional file 1: Table S6**.

Discussion

In this study we report four trematode species that infect the common hippopotamus and show that they are transmitted by six snail species that thrive in Lake Kariba and Mwenje reservoir. We are able to complete the life cycle for three of these species by identifying them in both the final and the intermediate host. Below, we discuss the biological phenomena behind parasite transmission in artificial lakes and the possible implications for threatened hippopotamus populations.

1. Completing the *Fasciola* phylogeny

The adult liver flukes sampled in this study match the morphological descriptions of *Fasciola nyanzae* made by Leiper in 1910 [27]. Since then, only two scientific reports have elaborated on the morphological description and life cycle of *F. nyanzae* [27, 28]. Along with morphological measurements of the cercariae that were genetically identical to the adult liver flukes, these morphological descriptions enable us to identify *F. nyanzae* and provide the first genetic record of this species. As a result, we can provide the last missing link in the molecular phylogeny of the *Fasciola* genus and show that *F. nyanzae* is most closely related to *Fasciola gigantica* based on partial *COI* mtDNA (**Figure 3a** and **Additional file 1: Table S1**). This close relationship is also supported by the observation that both species can infect the intermediate snail host *R. natalensis* and the definitive mammalian host *H. amphibius*, while no records exist of *Fasciola hepatica* in hippopotami [27, 43]. This phylogenetic relationship is however less clear based on the more conserved nuclear markers, where the distances between *F. nyanzae* and both *F. hepatica* and *F. gigantica* are of the same order and below 1% (**Figure 3c** and **Additional file 1: Table S2**). This apparent mito-nuclear discordance can either be explained by drastically different evolutionary rates between nuclear and mitochondrial DNA [44], or by introgressive hybridization [24, 45]. A higher genomic coverage is needed to conclude on this, as concerted evolution in the rDNA region can blur biological phenomena, such as a bias towards one of the two parental species after hybridization [46]. Our phylogenetic analyses also enable us to contribute to the taxonomic discussion on whether or not *Fasciola jacksoni* should be reclassified as *Fascioloides jacksoni,* as suggested by Lotfy *et al*. [47]. Mas-Coma *et al*. [43] opposed this reclassification, because: the reference sequences used by Lotfy *et al*. [47] originated from regions where *F. gigantica x F. hepatica* hybrids are known to occur, *F. nyanzae* and *Tenuifasciola tragelaphi* were missing from the phylogenetic analysis and the mitochondrial marker (*nad1*) used in the study of Lotfy *et al*. [47] is too variable, leading to substitution saturation. The inclusion of well-selected reference sequences from regions where no hybridization has been reported, the addition of *F. nyanzae,* and the use of a less variable mtDNA marker [48] in this study,

results in a dataset without substitution saturation and resolves most of the aforementioned issues. Our maximum likelihood and Bayesian phylogenies of mitochondrial and nuclear markers strongly support the position of *F. jacksoni* within the genus *Fascioloides* (**Figure 3a** & **c** and **Additional file 1: Table S1** & **S2**, respectively).

2. Parasite spillback of the hippo liver fluke *Fasciola nyanzae* in Lake Kariba.

Fasciola nyanzae infections were detected in three lymnaeid snail species: the endemic *Radix natalensis,* the Asian *Radix* aff*. plicatula,* and the North American *Pseudosuccinea columella*. The latter two species are invasive non-endemic snails that could affect parasite transmission in three distinct ways: parasite spillover, parasite spillback or a dilution effect (see 'Background' section for explanations). Co-invading parasites, like other invading fauna and flora, typically go through a genetic bottleneck, resulting in low genetic diversity in the invasive population [49]. In contrast, our *F. nyanzae* samples show a high genetic diversity with 9 out of the 11 sequenced *COI* haplotypes being unique. Moreover, their definitive host (*H. amphibius*) and at least one of their intermediate hosts (*R. natalensis*) are endemic to Zimbabwe [50, 51], supporting the endemicity of *F. nyanzae* to this region. In addition, another requirement to comply with a 'parasite spillback' hypothesis was met, as a higher *F. nyanzae* infection prevalence was detected in the invasive snail *P. columella* compared to native snails. We can therefore confirm that the cascade of biological invasions, in which the invasion of *P. columella* was facilitated by the introduction and subsequent colonization of water hyacinth from South America as described in Carolus *et al.* [5], has led to 'parasite spillback' of the endemic parasite *F. nyanzae* in Lake Kariba. Water hyacinth [52] and lymnaeid snails such as *P. columella* [53] generally thrive in nutrient rich, still or slow-moving water, but not in fast moving riverine systems, like the Zambezi river, which occupied the Kariba gorge before the construction of Kariba dam. Therefore, we hypothesize that the parasite spillback phenomenon we witness here, is a result of this man-made impoundment.

3. Two hippopotamus stomach flukes, one case of parasite spillover?

Although amphistomiasis, or stomach fluke disease, is one of the most prevalent and pathogenic animal trematodiases, there is a significant knowledge gap concerning the life cycle, host compatibility, prevalence and geographic range of many amphistome species, especially in Africa [54-56]. Amphistomes are most commonly studied in livestock, especially ruminants, but many species infect wild animals. Here we provide sequences, the intermediate host identity and high–resolution photographs of two amphistome species of the common hippopotamus (i.e. *Carmyerius cruciformis* and an unidentified amphistome species). We link these stomach fluke species to infections in the endemic snail *B. truncatus* reported by Muzarabani *et al*. (preprint, [33]) and the non-endemic snail *R.* aff*. plicatula,*

respectively. Based on its morphology (**Figure 1b** and **Additional file 1: Figure S1**), its final and intermediate hosts and close phylogenetic affinity to *Carmyerius exoporus* (**Figure 2**), we tentatively identify the amphistome Hippo stomach fluke type 1, genotyped as '*Carmyerius* sp.' in this study, as *Carmyerius cruciformis*. First of all, the reported size, shape and the distinct color change upon fixation in our specimens match prior descriptions [29, 57]. Secondly, it is the only *Carmyerius* species described from the hippopotamus so far [58]. Thirdly, members of the *Bulinus* genus are known intermediate hosts of this parasite [59], which corresponds with the prior infections detected in *B. truncatus* ("GenBank: MT013350, MT013355"; preprint, [33]), that match our genotype. Fourthly, the oral opening and tegumental papillae (**Additional file 1: Figure S1**) correspond to prior reports of *C. cruciformis* [57,60]. And finally, no reference sequence exists of *C. cruciformis,* probably explaining the failed attempt to identify this species through molecular barcoding. To our knowledge, this is the first molecular record and southernmost report (other reports are from Kenya, Uganda, Benin and Chad [58]) of *C. cruciformis*.

The amphistome Hippo stomach fluke type 2 (**Figure 1c**) could not be identified based on the *COI* phylogeny that includes all families of the superfamily Paramphistomoidea present in online molecular databases (**Figure 2**). The absence of a morphologically similar hippopotamus trematode described in literature and the lack of reference sequences for many amphistome families (e.g. family Brumptinae) make the identification of these specimens currently impossible. To further elucidate its phylogenetic position and identify it to species level, future research should study the morphological characteristics such as the tegumental surface and the genital pore through scanning electron microscopy [60] and transversal sections [57], respectively. Furthermore, an integrative taxonomic approach could be applied to generate reference sequences for the missing amphistome families [26, 41]. For the Hippo stomach fluke type 2, sampled from the Asian snail *R.* aff*. plicatula,* no prior genetic and morphological records exist*,* which raises the question whether this parasite is endemic to Africa. The answer will determine whether this is a case of a potential 'parasite spillback' (like for *F. nyanzae*), or a 'parasite spillover', as a result of co-introduction of a non-endemic parasite with a non-endemic snail host.

4. New intermediate hosts for the hippopotamus blood fluke *Schistosoma edwardiense*?

Three endemic planorbid snail species, namely *B. pfeifferi, B. globosus* and an unidentified *Bulinus* sp. from the *Bulinus truncatus/tropicus* complex, were found to be infected with the hippopotamus blood fluke *S. edwardiense.* Although schistosome blood flukes are among the most studied trematode species due to their public health importance, wildlife schistosomes are severely understudied. *S. edwardiense,* has so far only been reported in

Uganda [61] and South Africa [62]. The species is known to be transmitted through *Biomphalaria* snails [61], and so far, *Bulinus* snails have not been reported as an intermediate host. We cannot conclude whether the molecular detection of *S. edwardiense* in the two *Bulinus* species reflects true, compatible host-parasite combinations or whether they are a result of aborted infections, since we did not observe cercarial shedding in these samples (neither in the infected *Biomphalaria* snails). However, we found a positive PCR signal in seven bulinid specimens, and the closest known relative of *S. edwardiense, Schistosoma hippopotami,* is also transmitted through bulinid snails [61]. These facts strengthen our hypothesis that apart from *B. pfeifferi*, bulinid snails are compatible hosts for *S. edwardiense* too. Our results strengthen the statement of Morgan *et al*. [61], that *S. edwardiense* and *S. hippopotami* belong to a different group, ancestral to the *S. mansoni* and *S. haematobium* species groups. The ability to use members of both the *Biomphalaria* and *Bulinus* genera appears as a 'primitive' trait, which is lost in the derived species groups that specialized in only one of the two snail genera, respectively.

5. The potential health burden of hippopotamus trematodiases

Trematode parasites can have detrimental effects on the health, lifespan, and reproduction of wild and domestic animals. For instance, liver flukes cause hepatic fibrosis, cirrhosis, internal hemorrhage and calcification of the bile duct [63]. As a result, fascioliasis in livestock is associated with an increased mortality and abortion rate, reduced growth and a lower productivity [64, 65], which accounts for global economic losses exceeding 3.2 billion US dollars [64]. *Fasciola jacksoni* and *Protofasciola rubusta* can form a major health burden and source of (calf) mortality in Asian [66] and African [67] elephants respectively. Additionally, *F. jacksoni* has been suggested to be a major contributor to the drastic decline of endangered elephant populations in Asia [66]. Another fluke, *Fascioloides magna*, is thought to be the single major cause of mortality in a declining population of moose in the United States [68]. Schistosomes or blood flukes are also thought to be an important yet overlooked cause of animal mortality and productivity losses in the livestock industry, with a major underestimated economic impact [69]. Certain schistosome species have also been suggested to be of concern to conservation of endangered animals such as rhinos and elephants in Asia [70] and chimpanzees in Africa [71]. In addition to liver and blood flukes, immature stomach flukes, or amphistomes, inflict damage to the intestine, leading to anorexia and diarrhea, while severe infections can be lethal [72, 73]. Amphistome infections are regarded as one of the most prevalent parasitic disease in livestock [55]. Due to their high morbidity and mortality in young animals, some authors hypothesize that the economic losses due to amphistomiases are greater than many other parasitic diseases in livestock [74].

Few records exist on the pathology and impact of trematode infections in hippopotami, but a similar impact as described above can be expected. The most elaborate study on hippopotamus trematodiases dates back to 1967 and reports one hundred culled hippopotami of the Kruger National Park in South Africa [75]. It describes unknown fasciolids that colonized the bile duct in large numbers, causing lesions and fibrosis of the duct wall and damage extending into the liver parenchyma. The same study reports a high load of schistosomes in the heart and most major blood vessels, especially in the venae cavae, pulmonary and hepatic veins, associated with inflammation, macroscopic lesions and endocarditis [75]. Since then, no further pathological reports on trematode infections in the common hippopotamus have been published to the best of our knowledge.

Here we provide a parasitological survey of a single culled hippopotamus from Lake Kariba, in which only parts of the liver and stomach lining were studied. This revealed three different trematode species (i.e. *F. nyanzae, C. cruciformis* and an unidentified amphistome) with a very high infection intensity of the stomach fluke *C. cruciformis*. In addition, the high trematode infection prevalence in snail intermediate hosts, reported here and in Carolus *et al*. [5], indicates that the exposure risk of hippopotami to these parasites is high.

6. Do artificial lakes and biological invasions pose a burden on *Hippopotamus amphibius*?

Considering our findings and the aforementioned burden of trematodes on wildlife populations, we argue that artificial lakes can act as a breeding ground for (non-)endemic intermediate snail hosts that transmit hippopotamus infecting parasites, and thereby indirectly affect hippopotamus (and possibly other wildlife) populations. Nevertheless, the actual impact of dam construction on hippopotami cannot be directly verified. No data is available on hippopotamus populations and their trematode fauna prior to dam construction or the arrival of invasive snail species. However, key historical reports do show a link between the Kariba dam construction and altered snail and trematode dynamics in the area. A health risk assessment prior to the Kariba dam construction concluded that the chance for a schistosomiasis outbreak was minimal given the rocky substrate of Kariba gorge, which is unsuitable for freshwater snails [76]. At that time, only urinary schistosomiasis was present in the surrounding communities at low prevalence, while *B. pfeifferi* and thus intestinal schistosomiasis was absent. However, soon after dam construction, urinary schistosomiasis increased and for the first time, intestinal schistosomiasis was reported [77]. Furthermore, *Bulinus, Biomphalaria* and lymnaeid species, all potentially suitable intermediate snail hosts for the hippopotamus parasites we describe here, colonized the lake [77]. Later studies confirmed the presence of planorbid snails and increased levels of schistosomiasis in nearby towns [78-81]. Ultimately, the invasive snails *P. columella* and *Radix* sp. (here identified as

R. aff. plicatula), which we find to be compatible intermediate hosts for hippopotamus parasites, were first reported in the lake in 2017 [5]. In conclusion, these historical records and our findings strongly suggest that the creation of Lake Kariba has drastically altered parasite transmission dynamics by favoring the colonization and proliferation of (non-)endemic snail species that transmit trematode parasites, including those infecting hippopotami.

Large herbivores such as hippos play pivotal roles in their ecosystems and are therefore crucial targets for conservation efforts, which rely on a profound understanding of the factors that put the target population at risk [82-85]. Among the major anthropogenic disturbances, such as habitat loss, hunting and poaching [82, 86], the impact of parasitic diseases remains hard to assess due to knowledge gaps in the diversity and epidemiology of most wildlife parasites [87]. Therefore, the impact of trematode parasites on wildlife populations might be severely underestimated [88]. The common hippopotamus is listed as vulnerable to extinction [50], yet remains populous in southern and eastern Africa with an estimated 5,000 specimens in Zimbabwe [50, 85]. However, models show that these populations are two to five times more likely to cross the risk threshold of drastic decline in the next 60 years, if mild human-induced perturbations continue, alongside the present natural disturbance [85]. The combination of disease with other environmental or anthropogenic stressors can impose significant conservation threats [88-90]. This is illustrated by Obanda *et al*. [91], which suggest that infections with the liver fluke *P. robusta* have a synergetic effect with drought-related starvation in mass-mortality of African elephants in Kenya. Parasitic diseases can thus have major consequences for threatened populations, making it a critical, although understudied issue for the conservation of species [92].

Conclusions

The data presented here suggests that the construction of artificial lakes along with the introduction and establishment of endemic and non-endemic species, might have increased or at least altered the burden of snail-borne diseases on hippopotami. Still, to quantify what the exact impact might be, we lack historic and current information on the prevalence and infection intensities of trematode species in the hippopotamus populations in this region. Such information is hard to obtain and requires collaborations between researchers in the fields of ecology, veterinary science and conservation biology as suggested by previous studies [92, 93]. Nevertheless, enriching the genetic database of wildlife trematodes to design non-invasive sampling methods can generate valuable resources in conservation studies [92]. For example, by providing the genetic reference of parasites like *F. nyanzae*

and *C. cruciformis,* its prevalence can be monitored by designing species-specific diagnostic PCRs to screen stool samples from hippos and other wildlife species.

Methods

Adult fluke collection and morphometric characterization

A subadult male *Hippopotamus amphibius* was culled by rangers as part of the wildlife governance quota set for population control, problem animal management, community benefits or other aspects of sustainable utilization by Zimbabwe Parks and Wildlife Management Authority. The hippopotamus was culled near Kariba Town (**Figure 4**) and the liver, bile ducts and stomach were dissected and inspected for adult trematode parasites. The entire liver and a 15 by 15 cm part of the stomach were screened for liver and stomach flukes, respectively. Fresh adult flukes were stored in 70% ethanol for later DNA analysis and morphometrics. The fixed flukes collected in this study, along with museum (Royal Museum for Central Africa, RMCA) specimens of *Fasciola nyanzae, F. gigantica* and *F. hepatica* ("RMCA tissue vouchers: 31048, 29430 and 22595 resp."), that were collected in the Democratic Republic of Congo in 1956, 1952 and 1938, respectively, were photographed using a Canon EOS 600D camera equipped with a Macro Photo Lens according to Brecko *et al.* [94]. Pictures were taken at different focal depths while the specimens remained submerged in 70% ethanol and stacked using the Zerene Stacker™ software. Stacked pictures of the adult flukes were used for measurements in the ImageJ software (version 1.8.0). Morphometric measurements were taken of body width at the widest point (BW), body length (BL), cephalic cone length until shoulders (CL), width at 2 mm from the posterior end (BW2) and the anteroposterior diameter of the ventral sucker (VS) (see **Additional file 1: Figure S2** for a detailed visualization of how the measurements were done). Specimens for scanning electron microscopy were transferred in acetone for 24 hours, then airdried, mounted on aluminium stubs, coated with gold and studied using a JEOL JSM-6480LV scanning electron microscope.

Snail sampling and shedding experiments

The study area constituted of Mwenje reservoir, and the northeastern shoreline of Lake Kariba, which are two artificial reservoirs of the Zambezi river basin in Zimbabwe. Mwenje dam was erected in 1970, covers roughly 5 km² when full and has a catchment area of approximately 557 km² [95]. The Kariba dam was completed in 1959 and Lake Kariba covers about 5,580 km² when full and has an approximated catchment area of 815,000 km² [96]. Sampling sites are displayed in **Figure 4**. Locations sampled at Lake Kariba were adopted from prior studies [5, 79]. The Mwenje sampling site was located at S 17°14'47.9" E 31°01'07.7" in close vicinity to the reservoir. Snails were collected in October 2018 from

water, aquatic vegetation, and sediment by two persons for 30 minutes per site, using both a scooping net and manual collection. Collected snails were stored in jars with lake water from the site of origin and kept at a constant temperature during transport. Upon arrival, snails were sorted per morphotype, counted and preliminary identified based on the morphological identification keys of Brown [97] and Mandahl-Barth [98]. Next, shedding experiments were conducted: snails were separated in 12-well cell culture plates and incubated in filtered and aged lake water in complete darkness overnight, followed by bright light exposure for five hours from 7 am to 12 pm. This treatment induces the natural emergence (i.e. *shedding*) of larval stages, the so-called cercariae, of several trematode species [55, 99]. Well contents and surfaces were inspected with a stereomicroscope for the presence of cercariae and/or metacercariae respectively, as cercariae from liver flukes are known to rapidly encyst and form metacercariae on biotic or abiotic surfaces [100]. This was done at the start of the shedding experiment, 10 pm, 7 am and hourly between 7 am and 12 pm during the bright light exposure experiment to detect nocturnal and diurnal shedding, respectively. All snails were sacrificed by heat shock in ~70°C water to prevent contraction and simplify later DNA extraction, followed by fixation in 80% ethanol, then we added the released cercariae to the same tube in the event of shedding. Snails that released cercariae were individually stored together with the emerged cercariae, while non-shedding snails were pooled per species per site. One representative of each snail morphotype per site was, prior to DNA extraction, fixed on black clay and photographed from its ventral and dorsal side according to Brecko *et al.* [94] using the focus stacking system described above. The obtained pictures were processed in Microsoft PowerPoint® by removing the image background and combining the front and rear perspective of the shell in one picture while ensuring a constant scale size. The (meta)cercariae were placed in 80% ethanol on a glass slide with a glass cover. For taking and compiling photos, we used a digital camera QImaging MicroPublisher 5.0 RTV mounted on a Leitz Dialux 22 stereomicroscope and piloted with the Syncroscopy's Image Reconstruction Software Auto-Montage Pro (version 5.03.0061). The resulting images of (meta)cercariae were used for measurements in the ImageJ software (version 1.8.0).

DNA extraction

DNA of ethanol preserved **adult trematodes** was extracted using the DNeasy Blood and Tissue kit (Qiagen™) according to the manufacturer's protocol. For each DNA extraction we used a small piece of tissue isolated from the posterior side of the specimen with a sterile scissor. The DNeasy Blood and Tissue kit (Qiagen™) was not successful for the museum samples of adult trematodes. The specimens are currently stored in 70% ethanol yet were initially fixed in formaldehyde at the time of collection. Formaldehyde, a storage medium often used in the past by museums, is known to inhibit proteinase *K*-based DNA extractions

[101]. Therefore, Chelex® (Biorad™) and phenol chloroform isoamyl alcohol (PCI) based DNA extraction methods were assessed for the same museum samples, which do not rely on proteinase *K* for lysis. For the Chelex-based extraction, tissue was first incubated for 24h in ATL buffer (Qiagen™) to flush excess formaldehyde as suggested by Shedlock *et al*. [101]. Next, samples were homogenized with a sterile scalpel on a glass plate. Tissue homogenate was incubated for one hour at 56°C and 30 minutes at 95°C in 200 µL of 5% Chelex® (Biorad™) solution in a shaking Thermocycler™. Next, samples were centrifuged for 7 minutes at 13,000xg and supernatant DNA extract was collected and stored at -20°C. Regarding the PCI-based DNA extraction, tissue samples were dried with sterile absorbent paper, 300 µL Tris-EDTA (TE) buffer and 300 µL PCI solution (phenol pH 6.7-chloroform-isoamylalcohol [25:24:1]) was added and tissue was lysed by micro-bead shearing in a FastPrep-24™ Classic lysis system (20 sec, 6 m/sec) (MP biomedical™). The DNA containing fraction was separated from lipids and cellular debris by 10 minutes of centrifugation at 14,000 rpm. Next, 15 µL sodium acetate (3M, pH 5,2) and 450 µL ethanol (100%) were added and the mixture was placed in a freezer at -20°C for 20 minutes followed by centrifugation for 10 minutes at 4°C (14,000 rpm) for DNA precipitation. Pellets were washed by adding ice cold ethanol (70%) followed by centrifugation (10 minutes at 4°C, 14,000 rpm). The resulting DNA was dissolved in TE buffer and stored at -20°C. Successful DNA isolation was verified by gel electrophoresis (0.8% agarose gel, SYBR™ Safe).

The DNA of (**meta**)**cercariae** was extracted using a proteinase *K*-based lysis buffer as described in Ziętara *et al.* [102]. In short, 10 µL of the prepared proteinase *K* lysis buffer was added to a single cercaria in 10 µL of MilliQ water (Merck, Darmstadt, Germany). A first and second incubation step followed at 65°C for 25 minutes and 95°C at 10 minutes, respectively. After the protocol, DNA extracts were stored undiluted at -20°C.

For DNA extractions from ethanol preserved **snail samples**, soft tissue was isolated from the shell by using a sterile needle to make a hole in the apex to push the soft tissue out of the shell. Next, the isolated tissue was dried using absorbent paper and homogenized using a sterile scalpel. DNA was extracted using the E.Z.N.A.® Mollusc DNA Kit (OMEGA Bio-tek, Norcross, GA, USA) according to the manufacturer's protocol and eluted through two elution steps of 75µL, totaling to 150µL of DNA extract. Additionally, the more cost- and time efficient Chelex® (Biorad™) method [5, 103] was used on 24 non-shedding *P. columella* specimens per site in Lake Kariba, as described previously. All DNA extracts were diluted 1:10 with MilliQ water (Merck, Darmstadt, Germany) to prevent inhibition of PCR reactions.

Molecular diagnosis of infected snails

The DNA extracts of a maximum of 24 snails per species per site were subjected to the diagnostic multiplex PCR methods described in Schols *et al.* [42] and Carolus *et al.* [5].

These approaches enable rapid and cost-efficient diagnosis of trematode infections in snail DNA extracts, with simultaneous detection of *Schistosoma* spp. [42] or *Fasciola* spp. [5]. Both methods are based on the amplification of multiple amplicons of different lengths in a single PCR reaction: an internal control that amplifies *18S* snail rDNA to confirm the success of the DNA extraction and PCR reaction, a general trematode primer pair designed to amplify the *18S* rDNA of all trematode genera that have a reference sequence available in GenBank and S*chistosoma*- [42] or *Fasciola*- [5] specific internal transcribed spacer 2 (*ITS2*) or mitochondrial cytochrome c oxidase subunit I (*COI*) primers respectively. All used primers and PCR protocols are described elsewhere [5, 42]. Samples that were positive for a *Schistosoma* spp. infection were subsequently analyzed using a second multiplex PCR according to the two-step approach described by Schols *et al*. [42]. This PCR differentiates between several schistosomes of veterinary and medical importance (i.e. *S. haematobium, S. mansoni, S. mattheei* and *S. bovis/S. curassoni/S. guineensis*) by generating species-specific *COI* amplicons of different lengths. By selectively screening all snail DNA extracts using either of the two methods (i.e. Lymnaeidae with the method of Carolus *et al.* [5] and Planorbidae with the method of Schols *et al*. [42] we identified *Schistosoma* spp. and *Fasciola* spp. infections that were used for further analysis.

