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Drivers behind co-occurrence patterns between pathogenic bacteria, protozoa, and helminths in populations of the multimammate mouse, *Mastomys natalensis*

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ABSTRACT

Advances in experimental and theoretical work increasingly suggest that parasite interactions within a single host can affect the spread and severity of wildlife diseases. Yet empirical data to support predicted co-infection patterns are limited due to the practical challenges of gathering convincing data from animal populations and the stochastic nature of parasite transmission. Here, we investigated co-infection patterns between micro- (bacteria and protozoa) and macroparasites (gastro-intestinal helminths) in natural populations of the multimammate mouse (*Mastomys natalensis*). Fieldwork was performed in Morogoro (Tanzania), where we trapped 211 *M. natalensis* and tested their behaviour using a modified open-field arena. All animals were checked for the presence of helminths in their gastro-intestinal tract, three bacteria (*Anaplasma, Bartonella,* and *Borrelia*) and two protozoan genera (*Babesia* and *Hepatozoon*). Besides the presence of eight different helminth genera (reported earlier), we found that 19% of *M. natalensis* were positive for *Anaplasma,* 10% for *Bartonella,* and 2% for *Hepatozoon* species. Hierarchical modelling of species communities was used to investigate the effect of the different host-related factors on these parasites' infection probability and community structure. Our results show that the infection probability of *Bartonella* increased with the host's age, while the infection probability of *Anaplasma* peaked when individuals reached adulthood. We also observed that less explorative and stress-sensitive individuals had a higher infection probability with *Bartonella*. Finally, we found limited support for within-host interactions between micro-and macroparasites, as most co-infection patterns could be attributed to host exposure time.

1. Introduction

In natural populations, hosts are continuously exposed to multiple parasites while being acutely infected with others at the same time. The association between different parasite species or strains within the hosts (i.e., co-infections) is, however, not random (McArdle et al., 2018). Co-infection patterns can arise due to variation in the encounter rate with the different parasites depending on the host's sex (Zuk and Mckean, 1996), behaviour(Barron et al., 2015; Ezenwa et al., 2010), age or spatial variation in the distribution of the parasites (Abbate et al., 2020; Cattadori et al., 2008). They can also arise due to interactions between the parasites within the host, either direct or indirect, where infection with one parasite affects the parasitic infection probability and load of other parasites, which has important implications for the spread and pathogenicity of a disease (Clark et al., 2016; Henrichs et al., 2016; McArdle et al., 2018; Pedersen and Fenton, 2019) For example, viral loads of ranavirus in Cuban tree frogs (*Osteopilus septentrionalis*) were significantly higher in frogs that were previously exposed to the fungus *Batrachochytrum dendrobatidis* or the nematode *Aplectana hamatospicula* than individuals that were injected with the virus only (Ramsay and Rohr, 2021).

Direct interactions between parasites can occur due to resource

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competition or predation of one parasite on the other (Pedersen and Fenton, 2019; Vaumourin et al., 2015). For example, the concentration of malaria parasites inside the host has been found to increase significantly in populations that were dewormed from bloodsucking hookworms since they both compete for red blood cells (Budischak et al., 2018). Indirect interactions are often mediated through the host's immune response and can be facilitative or inhibitory (Ramsay and Rohr, 2021). Microparasites (e.g., viruses and bacteria) generally trigger the T helper 1 (Th1) branch of the adaptive immune response, whereas macroparasites (e.g., protozoa and helminths) trigger the T helper 2 (Th2) branch (Blanco and Garcia, 2008; McArdle et al., 2018). By activating one of these two branches, the host's resources will become depleted so much that it cannot properly ward off another infection. Hosts are therefore assumed to be more susceptible to microparasitic infections after infection with macroparasites. Indeed, African buffalos have a weaker Th1 response after nematode infections, facilitating the invasion of bovine tuberculosis (Ezenwa et al., 2010). In contrast, co-infections between two micro- or two macroparasites are more likely to be inhibitory because of cross-immunity and the activation of the same T helper immune response (Ramsay and Rohr, 2021). For example, a pre-existing trematode infection of *Clonorchis sinensis* is suggested to reduce the establishment of the nematode *Trichinella spiralis* in laboratory mice (Chen et al., 2013). To better understand the causal relationships between parasites, it will be crucial to disentangle the different factors that affect the presence of multiple parasites in the host. While the strongest evidence for direct parasite-parasite associations comes from long-term manipulative experiments (Ezenwa, 2016), hierarchical models can also unravel the different drivers of co-infection patterns from cross-sectionally sampled populations (Dallas et al., 2019).

Studies on rodent-borne parasites have improved our understanding of the importance of co-infections over the past decades (Pedersen and Fenton, 2019). While laboratory experiments typically inoculated mice with controlled doses of two or more parasites, field experiments allowed us to investigate the importance of co-infections under natural conditions. Those field studies mainly focused on micro-micro or macro-macroparasite interactions (Pedersen and Fenton, 2019). For example, one of the most convincing studies found that field voles (*Microtus agrestis*) were more likely to be infected with *Anaplasma, Babesia*, and *Bartonella* bacteria after a cowpox virus infection, but less likely to be infected with *Bartonella* after a *Babesia* infection (Telfer et al., 2010). Macro-microparasites interactions are less studied in rodent populations (Pedersen and Fenton 2019). One study found that bank voles (*Myodes glareolus*) infected with the nematode *Heligmosomoides mixtum* were more likely to have antibodies against Puumala hantavirus, which was explained by the immunomodulating effects of the helminth infection on the Th1 immune response (Salvador et al., 2011). In contrast, microparasites had no significant impact on the presence of the nematode *Heligmosomoides polygyrus*, suggesting that direct micro-macroparasite interactions are relatively rare in natural conditions (Luong et al., 2010).