PCR and DNA sequencing

A fragment of the mitochondrial *COI* and a nuclear rDNA region covering the *18S* rDNA marker, the internal transcribed spacer 1 (*ITS1*), the *5.8S* rDNA marker, the internal transcribed spacer 2 (*ITS2*) and/or the partial *28S* rDNA marker were amplified to assess the taxonomic position of trematode species sampled in this study. **Additional file 1: Figure S4** indicates the annealing sites of the used primers for amplification of the rDNA region of adult liver flukes. The primers and resulting amplicon lengths are listed in **Table 3**, together with those for snail and trematode identification. We barcoded (*COI* sequencing) two random specimens per snail species and all *Schistosoma* and *Fasciola* spp. infected snails. For the parasites, we selected all *Schistosoma* and *Fasciola* spp. infections, all adult fasciolids and one adult amphistome specimen per morphotype. A new *Fasciola*-specific reverse primer COI_FAS_R (‘5-GACAAACAAACACAAGCAGG-3’), targeting a shorter *COI* fragment of 148 nucleotides when combined with the COI1_DIG_F primer (see **Table 3**), was designed to amplify DNA of the *Fasciola* museum samples, as no successful PCR amplification was obtained using the longer amplicon. It has become apparent that shorter amplicons (< 400 bp) outperform longer amplicons due to severe DNA fragmentation and/or degradation in old samples [104-106], such as the museum specimens in this study. Simplex PCR reactions and programs for sequencing were performed, according to *Carolus et al.* [5], in a 15 µL volume with 1.5 µL of 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 MgCl2, 0.45 units of Taq Polymerase (Qiagen™), 0.8 µM forward primer and 0.8 µM reverse primer. The PCR reactions were performed as follows: initial denaturation at 94 °C for 15 min, 39 cycles of 94 °C for 30 sec, annealing temperature depending on primer pair as listed in **Table 3** for 45 sec and 72 °C for 45 sec and a final elongation step at 72 °C for 10 min in a Tprofessional Thermocycler (Biometra™). PCR products were visualized by gel electrophoresis on a 2% agarose gel with Midori Green Direct® staining and UV light. Samples that showed a clear amplified PCR product of the expected length were purified using the ExoSAP (Fermentas™) PCR purification protocol and sequenced using BigDye® chemistry (Macrogen™). The resulting sequences were processed (quality control, annotation, and generation of consensus sequences from forward and reverse primer sequencing) with Geneious® (R10). The *Trim Ends* command with an error probability limit of 5% in Geneious® was used to trim the obtained sequences. The complete chromatogram was inspected before and after consensus generation to resolve any additional ambiguities and ensure high sequence quality.

Phylogenetic analysis

All sequences of sufficient quality were compared to the GenBank (https://blast.ncbi.nlm.nih.gov/) and BOLD (http://v3.boldsystems.org/) databases using the Basic Local Alignment Search Tool (BLAST) and BOLD Identification System (IDS) respectively. In case the species could not be clearly identified (i.e. no highly similar reference sequence or high identity matches to several species), a phylogenetic analysis was performed in which sequences of all closely related taxa, with an available sequence in the GenBank database, were aligned with the MUSCLE alignment algorithm [107] in AliView (version 1.26). Reference sequences of *Fasciola nyanzae, Tenuifasciola tragelaphi,* several amphistome families and several members of the *Bulinus truncatus/tropicus* species complex [39] were absent from the database and could not be included in the analyses. The alignment was visually inspected for anomalies, trimmed to the largest possible consensus and the model selection analysis was run in MEGA (version 10.2.5) to assess the suitability (i.e. best model or only marginally different AIC and BIC values from the best model) of the General Time Reversible (GTR) substitution model. This model proved suitable for all datasets with the exception of Lymnaeidae phylogeny, which required the Hasegawa-Kishino-Yano (KHY) substitution model. The DAMBE software (version 7.2.152, [108]) was used to assess substitution saturation in all datasets through the Xia's [109] and Steel's method [110] according to Xia and Lemey [111]. For the Steel's method, mean phi values below 0.04 were assumed to lack phylogenetic information and were removed following Xia and Lemey [111]. For the *COI* phylogeny we tested the datasets for saturation in (1) the first

and second codon position and (2) the third codon position. For the fasciolid rDNA dataset we only assessed substitution saturation for the non-coding *ITS1* and *ITS2* markers as *18S, 5.8S* and *28S* rDNA are slowly evolving markers. Possible saturation signals were evaluated by constructing a Maximum likelihood tree from DNAML in DAMBE and assessing the tree's topology. The MEGA software was also used to estimate the corrected pairwise distances among *COI* sequences to correct for substitution saturation. For this, the Tamura-Nei (TN93) substitution model with gamma correction was applied. Gamma values were obtained through the model selection analysis in MEGA as mentioned above. A Bayesian inference of phylogeny was calculated using MrBayes (version 3.2.6) on the CIPRES portal (version 3.3) [112] (https://www.phylo.org/portal2) using the same model, for 10,000,000 generations while sampling the Markov chain every 1,000 steps. The first 25% of the sampled values were discarded as burn-in and the analysis stopped if the convergence diagnostic fell below 0.01. Convergence was confirmed by checking whether the Effective Sampling Size (ESS) was above 100 and by visually inspecting the absence of a trend in the convergence plots in MrBayes through the *sump* command and Tracer software (version 1.7.1). Finally, a Maximum Likelihood (ML) tree was also calculated through the IQ-tree web servers (http://iqtree.cibiv.univie.ac.at) and nodal support was assessed through 1,000 bootstrap replicates [113]. The resulting trees were rooted on the respective outgroup in Figtree (version 1.4.4). The same software was used to create a consensus tree, containing nodal support values of Bayesian inference and ML phylogenies, that was exported in PDF format to Adobe Acrobat Pro DC (version 2020.012.20048), in which we adjusted the position of ill placed nodal support values and italicized species names. All markers, alignment lengths and obtained sequences used for phylogenetic analyses are provided in their respective section in the results.

Haplotype network of *Fasciola nyanzae*

A *COI* haplotype network based on 412 bp was constructed to study the genetic intraspecific variation of *Fasciola nyanzae*. The obtained *COI* sequences from two adult liver flukes and six snail infections were aligned with those obtained by Carolus *et al.* [5] ("GenBank: MK330623, MK330624, MK330625") using the MUSCLE alignment algorithm [107] in AliView (version 1.26) and trimmed to the longest common overlapping sequence. Unique haplotypes were defined using the DnaSP® software (version 6.12) [114] and subsequently mapped in PopART® (version 1.7) (http://popart.otago.ac.nz) using the TCS network inference model [115].

Table 3: Primers used to obtain mitochondrial (*COI*) and nuclear (*18S*, *ITS1*, *5.8S*, *ITS2* and *28S*) amplicons for sequencing. The primer name is listed with the target organism, the targeted marker, the annealing temperature, the amplicon length, the primer sequence, and the literature reference from which primers were obtained. *COI*: mitochondrial cytochrome c oxidase subunit 1, *ITS1* and *ITS2*: Internal transcribed spacer 1 and 2 resp, bp: base pairs and *F. nyanzae*: *Fasciola nyanzae*. The rDNA region and *COI* marker of the metacercariae were amplified with the 18S_Dig_F - 1270R, ITS4 - ITS5 and COI1_Dig_F - NasmitR primer combinations.

Primer name	Used on	Marker	Annealing Temp.	Length (bp)	Primer sequence (5'-3')	Reference
WormA	*F. nyanzae*	*18S*	54°C	1870	GCGAATGGCTCATTAAATCAG	[116]
WormB					CTTGTTACGACTTTTACTTCC	[116]
18S_Dig_F	*F. nyanzae*	*18S*	50°C	1161	CAGCTATGGTTCCTTAGATCRTA	[5]
1270R					CCGTCAATTCCTTTAAGT	[117]
ITS5	*F. nyanzae*	*ITS1-5.8S-ITS2*	50°C	1065	GGAAGTAAAAGTCGTAACAAG	[118]
ITS4					TCCTCCGCTTATTGATATGC	[118]
ITS2_Dig_F	*F. nyanzae*	*ITS2-28S*	50°C	647	CAAHAAGTCGTGGMTTGG	[119]
ITS2_Dig_R					AACAACCCGACTCCAAGG	[119]
COI1_Dig_F	*F. nyanzae* & Stomach flukes	*COI*	53°C	943	CNATGATNTTNTTTTTTTTRATGCC	[119]
Nasmit R					ACATAATGAAARTCAGCNAYMACRA	[119]
COI1_Dig_F	Museum fasciolids	*COI*	53°C	148	CNATGATNTTNTTTTTTTTRATGCC	[119]
COI_FAS_R					GACAAACAAACACAAGCAGG	This study
Fasc-ITS1 F	*Fasciola* sp. from infected snails	*ITS1-5.8S-ITS2*	55°C	716	TCTACTCTTACACAAGCGATACAC	[7]
Fasc-ITS1 R					GGCTTTCTGCCAAGACAAG	[7]
COI1_Dig_F	*Fasciola* & *Schistosoma* sp. from infected snails	*COI*	50°C	451	CNATGATNTTNTTTTTTTTRATGCC	[119]
COI1_Dig_R					GMASWACCAAAWTTHCGATCAAA	[119]
ITS2_Schisto_F	*Schistosoma* sp. from infected snails	*ITS1-5.8S-ITS2*	62°C	369	GGAAACCAATGTATGGGATTATTG	[42]
ITS2_Schisto_R					ATTAAGCCACGACTCGAGCA	[42]
COI1_snail_F	Snails	*COI*	50°C	536	TAATTWATTGTTACDGCWCATGC	[119]
COI1_snail_R					CWCCTCCTGCWGGATCAAA	[119]

Declarations

Ethics approval and consent to participate

Research permits were obtained through the University of Zimbabwe (UZ), Faculty of Science Research Ethics Subcommittee in Harare, Zimbabwe. The Lake Kariba Research Station managed by the University of Zimbabwe has a memorandum of understanding with the Zimbabwe Parks and Wildlife Management Authority for research cooperation on Lake Kariba. It is within the context of this cooperation that permission was granted for sample collection. Permits for export and import were issued by the head of the Permits Section of the Directorate of Veterinary Services, Dr. Uchirai Chindezwa, organized through Prof. Maxwell Barson.

Consent for publication

Not applicable

Availability of data and materials

Lead Contact

Further information requests should be directed to and will be fulfilled by the Lead Contact, Ruben Schols (ruben.schols@africamuseum.be; ruben.schols@kuleuven.be).

Materials availability

DNA and tissue samples of all studied parasites, snails and the culled *H. amphibius* are stored in the RMCA's collection and are available with a completed Materials Transfer Agreement, with the exception of Hippo stomach fluke type 2 as the complete worms were used for DNA extraction.

Data and code availability

The sequences generated in this study have been deposited in the public repository NCBI GenBank under: MT909560-MT909561, MT909545-MT909550, MT909542-MT909543, MT893586-MT893595, MT888842-MT888847, MT886702-MT886703 and MT884914-MT884924. All GenBank identifiers generated by this study, and those of Carolus *et al*. [5] and Muzarabani *et al.* (preprint, [33]) involved in the analyses, are listed together with the sample type, marker, taxonomic identification, region of origin in Additional file 1: Table S6.
The sequence datasets used in this study are available in the supplementary file "Raw_data".

Competing interests

The authors declare no competing interests.

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Authors' contributions

Conceptualization R.S., H.C. and T.H.; Formal Analysis R.S.; Investigation R.S., H.C. and K.M.; Resources M.B and T.H.; Funding TH; Data Curation R.S.; Writing-Original Draft R.S. and H.C.; Writing-Review & Editing R.S., H.C., C.H., K. M., M.B. and T.H.; Supervision C.H., M.B. and T.H. All authors read and approved the final manuscript.

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Fasciola nyanzae

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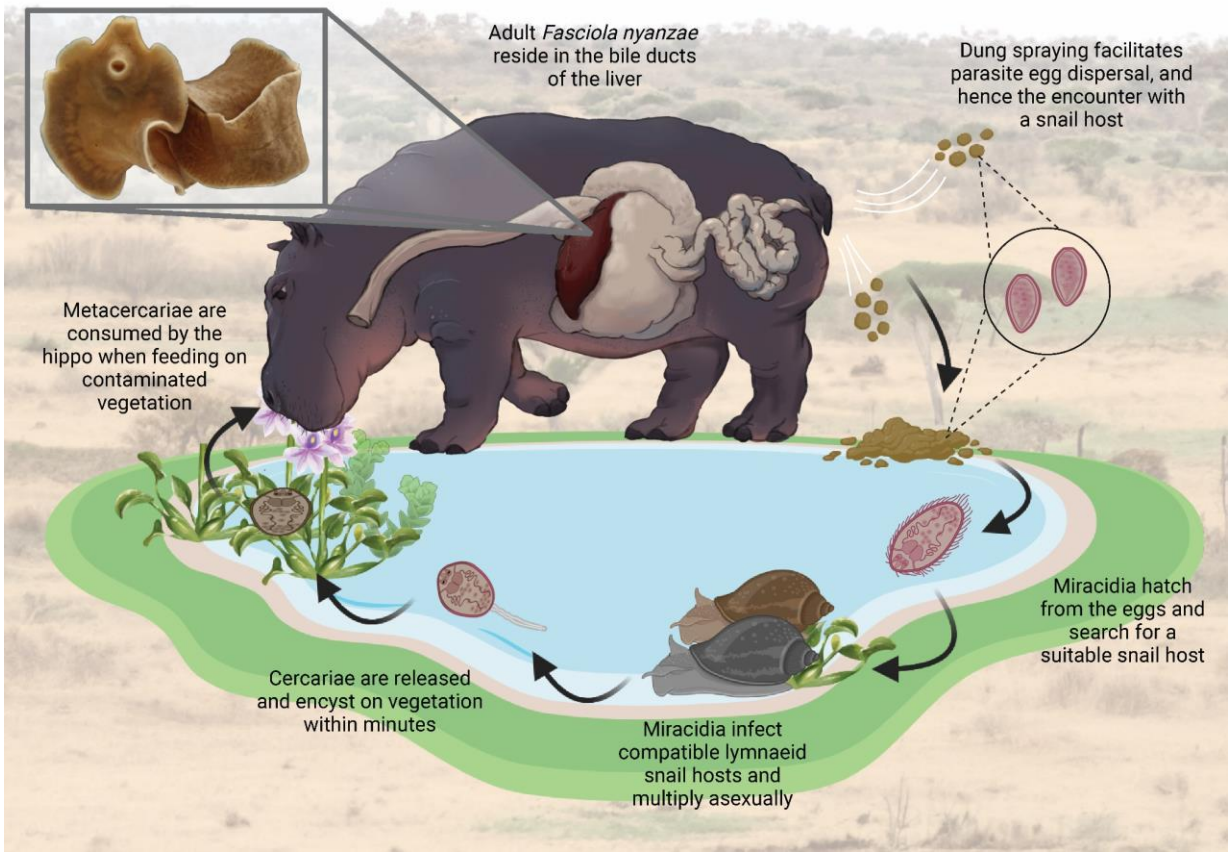
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Summary

The liver fluke *Fasciola nyanzae* infects the bile ducts of the enigmatic hippo (*Hippopotamus amphibius*) and can grow up to 9 cm in length. Its main intermediate host is the freshwater snail *Radix natalensis*, but invasive exotic species like *Pseudosuccinea columella*, and to a lesser extent *Radix* aff. *plicatula,* can also act as hosts. Historical studies describe notable liver damage, and a high infection prevalence in hippo populations. However, recent data on distribution and morbidity are lacking due to the vulnerable status of hippos, hindering post-mortem investigations. Optimized molecular tools to monitor infections in snails and hippo dung present non-invasive alternatives to collect epidemiological data on *F. nyanzae*. The combined effect of invasive exotic snail species, climate change and increased use of agrochemicals can amplify disease transmission and thereby impact hippo populations throughout Africa.



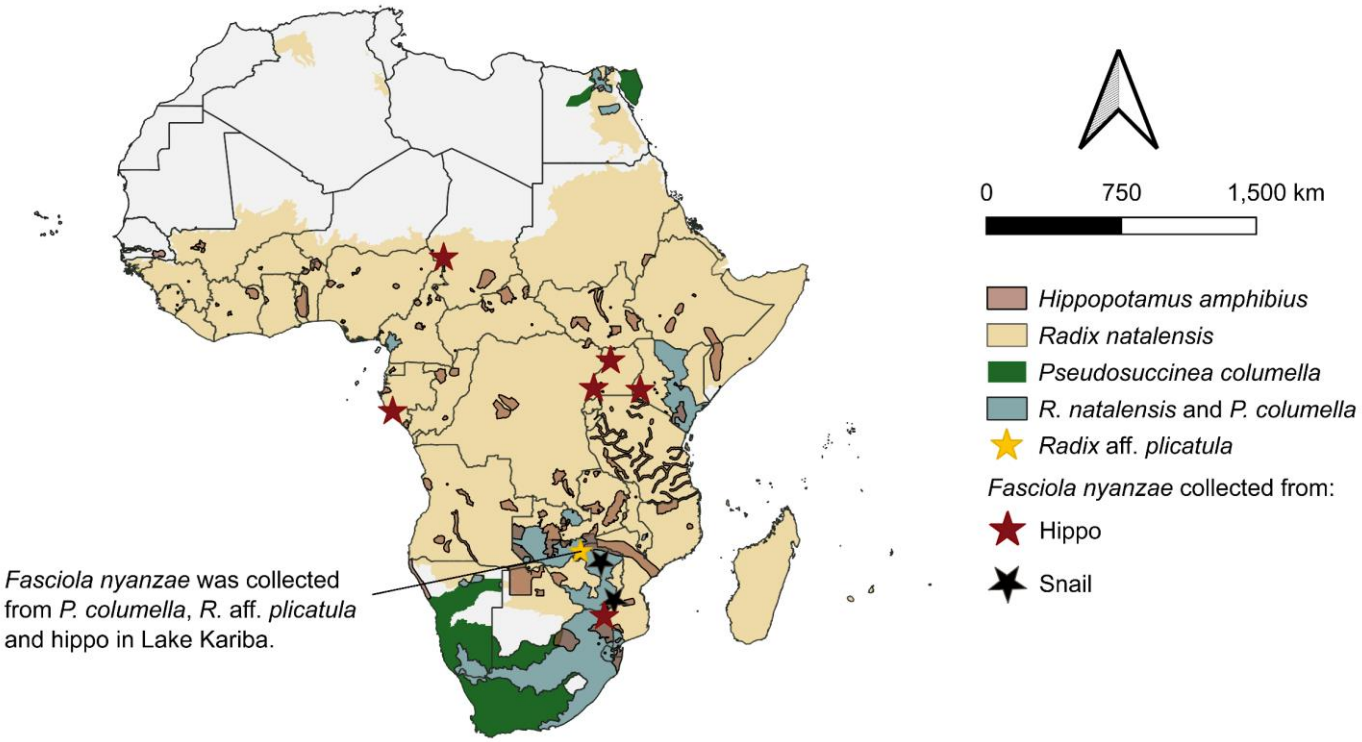
Key facts

F. nyanzae was described in 1910 but the first pictures and molecular data were only published in 2021.

It has currently been reported from six African countries, but its range is expected to be wider, encompassing all areas where hippos and snail hosts overlap.

Its only known final host, the hippo, is vulnerable to extinction, indicating that *F. nyanzae* is facing similar or even higher extinction risks in some areas.

Discordance between nuclear and mitochondrial phylogenies confounds the evolutionary history reconstruction of *F. nyanzae*.



Disease facts

Nearly 90% of 400 autopsied hippos in the ‘60s were infected with one to five specimens per animal.

Pathology reports include severe bile duct fibrosis, bile stasis, necrosis of the hyperplastic epithelium, adenomatous hyperplasia, and pericholangitis, indicating severe liver disease in affected hippos.

Information on *F. nyanzae* maturation within the final host is lacking since experimental infections of calves, goats, and rabbits failed.

Experimentally infected *R. natalensis* released cercariae for over two and a half months.

Fasciola spp. are known to hybridize, affecting fasciolosis epidemiology. No hybrids involving *F. nyanzae* have been reported, but co-infection with *F. gigantica* in hippos suggests the potential for hybridisation.

Taxonomy and Classification

PHYLUM: Platyhelminthes

CLASS: Trematoda

ORDER: Plagiorchiida

FAMILY: Fasciolidae

GENUS: *Fasciola*

SPECIES: *F. nyanzae*

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Declaration of interest

The authors declare no competing interests.

Resources

https://www.iucnredlist.org/species/10103/18567364

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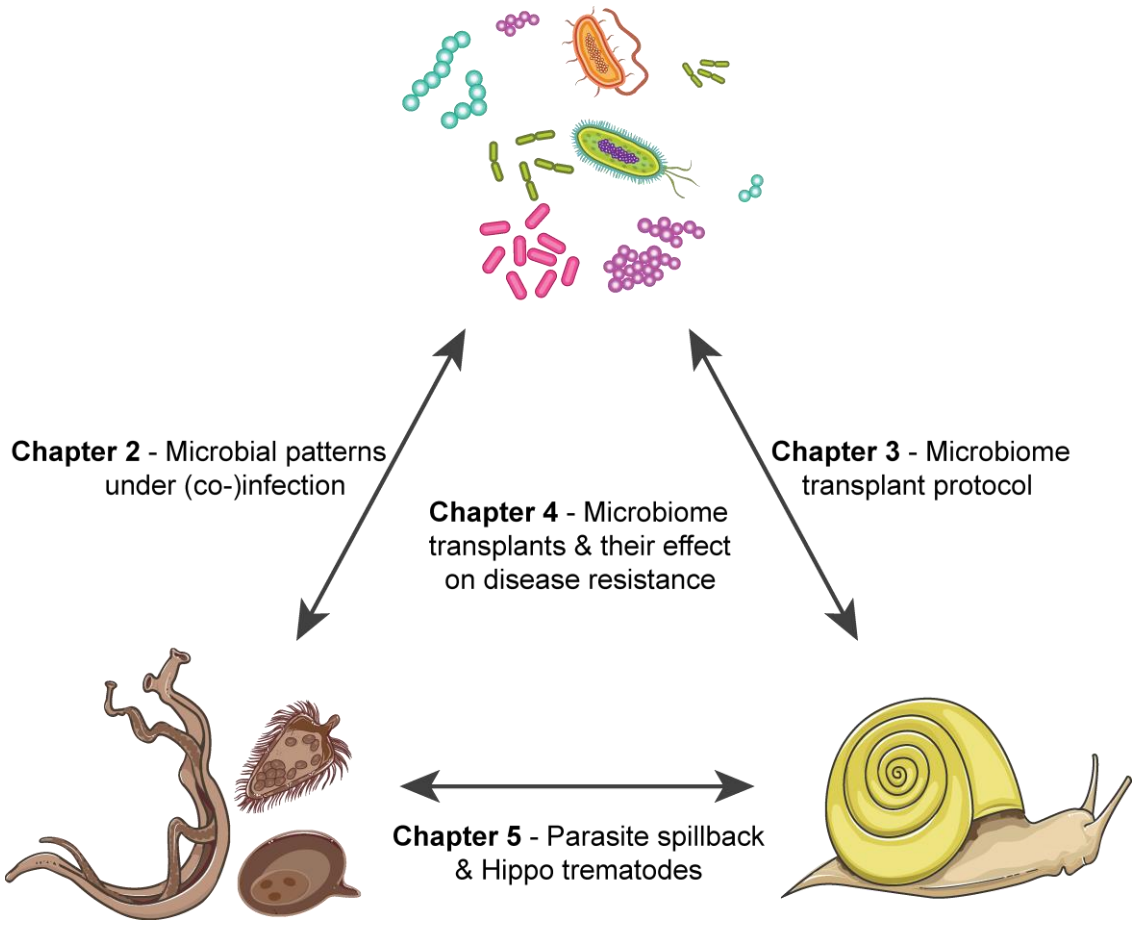
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Chapter 6

General discussion



Overview

Anthropogenic climatic change as well as globalization facilitate the spread of parasites and their hosts (Mora *et al.*, 2022). Consequently, the distribution and epidemiology of important diseases of humans, livestock and wildlife are dramatically changing (e.g., Appleton, 2003; Gubler, 2010; Boissier *et al.*, 2016; Mora *et al.*, 2022). This can be exemplified by snail-borne diseases such as schistosomiasis, a tropical disease that recently became established in southern Europe (Boissier *et al.*, 2016). Lately, the microbiome has been put forward as an important driver of snail host resistance to parasite infection and therefore disease transmission (Portet *et al.*, 2019; Huot *et al.*, 2020; Le Clec'h *et al.*, 2022; Sun *et al.*, 2024). The recent advancements in the control of mosquito borne-diseases *via* modified microbiomes presents hope for microbiome-mediated snail resistance as a sustainable alternative to molluscicides (Wang *et al.*, 2012; Gomes *et al.*, 2017; Pinto *et al.*, 2021; Shi *et al.*, 2023).

The core aim of this thesis was to investigate the probability of microbiome-mediated snail resistance by applying an integrative approach. In doing so, we expanded the limited knowledge of the tripartite interaction between freshwater snails, the bacterial aspect of their microbiome and the trematode parasites they transmit. First, we wanted to build additional correlative data, whereby the snail bacterial profiles were monitored throughout infection development of various parasites (**Chapter 2**). This allowed us to identify putative resistance-inducing bacterial strains or key moments in development whereby bacterial manipulations might be effective. Second, we developed a reliable and reproducible set of manipulations to create germ-free snail specimens and introduce new bacterial communities (**Chapter 3**). This new protocol optimized for schistosomiasis intermediate host snails will be instrumental to subsequent transplant experiments, and can be adapted to other freshwater snail species (**Chapter 4**). Apart from these laboratory-based experiments, we also included field surveys to study the consequences of environmental change and globalization on snail-borne disease transmission. For this part we focused on one of the largest artificial lakes in the world, and could demonstrate how a cascade of biological invasions involving freshwater snails, fueled disease transmission to the common hippopotamus in lake Kariba (**Chapter 5**). The respective snail species introduced from North America appeared to be highly susceptible to the local liver fluke of the hippopotamus. Therefore, in **Chapter 4** we adapted the transplant experiment of **Chapter 3** to test whether the high susceptibility of this invasive snail could be linked to its specific bacterial profile. The results of **Chapter 4**, like **Chapter 2**, suggested that the bacterial aspect of the microbiome is rather immunomodulated by the snail in response to parasite infection than directly related to snail susceptibility or resistance.

In this final chapter, **Chapter 6**, I integrate the findings of each of the previous chapters of this thesis. I discuss their implications and argue why the microbiome remains a valuable research focus to better understand snail-parasite interactions. I start by focusing on the specific case of the invasive snail *Pseudosuccinea columella* (integrating **Chapter 3, 4** and **5**) together with potential future avenues of research with this snail. Then, I examine what happens to the bacterial aspect of the snail microbiome after parasite exposure by integrating **Chapter 2** and **Chapter 4**. Next, I discuss why microbiome-mediated control of trematodes is not a dead end, despite not being linked to trematode resistance in **Chapter 4**. I do so by focusing on aspects of the microbiome beyond the scope of this thesis. Next I explore the practical considerations of mediating snail resistance to trematodes through microbiome manipulations in the field. Then, I step away briefly from the microbiome to explore how invasive snails may threaten the enigmatic hippopotamus as indicated in **Chapter 5**. From this, I built suggestions for research priorities to streamline conservation efforts in relation to invasive snails and trematode parasites. I conclude by discussing the methodological limitations of my PhD research and give ideas for future research avenues to tackle these limitations.

The high susceptibility of an invasive snail – what can the microbiome tell us?

Pseudosuccinea columella can be a highly suitable host for the transmission of *F. hepatica* (Krull, 1933; Dar *et al.*, 2015; Alba *et al.*, 2019)*, F. gigantica* (Grabner *et al.*, 2014) and *F. nyanzae* (Carolus *et al.*, 2019; **Chapter 5**). However, some *P. columella* populations from Cuba are resistant to *F. hepatica* infection (Gutiérrez *et al.*, 2003; Alba *et al.*, 2018, 2020, 2022). Moreover, while susceptible *P. columella* populations in Cuba contribute less to *Fasciola* transmission than the local snail *Galba cubensis* (Vázquez *et al.*, 2014), the introduced populations in Africa play a bigger role in transmission compared to the local African species, displaying unusually high infection rates (Carolus *et al.*, 2019; **Chapter 5**). This suggests an increased susceptibility of the invasive populations. Indeed, sympatric and allopatric combinations of *P. columella* with *F. hepatica* show highly variable compatibility patterns (Vázquez *et al.*, 2019). This could fit with earlier findings where the host-associated microbiome can shape host phenotypic traits and facilitate adaptation to novel environments (Macke *et al.*, 2017a; Henry *et al.*, 2021). On a higher level, if the host is a keystone species, the microbiome can indirectly structure environments acting as an important contribution for ecosystem engineering and eco-evolutionary interactions (Decaestecker *et al.*, 2024). Although not being described as keystone species, freshwater snails are important for aquatic ecosystems (Brönmark, 1989). Moreover, freshwater snails have been shown to acquire novel bacterial members within their invasive range (Bankers *et al.*, 2021). Combined these results suggest that *P. columella* might assimilate a new microbial community within its invasive range, potentially facilitating its invasion and its high transmission of *Fasciola* parasites. Although

Chapter 4 contained specimens from its invasive range in France, it did not contain wild-caught snail specimens, leaving this question unanswered (Alba *et al.,* in prep.). However, our reciprocal transplant experiment argues against this hypothesis since resistance could not be modulated through microbiome transplantation (**Chapter 4**). Nevertheless, the hypothesis that snail adaption to the environment and parasite infection is mediated through the microbiome remains open.

Resistant snails show a consistently increased immune response compared to susceptible snails (Gutiérrez *et al.*, 2003; Alba *et al.*, 2020), yet it does not protect them from infection by echinostomes and *Trichobilharzia* sp., suggesting a parasite-specific response (Alba *et al.*, 2022). Depending on the specificity of this response, transplantations might still induce resistance to *F. gigantica* or *F. nyanzae*. Therefore, future research should first discern if resistance in *P. columella* extends to these two fasciolids. Moreover, future research should investigate if the microbiome can be linked to this increased immune response in resistant snails. Nevertheless, microbial transplants do not always depend on the upregulation of the host immune system (Chrostek *et al.*, 2014; Mazuecos *et al.*, 2023), potentially circumventing the snail's immune response to induce resistance to trematode infection. Moreover, overwhelming evidence exists for *Wolbachia*-reduced transmissibility of pathogens by insects (Moreira *et al.*, 2009; Pinto *et al.*, 2021; Shi *et al.*, 2023), yet occasionally the opposite is found (Hughes *et al.*, 2012; Dodson *et al.*, 2014; Zélé *et al.*, 2014). Moreover, this response appears to be strain-specific (Osborne *et al.*, 2009). Combined with the strain-dependence of compatibility in the snail-trematode system (Webster and Woolhouse, 1998; Lockyer *et al.*, 2012; Nacif-Pimenta *et al.*, 2012), other strain or species combinations might be amendable for microbial transplants.

If anything, the high specificity of resistance holds promise for a sustainable solution within the *One Health* framework, as it would enable the targeted elimination of human pathogens without affecting other parasite fauna and thereby ecosystem functioning. The *One Health* paradigm is especially relevant for trematodiases as they are involved in many biological interactions, across animal kingdoms and environmental conditions (Loker *et al.*, 2022). Although the removal of one parasite can alter the transmission pattern of another (Laidemitt *et al.*, 2019), it can be argued that these effects are more desirable than completely removing all trematodes, snails and other aquatic life through the use of frequently toxic molluscicides (WHO, 1965; Dai *et al.*, 2008). Even so, it will be important to obtain a targeted control effort as parasites can also protect their host from viral infections (Wuerthner *et al.*, 2017). Nevertheless, close monitoring of these systems and a profound understanding of the biology will be vital to detect and intervene at an early stage of a trematode outbreak.

The effect of trematode infection on the snail-associated microbiome

Earlier works report the effect of pathogen or parasite infections on the host-associated microbiome (Jani *et al.*, 2021; Rajarajan *et al.*, 2022). Jani *et al.* (2021) revealed that the skin bacterial profile of frogs shifted after fungal infection, while Rajarajan *et al.* (2022) showed that the gut bacteria of *Daphnia longispina* changed after infection with an eukaryotic gut parasite. Likewise, trematodes consistently drove changes in the bacterial aspect of the snail microbiome, although some more than others and differently throughout bacterial metrics (**Chapter 2** and **Chapter 4**). For example, *S. mansoni* strains were more likely to drive bacterial dysbiosis compared to *S. rodhaini* in *B. glabrata,* while in co-exposure *S. rodhaini* managed to stabilize the effect of a dominant *S. mansoni* strain on the bacterial profile (**Chapter 2**). Most importantly, we revealed that these shifts correlate with key moments in the intramolluscan development of the parasite (**Chapter 2**). In **Chapter 4** we hint to alternative stable states or the checkerboard pattern potentially explaining the observed clusters in our bacterial dataset. Similarly, *Batrachochytrium dendrobatidis* infection can lead to alternative stable states in the skin bacterial communities of frogs (Jani *et al.*, 2021). It appears to be a rather ubiquitous pattern as nematode infections in both terrestrial slugs and one of our used model organisms, *B. glabrata,* also show a shift in the bacterial aspect of their microbiome (Osório *et al.*, 2020). Whether this bacterial shift occurs due to the snail's immune response, the parasite multiplying and damaging host tissue, or an interplay between the snail and parasite, remains to be investigated. Future experiments could compare signals in the microbiome across artificial (Ittiprasert and Knight, 2012; Pila *et al.*, 2016) or parasite-induced (Núñez *et al.*, 1994; De Jong-Brink *et al.*, 2001) snail immune responses. These experiments supplemented by histopathological examination of infected snails, which also focus on tissue-specific microbiota, should reveal what drives the observed bacterial shifts reported throughout this thesis.

Indeed, bacterial shifts occur during mechanical damage and biochemical changes at the onset of shedding, and at the onset of migration to the gonad-digestive gland region (**Chapter 2**), suggesting the importance of tissue damage in this pattern. However, Allan *et al.* (2018) linked the presence of a genomic region that confers schistosome resistance, to the presence of different bacterial species in or on snails, which suggests that the snail immune response might also be a major driver. Trematodes can modulate the snail's hemocytes to avoid destruction (Núñez *et al.*, 1994; De Jong-Brink *et al.*, 2001), which affects its capacity for bacterial clearance, providing a parasite-induced effect on the host's immunomodulation of the associated microbiome (Núñez *et al.*, 1994). Across host-parasite systems, parasites have been shown to modulate the epigenome of their parasitized host (McMaster *et al.*, 2016). Specifically, *S. mansoni* subverts the stress response of *B. glabrata* by modulating the epigenome to ensure successful infection development (Knight *et al.*, 2016; Geyer *et al.*,

2017). The microbiome is being put forward as potentially shaping a host's phenotype through epigenetic regulation by, for example, influencing the availability of chemical building blocks involved in epigenetic regulation, affecting host-associated processes directly involved in epigenetic pathways, or influencing the expression or activity of epigenetic regulatory enzymes (Chernov *et al.*, 2015; Woo and Alenghat, 2022). Combined, the potential interaction between the microbiome and the parasite-mediated epigenetic modulation of the snail showcases the importance of epigenetic studies in trematode resistance. In conclusion, all the abovementioned factors might be interacting and driving the observed patterns in the snail bacterial profiles.

Microbiome-mediated control of trematodes – a dead end?

In **Chapter 2** we showed the possibility of transplanting the bacterial aspect of the microbiome in and between freshwater snails. Indeed, from earlier works, it became apparent that it should be feasible due to the plasticity of the snail associated bacteria (Chernin, 1957, 1960; Ducklow *et al.*, 1981). Chernin (1957), like us, was able to obtain high proportions of germ-free snails by treating the external egg surface. This suggests that the parental snail does not, or at least not consistently, transmit bacteria vertically within the egg (Ducklow *et al.*, 1981). This conflicts with the argument of consistent vertical transmission made by Lin *et al.* (2023). Nevertheless, pseudo-vertical transmission (Alberdi *et al.*, 2016) can still play a major role in the snail microbiome as the gelatinous matrix of the egg mass contains a significant microbial community (own observations). Indeed, other studies have shown that snails contain some overlapping bacterial species between their invasive range and their native region (Bankers *et al.*, 2021). For now, it remains unknown whether the bacteria co-invaded with the snails out of the native region, favoring the importance of (pseudo-)vertical transmission, or whether these bacteria are consistently accrued form the environment (Bankers *et al.*, 2021). Either way, this reveals a readily amendable microbiome that is key for microbial transplants to work.

Temperature changes can modulate snail resistance to trematode infections (Ittiprasert and Knight, 2012) and have been shown to affect the efficiency of symbiont-mediated protection, e.g. from viral infections in an insect host (Chrostek *et al.*, 2021). Before exposure, the snail immune response can be artificially modulated by injecting a granulin growth factor, which induces hemocyte proliferation, making snails resistant to parasite infection (Pila *et al.*, 2016). During infection development, the reproductive capacity of the parasite can be impaired by introducing *Nosema algerae*, a microsporidium isolated from mosquitoes, which infects the parasite sporocysts and effectively reduce the eventual shedding load of *B. glabrata* (Lai and Canning, 1980). These examples underline the possibility of bioengineering resistance (Le Clec'h *et al.*, 2022; Sun *et al.*, 2024). However, transplanting the microbiome from resistant

snails to susceptible snails does not confer resistance to trematode infection (**Chapter 4**). Supporting this finding, an ichtyosporidean pathogenic to *Schistosoma mansoni* occurred more in resistant than susceptible snails yet was not involved in conferring that resistance (Hertel *et al.*, 2004). These results might be interpreted as a dead end for microbiome-mediated trematode control. However, some host phenotypes are not driven by the microbiome nor do all microbes shape host phenotypes (Henry *et al.*, 2021). Moreover, host-microbiome interactions can be genotype specific (Macke *et al.*, 2017b; Decaestecker *et al.*, 2024). This suggests that we might have excluded host genotype-microbiome combinations which would have shown a phenotypic response. A case can be made that recent works have only scratched the surface of the snail-trematode-microbiome interaction. Snails have close associations with a complex set of symbiotic bacteria, fungi, ichtyosporideans, microsporidia, protozoa, nematodes, Oligochaeta, viruses and rotifers (Fashuyi and Williams, 1977; Hertel *et al.*, 2004; Galinier *et al.*, 2017; Clerissi *et al.*, 2023), each offering avenues for snail control (Duval *et al.*, 2015) or reducing parasite load (Lai and Canning, 1980). Our work focused on an amplicon-based approach of the bacterial aspect of the complex microbiome. It paves the way for more in-depth techniques as well as various other aspects of the microbiome whereby bacteria could still be critical for the complex interactions surrounding trematode resistance.

All these potential microbiome-mediated control measures to increase snail resistance still hinge on the fact that some level of parent offspring transmission occurs, while ideally providing a fitness benefit so that it spreads naturally throughout snail populations. Given the incomplete inheritance (**Chapter 3**) this might become problematic. However, since snails even in different continents accrue similar bacteria (Bankers *et al.*, 2021), there is indeed hope that it is possible to get (pseudo-)vertical transmission when a trematode resistance-conferring microbe is found. Alternatively, snail-resistance could be achieved if resistance-inducing strains enter the environment and hosts accrue these environmental bacteria in their microbiome (Decaestecker *et al.*, 2024). This approach holds promise, since snails have a modifiable microbiome (see earlier). However, snails raised in a common garden showed a phylosymbiosis pattern with their bacterial profiles, indicating that they will not accrue just any bacterial community (Huot *et al.*, 2020). This specificity is an important factor to consider for future applications of microbiome manipulations.

More attempts are to be made on the reciprocal transplant experiment, focusing now also on pre-exposed microbiome transfers. Hosts previously exposed to pathogens have a microbiome that shows enhanced resistance to infection (Stacy *et al.*, 2021). Transplanting this microbiome to unexposed hosts subsequently increases their tolerance (Macke *et al.*, 2017a). Indeed, a

significant shift is noted after parasite exposure (**Chapter 2** and **Chapter 4**), suggesting the potential of pre-exposed microbiota to confer resistance.

If eventually resistance-inducing bacteria do not exist within the snail-trematode system under study, we might still find them in other systems. As mentioned earlier, microsporidia isolated from mosquitoes can reduce parasite load in snails (Lai and Canning, 1980). Similarly, *Wolbachia* from *Drosophila melanogaster* has been introduced in *Aedes Aegypti* to reduce dengue transmission (Hoffmann *et al.*, 2011). These examples reveal the value and future interest in the inter-species exchange of microbial components.

Alternatively, the microbiome can be engineered to confer resistance (Mueller and Sachs, 2015). Bacteria can have intrinsic anti-tumor activity or stimulate the host's immune response to tumor cells, providing an important treatment option to certain cancer types (Zhou *et al.*, 2018). Bioengineering these bacteria to improve safety, increase tumor targeting, and heighten effector molecule production should improve the applicability of this therapeutic approach (Zhou *et al.*, 2018). The microbiome is intimately linked to the host immune system through the production of outer membrane vesicles, antigens and metabolites (Brestoff and Artis, 2013; Kaparakis-Liaskos and Ferrero, 2015; Rooks and Garrett, 2016). Therefore, the resistance obtained through hemocyte boosting with granulin injections (Pila *et al.*, 2016) might be achievable through microbiome manipulations. Indeed, bacterial and viral pathogens can readily modulate host immune responses (Finlay and McFadden, 2006), and endosymbionts can manipulate host gene expression affecting disease resistance (Hussain *et al.*, 2011). These examples suggest that we could apply this microbiome-modulation to stimulate immune responses as discussed for outer membrane vesicles (Kaparakis-Liaskos and Ferrero, 2015).

In contrast to the bacterial aspect of the snail microbiome, it appears that the trematode bacterial profiles have some core members that can be transmitted across and between life stages (Fischer *et al.*, 2017; Jorge *et al.*, 2020). Therefore, future research could focus on the microbiome of the disease agent itself, rather than that of the intermediate host. Indeed, microbial symbionts can be critical for pathogen survival (Taylor *et al.*, 2005), while also determining disease severity (Ives *et al.*, 2011). We might be able to find and target a vital symbiont of trematodes, similarly to how antibiotics kill the *Wolbachia* symbiont and consequently the filarial nematode within the human body (Taylor *et al.*, 2010; Landmann *et al.*, 2011). It is promising that certain bacteria are transmitted across generations and life cycle stages (Jorge *et al.*, 2020). Moreover, antibiotic treatment changes the ratio of soldier to reproductive stages within a snail host, suggesting a potentially important role of bacteria in the lifecycle biology of trematodes (Jorge *et al.*, 2022). However, another study found only a

weak correlative link between bacteria and alternative lifecycles within a trematode species (Salloum *et al.*, 2023). Either way, this aspect of the snail-trematode-microbiome interaction appears promising and should be further investigated.

Miracidia respond to chemical cues released in the water by compatible freshwater snail hosts while ignoring those of incompatible sympatric snail species (Kalbe *et al.*, 1997; Hertel *et al.*, 2006; Allan *et al.*, 2009). Consequently, miracidia transmission success is increased markedly (Hertel *et al.*, 2006). However, transmission frequently does not depend on miracidia failing to locate the intermediate snail host (Gutiérrez *et al.*, 2003). Nevertheless, there might be value in making decoy snails attractive or compatible snails unattractive to miracidia by manipulating the cues their release (discussed in Loker *et al.*, 2022). Perhaps microbial organisms can be applied to achieve this (Archie and Theis, 2011). Indeed, bacteria associated with caterpillars release attractant molecules, aiding their localization by hyperparasitoid wasps (Bourne *et al.*, 2023). Moreover, this attraction was successfully induced artificially (Bourne *et al.*, 2023), revealing the potential of this approach. Similarly, the human skin microbiome determines our attractiveness to malaria-transmitting mosquitoes (Verhulst *et al.*, 2011).

This reveals another target for microbiome-mediated control of trematodiases – hiding the final host. Indeed, cercariae require certain chemical compounds, in combination with other stimulants like temperature and water turbulence, to attach to their final host (Haas *et al.*, 2002). Notably, the drivers of this response can be constant across taxonomic levels yet also variable, even within genera (Haas *et al.*, 2002; Horák *et al.*, 2015). Nevertheless, it is a promising avenue since an adapted DEET formula applied to the skin has been shown to prevent cercarial penetration (Salafsky *et al.*, 1999). However, this effect is most likely induced by preventing penetration or killing cercariae on the skin (Salafsky *et al.*, 1999). Similarly, symbiotic rotifers living in close association with *B. glabrata* have been shown to produce a chemical capable of paralyzing schistosome cercariae (Gao *et al.*, 2019).

As discussed in **Chapter 2**, targeting key development moments within the snail might provide an interesting alternative to resistance at the initial infection stage. We will not go into that aspect here as it has been extensively discussed in **Chapter 2**. Alternatively, we might not focus on making susceptible snails more resistant but on making resistant snails better competitors. Typically, resistance and competitive ability are at a trade-off (Wolinska and King, 2009), yet the microbiome can alter the mean and variation of phenotypic traits (Henry *et al.*, 2021). As a result the microbiome members and their interactions have been critical in shaping host fitness and lifespan (Gould *et al.*, 2018). As organisms age, the immune system and microbiome change composition and function, increasing susceptibility to pathogens (Bosco

and Noti, 2021). Moreover, transplanting the microbiome of old mice to young ones reduces their lifespan (Lee *et al.*, 2020). The opposite is also true whereby microbial transplants can reverse symptoms of aging (Parker *et al.*, 2022; Meng *et al.*, 2023). Perhaps this can lay the basis for increased fitness avenues for resistant snail genotypes. If the microbiome eventually does not induce resistance, it might still be valuable as control efforts to target and kill invasive snails (Lin *et al.*, 2023). Nevertheless, extrapolating such interventions to the field can be complex and is not without risk (Greyson-Gaito *et al.*, 2020), warranting a deeper understanding of the snail microbiome before any field interventions are considered.

Microbiome manipulations in the field – things to consider

Critical insights have come from controlled experiments investigating the importance of the microbiome in host biology (Greyson-Gaito *et al.*, 2020). The isolation of microbiome-mediated effects from other factors, such as environment and host physiology, has been critical in eliminating potentially confounding factors and discern a true microbiome-driven effect. Consequentially, the ecological relevance of many of these experiments has remained limited (Greyson-Gaito *et al.*, 2020). Therefore, putative resistance-inducing bacteria should be tested in different ecological and host physiological contexts. However, these experiments should first occur in controlled settings as a stepping-stone to field manipulations since biological control can have unexpected impacts on an ecosystem (Howarth, 1991; Appleton *et al.*, 2009; Wong *et al.*, 2009; Ip *et al.*, 2014) and incomplete resistance may lead to increased pathogen virulence (Gandon *et al.*, 2001; Fleming-Davies *et al.*, 2018). Nevertheless, we can take inspiration and caution from, for example, aquaculture, agriculture and mosquito-borne disease control efforts once these experiments are on the radar.

The agricultural sector has been leveraging microorganisms in the field for decades by treating seeds, plants and soils with microorganisms for nitrogen fixation, killing pest insects, and increasing stress-tolerance (French *et al.*, 2021). Similarly, incorporating prebiotic or probiotic treatment of the environment or food could drive snail-microbiome associations, as it does for the microbiome of fish in aquaculture (Denev *et al.*, 2009; Foo *et al.*, 2017). Its efficacy could be augmented by customized microbiome manipulations tailored to the pond or snail population, similar to the field or crop specific microbiome management in agricultural systems (Sergaki *et al.*, 2018; French *et al.*, 2021). However, the immense amount of environmental bacteria may interfere with or dilute the desired effect of inocula (Čaušević *et al.*, 2024), suggesting that directly manipulating (pseudo-)vertically-inherited endosymbionts of snails remains a preferred mode of action. Indeed, mosquito-borne diseases have been successfully reduced by placing buckets with manipulated eggs in the desired region (Anders *et al.*, 2018) to release endosymbiont-manipulated mosquitoes in the environment (Pinto *et al.*, 2021;

Ogunlade *et al.*, 2023). The simplicity of this release method contributes to the high cost-efficiency of this control measure against mosquito-borne diseases (Zimmermann *et al.*, 2024).

For vector symbiont-manipulated control measures to work and be cost-efficient, replacement of wildtype vectors by resistant vectors should be feasible (Pinto *et al.*, 2021; Zimmermann *et al.*, 2024). Population replacement or persistence of the introduced vectors requires an augmented or at least comparable fitness relative to the native vectors (Manoj *et al.*, 2021; Pinto *et al.*, 2021). Resistance-competition trade-offs might make this difficult (Wolinska and King, 2009), yet has been shown to be surmountable for mosquitoes (Pinto *et al.*, 2021). Trematode infections impose a severe fitness cost on their snail host (King *et al.*, 2011; Faro *et al.*, 2013; Tavalire *et al.*, 2016; Paczesniak *et al.*, 2019), theoretically boosting the fitness of resistant snails. However, in the wild, other factors are at play since resistant snails rarely dominate local populations (Webster *et al.*, 2003; Alba *et al.*, 2019, 2022). Additionally, parasites and snails are in a constant evolutionary arms race (Jokela *et al.*, 2009). Combined with the strong selective pressure of releasing resistant snails, parasites might adapt and circumvent the introduced resistance factor. Therefore, continuous work on discovering or developing additional resistance-inducing microbiota will be critical. Nevertheless, combined with integrated control measures involving improved sanitation and drug treatment, resistance-inducing microbiota might contribute to interrupting the transmission of detrimental trematode parasites.

How one snail can threaten the enigmatic hippopotamus

Large herbivores like *Hippopotamus amphibius,* the common hippopotamus, have a disproportionately large effect on the ecosystems they inhabit (Stears *et al.*, 2018; Ripple *et al.*, 2023). In contrast, parasites can drastically alter the impact an organism has on its environment (Collinge *et al.*, 2008; Smith *et al.*, 2009; Tompkins *et al.*, 2011). Despite the importance of large herbivores and the impact of parasitic infections, the knowledge of parasites of large herbivores remains fragmentary (Polley, 2005; Smith *et al.*, 2009; Schols *et al.*, 2020). Moreover, in the Anthropocene, habitat change, pollution, over-exploitation, climate change, and invasive species can all exacerbate the impact of diseases by directly altering parasite abundance and distribution (Lafferty and Kuris, 2005) or indirectly by reducing host population size and genetic diversity (Heard *et al.*, 2013; Budria and Candolin, 2014).

Combined with earlier work, we here showed that human alteration to the environment has led to a surprising cascade of biological invasions, inducing parasite spillback and thereby threatening hippopotamus conservation efforts (Carolus *et al.*, 2019; **Chapter 5**). A key aspect of this parasite spillback is the unusually high susceptibility of the intermediate snail host *P.*

columella to *F. nyanzae*. Moreover, as this snail continues to invade new areas, animals and humans across Africa are at increased risk of fasciolosis (Grabner *et al.*, 2014; Lounnas *et al.*, 2017; **Chapter 5**). One solution might be harvesting the invasive water hyacinth, which is the main substrate for *P. columella* in lake Kariba, for composting and agricultural fertilizer (Rohr *et al.*, 2023), controlling both invasive snails and their substrate. Especially since natural predators preferentially feed on native snails compared to *P. columella* (Appleton *et al.*, 2004). However, the vastness of the lake and broad distribution throughout Africa of both the plant and snail will undoubtedly complicate such an effort.