This study focuses on micro-macroparasite co-infection patterns in the multimammate mouse (*Mastomys natalensis*) and the different factors that drive these co-infection patterns. *Mastomys natalensis* is the most important rodent pest species in sub-Saharan Africa (Leirs, 1995; Mulungu, 2017). While its natural habitat consists of savannah and grassland areas, the animal currently thrives in agricultural fields and human dwellings where outbreaks can cause crop losses of up to 80% (Mulungu, 2017; Mwanjabe et al., 2002). The rodent also hosts several zoonotic diseases, including *Yersinia pestis* (bubonic plague), *Leptospira interrogans* (leptospirosis), *Leishmania major* (cutaneous leishmaniasis) and Lassa virus (Lassa fever) (Holt et al., 2006; Mariën et al., 2019b; Meerburg et al., 2009; Monath, 1987; Neerinckx et al., 2008; Sadlova et al., 2019) as well as several ecto- and endoparasites(Brouat and Duplantier, 2007; Diagne et al., 2016; Diouf et al., 2013; Oguge et al., 1997; Ribas et al., 2017, 2013, 2012a). We recently used *M. natalensis* as a model system to investigate how host characteristics and behaviour affect the community structure of gastro-intestinal helminths (Vanden Broecke al., 2021). We observed that helminth richness was higher in adults and females than juveniles and males. Additionally, we found that less explorative individuals (observed in a modified open-field arena) had a higher infection probability with different helminths.

Here, we screened the same individuals on the presence of five microparasite genera known to cause disease in humans and livestock: (i) *Bartonella*, a gram-negative intracellular, flea-borne bacteria with a worldwide distribution (Dehio, 2004). At least 13 *Bartonella* species are responsible for human diseases such as Carrion disease, trench fever and cat-scratch disease (Gutiérrez et al., 2014). (ii) *Anaplasma*, a genus of gram-negative intracellular, tick-borne bacteria with vertebrates as their reservoir hosts. Anaplasmosis is an important issue for farmers because the disease frequently causes abortions, reduced milk production, and increased livestock mortality (Rymaszewska and Grenda, 2008). The species *Anaplasma phagocytophilum* is pathogenic for humans, as it causes human granulocytic anaplasmosis (Camprubí-Ferrer et al., 2021; Jin et al., 2012). (iii) *Borrelia*, a gram negative bacteria transmitted by ticks and lice, which can cause lyme disease and relapsing fever (Samuels and Radolf, 2010). (iv) *Babesia*, a genus of tick-borne protozoa with over 100 species, many of which are well-established threats to domestic animals and livestock (Young et al., 2019), and (v) *Hepatozoon*, a genus of apicomplexan protozoans that parasitize on a wide diversity of hosts (Smith, 1996). Veterinarians mainly know the species of *Hepatozoon* that infect cats and dogs to cause hepatozoonosis, a disease with symptoms ranging from sub-clinical in healthy pets to life-threatening in animals with extreme lethargy and anaemia (Baneth, 2011).

The main focus of this study is to investigate the micro-macroparasite co-infection patterns in a wild rodent species and to disentangle the potential factors that drive these co-infection patterns under natural conditions. We hypothesize that the host's gastro-intestinal helminth community has a significant effect on the presence of microparasites due to immunomodulation. More specifically, we predict that infection with helminths will increase the probability of becoming infected with microparasites, even after correcting for host characteristics such as sex and age. Additionally, we expect that highly explorative individuals are more likely to be infected with microparasites compared to less explorative individuals due to the higher chances of encountering an infected vector (e.g., tick, fleas, mites) throughout their life (Barber and Dingemanse, 2010; Bohn et al., 2017; Boyer et al., 2010).

2. Material and methods

2.1. Study site and trapping

The field setup, rodent collections, behavioural measurements, and helminth screening are described in detail in Vanden Broecke et al. (2021a). In brief, rodents were trapped on eleven different sites in both maize fields and fallow lands on the Sokoine University of Agriculture farm (SUA) in Morogoro, Tanzania, from July until September 2019. Traps were placed in the late afternoon and checked in the early morning, and captured rodents were brought to the Institute of Pest Management Centre (IPM).

The behaviour of the rodents was measured immediately after they arrived at the IPM using a hole-board test, which is used to study exploration and stress-sensitivity behaviour in *M. natalensis* (Vanden Broecke et al., 2018, 2019, 2021a, b, c). Behavioural recordings started when the individual was inside the box and lasted for 10 min. During this period, we measured five different behaviours: activity, the number of times they sniffed one of the blind holes, number of head dips, the time they spent grooming and the number of jumps. After each recording, the box was cleaned with 70% ethanol to remove animal scent and dirt. For all animals, we noted their body weight, sex, and reproductive status where we considered the individuals as juvenile if signs of sexual activity could not be observed (scrotal testes in males;

perforated vagina or pregnancy in females), following a study by Leirs in 1994. Blood samples were taken from the retro-orbital sinus when the animal was still alive and preserved on pre-punched filter paper (Serobuvard, LDA 22, Zoopole) (Mariën et al., 2017). We then euthanized the rodents using a halothane overdose followed by cervical dislocation. We collected a small piece of the kidney, liver, lung, salivary glands, and brain and stored it in 100% ethanol. Afterwards, we removed the whole gastro-intestinal tract and kept it in a 50 ml tube with 100% ethanol for further analysis at the parasitology lab at the University of Barcelona (Ribas et al., 2011). The helminths were identified to genus or species level using morphological characteristics previously described in Ribas et al. (2012a, 2017), Vanden Broecke et al. (2021a).