The limited knowledge of the biology of *F. nyanzae* confounds impact estimates of the increased transmission through *P. columella* on populations of the hippopotamus (**Chapter 5**). Infections by helminths can sometimes protect a host from subsequent infections (Karvonen *et al.*, 2004; Abruzzi and Fried, 2011; Rellstab *et al.*, 2013; Toledo and Fried, 2014). Indeed, up to a dozen liver flukes but not more can be retrieved from a single hippopotamus (reviewed in **Chapter 5**), suggesting some protective effect against subsequent infections. However, most of the time subsequent infections are worsened (Abruzzi and Fried, 2011; Louhi *et al.*, 2015). Although unknown for *F. nyanzae, F. gigantica* infection load can get problematic (Bindernagel, 1972 & own observations). As reports continue with unexplained mortalities of giraffes, buffaloes and eland antelopes across Zimbabwe, all occurring near artificial lakes where we confirmed the presence of *P. columella*, it raises suspicion of an unnoticed and ongoing epidemic threatening wildlife conservation efforts across the country and beyond. Therefore, it will be imperative for future projects to focus on mapping the spread of invasive snails, their relative contribution to disease transmission, inventorying parasite biodiversity and quantifying the impact of these diseases on animal health for effective and sustainable wildlife conservation efforts (Kelly *et al.*, 2009; Thompson *et al.*, 2010; Mudavanhu *et al.*, 2024).

An urgent need to increase monitoring efforts of trematode parasites and invasive snails, while also improving biological knowledge exists. It can be argued that this knowledge gap can only be filled through an integrative approach involving wildlife managers, hunters, citizen scientists, parasitologists, ecologists, and veterinarians. Increased financial investments and more experts would help considerably (Rodman and Cody, 2003; Vinarski, 2020). However, supplementary avenues can be explored to assess the impact of biological invasions on conservation efforts. First, citizen scientists can help expand the research area under study in addition to the sampling frequency (Ashepet *et al.*, 2021; Tumusiime *et al.*, 2024), which is vital to monitor the spread of invasive snails and to obtain temporal patterns. Second, the rise of approaches leveraging environmental DNA (eDNA) provides a rapid and efficient alternative to the standard and time-consuming sampling protocols to confirm the presence or absence

of a species (Sengupta *et al.*, 2019; Douchet *et al.*, 2022). Third, the clinical assessment of veterinarians will be critical to quantify the impact of trematodiases on individual animals (Smith *et al.*, 2009). Fourth, opportunistic sampling, as in **Chapter 5**, provides unique insights into the diseases affecting threatened wildlife species. However, it is not an easy feat and requires an intimate network with mutual trust between scientists, hunters and wildlife managers. Finally, close monitoring of wildlife populations will be vital to assess the effect at population level, especially to detect more subtle patterns in populations that do not involve the (near) complete local extinction of a species. This is, however, not an easy task as it requires dedicated and knowledgeable personnel capable of monitoring the wildlife populations in such detail to allow this analysis.

Notably, parasites can also support healthy and diverse ecosystem functioning, driving genetic and phenotypic diversity (Gómez and Nichols, 2013; Stringer and Linklater, 2014; Wood and Vanhove, 2023). Consequently, the realization that parasite conservation holds value is gaining traction (Carlson *et al.*, 2020; Kwak *et al.*, 2020). Trematode parasites, like those of the common hippopotamus, stand out, as roughly 90% of the species still await discovery and formal description (Hechinger, 2023). The unknown stomach fluke, transmitted by the exotic *Radix* aff. *plicatula*, from **Chapter 5** could be one of those undescribed species, as we were unsuccessful at identifying it despite an integrative taxonomic approach involving lifecycle information, morphology, and genetics (Will *et al.*, 2005; Schols *et al.*, 2020). Currently, the golden standard for stomach fluke identification relies on median sagittal sections and Scanning Electron Microscopy (SEM) imaging, both destructive approaches (Sey, 1991). Therefore, non-destructive identification methods will be imperative to leverage natural history collections and obtain historical perspectives. Micro-computed tomography (µCT), is a non-destructive 3D imaging technique that could offer this solution without altering the physical properties of a specimen (Faulwetter *et al.*, 2013). Moreover, because of the three-dimensional information, new diagnostic characteristics are frequently found (Ziegler *et al.*, 2010; Zimmermann *et al.*, 2011; Faulwetter *et al.*, 2013), which makes it especially valuable for stomach fluke taxonomy due to the complexity of species delimiting traits (Schols *et al.,* in prep.). Moreover, only a fraction of the described stomach fluke species have a genetic reference sequence available online, making the barcoding void especially pronounced for stomach flukes (Blasco-Costa *et al.*, 2016; Schols *et al.*, 2020). Therefore, parasite-parasite interactions within snails are undoubtedly going unnoticed since stomach flukes frequently interact with other trematode parasites in snail hosts, driving their transmission (Lim and Heyneman, 1972; Laidemitt *et al.*, 2019). The lack of knowledge of trematode biology, therefore, hinders biodiversity assessments, ecological studies, effective disease control and conservation efforts.

Future prospects

The lack of putative resistance-inducing bacteria throughout this thesis might have originated from methodological limitations that can be tackled in future experiments. Although bacterial community analysis provides invaluable insights, it fails to unfold the mechanisms behind the host-microbiome interaction. The diversity analysis applied throughout this thesis sets the stage for investigating the bacterial functionality through a metagenomics approach. In combination, metabarcoding and metagenomics enable us to address pressing challenges in understanding the snail-microbiome-trematode interaction (Arribas *et al.*, 2016; Knight *et al.*, 2018). Sequencing whole bacterial genomes should provide these much-needed high-resolution functional insights (Knight *et al.*, 2018).

Analysing DNA extracts removes the 3D-world organisms live and interact in (Jahn *et al.*, 2016), which turns out to be critical given the limited diffusion distance of microbial metabolites (on average 15 µm) (van Tatenhove-Pel *et al.*, 2021). As a result, we undoubtedly investigated vast numbers of bacteria irrelevant to the role of resistance, which increased the noise and potentially confounded true signals in our dataset. Similar to Jahn *et al.* (2016) a tool capable of obtaining high-resolution *in situ* visualization of bacterial species will be groundbreaking. Not only will it enable us to study microbial ecology within the snail host, but it will also allow us to identify which bacterial species might be near the trematode parasite during key development stages. Specifically, it could be used to identify bacteria that are intimately linked to hemocytes during the first 24 hours of infection, a period critical for infection establishment (De Jong-Brink *et al.*, 2001). These hemocyte-associated bacteria could then be further investigated to identify putative hemocyte-modulating pathways. The high-resolution *in situ* visualization could also identify where exactly in the snail the observed microbial shifts, in response to trematode infection, occur as reported throughout this thesis. One such tool is currently in the pipeline but it depends on the full characterization of the host's bacterial community by sequencing the entire 16S and 23S rRNA genes (Martin Jahn pers. comm.), a technique that only recently became feasible (Johnson *et al.*, 2019). Nevertheless, it holds great promise and its inclusion in future experiments will undoubtedly contribute to a profound understanding of the snail-trematode-microbiome interaction.

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Appendix



Appendix – Chapter 2

Schistosome species, onset of shedding, and co-infection dynamics determine bacterial profiles in the snail *Biomphalaria glabrata*

Table 1: Sample counts per experimental condition. Sub-experiment refers to the sub-experiment shown in Figure 1. Parasite exposure refers to the miracidia of which parasite the snails were exposed. Days post exposure refers to the number of days between miracidia exposure and sample collection. Infection status refers to the infection status determined by the infection RD-PCR of Schols *et al.* [1] with reduced cycle numbers as mentioned in Hammoud *et al.* [2] and the metabarcoding infection output from Hammoud *et al.* [2]. Sample count lists the number of snail samples that meet each of the previously listed conditions and for which the bacterial communities were characterized using 16S rRNA gene metabarcoding. Counts marked with an asterisk were included in the first run, samples with a '°' were included in the second run.

SUB-EXPERIMENT	PARASITE EXPOSURE	DAYS POST EXPOSURE	INFECTION STATUS	SAMPLE COUNT
TEMPORAL SIGNAL MONO-MIRACIDIAL INFECTION - FIGURE 1.B	*S. mansoni* Bre	2	Infected	0
			Uninfected	5*
		6	Infected	4*
			Uninfected	5*
		10	Infected	5*
			Uninfected	5*
		16	Infected	5*
			Uninfected	5*
		20	Infected	5*
			Uninfected	5*
		30	Infected	5*
			Uninfected	5*
		40	Infected	2*
			Uninfected	5*
10-MIRACIDIAL INFECTIONS - FIGURE 1.C	*S. mansoni* Bre	40	Infected	5*+19°
			Uninfected	2*
	S. mansoni LE	40	Infected	5*+19°
			Uninfected	2*
	S. rodhaini	40	Infected	5*+18°
			Uninfected	4*
CO-INFECTION 10 MIRACIDIA EACH S. *MANSONI* STRAINS – FIGURE 1.D	*S. mansoni* Bre + *S. mansoni* LE	2	*S. mansoni* Bre	0
			S. mansoni LE	0
			S. mansoni Bre + *S. mansoni* LE	0
			uninfected	5*
		10	*S. mansoni* Bre	5*
			S. mansoni LE	5*
			S. mansoni Bre + *S. mansoni* LE	5*
			uninfected	1*

		40	*S. mansoni* Bre *S. mansoni* LE *S. mansoni* Bre + *S. mansoni* LE uninfected	4* 5* 5* 1*
CO-INFECTION 10 MIRACIDIA EACH S. *MANSONI* AND *S. RODHAINI* – FIGURE 1.E	*S. mansoni* LE + *S. rodhaini*	2	*S. rodhaini* *S. mansoni* LE *S. rodhaini* + *S. mansoni* LE uninfected	3* 0 0 5*
		10	*S. rodhaini* *S. mansoni* LE *S. rodhaini* + *S. mansoni* LE uninfected	0 5* 3* 0
		40	*S. rodhaini* *S. mansoni* LE *S. rodhaini* + *S. mansoni* LE uninfected	1* 5* 2* 2*
SNAILS NEVER EXPOSED TO MIRACIDIA – NAÏVE SNAILS	NA NA	0 40	NA NA	5* 5*

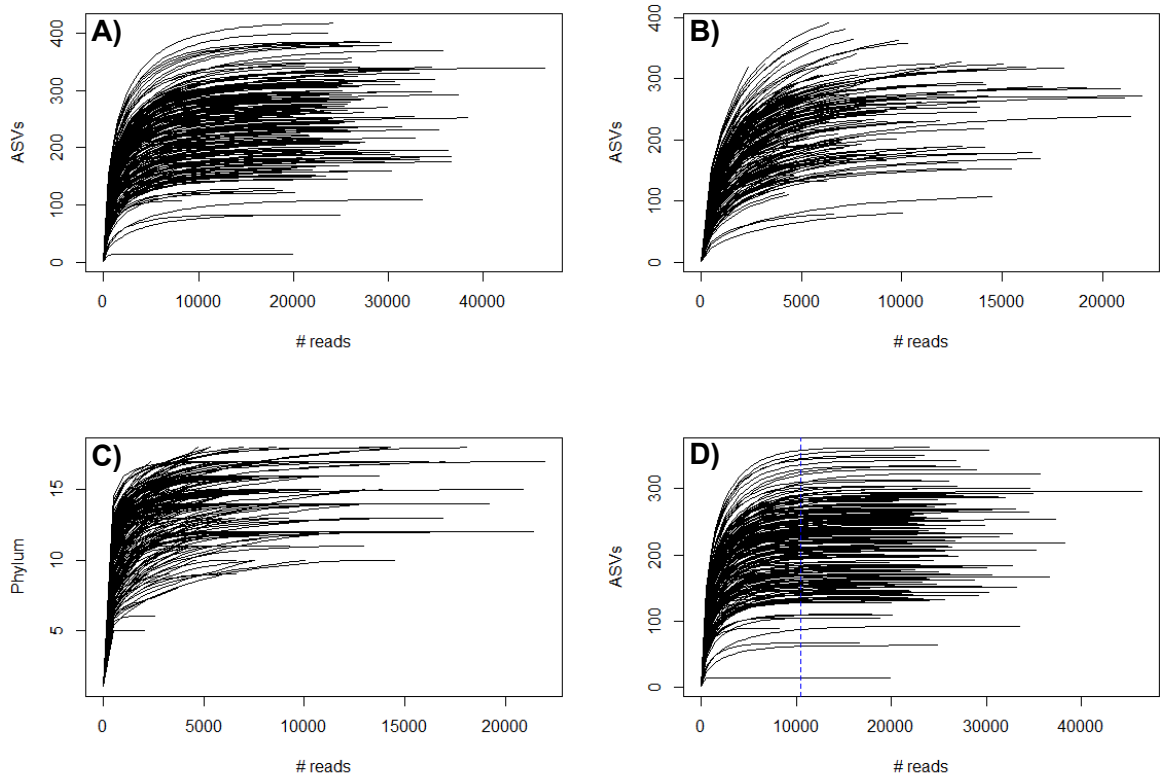


Figure 1: Rarefaction curves of the various datasets investigated for suitability. The subsequent analysis were done with the dataset associated with rarefaction curve D. A) Unedited dataset, B) qPCR correction ASV level, C) qPCR correction phylum level, D) Tripleton removed.

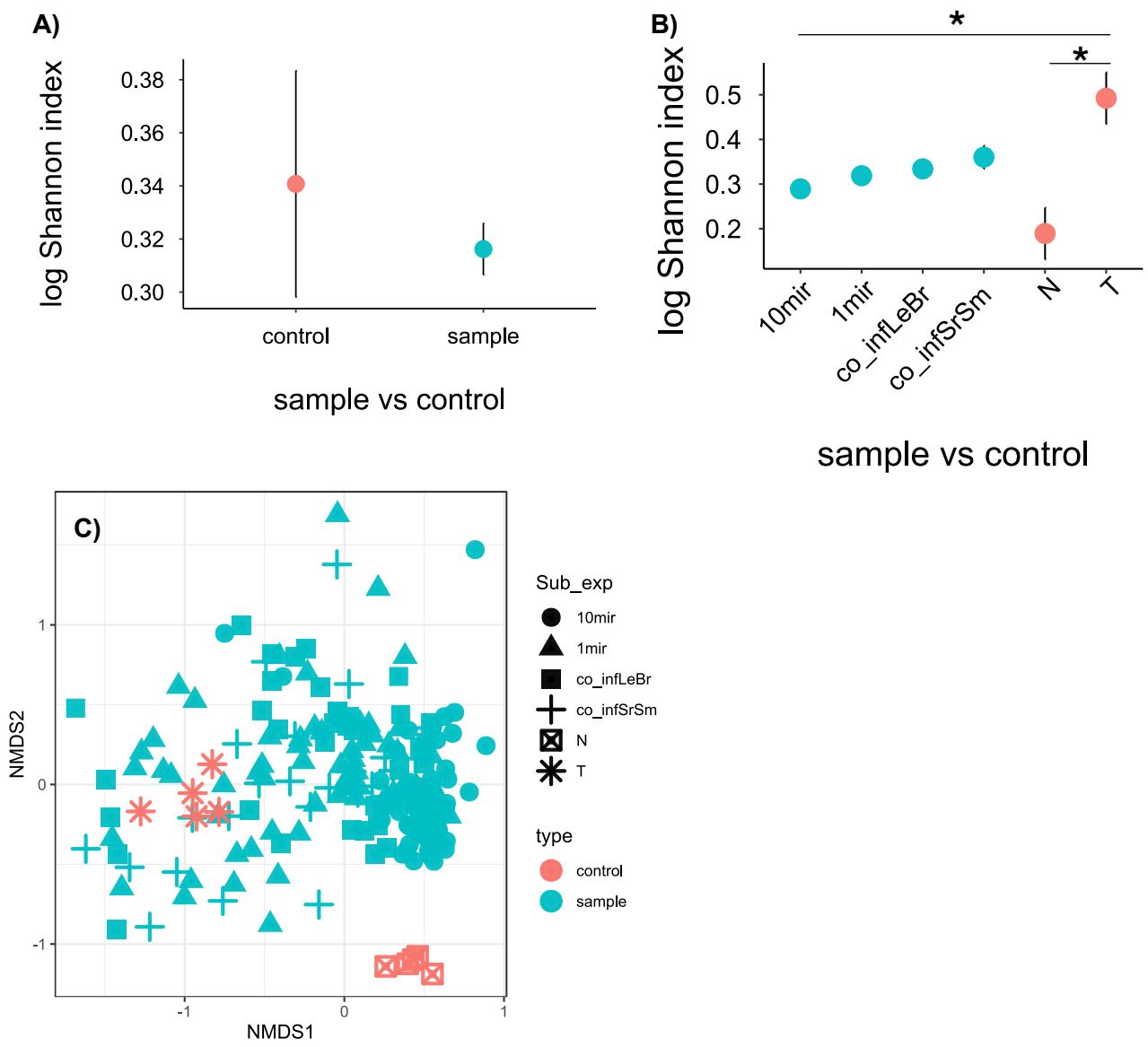


Figure 2: Comparing miracidia exposed snail samples to naïve snails. A) log-transformed Shannon diversity metric comparing unexposed (naïve) to exposed (sample) snails. B) log-transformed Shannon diversity metric comparing the various sub experiments (see below). C) NMDS analysis of the Bray-Curtis dissimilarity measure across the entire dataset of parasite exposed (sample) and unexposed (naïve) snails. Sub-experiment is indicated by different shapes: '10mir' -Figure 1C – Exposure to 10 miracidia of either SmBRE, SmLE, or Sr; '1mir' – Figure 1B -exposure to a single miracidium of SmBRE; 'co_infLeBr' – Figure 1D – co-exposure to 10 miracidia of both SmLe and SmBRE; 'co_infSrSm' – Figure 1E – co-exposure to 10 miracidia of both SmLe and Sr; 'N' – unexposed snails collected at the start of the experiment day 0; 'T' – unexposed snails collected at the end of the experiment day 40.

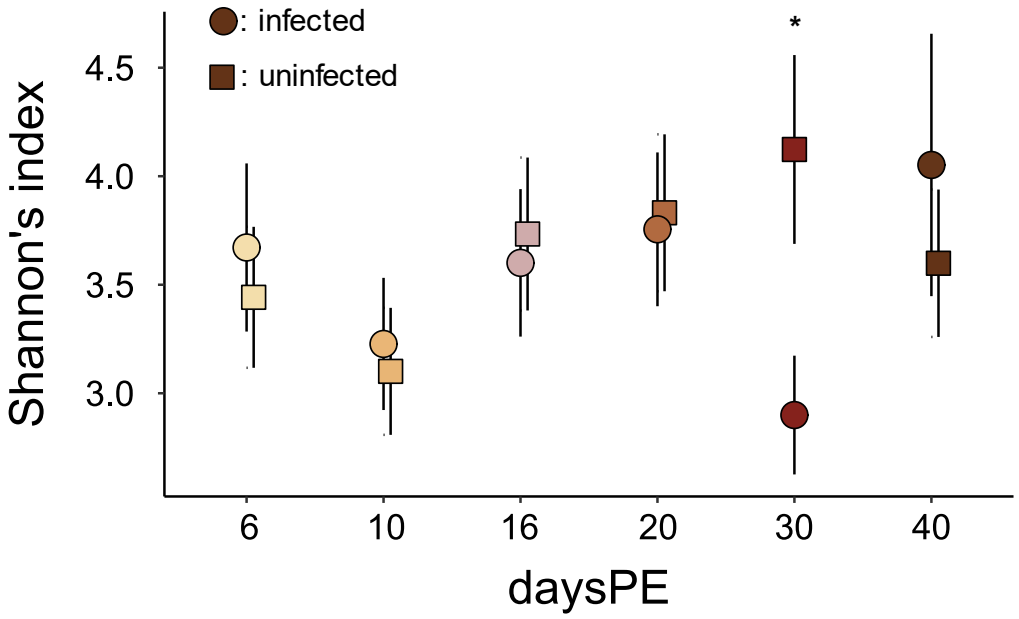


Figure 3: The temporal pattern throughout schistosome infection development. Shannon diversity metric for infected vs uninfected samples across various days post miracidium exposure (daysPE). Circles indicate infected samples, squares uninfected samples. The asterisk indicates the significant pairwise difference between infected and uninfected samples at 30 daysPE.

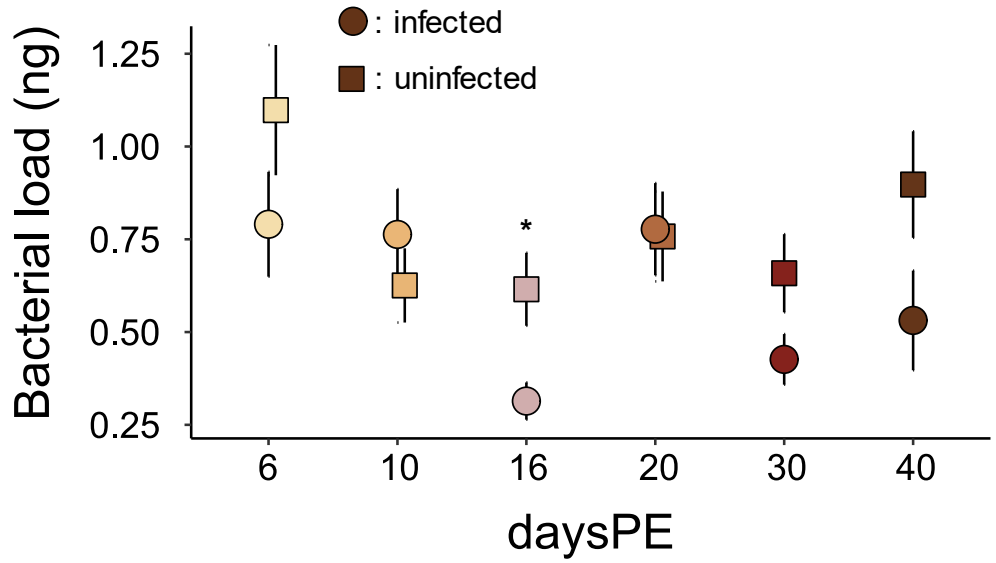


Figure 4: Bacterial load (ng) as determined through qPCR measurements. This quantity of DNA was used as PCR-input from the 10 ng of total DNA used. Total DNA encompasses snail, parasite, bacterial, and other organisms' DNA. Circles indicate infected samples, squares uninfected samples. The asterisk indicates the significant pairwise differences. DaysPE = days post exposure.

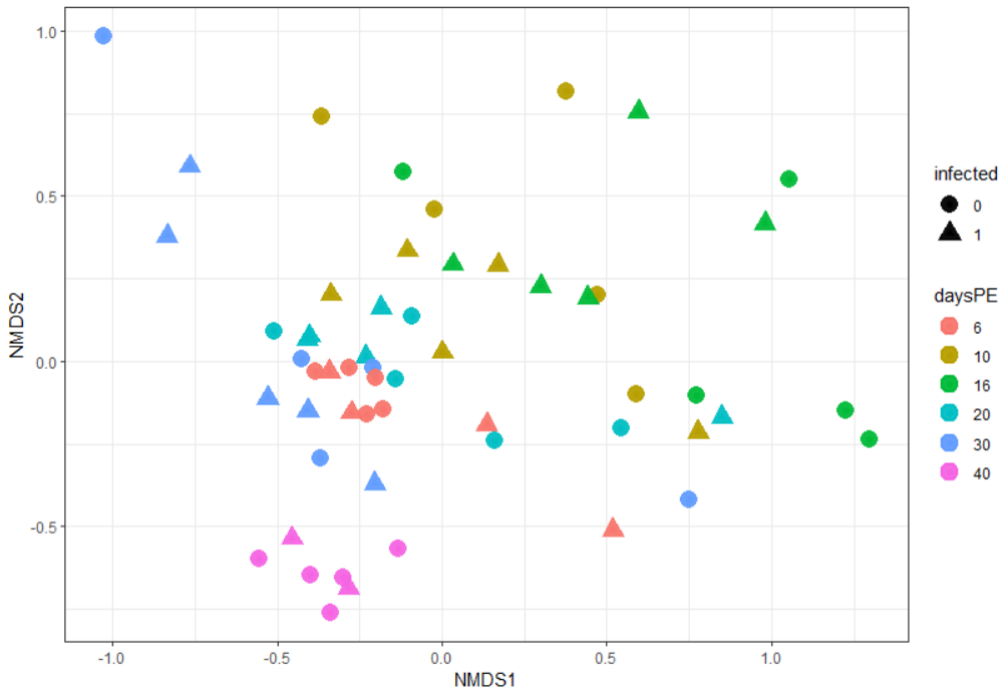


Figure 5: NMDS plot showing the Bray-Curtis Dissimilarity throughout schistosome development (single miracidium exposures with SmBRE). Colors indicate days post exposure while shapes indicate infection status, triangles are infected samples circles are uninfected samples.

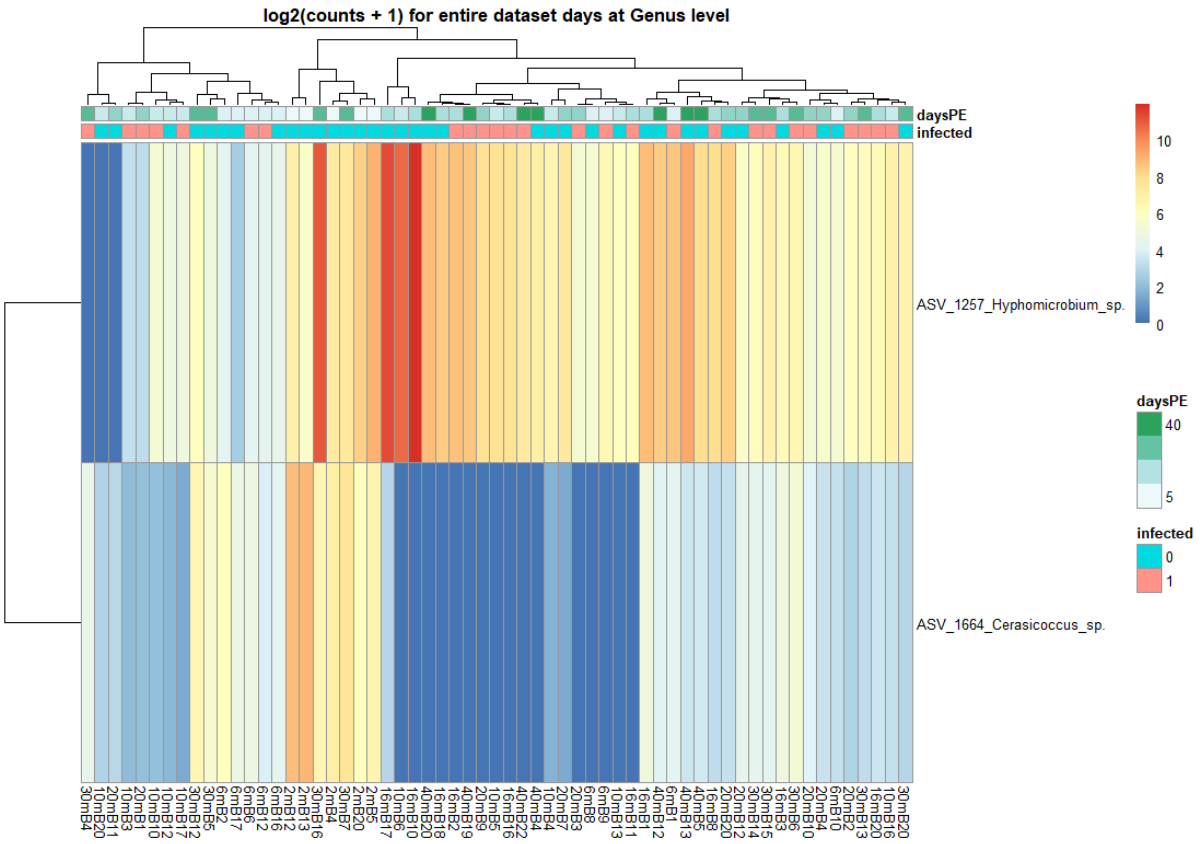


Figure 6: A heatmap showing the Log2(read counts+1) for each sample at the genus level as selected for through DeSeq2 for significantly changing read counts with adjusted p-values <0.05. Clustering on top is based on sample similarity based on significantly different ASVs at genus level.

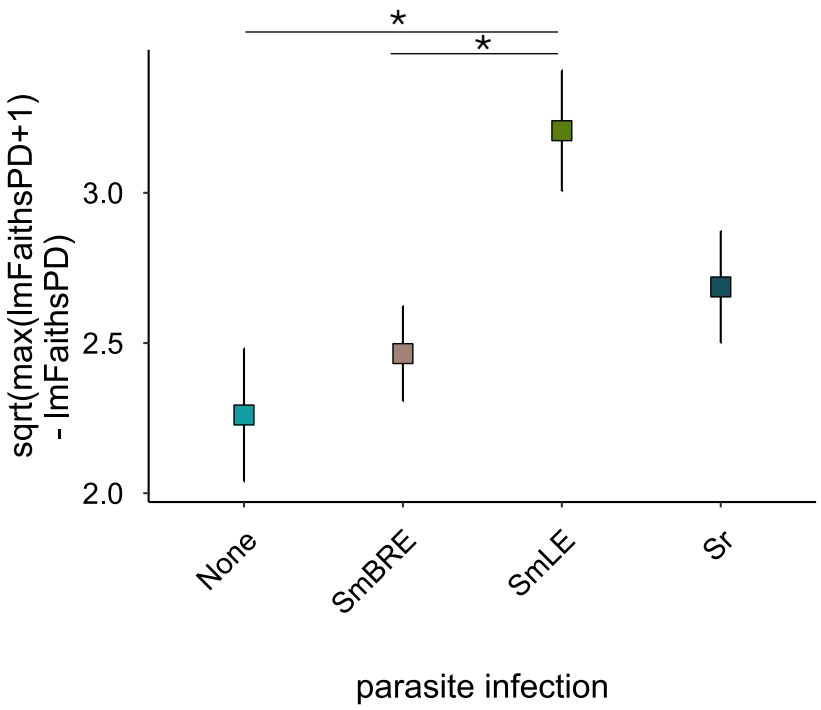


Figure 7: Square root transformed Faiths Phylogenetic diversity metric (alpha diversity) for snails 40 days post exposure to S. mansoni (SmBRE, low shedder), S. mansoni (SmLE, high shedder), and S. rodhaini (Sr). Uninfected samples from all exposure experiments are combined under "None" as selected for by the model. Squares indicate mean Square root transformed Faiths Phylogenetic diversity. Error bars indicate a single standard deviation. Significant differences are indicated by an asterisk.

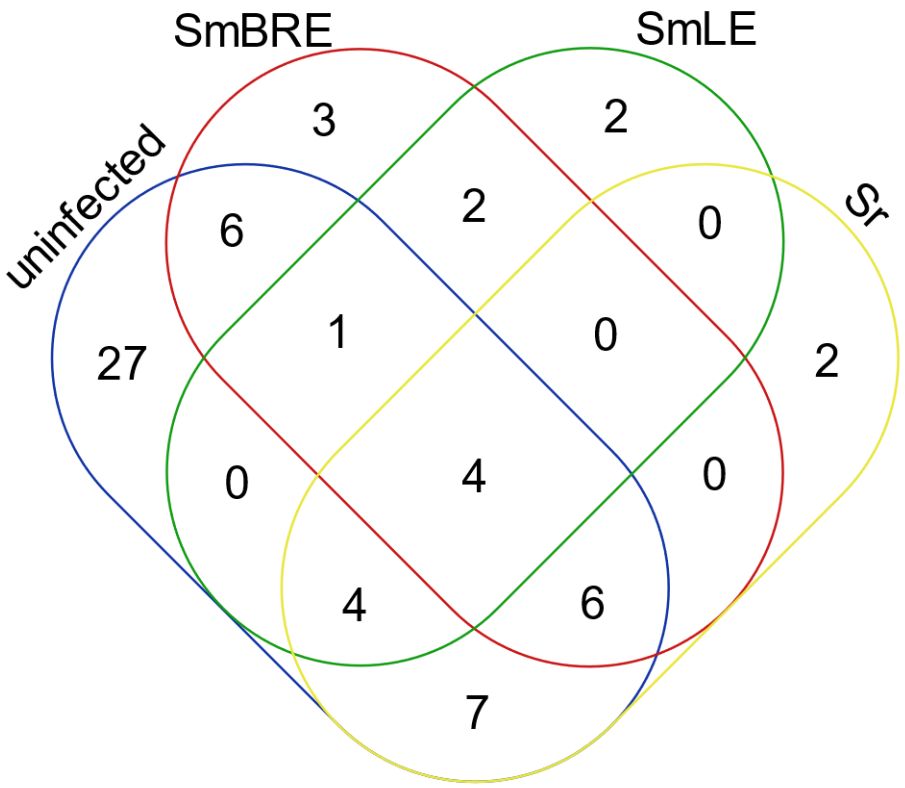


Figure 8: Venn diagram showing the shared and unique core ASV members across the different parasite infections (i.e., uninfected N=9, SmBRE N=21, SmLE N=22, and Sr N=18). Core members were determined based on a minimum detection threshold of 0.001 and maximally missing from one sample for that treatment. Venn diagram was generated through https://bioinformatics.psb.ugent.be/webtools/Venn/.

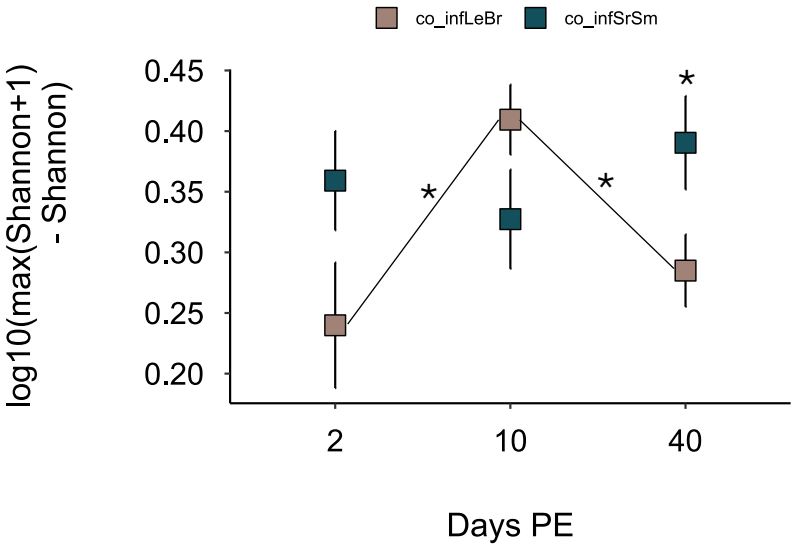


Figure 9: Log transformed Shannon diversity metric for the two co-infection experiments across 2, 10, and 40 days post miracidium exposure (DaysPE). Significant differences as determined through pairwise comparisons are indicted with an asterisk.

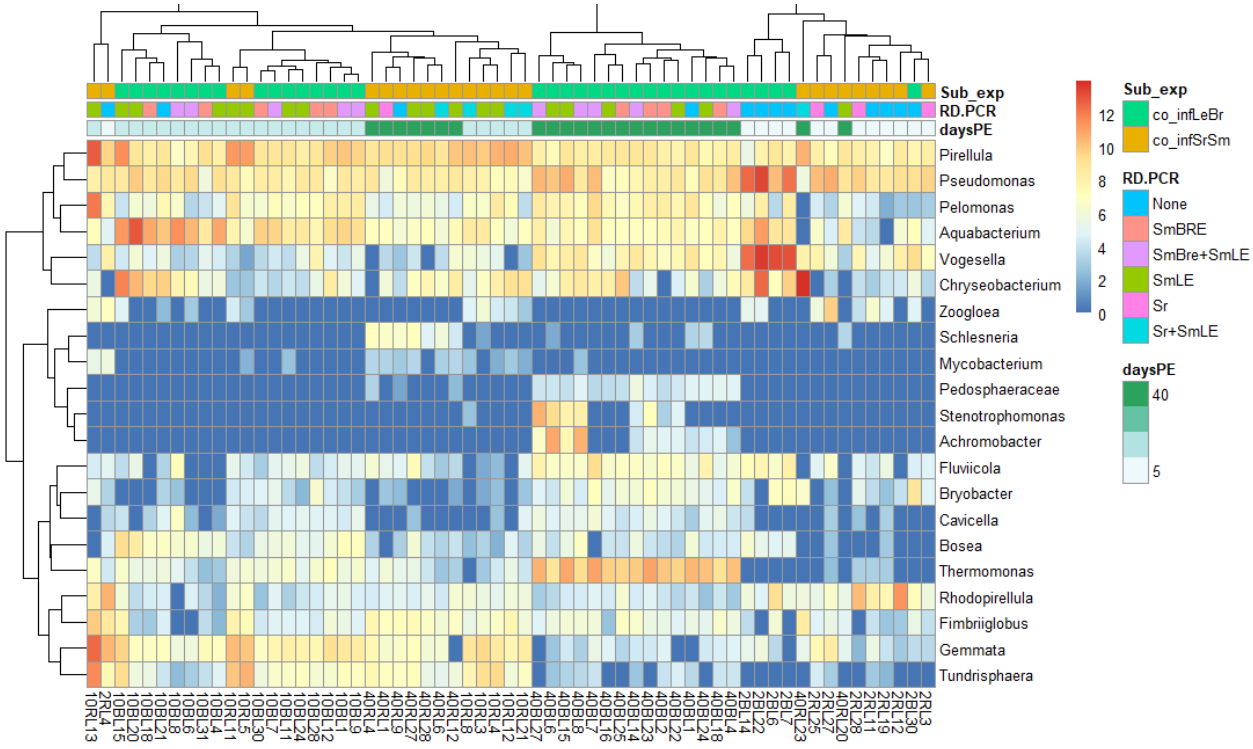


Figure 10: Heatmap showing the Log2(read counts+1) for each sample at the Genus level as selected for through DeSeq2 for significantly changing read counts with adjusted P-values <0.05. Sub_exp: co-infection experiment (S. rodhaini and S. mansoni =co_infSrSm and two strains of S. mansoni =co_infLeBr). daysPE: Days post miracidia exposure (2, 10 and 40). Samples are listed in the X-axis while significantly changing genera are listed on the Y-axis.

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Appendix – Chapter 3

Host-bacteriome transplants of the schistosome snail host *Biomphalaria glabrata* reflect species-specific associations

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Donor snail host identification

DNA was extracted with the E.Z.N.A.® Mollusc DNA Kit (OMEGA Bio-tek, Norcross, GA, USA) according to the manufacturer's protocol and eluted through two elution steps of 75 µL, totalling to 150 µL of DNA extract. DNA concentrations were determined using the Qubit dsDNA high-sensitivity assay (Thermo Fisher Scientific) for all DNA extracts and diluted until a concentration of 10 ng/µL was obtained. We amplified part of the COI marker for all three snail species using the LoboF1 (5'-KBTCHACAAAYCAYAARGAYATHGG-3') and LoboR1 (5'-TAAACYTCWGGRTGWCCRAARAAYCA-3') primers based on Lobo *et al.* (2013) and Aksenova *et al.* (2018). Furthermore, part of the rDNA region spanning the ITS2 marker was amplified according to Vinarski *et al.* (2011) and Schniebs *et al.* (2016) for the *Stagnicola* sp. specimen using the LT1 (5'-TCGTCTGTGTGAGGGTCG-3') and ITS2_Rixo (5'-TTCTATGCTTAAATTCAGGGG-3') primers. PCR reactions were done with the Qiagen™ Taq DNA polymerase kit in 20 µL and consisted of 2 µL DNA extract, 2 µL PCR buffer (x10), 0.5 µL dNTP, 1 µL MgCl2 (25 mM), 0.8 µL of each primer (20 µM), 12.78 µL milli-Q water and 0.12 µL of Taq Polymerase. The PCR reaction to amplify COI was performed as follows: initial denaturation at 94 °C for 5 min; 5 cycles of 94 °C for 30 s, 45 °C for 90 s and 72 °C for 60 s; followed by 45 cycles of 94 °C for 30 s, 54 °C for 90 s and 72 °C for 60 s; and a final elongation step at 72 °C for 10 min in a Tprofessional Thermocycler (Biometra™). The PCR reactions amplifying the ITS2 marker was performed as follows: initial denaturation at 94 °C for 5 min, 40 cycles of 94 °C for 30 s, 50 °C for 30 s and 72 °C for 45 s, and a final elongation step at 72 °C for 10 min. The resulting amplicons were cleaned using the ExoSAP-IT™ PCR Product Clean-up protocol and shipped to Macrogen™ Europe located in Amsterdam for Sanger Sequencing. All

sequences were quality checked, cleaned, and consensus sequences assembled with the Geneious software (version 10.0.9). The sequences were then BLAST-searched on the GenBank (https://www.ncbi.nlm.nih.gov/) database. This provided conclusive identifications for the *Biomphalaria glabrata* and *Planorbarius corneus* specimens (BLAST ID > 98% and no other species in the top BLAST hits, GenBank Accession number OQ954055 and OQ954057, respectively). However, the *Stagnicola* sp. required further identification efforts. For this species the resulting sequences for COI and ITS2 were, per marker, aligned with the MUSCLE alignment algorithm (Edgar 2004) in AliView (version 1.26), visually inspected and trimmed homogeneously for phylogenetic tree construction together with other *Stagnicola* species in accordance with Schniebs *et al.* (2016). *Lymnaea stagnalis* was used as an outgroup. The resulting alignments were subsequently imported into MEGA-X (version X) to determine the optimal substitution model and construct corrected pairwise genetic distance matrices. The Tamura 3-paramater model with a discrete Gamma distribution of 0.19 and the Tamura 3-paramater model were most suitable for the COI and ITS2 datasets, respectively. Next, maximum likelihood analyses were computed with 1,000 bootstraps through the IQ-tree web servers (http://iqtree.cibiv.univie.ac.at). Nodes with a Nodal support value below 70% were collapsed in TreeGraph2 (version 15.0-887 beta; Stöver and Müller, 2010). The resulting tree was edited in the FigTree software (version 1.4.4). Finally, nodal values were repositioned, and species names italicized in Adobe Acrobat DC (version 21.011.20039).

The COI-based phylogenetic analysis (523 bp) was not able to assign our *Stagnicola* specimen to either *Stagnicola fuscus* or *Stagnicola palustris* (**Fig. S1**). In contrast, the ITS2-based phylogenetic analysis (362 bp) indicated that our *Stagnicola* specimen

unambiguously clusters with *S. palustris* (**Fig. S2**). This mito-nuclear discordance in *Stagnicola* spp. has been reported and extensively discussed by Schniebs *et al.* (2016). The mitochondrial DNA of *S. fuscus* appears to have introgressed into certain *S. palustris* populations without leaving a nuclear signal. Therefore, we further refer to the *Stagnicola* sp. specimen here and in the main manuscript as '*Stagnicola fuscus x S. palustris'.*

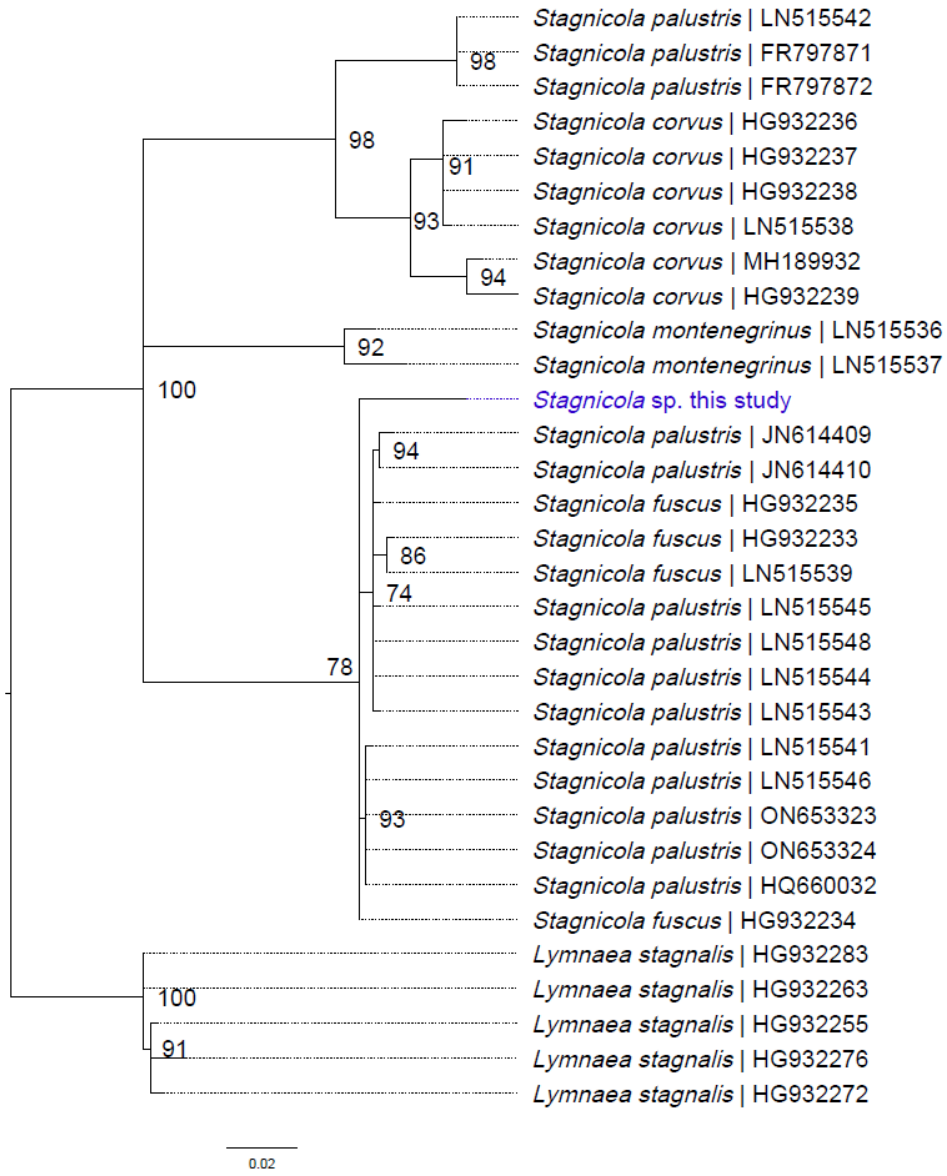


Fig. S1: The Tamura 3-paramater model with a discrete Gamma distribution of 0.19 was used to construct the phylogeny of *Stagnicola* spp. based on 523 bp of the COI marker. *Lymnaea stagnalis* was used as on outgroup. The *Stagnicola* sp. from this study is indicated in dark blue and was uploaded to GenBank under accession code OQ954056.

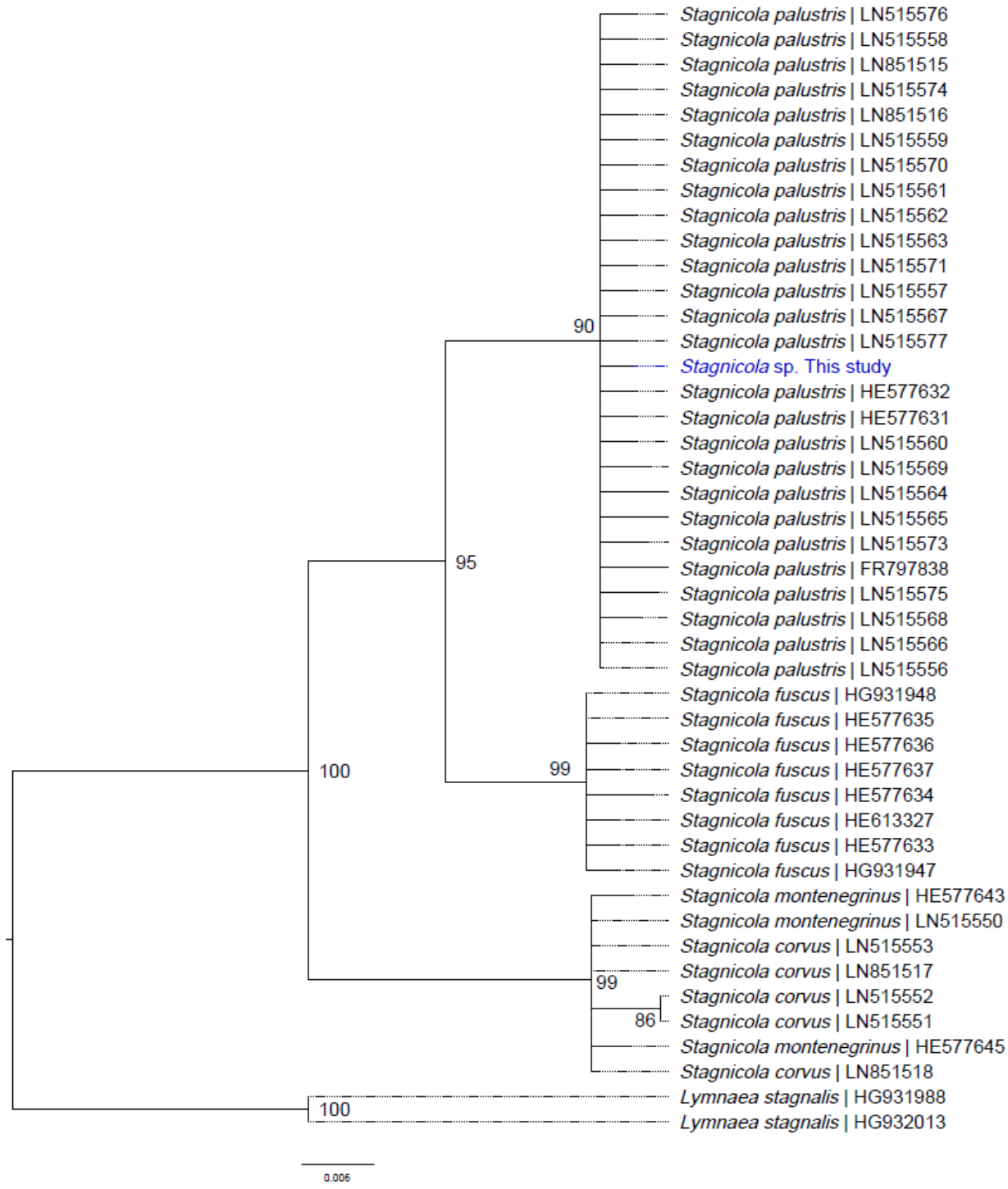


Fig. S2: The Tamura 3-paramater model was used to construct the phylogeny of *Stagnicola* spp. based on 362 bp of the rDNA region spanning the ITS2 marker. *Lymnaea stagnalis* was used as on outgroup. The *Stagnicola* sp. from this study is indicated in dark blue and was uploaded to GenBank under accession code OQ955479.