2.2. Age estimation

The eyes of the rodents were stored in 10% formaldehyde and used for age determination (Leirs et al., 1990b; Morris, 1972). Eye lenses were extracted with forceps, cleaned, dried for 2 h at 100 °C and weighed to the nearest 0.1 mg (Leirs et al., 1990b). We used the equation, published in Leirs et al. (1990b) to estimate the age of each individual (see Figure 1 in supplementary information). We noticed that the age distribution of the individuals had some outliers, as 23 individuals were older than 200 days (see Figure 1 in supplementary information). These individuals would significantly distort the regression analysis if we included the individual's age as a continuous covariate based on the dry eye lens weight. We therefore decided to divide the individuals into three separate age categories: (i) younger than 50 days ($N = 100$), (ii) between 50 and 200 days ($N = 88$) and (iii) older than 200 days ($N = 23$). Individuals in the first category are less than two months old and generally considered as juveniles (Coetzee, 1975; Leirs et al., 1993). The second category consists of reproductive active animals between 2–6 months old. These individuals were born in the same year when the fieldwork was performed since the fieldwork was conducted from July until September, and the breeding season of *M. natalensis* starts in March-May (Leirs, 1995). The third category consists of individuals older than 6 months (mean = 304 days, max = 491 days old) who are most likely born during the previous year's breeding season.

2.3. Pathogen DNA detection

Genomic DNA from the tissues was extracted using the NucleoSpin® Tissue DNA Extraction kit (MACHEREY-NAGEL GmbH & Co. KG, Germany) according to the manufacturer's protocol. We pooled tissues of the kidney, liver, and lungs per individual to have 25 mg in total. Instruments used in the dissection of each rodent to obtain samples were cleaned with 5 % Virkon® (Antec International, United Kingdom) and dried after each rodent to avoid contamination. DNA quality and quantity were checked for each sample using a Qubit fluorometer (Thermo Fisher Scientific, Germany). The DNA extracts were then stored at −20 °C until PCR analysis.

First, all DNA extracts were screened for the presence of *Bartonella, Anaplasma, Borrelia*, and *Babesia* using real-time qPCR systems with primers and probes (Dahmana et al., 2020). Amplification reactions were conducted in a final volume of 20 μL containing 10 μL of 2xEurogentec TakyonTM Mix (Eurogentec, Liège, Belgium), 1 μL of each primer (0.5 μM), 2.5 μL of DNase-free water, and 5 μL of DNA template. The qPCR was performed on the StepOne™ Real-Time PCR system (by Thermo Fisher Scientific, Germany) using the following thermal profile: an incubation step at 50 °C for two minutes for eliminating PCR amplicons' contaminant, then an activation step at 95 °C for three minutes followed by 40 cycles of denaturation at 95 °C for 15 s and an annealing-extension at 60 °C for 30 s. Samples with a Ct value below 35 were screened a second time (duplicates). An individual was considered positive on the qPCR if tested positive during both runs.

We then screened all extracts for the presence of *Hepatozoon* using a broad-range conventional PCR system (Dahmana et al., 2020). We also used conventional PCRs to amplify and sequence all qPCR-positive samples for *Anaplasma* targeting the 23S gene (Dahmana et al., 2020) and *Bartonella* targeting the gltA and 16S-23S rRNA ITS region (Böge et al., 2021) The amplification reactions were conducted in a final volume of 25 μL, containing 12.5 μL of Amplitaq Gold master mix (Thermo Fisher Scientific, Germany), 0.5 μL of each primer, 2.5 μL of DNA template, and 9 μL of DNA free water. Reactions were performed in an automated thermal cycler (TProfessional Basic Thermocycler by Biometra) following the specific thermal cycling profile. For *Anaplasma* and *Hepatozoon*: one incubation step at 95 °C for 15 min, 40 cycles of 60 s at 95 °C, 30 s at annealing temperature and 1 min at 72 °C and a final extension step of 5 min at 72 °C. Amplification of the gltA *Bartonella* gene consisted of 45 cycles of denaturation at 95 °C for 30 s, annealing at 53 °C for 30 s and elongation at 72 °C for 1 min. Amplification of the ITS *Bartonella* gene consisted of 40 cycles for 30 s at 94 °C, for 30 s at 66 °C, for 50 s at 72 °C. PCR products were prepared with DNA Gel Loading Dye (6 ×) (Thermo Fisher Scientific Baltics UAB, Vilnius, Lithuania) for gel electrophoresis in 2% agarose. The results were visualized by UV light using the Syngene Geneflash Network Bio-Imaging device. Amplicons of positive samples were purified and sent to the Vlaams Institute of Biotechnologie for Sanger Sequencing with forward and reversed primers. Sequences were trimmed using Geneious and compared to available sequences in GenBank with BLASTn. Individuals were considered to be positive for *Anaplasma* or *Bartonella* if they were positive on the qPCR after replication, and we obtained Sanger sequences from the conventional PCR for at least one gene. If no sequences were obtained, they were considered uncertain if we obtained at least repeated positive results on the qPCR. The categorization was made because the qPCR is generally a more sensitive approach than the conventional PCR (which targets larger DNA amplicons).

2.4. Statistical analysis

We used Hierarchical Modelling of Species Communities (HMSC) to test which host characteristics and behaviours affect the infection probability of *Bartonella* and *Anaplasma* and if co-infection patterns exist with the different helminths (Ovaskainen et al., 2017; Ovaskainen and Abrego, 2020). We did not consider the other microparasites because they were either not found (*Borrelia* and *Babesia*) or were present at a too low prevalence (*Hepatozoon*; see results). HMSC is a joint species distribution model that includes a hierarchical layer to investigate how species respond to environmental covariates depending on different species traits and phylogenetic relationships (Abrego et al., 2017; Warton et al., 2015). To correct for the presence of uncertain samples (i.e., individuals that were repeatedly positive on the qPCR but negative on sequencing), we ran two different models where we considered all uncertain samples either as negative or positive. This allows testing if the assignment of the uncertain status affects the main results and conclusions.