Egg clutch selection

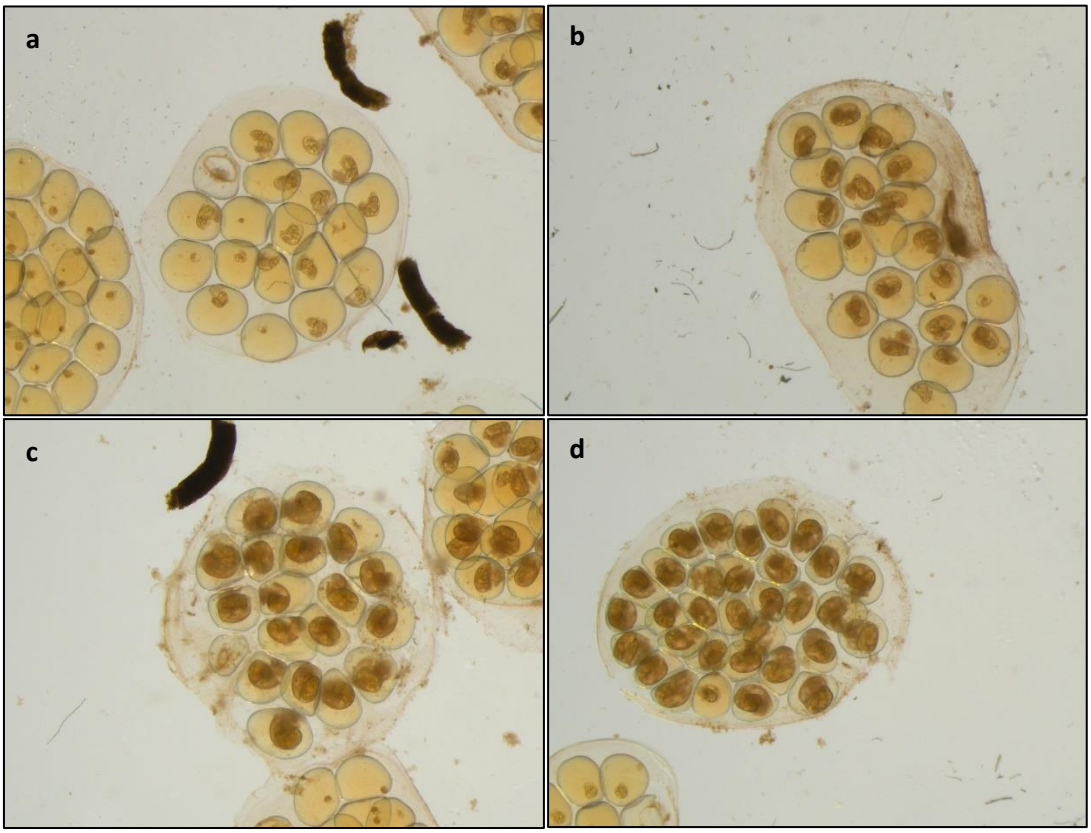


Fig. S3: **Selecting suitable egg clutches for the transplant experiment. a** and **b** unsuitable egg clutches due to the early developmental stage, increasing mortality during the sterilization protocol. **c** and **d** suitable egg clutches due to the late developmental stage, decreasing mortality during the sterilization protocol. Developmental stages beyond **c** and **d** are not preffered due to the fragile egg shell and consequential inability to survive the bleach exposure and pipetting steps.

Mock communities

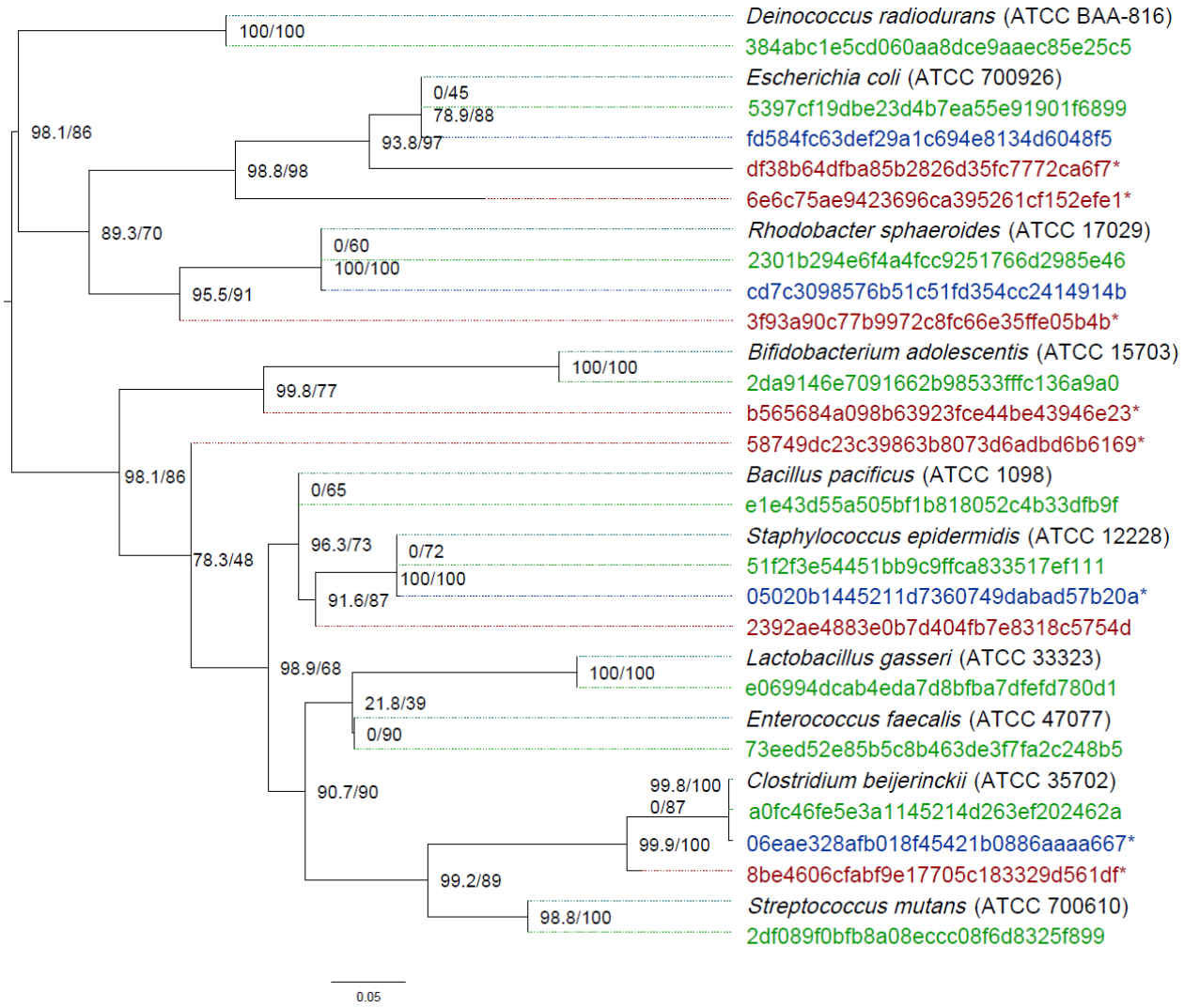


Fig. S4: Maximum likelihood midpoint rooted phylogenetic tree using 16S rDNA (453 bp) sequences of bacterial mock communities using the K3P+ G model (G = 0.46). Nodal support is indicated as bootstrap percentages (1 000 bootstrap replicates, before the '/'). Tree branch support by SHaLRT with 1 000 replicates (after the '/'). Black indicates reference sequences from both DNA and cell-based mock communities. Their respective identifier from the ATCC website is provided between parentheses. Green indicates ASVs perfectly matching the respective reference sequence. Blue indicates ASVs with one bp mismatch with the respective reference sequence. Red indicates ASVs that do not cluster with any reference sequence. ASVs removed after increasing the relative abundance threshold to 0.5 % are indicated by '*'.

Table S1: Cumulative read abundance (%) of each ASV across all bacterial mock community samples. ASVs exceeding the 0.5% threshold are indicated as such in the last column. '°' indicates a sample that would ideally have been removed from the dataset based on **Fig. S4**. However, increasing the threshold further would risk losing valid data.

ASV	Cumulative relative read abundance (%) across mock communities	Exceeds threshold?
b565684a098b63923fce44be43946e23	0.028	No
e1e43d55a505bf1b818052c4b33dfb9f	52.031	Yes
2392ae4883e0b7d404fb7e8318c5754d	0.642	Yes°
73eed52e85b5c8b463de3f7fa2c248b5	0.926	Yes
e06994dcab4eda7d8bfba7dfefd780d1	3.599	Yes
2df089f0bfb8a08eccc08f6d8325f899	14.714	Yes
51f2f3e54451bb9c9ffca833517ef111	1.147	Yes
05020b1445211d7360749dabad57b20a	0.106	No
58749dc23c39863b8073d6adbd6b6169	0.014	No
8be4606cfabf9e17705c183329d561df	0.013	No
a0fc46fe5e3a1145214d263ef202462a	19.361	Yes
06eae328afb018f45421b0886aaaa667	0.305	No
384abc1e5cd060aa8dce9aaec85e25c5	19.938	Yes
2301b294e6f4a4fcc9251766d2985e46	135.290	Yes
cd7c3098576b51c51fd354cc2414914b	2.689	Yes
3f93a90c77b9972c8fc66e35ffe05b4b	0.050	No
2da9146e7091662b98533fffc136a9a0	1.203	Yes
5397cf19dbe23d4b7ea55e91901f6899	244.204	Yes
fd584fc63def29a1c694e8134d6048f5	3.707	Yes
df38b64dfba85b2826d35fc7772ca6f7	0.013	No
6e6c75ae9423696ca395261cf152efe1	0.019	No

Sterilization experiment

Table S2: **Results from the Sterilization experiment**. Optical density measurements at 600 nm (OD), staining method (Syto 9) and qPCR (Cp) were done on the negative and positive samples. The question mark indicates samples where the interpretation was inconclusive. The '*' indicates samples that had Cp values not-significantly different from qPCR negatives.

Sample	OD	SYTO 9	qPCR (Cp+-Sd)
0A7-	0.178	bacteria	30.08 +- 0.27
0A8-	0.028	bacteria but most look dead	30.24 +- 0.01
0A2-	0	no bacteria	32.58 +- 0.41*
24A7-	0.359	some bacteria	26.43 +- 0.14
24A8-	0	no bacteria (?)	33.03 +- 0.22*
24A2-	0.428	some bacteria	28.04 +- 0.05
48A7-	0	no bacteria (?)	21.31 +- 0.17
48A8-	0	no bacteria (?)	34.06 +- 0.60*
48A2-	0	no bacteria	34.28 +- 0.32*
72A7-	0.417	bacteria	19.99 +- 0.21
72A8-	0	no bacteria (?)	32.71 +- 0.11*
72A2-	0.005	bacteria	30.52 +- 0.18
0A7+	0.52	bacteria	25.99 +- 0.09
0A8+	0.507	bacteria	24.08 +- 0.02
0A2+	0.406	bacteria	24.10 +- 0.14
72A7+	0.367	bacteria	28.65 +- 0.49
72A8+	0.361	bacteria	26.14 +- 0.32
72A2+	0.382	bacteria	27.61 +- 0.19
10^1	/	/	30.56 +- 0.12
10^2	/	/	29.99 +- 0.22
10^3	/	/	27.43 +- 0.17
10^4	/	/	23.83 +- 0.09
MILLI-Q1	/	/	33.89 +- 0.13
MILLI-Q2	/	/	33.63 +- 0.23

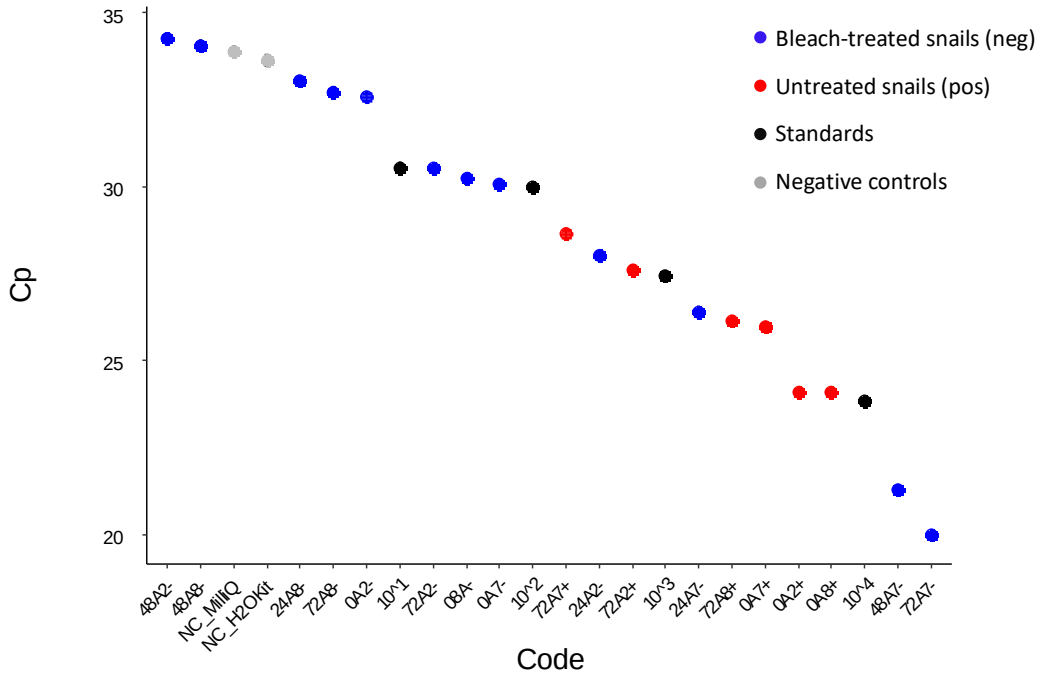


Fig. S5: **Cp values for all samples tested in qPCR.** Sample codes were constructed as follows: first, 0, 24, 48, and 72 indicate whether the sample was bacteriome-disturbed at the day of, or, one, two or three days prior to being sacrificed. Second, A2, A7 and A8 refer to either of the three maternal lines used during this experiment. Finally, '+' or '-' refers to the sample being a positive or a negative control (untreated or bleach-treated without donor inoculum exposure, respectively). For example, '48A2-' refers to the sample of Maternal line 2 (A2) that was sterilized two days (48) before being sacrificed and did not receive a donor bacteriome (-). 'NC_MilliQ' refers to the negative PCR control. 'NC_H20Kit' refers to a Milli-Q extraction using the E.Z.N.A.® Mollusk DNA Kit (OMEGA Bio-tek, Norcross, GA, USA) used for all DNA extractions. Negative controls, bleach-treated samples, untreated samples and standards are, respectively, colored as grey, blue, red and black. Standards contained 10^1, 10^2, 10^3 and 10^4 genomic copies of *Escherichia coli.*

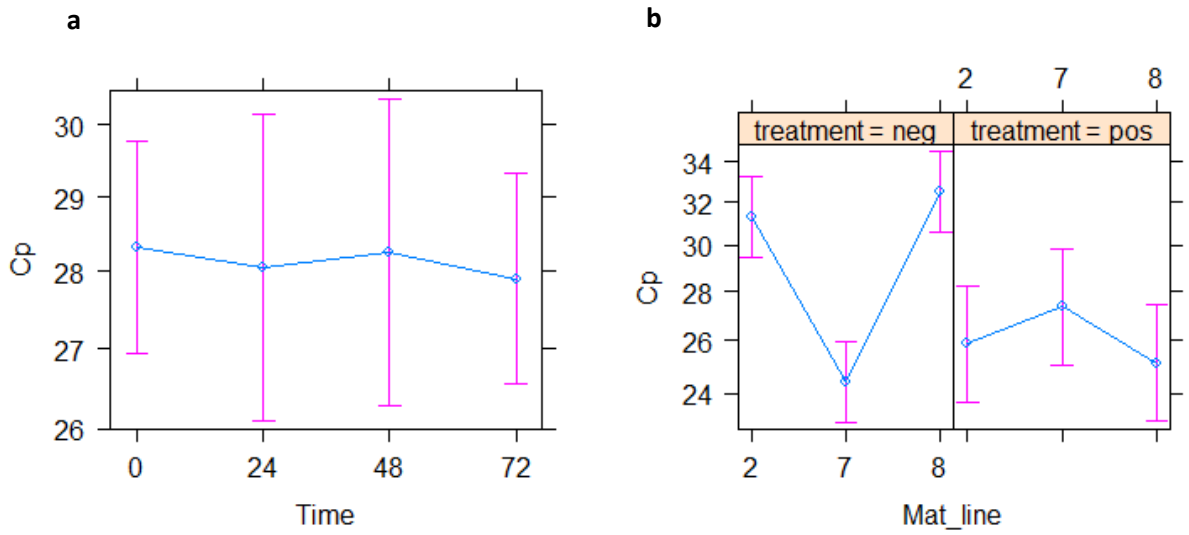


Fig. S6: Effects on Cp values. a The effect of time between sterilization and donor inoculum exposure on Cp values. Snails were sterilized on the same day as ('0'), and one day ('24'), two days ('48') and three days ('72') prior to, receiving a donor inoculum. **b** Cp values for the interaction effect between the maternal line and the treatment. **'**Neg' refers to negative controls (bleach-treated specimens without donor inoculum), 'pos' to positive controls (untreated specimens). There was a significant effect of the maternal line on the Cp value for samples without a donor inoculum ('neg', $p<0.001$). Moreover, Maternal line '7' had a significantly lower Cp value compared to the other two maternal lines ($p<0.001$), which were not significantly different amongst each other. Cp values were obtained by running all samples in triplicate in a qPCR reaction targeting bacterial 16s rRNA (see main manuscript for protocol). The maternal lines (2, 7 and 8) were started from individual egg clutches in October 2020 from the *Biomphalaria glabrata* Brazil strain that arrived in our lab in June 2020, and conducted the qPCR experiment reported here in April 2022.

Kaplan-Meier survival curves

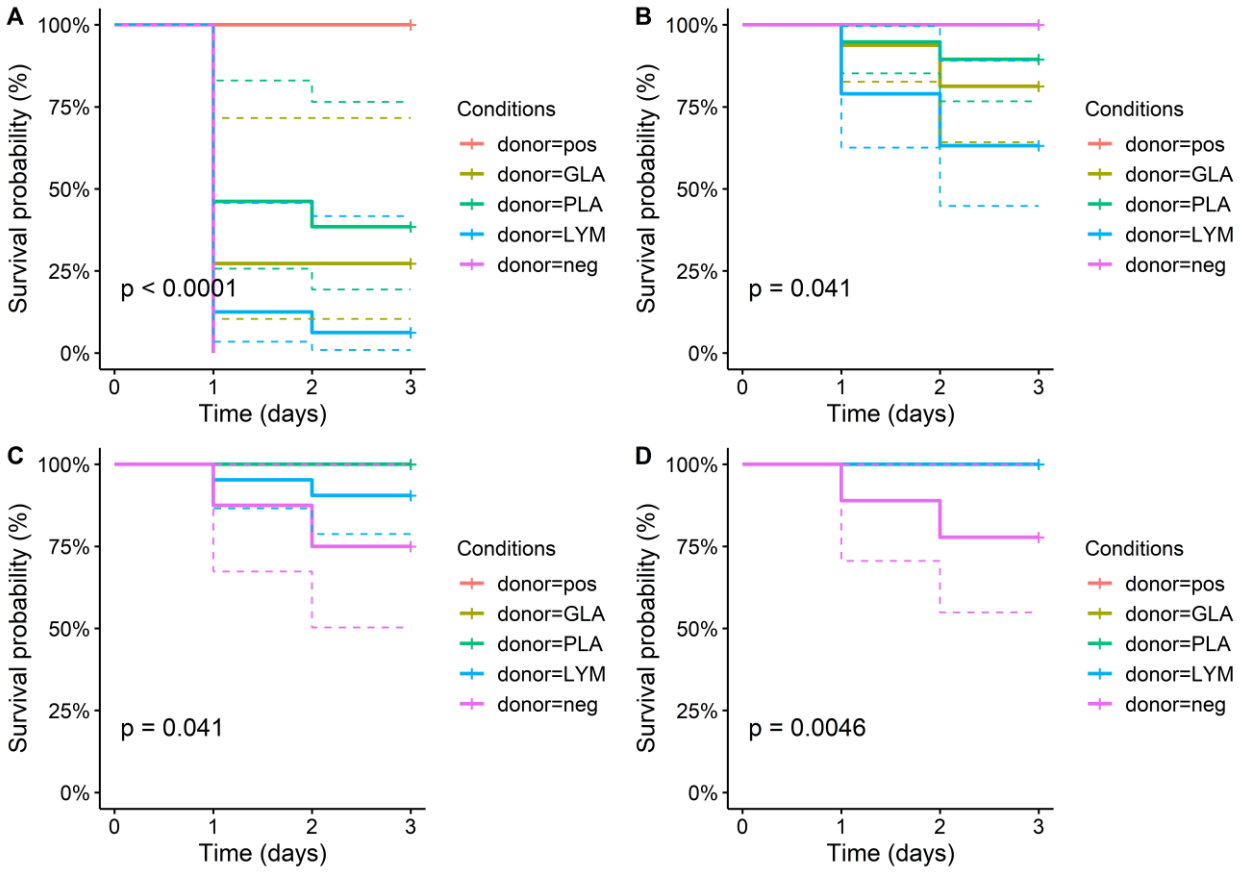


Fig. S7: The survival probability of all treatments across the days between sterilization and inoculum exposure. **A** Sterilization and inoculum exposure on the same day. **B** Sterilization one day before inoculum exposure. **C** Sterilization two days before inoculum exposure. **D** Sterilization three days before inoculum exposure. 'Pos' refers to positive controls (untreated specimens), 'GLA' indicates the samples that received a donor inoculum from *Biomphalaria glabrata*, 'PLA' indicates the samples that received a donor inoculum from *Planorbarius corneus*, 'LYM' indicates the samples that received a donor inoculum from *Stagnicola fuscus x S. palustris* and 'neg' refers to negative controls (bleach-treated specimens without donor inoculum exposure). The difference in survival probability between the different donor treatments was significant for all dissection days.

Alpha diversity

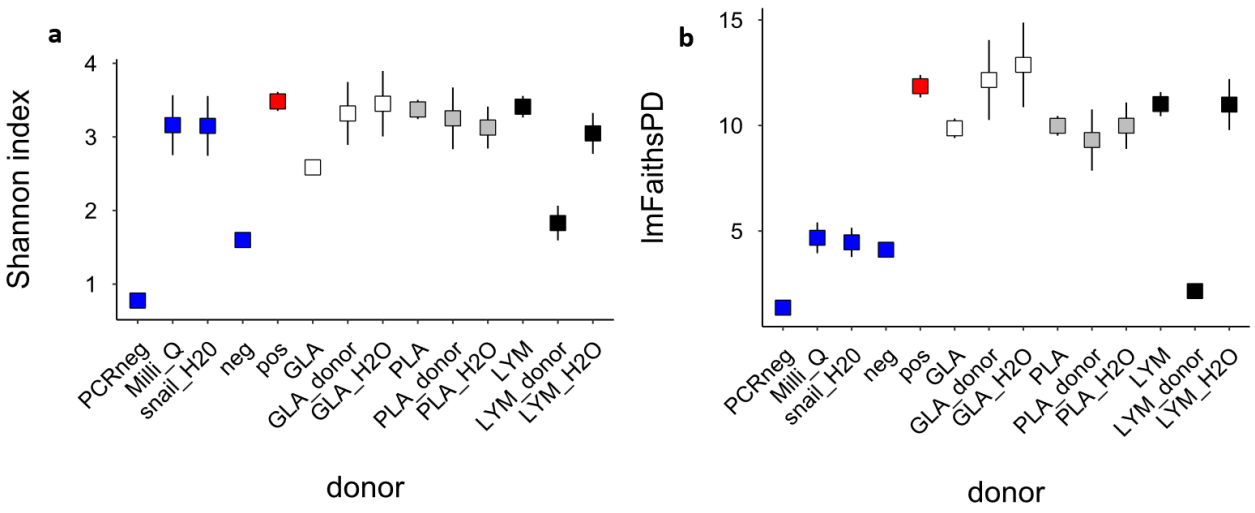


Fig. S8: Alpha diversity across the transplant experiment. **a** Shannon diversity index **b** Faith's PD per treatment group. GLA indicates the samples that received *Biomphalaria glabrata* (different maternal line) donor bacteriome, PLA indicates the samples that received *Planorbarius corneus* donor bacteriome, LYM indicates the samples that received *Stagnicola fuscus x S. palustris* donor bacteriome. 'pos' refers to positive controls (untreated samples). 'neg' refers to negative controls (bleach-treated samples without donor inoculum). Sample names ending with '_donor' refer to the respective donor bacteriome inoculum. Samples ending with '_H2O' refer to a water sample taken from the respective donor's aquarium. 'Milli-Q' refers to a Milli-Q extraction using the E.Z.N.A.® Mollusc DNA Kit (OMEGA Bio-tek, Norcross, GA, USA) used for all DNA extractions. 'snail_H20' refers to an extraction done on autoclaved tap water with the E.Z.N.A.® Mollusc DNA Kit (OMEGA Bio-tek, Norcross, GA, USA). 'PCRneg' is the PCR negative included in sequencing.