We considered the individuals' identity as the sample unit which was then subsequently nested as a random effect within the site in which the individual was trapped (which was added as another random effect) in both models. Three fields were less than 100m from each other and considered one (see Figure 2 supplementary information). As fixed effects (the n × n_c matrix X of HMSC; see Ovaskainen et al., 2017; Ovaskainen & Abrego, 2020); where n_c is the number of individual-specific regression parameters to be estimated, we included the individuals' sex (male or female) and their exploration and stress-sensitivity behaviour expressed in the hole-board test (more information in Vanden Broecke et al. (2019)).

We created two models to control for the individual's age (exposure time). In the first model we divided the individuals into two age categories based on their reproductive state. This distinction is commonly used as a proxy for age in Capture-Mark-Recapture studies (Leirs et al., 1990b; Mayamba et al., 2020; Morris, 1972). Eye lens weight is a more accurate estimation of age in *M. natalensis*, but this data is unavailable

during capture-recapture studies. We, therefore, created a second model where we used the three age categories (described previously) derived from the individual's eye lens weight to correct for the individual's age. Comparing these two models will allow us to assess the effect of a more accurate age description, and thus exposure time, on the results.

In total, we ran 4 different models: Two models where the individual's age was defined by reproductive state (adult or juvenile) in which we considered the uncertain *Anaplasma* and *Bartonella* samples either as negative or positive; and two models where the individual's age was defined by eye lens weight (<50 days old, 50 < X < 200 days and X > 200 days old) where the uncertain samples were considered to be either positive or negative. These 4 HMSC models were fitted with the R-package Hmsc (version 3.0-9; Tikhonov et al., 2020) using prior default distributions (Ovaskainen and Abrego, 2020). We sampled the posterior distribution with five Markov Chain Monte Carlo (MCMC) chains, each run with 3,000,000 iterations of which the first 1,000,000 were removed as burn-in. The chains were thinned by 1,000 to yield 2,000 posterior samples per chain, resulting in 10,000 posterior samples in total. We examined MCMC convergence using the potential scale reduction factors of the model parameters (Ovaskainen and Abrego, 2020). The explanatory and predictive power of all four models were analyzed using the species-specific AUC and Tjur's R^2 (Tjur, 2009) values (Pearce and Ferrier, 2000). Explanatory power was computed by making predictions based on models fitted to all data. Predictive ability was calculated by performing 5-fold cross-validation. The sampling units were assigned randomly to five-folds, and predictions for each fold were based on models fitted to data on the remaining four-folds. All statistical analyses were executed using R software 3.6.0 (R Core Team, 2016) summarized in the R-markdown file in the supplementary information.

3. Results

3.1. PCR results

We had a total sample size of 211 individuals ($N_{male\ adult} = 54$, $N_{male\ juvenile} = 21$, $N_{female\ adult} = 112$, $N_{female\ juvenile} = 24$). Overall 22 individuals tested two times positive for *Bartonella* on the qPCR (10.4% CI 6.7-15.3%). A complete sequence for the gltA and ITS genes was obtained for 14 and 15 individuals, respectively. For seven individuals, we did not obtain any sequence, despite being repeatedly positive in the qPCR (these individuals were given the uncertain *Bartonella* status). All sequences belonged to the same *Bartonella* strain (identical ITS sequences represented by OL984911) with the highest similarity in GenBank to *Bartonella mastomydis* (KY555067, 95.53%, Senegal, *Mastomys erythroleucus*) closely followed by *Bartonella elizabethae* (LR134527, 95.48%, human, United Kingdom) based on both genes, thus probably representing a new species. For the citrate synthase gene (gltA), all sequences confirmed the *Bartonella* genus (identical sequences represented by KM233487, 100%, Kenya, *Mastomys natalensis*). For *Anaplasma*, 40 individuals tested positive (18.9% CI 13.9-24.9%). A complete amplicon sequence of the 23S gene was obtained for 31 individuals. The sequences belonged to two different strains (represented by accession numbers OL982744 and OL982748). One strain was most similar to an uncultured *Anaplasma* strain (MT269273, 96.9%, *n*=29, Gabon, *Lemniscomys striatus*) in GenBank; the other strain was most similar to an uncultured *Ehrlichia* strain (MK942592, 98.13%, *n*=2, Democratic Republic of Congo, *Rhipicephalus complanatus*). We did not obtain any sequence for nine individuals, although they were repeatedly positive on the qPCR (*Anaplasma* uncertain status). For *Hepatozoon*, five individuals tested positive (2.4% CI 1.0-5.4%), and all belonged to the same strain (represented by acc. No: OL982745), which was highly similar to *Hepatozoon* cf. *ophisauri* (MN723845, 99.1%, Iran, *Pseudopus apodus*) in GenBank. All animals tested negative for *Borrelia* and *Babesia*.

3.2. Hierarchical modelling of species

To investigate if co-infections between the micro and macroparasites occurred and to test if any host characteristics and behaviours could affect the infection probability of *Bartonella* and *Anaplasma*, we constructed four HMSC models. The MCMC convergence of the four models was satisfactory. The potential scale reduction factors for the β-parameters (which measured the responses of the different parasitic species to the other intrinsic covariates; Ovaskainen et al., 2017) of the two models in which the age classes were categorized based on eye lens weight were, on average 1.000 (max = 1.002) for the positive model and 1.001 (max = 1.005) for the negative model. The β-parameters of the two models in which the age classes were categorized based on the individual's reproductive state were on average 1.001 (max = 1.009) for the positive model and 1.003 (max = 1.021) for the negative model. All four models fitted the data adequately (Supplementary Table 4, Figures 3 & 4). The explanatory power was similar for all four model, but the preditive power was slighltly higher in the models where the age classes were defined by eye lens weight (Supplemenatary table 4).

The host's age explained a large amount of variation in all four models, but the proportion was substantially higher in the models where age was defined based on the eye lens weight (Supplementary Table 2 & Figure 3). The infection probability for *Bartonella* increased significantly with age (Figure 1 & Supplementary Table 3). Indeed, the model with age classes defined by reproductive state revealed that adults were more likely to be infected with *Bartonella* than juveniles (Figs. 1 & 2D). This effect was even stronger in the model where the age classes were defined by eye lens weight (Figs. 1 & 2C). The individual's age had also an effect on the infection probability of *Anaplasma.* The model with reproductive state revealed that adults were more likely to be infection with *Anaplasma* than juveniles (Figs. 1 & 2B). However, the models based on eye lens weight showed that the infection probability was the highest for individuals that were 50 and 200 days old compared to those that were younger than 50 days or over 200 days (Figs. 1 & 2A).

Variance partitioning showed that the proportion of variation explained by the host's sex was similar in the four models (Supplementary Table 2 & Figure 3). We found that females were more likely to be infected with *Bartonella* than males. This effect, however, was only present in 1 model, where the age classes were defined based on eye lens weight and the uncertain samples were considered to be positive (Figure 1 & supplementary Table 3). There were no differences between males and females in infection risk with *Anaplasma* in all four models (Figure 1 & supplementary Table 3). The proportion of variation explained by the hosts' behaviour was almost similar in all four models (Supplementary Table 2 & Figure 3). The host's behaviour affected the infection probability of *Bartonella* (Figure 1 & Supplementary Table 3). Here, we observed a significant negative effect of both exploration (Figs. 1 & 3A, B) and stress-sensitivity (Figs. 1 & 3C, D) on the infection probability of *Bartonella* in all four models (Figure 2, supplementary table 1).

The models where age classes were defined based on reproductive state revealed that all parasite species, except *Anaplasma*, were positively correlated with each other at the within-individual level, after controlling for host-associated and spatial confounding factors (Fig. 4B, C). This positive co-infection pattern on the within-individual level was significant (>95 posterior support) for three helminths: *Protospirura muricola, Trichuris mastomysi*, and *Trichostrongylidae*; and *Bartonella* (Fig. 4B, C). The latter, however, was only significant in the model where the uncertain *Bartonella* samples were considered as positive (Fig. 4B, C), suggesting that this effect is not well supported. This positive co-occurrence pattern between parasite species was absent in the models where the age classes were defined based on eye lens weight (Fig. 4A, C). These models revealed a negative co-infection pattern between *Davaineidae* and *Hymenolepis* sp. which was only significant in the model where the uncertain *Anaplasma* and *Bartonella* samples were considered as negative (Fig. 4A, C).

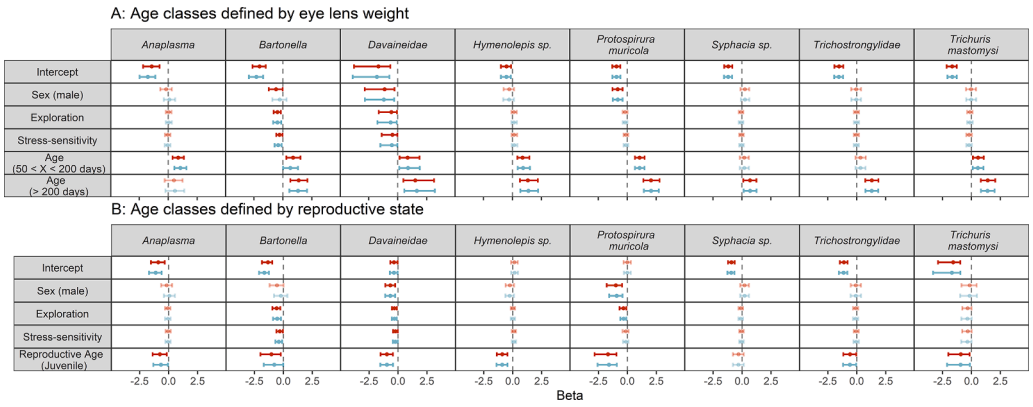

Fig. 1. Posterior mean responses of the different parasite species to the fixed effects derived from (A) the models where the age classes were defined based on the dry eye lens weight and (B) the models where age classes were defined based on reproductive state. The points are the posterior mean and the error bars represent the 95% credible interval. The colors represent two different models, where the uncertain Anaplasma and Bartonella samples were either classified as positive (red) or negative (blue). Estimates where the credible intervals do not overlap with zero are made more visible. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