Beta diversity

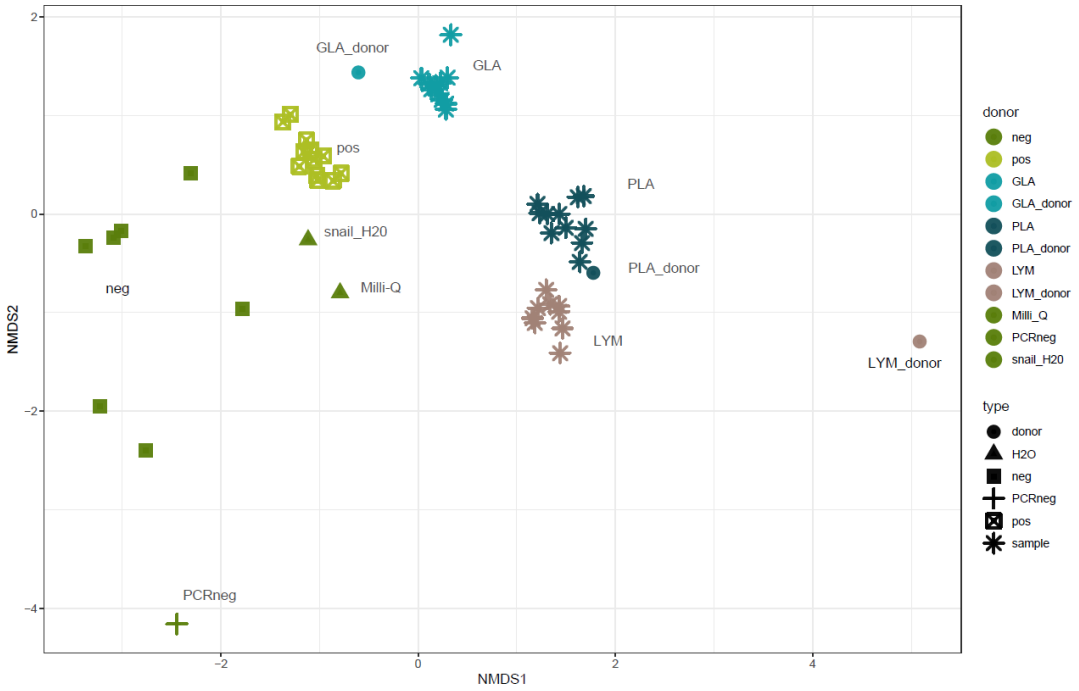


Fig. S9: NMDS plot showing the Bray-Curtis dissimilarity across all samples. 'pos' refers to positive controls (untreated samples). 'GLA' indicates the samples that received a donor inoculum from *Biomphalaria glabrata*. 'PLA' indicates the samples that received a donor inoculum from *Planorbarius corneus*. 'LYM' indicates the samples that received a donor inoculum from *Stagnicola fuscus x S. palustris*. 'neg' refers to negative controls (Bleach-treated samples without donor inoculum exposure). 'Milli-Q' refers to a Milli-Q extraction using the E.Z.N.A.® Mollusc DNA Kit (OMEGA Bio-tek, Norcross, GA, USA) used for all DNA extractions. 'snail_H2O' refers to an extraction done on autoclaved tap water with the E.Z.N.A.® Mollusc DNA Kit (OMEGA Bio-tek, Norcross, GA, USA). 'PCRneg' is the PCR negative included in sequencing.

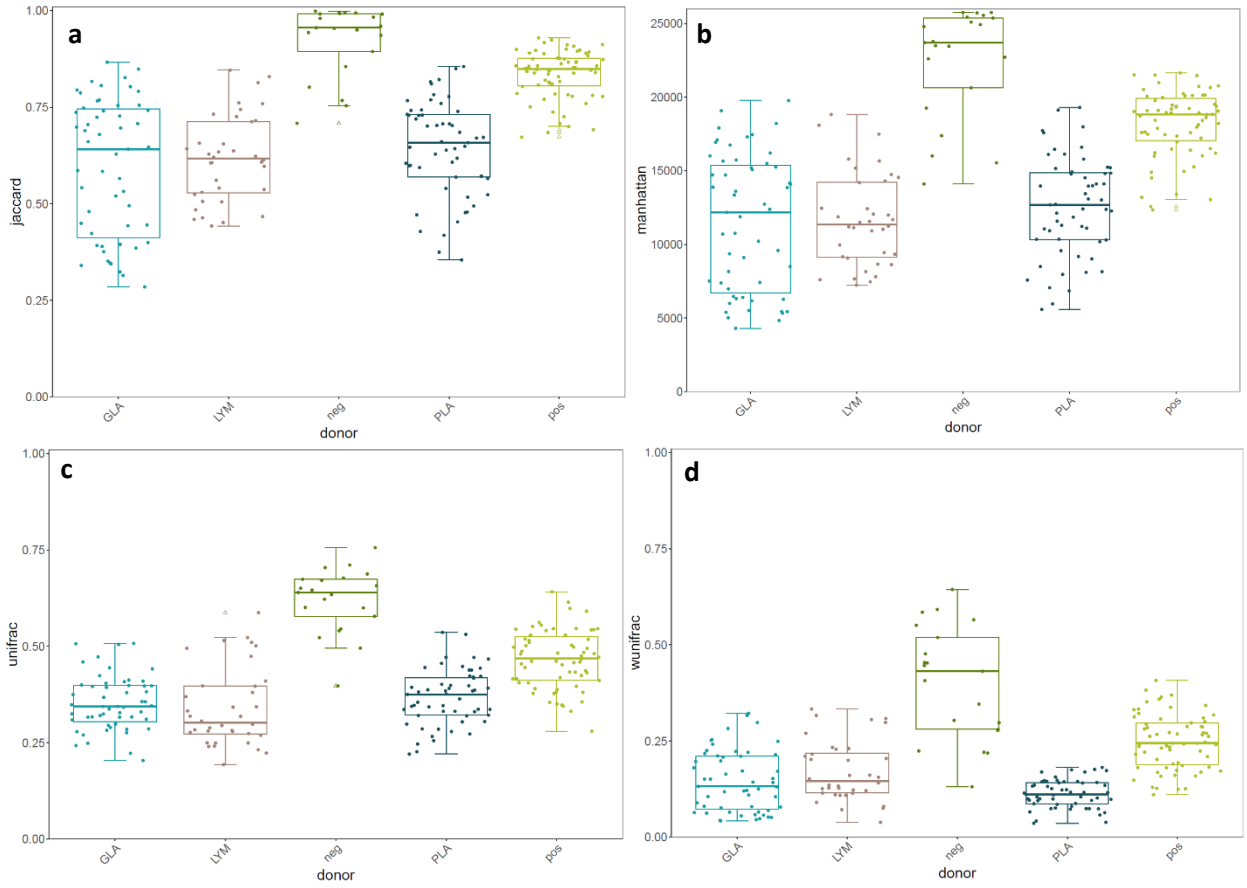


Fig S8: The within group beta dissimilarities (n=233). **a** Jaccard coefficient. **b** Manhatten distance. **c** unweighted UniFrac distance. **d** weighted UniFrac distance. 'pos' refers to positive controls (without bleach exposure). 'GLA' indicates the samples that received a donor inoculum from Biomphalaria glabrata. 'PLA' indicates the samples that received a donor inoculum from Planorbarius corneus. 'LYM' indicates the samples that received a donor inoculum from Stagnicola fuscus x S. palustris. 'neg' refers to negative controls (without donor inoculum exposure). Outliers are indicated by triangles. The lower and upper hinges correspond to the first and third quartiles (the 25th and 75th percentiles).

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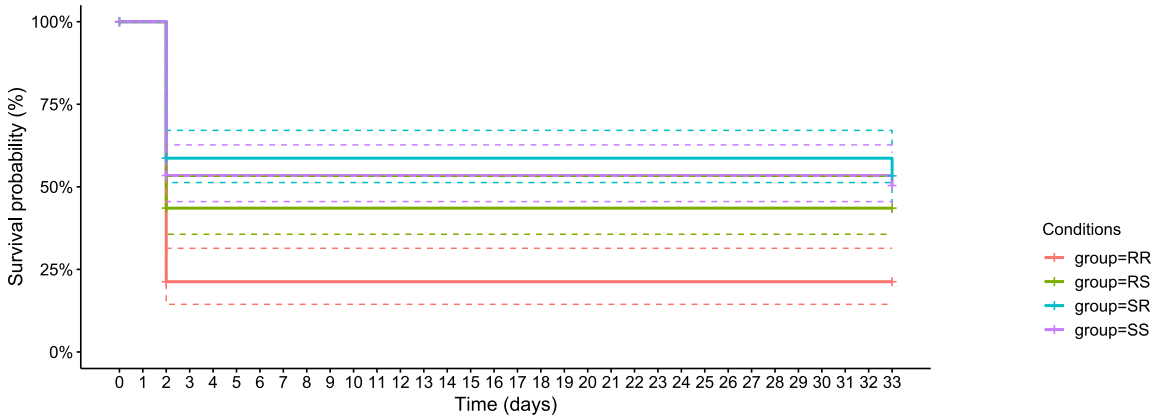
Schniebs K, Glöer P, Vinarski M *et al.* A barcode pitfall in Palaearctic Stagnicola specimens (Mollusca: Lymnaeidae): Incongruence of mitochondrial genes, a nuclear marker and morphology. *North West J Zool* 2016;**12**:239–54.

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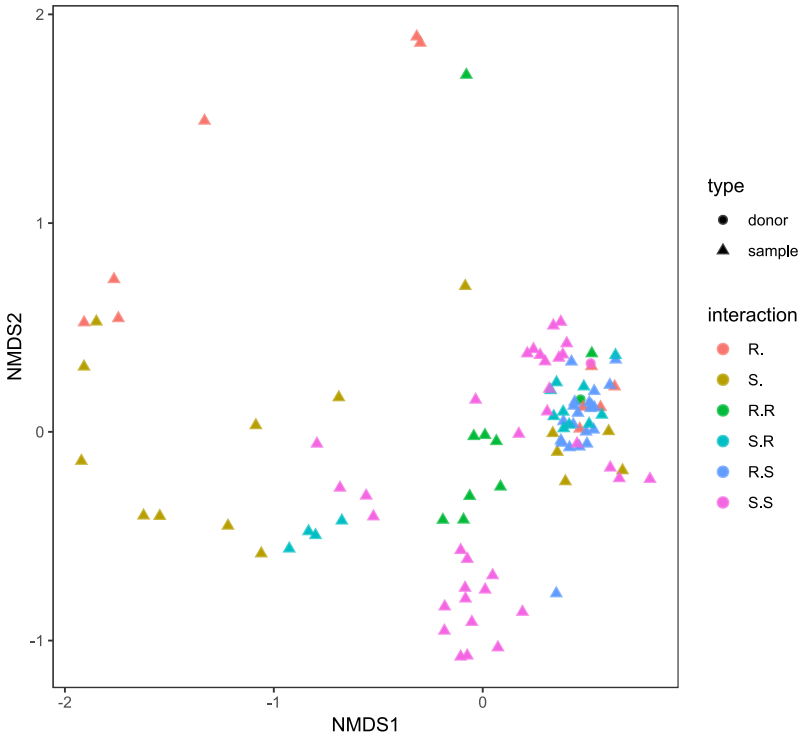
Vinarski M, Schniebs K, Glöer P *et al.* The taxonomic status and phylogenetic relationships of the genus Aenigmomphiscola Kruglov and Starobogatov, 1981 (Gastropoda: Pulmonata: Lymnaeidae). *J Nat Hist* 2011;**45**:2049–68.

Appendix – Chapter 4

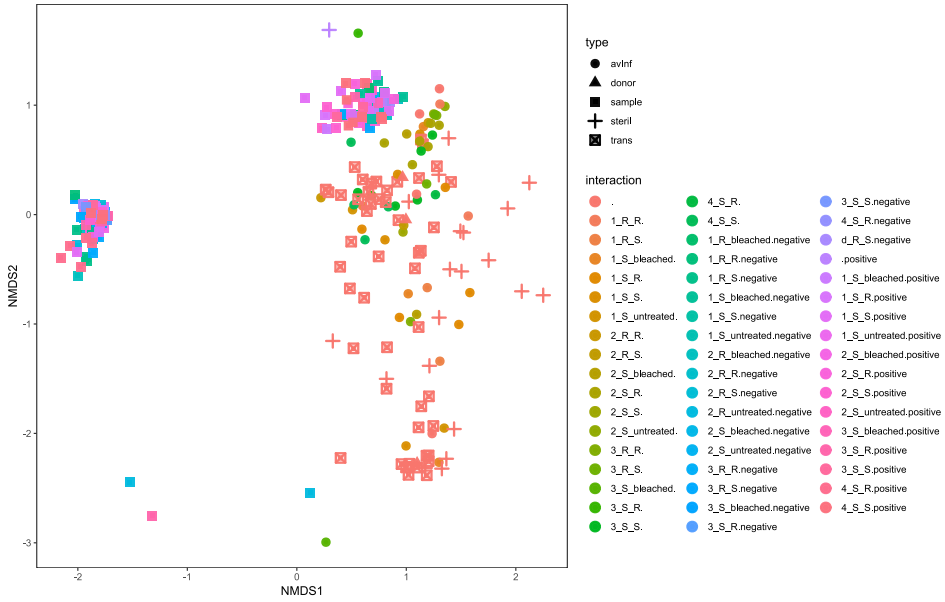
The bacterial aspect of the microbiome does not drive liver fluke resistance in some populations of the globally invasive snail *Pseudosuccinea columella*



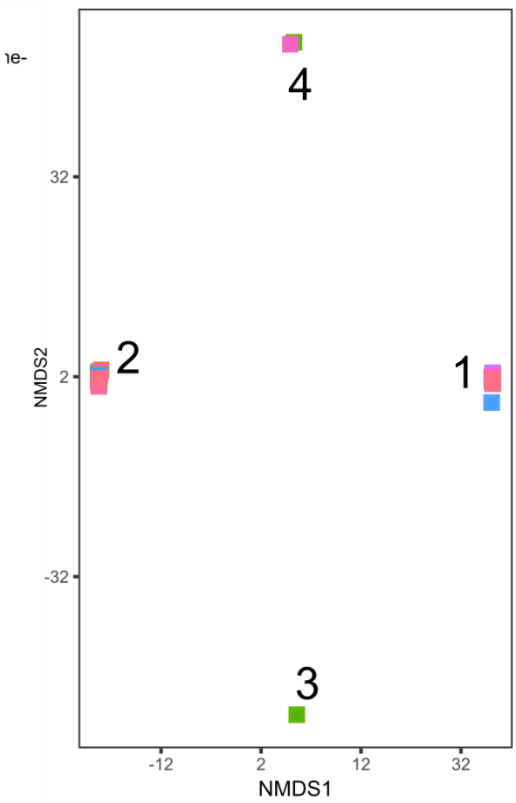
Suppl. Figure 1: Kaplan-Meier curve showing the survival probability (%) for each day survival was checked in transplant 2. Survival was assessed two days and 33 days after the first transplant occurred. Three days after the first transplant occurred snails were moves to glass petri dishes and fed unautoclaved algae. Colors indicate different genotype/microbiome combinations as explained in Figure 1. The dotted lines indicate 95% confidence intervals for each combination.



Suppl. Figure 2: NMDS plot based on the Bray-Curtis dissimilarity with sample type (recipient or donor) indicated by shape and the genotype-microbiome interaction as color.



Suppl. Figure 3: NMDS plot based on the Bray-Curtis dissimilarity with sample types indicated by shape and the genotype, treatment, batch number and infection status interaction as color. Compared to Figure 8 in the main manuscript, this figure makes the distinction between the different unexposed samples and their treatment.



Suppl. Figure 4: NMDS plot based on the Bray-Curtis dissimilarity with sample types indicated by shape and the genotype, treatment, batch number and infection status interaction as color. Compared to Figure 8 in the main manuscript, this figure makes the distinction between the different unexposed samples and their treatment.

Appendix – Chapter 5

Invasive snails, parasite spillback, and potential parasite spillover drive parasitic diseases of *Hippopotamus amphibius* in artificial lakes of Zimbabwe

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[+] Lead Contact

Corresponding author: Ruben Schols (ruben.schols@africamuseum.be)

Legend

Additional file 1: Figures S1-S5. Figure S1 - SEM imaging of “Hippo stomach fluke type 1”. Figure S2 - The morphometric approach to identify the hippo liver flukes. Figure S3 - *F. nyanzae* metacercariae isolated from *P. columella*. Figure S4 – Primers used to amplify the partial rDNA region of *F. nyanzae*. Figure S5 – *COI*-based Phylogeny of the *Radix* genus. Figure S6 – *COI*-based Phylogenies of the genera *Bulinus* and *Biomphalaria*. Tables S1-S6. Table S1 – Corrected *COI* genetic distances for the subfamily Fasciolinae. Table S2 – Corrected rDNA genetic distances for the subfamily Fasciolinae. Table S3 – Corrected *COI* genetic distances

for the genus *Radix*. Table S4 – Corrected *COI* genetic distances for the genus *Biomphalaria*. Table S5 – Corrected *COI* genetic distances for the genus *Bulinus*. Table S6 – Accession numbers generated in this study, Carolus *et al.* (2019), and Muzarabani *et al.* (2023).

Supplemental Figures

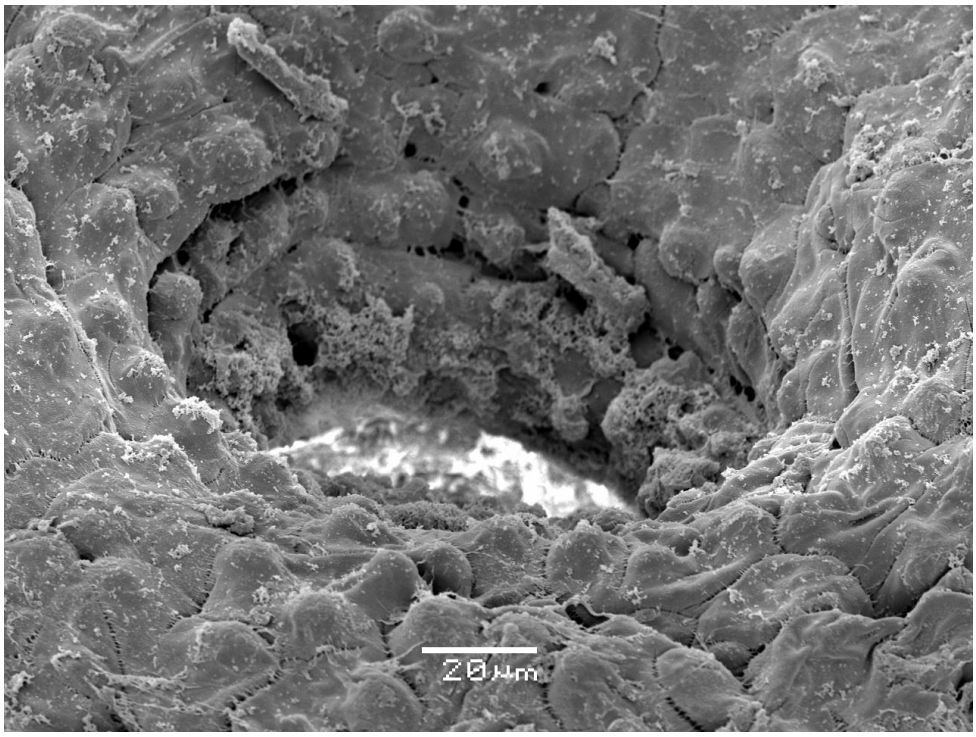


Figure S1: SEM imaging of the oral opening of “Hippo stomach fluke type 1”. The tegumental papillae are arranged in several rows and resemble the “*Dome to conical, nonciliated papilla*” described in Sey (1991).

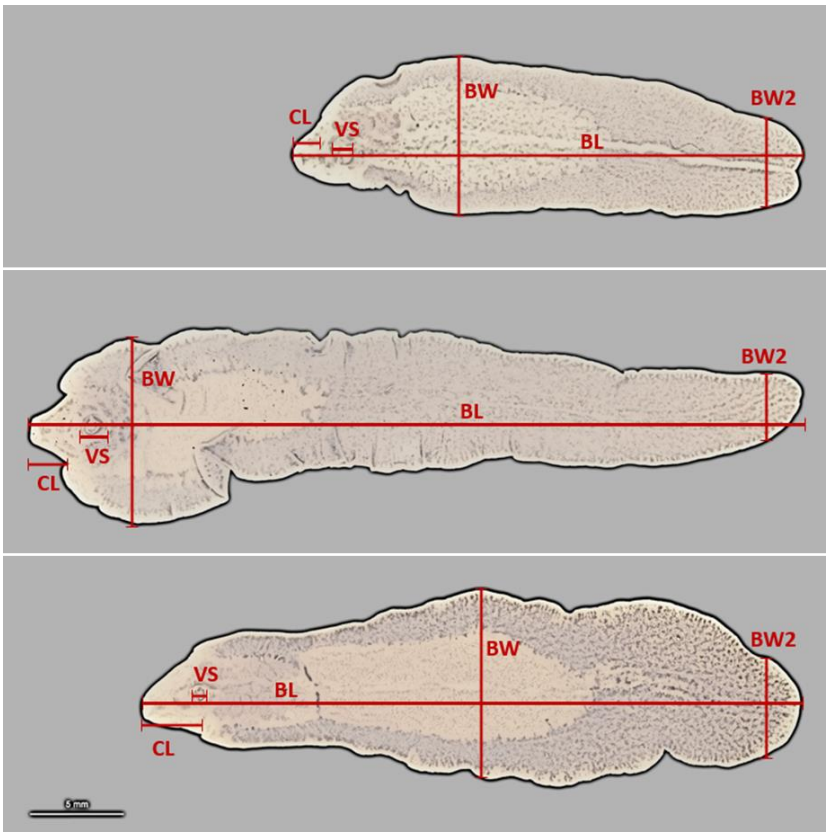


Figure S2: A visualization of the conducted measurements in the morphometric analysis of the hippo liver flukes. Samples included are, from top to bottom, *Fasciola hepatica*, *Fasciola nyanzae* and *Fasciola gigantica*. The following measurements were obtained: body width at widest point (BW), body length (BL), cephalic cone length until shoulders (CL), posterior width at 2 mm from the posterior end (BW2) and the anteroposterior diameter of the ventral sucker (VS). Scale bar represents 5 mm. A filter was added to the entire figure to enhance contrasts and improve interpretability of the conducted measurements.

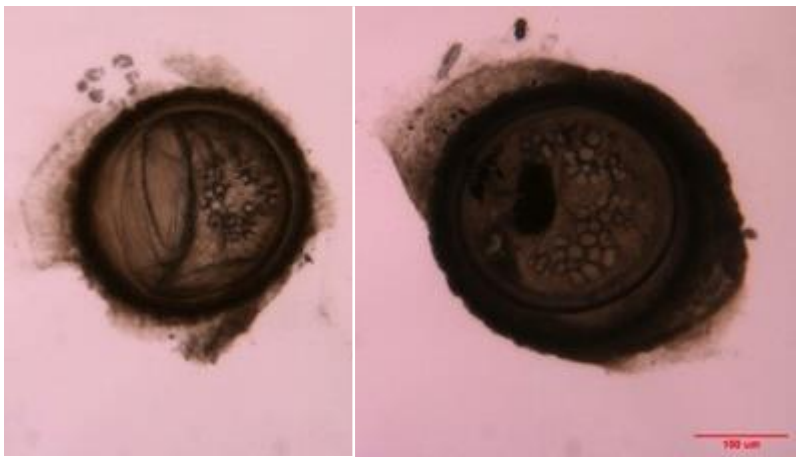


Figure S3: Encysted cercariae (i.e., metacercariae) used for morphometrics that encysted following emergence from two *Pseudosuccinea columella* snails from site 3 in Kariba. They are respectively listed under Hippo liver fluke 1 & 2 in **Table 1**. The scale bar represents 100 µm and accounts for both pictures.

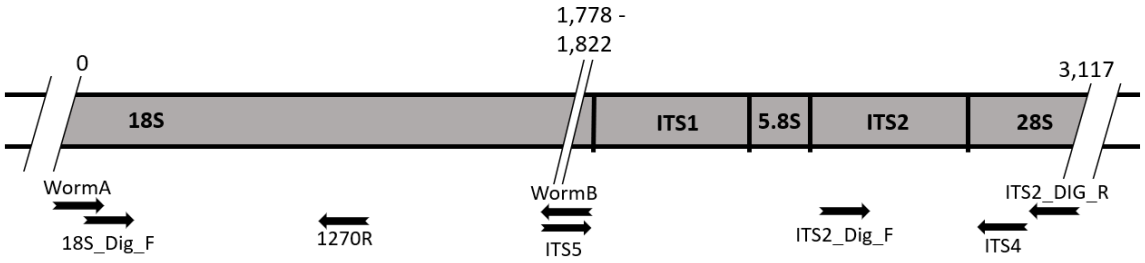


Figure S4: A visualization of the primers used for amplifying and sequencing the 3117 base pairs of the rDNA region of the Hippo liver flukes from Kariba. Base pair positions 1778 - 1822 are highlighted, as they could not be sequenced with the used primers. Primer information is listed in **Table 3**.