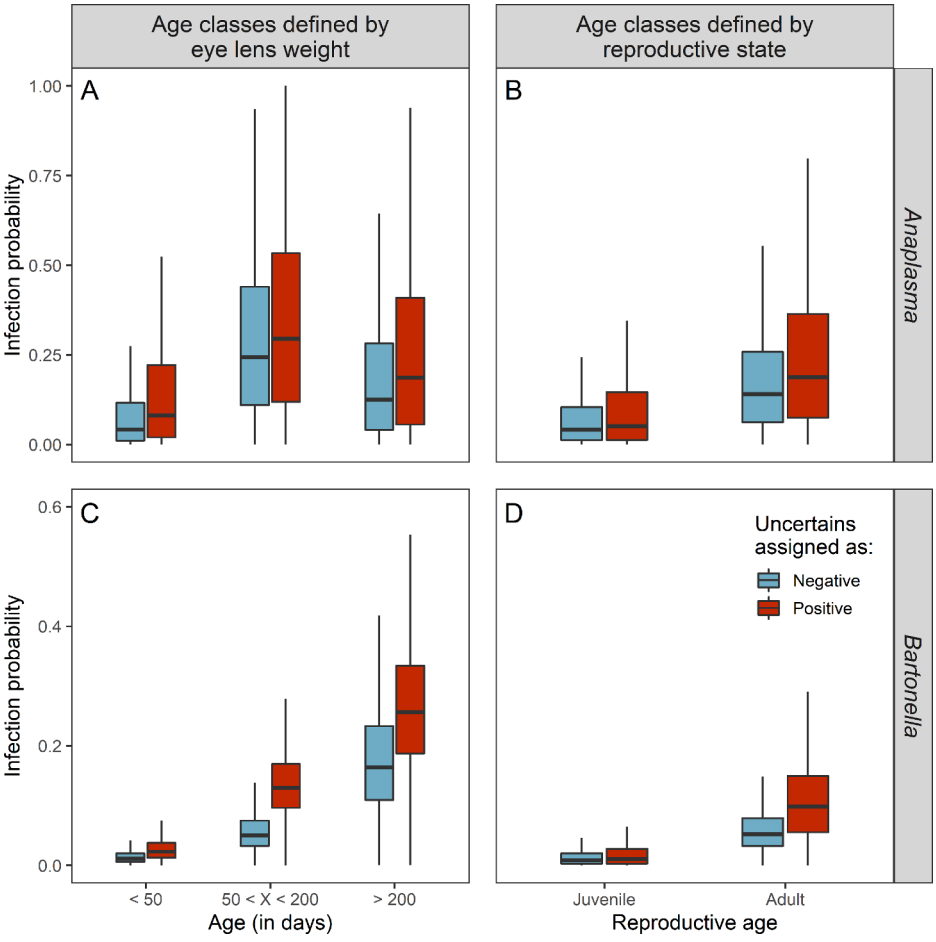

Fig. 2. Predicted infection probability of Mastomys natalensis for (A–B) Anaplasma and (C–D) Bartonella for the different age classes, which were either defined using (A–C) the individuals eye lens weight or (B–D) reproductive state. Different infection probabilities were estimated when considering uncertain samples to be positive (red) or negative (blue). Boxplots show the estimated infection probability of all posterior samples (n=10,000). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

4. Discussion

In this study, we investigated the link between the gastro-intestinal helminth community and the presence of different microparasites in *M. natalensis*. Three microparasites genera were detected from the five included in our screening panel: *Bartonella, Anaplasma*, and *Hepatozoon*, which is, to our knowledge, the first observation of these pathogens in rodent populations in Morogoro, Tanzania. We found that the host's behaviour correlated with *Bartonella* infection risk, but not *Anaplasma*. Additionally, we found that the individual's age, and consequently exposure time, was correlated positively with the infection risk for *Bartonella* and *Anaplasma.* Although for the latter it was the highest when the host was between 50 and 200 days old.

4.1. Hepatozoon

Our screening revealed that 2.3% (5/211) of *M. natalensis* were infected with *Hepatozoon ophisauri* (99.23% similarity). The protozoan species in this genus all have a life cycle that involves an intermediate vertebrate host and a definitive blood-feeding invertebrate host (ticks, mites, sandflies, and mosquitos) (Kamani et al., 2018). Unlike most vector-borne parasites that are transmitted during a blood meal, the

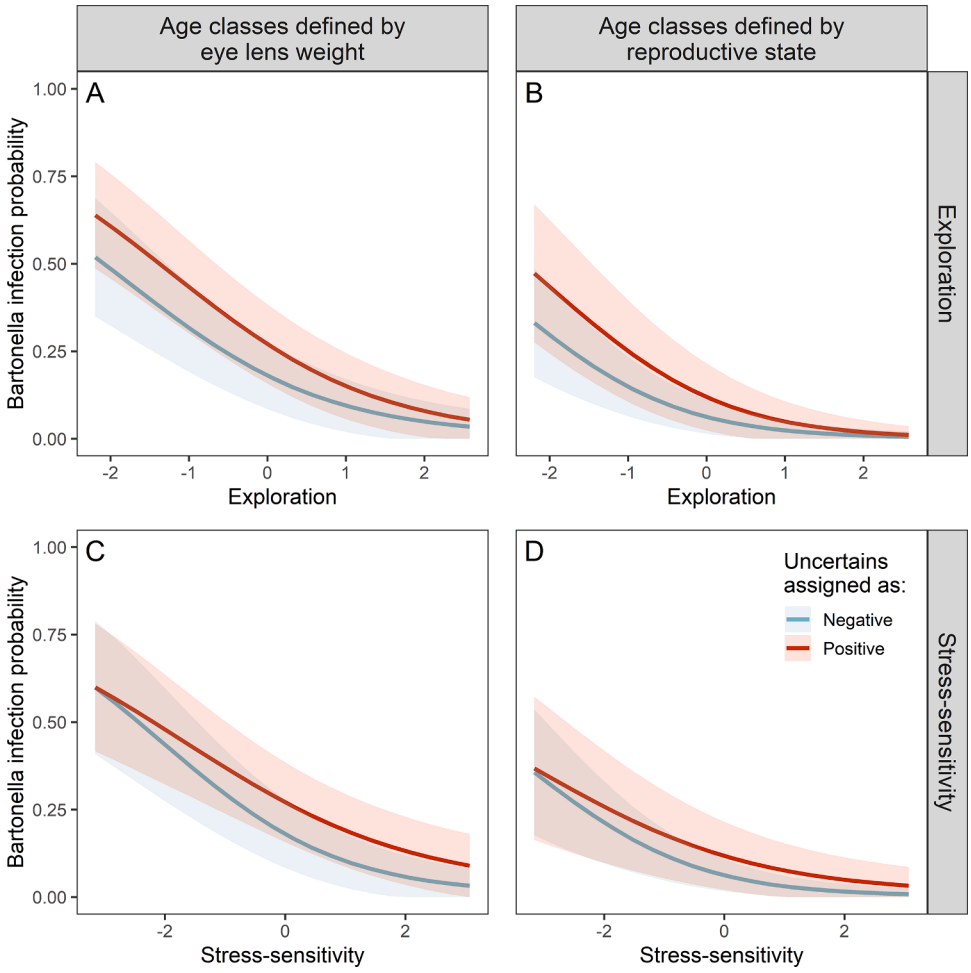

Fig. 3. Predicted infection probability of Bartonella as a function of exploration (A–B) and stress-sensitivity (C–D), based on the models where age classes were either defined using dry eye lens weight (A–C) or reproductive state (B–D). Different infection probabilities were estimated when considering uncertain samples to be positive (red) or negative (blue). Envelops represent standard deviation around the estimated infection probability of all posterior samples (n=10,000). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

transmission of *Hepatozoon* to vertebrates occurs by ingestion of the infected invertebrates (Smith, 1996) This species was previously found in Iranian lizards (*Pseudopus apodus*), which are suggested to serve as an intermediate host with snakes as a final vertebrate host (Zechmeisterová et al., 2021). Besides lizards, our data indicate that rodents can be additional intermediate hosts that are prey for snakes. Indeed, predation of rodents might represent an essential route for *Hepatozoon* transmission, as rodents are more likely to eat invertebrates and snakes can be successfully infected by consuming infectious rodents' tissues (Sloboda et al., 2008).

4.2. *Bartonella*

The *Bartonella* species that we detected in *M. natalensis* in Morogoro was genetically most closely related to *B. mastomydis*, which was previously isolated from *Mastomys erythroleucus* in Senegal (Dahmani et al., 2018). The infection probability was higher in adults compared to juveniles and increased with the host's age. This age-dependent infection risk is expected given that *Bartonella* infections are usually long-lasting in the natural reservoirs (up to several months in European rodent populations), and older individuals have more chance of acquiring an infection during their life (Gutiérrez et al., 2015) Additionally, we found that infection probability was higher in females compared to male mice, but this effect was only significant in one model (in which we used the reproductive state to define age classes and where the uncertain samples were considered as positive). This may indicate that sex has no, or only a small, effect on *Bartonella* infection risk and corresponds with most studies which reported no difference in *Bartonella* prevalence between sexes. However, minor differences were observed in *Apodemus sylvaticus* and *A. flavicollis*, in which males presented higher infection prevalence than female rodents (Gutiérrez et al., 2015; Welc-Faleciak et al., 2010).

Contrary to our expectations, we observed in all models that less explorative individuals are more likely to become infected with *Bartonella*. This pattern has been observed previously in *M. natalensis* for both helminth and viral infections, in which less explorative individuals are more likely to be infected with different helminths and to be antibody-positive against Morogoro arenavirus (Vanden Broecke et al., 2021a). There are three potential, non-mutually exclusive explanations for these results. The first explanation is that exploration behaviour, expressed in the hole-board test, relates to space use in the wild. Indeed, such an indirect correlation has been found in Siberian chipmunks (*Tamias sibiricus*), where exploration behaviour in an open-field arena correlated with activity in the field, resulting in a higher tick load (Boyer et al., 2010). It is, however, currently unclear whether this is the case in *M. natalensis*. The second explanation is that this link is driven by an underlying correlation between exploration behaviour and immunological investment, whereby highly explorative individuals are more immunologically competent than less explorative individuals. Indeed, several studies have shown that bolder and more explorative individuals have a better innate immune system or more MHC alleles, which reduces the risk of infection when exposed (Garamszegi et al., 2015; Kortet et al., 2010; Zylberberg et al., 2014). The third and last explanation is that parasitic infection risk leads to behavioural modification due to sickness effects, where energy is diverted to the host immune response rather than expressing certain behaviours (Poulin, 2013).