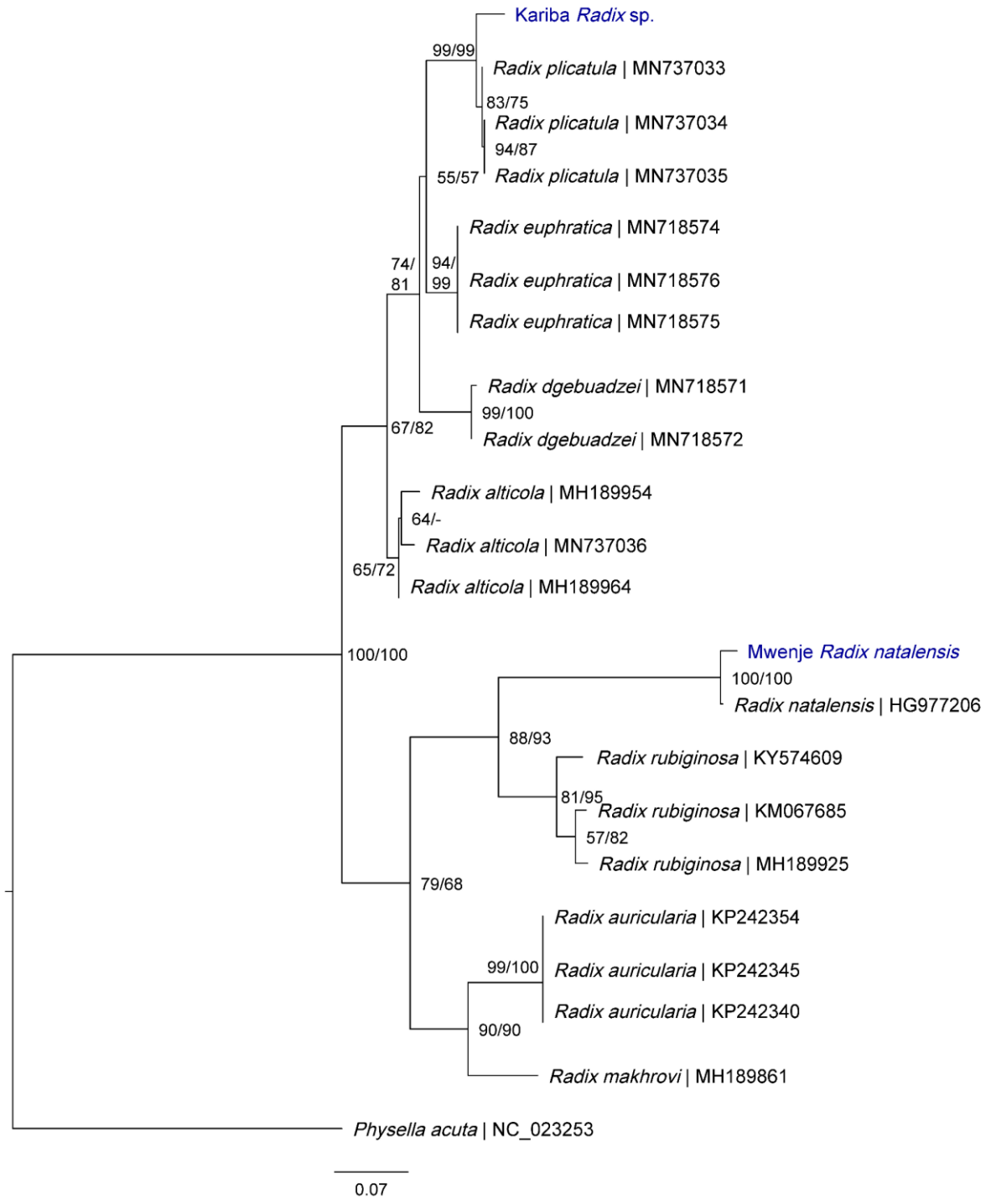


Figure S5: Maximum Likelihood analysis for the *Radix* species collected in this study, using the HKY + G (= 0.12) model on 463 bp of the *COI* marker. *Physella acuta* ("GenBank: NC_023253") was used as an outgroup. Nodal support is indicated as bootstrap percentages (1000 bootstraps) and posterior probabilities, respectively before and after the "/" separator. GenBank accession numbers are provided after the "|" separator. Specimens from this study are indicated in blue.

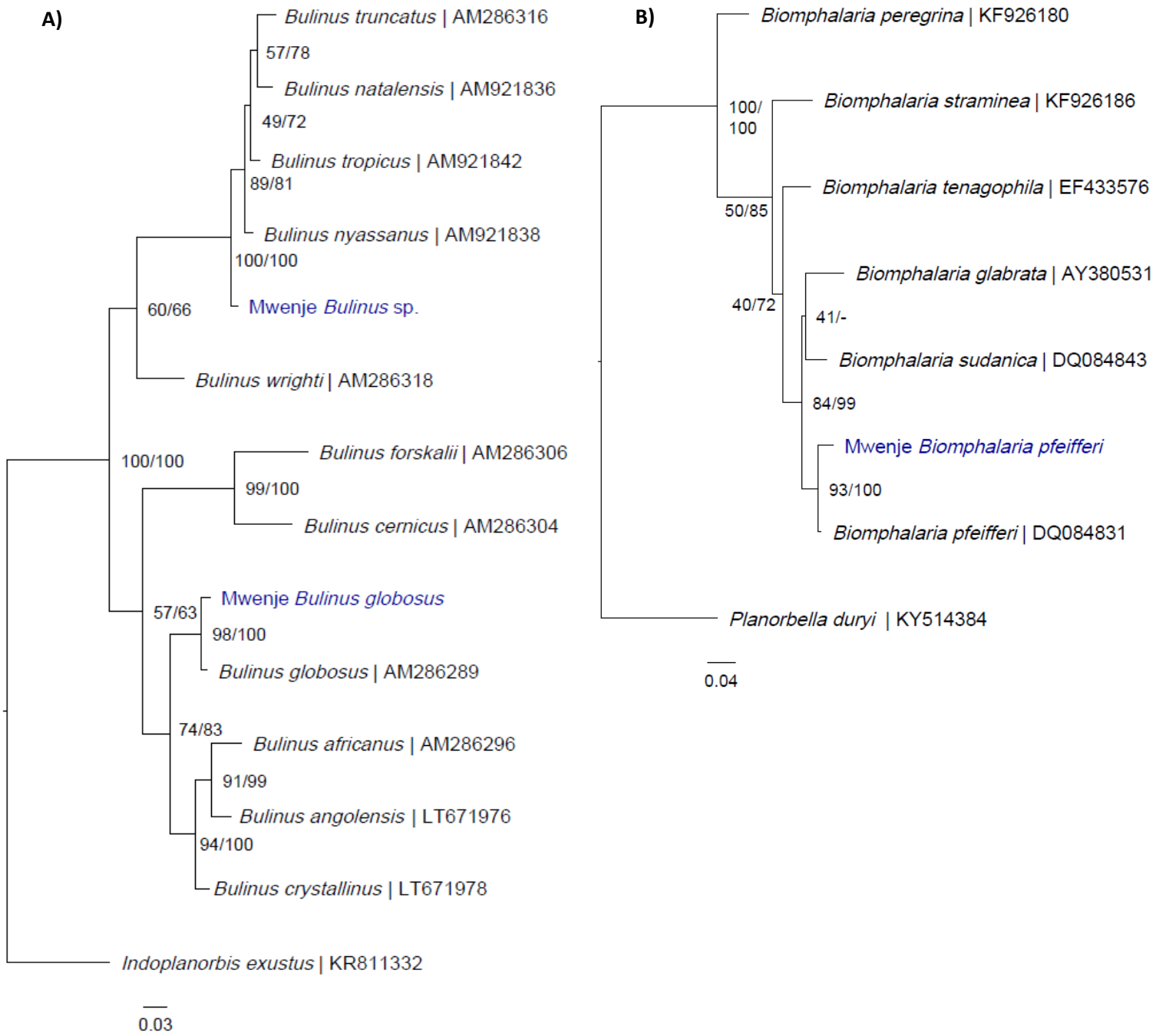


Figure S6: A) Maximum Likelihood analysis for the *Biomphalaria* species collected in this study, using the GTR + G (= 0.19) model on 446 bp of the *COI* marker. *Planorbella duryi* ("GenBank: KY514384"), from the tribe Helisomatini, was used as an outgroup. B) Maximum Likelihood analysis for the *Bulinus* species collected in this study, using the GTR + G (= 0.19) model on 446 bp of the *COI* marker. *Indoplanorbis exustus* ("GenBank: KR811332"), from the subfamily Bulininae, was used as an outgroup. Nodal support is indicated as bootstrap percentages (1000 bootstraps) and posterior probabilities, respectively before and after the "/" separator. GenBank accession numbers are provided after the "|" separator. Specimens from this study are indicated in blue.

Supplementary Tables

Table S1: **Corrected *COI* genetic distances for the subfamily Fasciolinae.** Corrected genetic distances (Tamura-Nei model with gamma correction [G=0.23]) between members of the subfamily Fasciolinae based on a 814 bp *COI* fragment. All sequences obtained during this study are indicated in bold together with important p-distances relevant for species identification. GenBank accession numbers are provided after the "|" separator. *Fasciolopsis buski* ("GenBank: NC_030528"), from the subfamily Fasciolopsinae, was used as outgroup.

Species		1	2	3	4	5	6
Fasciolopsis buski | NC_030528	1						
Fasciola jacksoni | KX787886	2	0,482					
Fascioloides magna | EF534996	3	0,401	0,239				
Fasciola hepatica | AP017707	4	0,291	0,331	0,224			
Fasciola gigantica | NC_024025	5	0,358	0,248	0,244	0,168		
Hippo liver fluke 1	6	0,401	0,328	0,225	**0,180**	**0,144**	
Hippo liver fluke 2	7	0,343	0,284	0,206	**0,164**	**0,106**	**0,025**

Table S2: **Corrected rDNA genetic distances for the subfamily Fasciolinae.** Corrected genetic distances (Tamura-Nei model with gamma correction [G=0.05]) between members of the subfamily Fasciolinae based on 2771 bp of the rDNA region. All sequences obtained during this study are indicated in bold together with important p-distances relevant for species identification. GenBank accession numbers are provided after the "|" separator. *Fasciolopsis buski* ("GenBank: MN970005"), from the subfamily Fasciolopsinae, was used as outgroup.

Species		1	2	3	4	5
Fasciolopsis buski | MN970005	1					
Fasciola jacksoni | MN970006	2	0,113				
Fascioloides magna | EF051080	3	0,155	0,034			
Fasciola hepatica | MN970007	4	0,113	0,030	0,044		
Fasciola gigantica | MN970009	5	0,109	0,030	0,046	0,005	
Hippo liver fluke 1 & 2	6	0,107	0,031	0,045	**0,002**	**0,004**

Table S3: **Corrected *COI* genetic distances for the genus *Radix*.** Corrected genetic distances (Tamura-Nei model with gamma correction [G=0.16]) between *Radix* species based on a 463 bp *COI* fragment. All sequences obtained during this study are indicated in bold together with

important p-distances relevant for species identification. GenBank accession numbers are provided after the "|" separator. *Physella acuta* ("GenBank: NC_023253"), from the family Physidae, was used as outgroup.

Species		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
Physella acuta | NC_023253	1																					
Mwenje *Radix natalensis*	2	0,720																				
Kariba *Radix sp.*	3	0,702	0,363																			
Radix natalensis | HG977206	4	0,770	**0,017**	0,394																		
Radix rubiginosa | KY574609	5	0,683	0,361	0,396	0,325																	
Radix rubiginosa | KM067685	6	0,632	0,265	0,299	0,243	0,057																
Radix rubiginosa | MH189925	7	0,678	0,269	0,309	0,243	0,058	0,020															
Radix plicatula | MN737033	8	0,681	0,410	**0,036**	0,405	0,306	0,276	0,269														
Radix plicatula | MN737034	9	0,704	0,426	**0,040**	0,421	0,318	0,287	0,277	0,002													
Radix plicatula | MN737035	10	0,704	0,426	**0,040**	0,421	0,318	0,287	0,277	0,002	0,000												
Radix dgebuadzei | MN718571	11	0,790	0,473	0,183	0,465	0,299	0,271	0,259	0,159	0,165	0,165											
Radix dgebuadzei | MN718572	12	0,790	0,435	0,162	0,431	0,299	0,271	0,259	0,139	0,145	0,145	0,005										
Radix euphratica | MN718574	13	0,660	0,472	0,125	0,437	0,337	0,265	0,265	0,091	0,095	0,095	0,101	0,091									
Radix euphratica | MN718575	14	0,660	0,472	0,125	0,437	0,337	0,265	0,265	0,091	0,095	0,095	0,101	0,091	0,000								
Radix euphratica | MN718576	15	0,660	0,472	0,125	0,437	0,337	0,265	0,265	0,091	0,095	0,095	0,101	0,091	0,000	0,000							
Radix alticola | MH189954	16	0,603	0,397	0,210	0,392	0,333	0,345	0,333	0,171	0,178	0,178	0,119	0,119	0,099	0,099	0,099						
Radix alticola | MH189964	17	0,541	0,384	0,157	0,381	0,293	0,277	0,266	0,124	0,129	0,129	0,088	0,076	0,076	0,076	0,076	0,020					
Radix alticola | MN737036	18	0,586	0,373	0,181	0,370	0,277	0,253	0,237	0,125	0,130	0,130	0,107	0,093	0,094	0,094	0,094	0,032	0,014				
Radix auricularia | KP242354	19	0,588	0,397	0,444	0,373	0,262	0,317	0,320	0,351	0,367	0,367	0,417	0,379	0,349	0,349	0,349	0,307	0,274	0,283			
Radix auricularia | KP242340	20	0,588	0,397	0,444	0,373	0,262	0,317	0,320	0,351	0,367	0,367	0,417	0,379	0,349	0,349	0,349	0,307	0,274	0,283	0,000		
Radix auricularia | KP242345	21	0,588	0,397	0,444	0,373	0,262	0,317	0,320	0,351	0,367	0,367	0,417	0,379	0,349	0,349	0,349	0,307	0,274	0,283	0,000	0,000	
Radix makhrovi | MH189861	22	0,851	0,494	0,461	0,461	0,345	0,305	0,350	0,495	0,514	0,514	0,460	0,460	0,433	0,433	0,433	0,502	0,411	0,360	0,159	0,159	0,159

Table S4: Corrected *COI* genetic distances for the genus *Biomphalaria*. Corrected genetic distances (Tamura-Nei model with gamma correction [G=2.00]) between *Biomphalaria* species based on a 446 bp *COI* fragment. All sequences obtained during this study are indicated in

bold together with important p-distances relevant for species identification. GenBank accession numbers are provided after the "|" separator. *Planorbella duryi* ("GenBank: KY514384"), from the tribe Helisomatini, was used as outgroup.

Species		1	2	3	4	5	6	7
Mwenje *Biomphalaria pfeifferi*	1							
Biomphalaria pfeifferi | DQ084831	2	0,**026**						

Species		1	2	3	4	5	6	7	8	9	10	11	12	13
Biomphalaria peregrina | KF926180	3	0,142	0,136											
Biomphalaria straminea | KF926186	4	0,120	0,105	0,135										
Biomphalaria tenagophila | EF433576	5	0,089	0,073	0,137	0,095									
Biomphalaria glabrata | AY380531	6	0,080	0,078	0,161	0,121	0,095								
Biomphalaria sudanica | DQ084843	7	0,067	0,056	0,149	0,112	0,076	0,076							
Planorbella duryi | KY514384	8	0,222	0,219	0,230	0,225	0,197	0,245	0,218						

Table S5: Corrected *COI* genetic distances for the genus *Bulinus*. Corrected genetic distances (Tamura-Nei model with gamma correction [G=0.21]) between *Bulinus* species based on a 446 bp *COI* fragment. All sequences obtained during this study are indicated in bold together with important p-distances relevant for species identification. GenBank accession numbers are provided after the "|" separator. *Indoplanorbis exustus* ("GenBank: KR811332"), from the subfamily Bulininae, was used as outgroup.

Mwenje *Bulinus globosus*	1													
Mwenje *Bulinus* sp.	2	0,252												
Bulinus forskalii | AM286306	3	0,282	0,310											
Bulinus cernicus | AM286304	4	0,227	0,333	0,179										
Bulinus truncatus | AM286316	5	0,289	**0,045**	0,316	0,329									
Bulinus natalensis | AM921836	6	0,274	**0,031**	0,359	0,304	0,038								
Bulinus nyassanus| AM921838	7	0,266	**0,029**	0,334	0,319	0,044	0,036							
Bulinus tropicus | AM921842	8	0,290	**0,031**	0,350	0,314	0,038	0,030	0,022						
Bulinus wrighti | AM286318	9	0,164	0,173	0,296	0,242	0,199	0,197	0,180	0,191					
Bulinus globosus | AM286289	10	**0,015**	0,245	0,280	0,244	0,298	0,283	0,276	0,300	0,153				
Bulinus africanus | AM286296	11	0,134	0,288	0,308	0,254	0,281	0,306	0,289	0,289	0,213	0,133			
Bulinus angolensis | LT671976	12	0,127	0,259	0,276	0,261	0,289	0,281	0,265	0,280	0,193	0,119	0,063		
Bulinus crystallinus | LT671978	13	0,091	0,208	0,226	0,258	0,264	0,252	0,245	0,252	0,167	0,084	0,066	0,054	
Indoplanorbis exustus | KR811332	14	0,316	0,369	0,432	0,407	0,474	0,395	0,435	0,409	0,330	0,284	0,332	0,340	0,289

Table S6: Accession numbers generated in this study, Carolus *et al.* (2019), and Muzarabani *et al.* (2023). Table listing all the GenBank accession numbers (Acc. Nr.) generated by this study and those included from Carolus *et al.* (2019), and Muzarabani *et al.* (2023). The table also lists information on the sample type and/or preliminary identification (Sample), the sequenced marker (Marker), the current identification (ID), the original locality (Locality) and an identifier to show which were generated in this study and which in Carolus *et al.* (2019) or Muzarabani *et al.* (2023). The '(partial)' nominator is used to indicate that the sequence only spans part of that genetic marker.

Acc. Nr.	Sample	Marker	ID	Locality	Reference
MT884914	*Schistosoma* sp. infection from snail	*28S* (partial)-*ITS1*-*5.8S*-*ITS2*-*18S* (partial)	*S. haematobium*	Mwenje	This study
MT884915	*Schistosoma* sp. infection from snail	*ITS1* (partial)-*5.8S*-*ITS2* (partial)	*S. edwardiense*	Mwenje	This study
MT884916	*Schistosoma* sp. infection from snail	*ITS1* (partial)-*5.8S*-*ITS2* (partial)	*S. edwardiense*	Mwenje	This study
MT884917	*Schistosoma* sp. infection from snail	*ITS1* (partial)-*5.8S*-*ITS2* (partial)	*S. edwardiense*	Mwenje	This study
MT884918	*Schistosoma* sp. infection from snail	*ITS1* (partial)-*5.8S*-*ITS2* (partial)	*S. edwardiense*	Mwenje	This study
MT884919	*Schistosoma* sp. infection from snail	*ITS1* (partial)-*5.8S*-*ITS2* (partial)	*S. edwardiense*	Mwenje	This study
MT884920	*Schistosoma* sp. infection from snail	*ITS1* (partial)-*5.8S*-*ITS2* (partial)	*S. edwardiense*	Mwenje	This study
MT884921	*Schistosoma* sp. infection from snail	*ITS1* (partial)-*5.8S*-*ITS2* (partial)	*S. edwardiense*	Mwenje	This study
MT884922	*Schistosoma* sp. infection from snail	*ITS1* (partial)-*5.8S*-*ITS2* (partial)	*S. edwardiense*	Mwenje	This study
MT884923	*Schistosoma* sp. infection from snail	*ITS1* (partial)-*5.8S*-*ITS2* (partial)	*S. edwardiense*	Mwenje	This study
MT884924	*Schistosoma* sp. infection from snail	*ITS1* (partial)-*5.8S*-*ITS2* (partial)	*S. edwardiense*	Mwenje	This study
MT886702	*Schistosoma* sp. infection from snail	*COI* (partial)	*S. edwardiense*	Mwenje	This study
MT886703	*Schistosoma* sp. infection from snail	*COI* (partial)	*S. haematobium*	Mwenje	This study
MT888842	snail	*COI* (partial)	*P. columella*	Kariba	This study
MT888843	snail	*COI* (partial)	*Bulinus sp.*	Mwenje	This study

MT888844	snail	*COI* (partial)	*B. globosus*	Mwenje	This study
MT888845	snail	*COI* (partial)	*B. pfeifferi*	Mwenje	This study
MT888846	snail	*COI* (partial)	*R. natalensis*	Mwenje	This study
MT888847	snail	*COI* (partial)	*R.* aff. *plicatula*	Kariba	This study
MT893586	*Fasciola* sp. infection from snail	*28S* (partial)-*ITS1-5.8S-ITS2-18S* (partial)	*F. nyanzae*	Mwenje	This study
MT893587	*Fasciola* sp. infection from snail	*28S* (partial)-*ITS1-5.8S-ITS2-18S* (partial)	*F. nyanzae*	Mwenje	This study
MT893588	*Fasciola* sp. infection from snail	*28S* (partial)-*ITS1-5.8S-ITS2-18S* (partial)	*F. nyanzae*	Mwenje	This study
MT893589	*Fasciola* sp. infection from snail	*ITS1-5.8S-ITS2*	*F. nyanzae*	Mwenje	This study
MT893590	*Fasciola* sp. infection from snail	*ITS1-5.8S-ITS3*	*F. nyanzae*	Mwenje	This study
MT893591	*Fasciola* sp. infection from snail	*ITS1-5.8S-ITS4*	*F. nyanzae*	Mwenje	This study
MT893592	*Fasciola* sp. infection from snail	*ITS1-5.8S-ITS5*	*F. nyanzae*	Mwenje	This study
MT893593	*Fasciola* sp. infection from snail	*ITS1-5.8S-ITS6*	*F. nyanzae*	Mwenje	This study
MT893594	*Fasciola* sp. infection from snail	*ITS1-5.8S-ITS7*	*F. nyanzae*	Mwenje	This study
MT893595	*Fasciola* sp. infection from snail	*28S* (partial)-*ITS1-5.8S-ITS2-18S* (partial)	*F. nyanzae*	Mwenje	This study
MT909542	Hippo liver fluke 1	*COI* (partial)	*F. nyanzae*	Kariba	This study
MT909543	Hippo liver fluke 2	*COI* (partial)	*F. nyanzae*	Kariba	This study
MT909545	*Fasciola* sp. infection from snail	*COI* (partial)	*F. nyanzae*	Kariba	This study
MT909546	*Fasciola* sp. infection from snail	*COI* (partial)	*F. nyanzae*	Mwenje	This study
MT909547	*Fasciola* sp. infection from snail	*COI* (partial)	*F. nyanzae*	Mwenje	This study
MT909548	*Fasciola* sp. infection from snail	*COI* (partial)	*F. nyanzae*	Mwenje	This study
MT909549	*Fasciola* sp. infection from snail	*COI* (partial)	*F. nyanzae*	Kariba	This study

MT909550	*Fasciola* sp. infection from snail	*COI* (partial)	*F. nyanzae*	Mwenje	This study
MT909560	Hippo stomach fluke Type 1	*COI* (partial)	*C. cruciformis*	Kariba	This study
MT909561	Hippo stomach fluke Type 2	*COI* (partial)	Unidentified amphistome species	Kariba	This study
MT909820	Hippo liver fluke 1	*28S* (partial)-*ITS1*-*5.8S*-*ITS2*-*18S* (partial)	*F. nyanzae*	Kariba	This study
MT909821	Hippo liver fluke 2	*28S* (partial)-*ITS1*-*5.8S*-*ITS2*-*18S* (partial)	*F. nyanzae*	Kariba	This study
MK330628	99,3% match to MT909543	*COI* (partial)	*F. nyanzae*	Kariba	(Carolus *et al.*, 2019)
MK330629	99,3% match to MT909543	*COI* (partial)	*F. nyanzae*	Kariba	(Carolus *et al.*, 2019)
MK330630	99,3% match to MT909542	*COI* (partial)	*F. nyanzae*	Kariba	(Carolus *et al.*, 2019)
MK330623	99.9% match to MT909820&MT909821	*ITS1* (partial)-*5.8S*-*ITS2* (partial)	*F. nyanzae*	Kariba	(Carolus *et al.*, 2019)
MK330624	99.9% match to MT909820&MT909821	*ITS1* (partial)-*5.8S*-*ITS2* (partial)	*F. nyanzae*	Kariba	(Carolus *et al.*, 2019)
MK330625	99.9% match to MT909820&MT909821	*ITS1* (partial)-*5.8S*-*ITS2* (partial)	*F. nyanzae*	Kariba	(Carolus *et al.*, 2019)
MK333465	*P. columella*	*COI* (partial)	*P. columella*	Kariba	(Carolus *et al.*, 2019)
MK333466	*Radix* sp.	*COI* (partial)	*R.* aff. *plicatula*	Kariba	(Carolus *et al.*, 2019)
MT013349	*B. truncatus*	*COI* (partial)	*B. truncatus*	Kariba	(Muzarabani *et al.*, 2023)
MT013355	*Carmyerius* sp.	*COI* (partial)	*C. cruciformis*	Kariba	(Muzarabani *et al.*, 2023)
MT013350	Unidentified amphistome species	*COI* (partial)	Unidentified amphistome species	Kariba	(Muzarabani *et al.*, 2023)

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