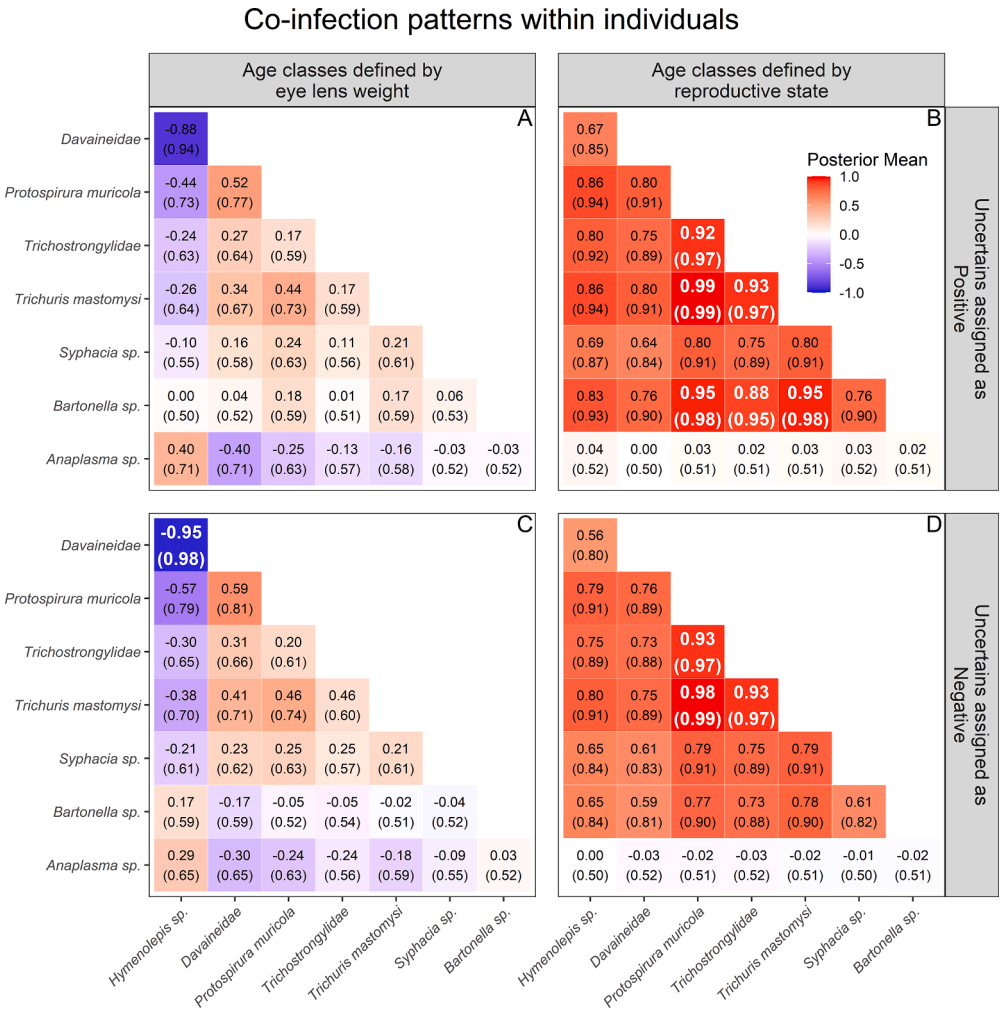

Fig. 4. Co-infewction patterns (posterior mean and support) of the different micro-and macroparasites detected in *Mastomys natalensis* on the within individual scale, derived from the models where age classes were defined either by eye lens weight (A–C) or reproductive state (B–D) where the uncertain Anaplasma and Bartonella samples were considered to be positive (A–B) or negative (C–D). Posterior means with more than 95 support are marked in white.

4.3. Anaplasma

The *Anaplasma* species detected in this study were most closely related to uncultured *Anaplasma* (14% prevalence) and *Ehrlichia* strains (1% prevalence) previously detected in rodents and ticks from Africa. The *Anaplasma* strain was most closely related to a strain found in *Lemniscomys striatus* in Franceville in Gabon (Mangombi et al., 2021). The *Ehrlichia* strain was mainly similar to a strain detected in a *Rhipicephalus* tick (family *Ixodidae*) collected from livestock in Kisangani, Democratic Republic of Congo (Ngoy et al., 2021). Similar to *Bartonella*, we found that adults were more likely to be infected with *Anaplasma* than juveniles. However, when we defined the age classes based on eye lens weight, we found that this correlation was nonlinear, as opposed to *Bartonella*. The results from those models revealed that individuals that were between 50 and 200 days old had a higher infection risk than individuals that were younger or older. This could suggest that *M. natalensis* is able to clear the bacteria more efficiently than *Bartonella* after which they become resistant. Similarly, *Peromyscus* mice were more effective at eliminating *Anaplasma phagocytophilum* than *Borrelia burgdorferi* (Barbour, 2017). This is supported by experimental inoculations, where the infection period of mice that were infected with *B. burgdorferi* can persist for a year or longer (Barthold et al., 1993), while *A. phagocytophilum* is cleared after 35 to 60 days (Massung et al., 2004).

4.4. Co-infections in M. natalensis

Vanden Broecke et al. (2021a) have shown that the prevalence of different helminths is relatively high in *M. natalensis* and co-infections with these macroparasites are common. In this study, we focused on microparasites, and we have shown that micro-macro parasite co-infections also occur. However, the causal relationship underlying these co-infection patterns is potentially more complex than initially considered and might depend on both extrinsic and intrinsic factors.

The models where we defined "age" based on their reproductive state showed that all parasites (except *Anaplasma*) co-occurred positively with each other. These results suggest that individuals infected with one parasite species were more likely to be infected with other species as well. A potential explanation is that infection with one parasite reduces the host's immune system, making them more vulnerable to subsequent infection (Boulouis et al., 2005; Telfer et al., 2010). Another explanation is these results were driven by unaccounted variation among the individuals (Vanden Broecke et al., 2021a). One potential source of unaccounted variation is the individual's exposure time to the parasite, i.e. age. To explore this explanation, we estimated the individual's age more accurately using their eye lenses (Leirs et al., 1990b; Morris, 1972). When we reran the HMSC models, using eye lens weight instead of reproductive state to define the age classes, we found that the proportion of variation explained by the individual's age almost doubled compared to the models based on reproductive state, while the residual variation

on the individual level halved. The positive co-infection patterns on the within-individual level, on the other hand, disappeared. Together, these results suggest that the positive co-infection patterns are partly the result of unaccounted variation in exposure time between individuals born in different years.

In conclusion, we found that *M. natalensis* are exposed to multiple parasites at the same time and can constitute multiple zoonotic pathogens, of which the epidemiology is still poorly studied, especially in Africa. The fact that *M. natalensis* is often present near human dwellings suggests that humans and livestock are likely to come into contact with these parasites (Mariën et al., 2018, 2019a, 2020). We have shown that both behaviour and exposure time are important in determining the infection risk for *Anaplasma* and *Bartonella*. While our data will allow designing further ecological studies on the risks associated to exposure with *M. natalensis*-borne parasites, medical and veterinary studies need to be conducted to elucidate the role of these parasites in human and animal pathology. Additionally, longitudinal studies (e.g. capture-mark-recapture) and experimental inoculation experiments are needed to gain more insight into the causal directionality of the underlying patterns (Mariën et al., 2019b; Pedersen and Fenton, 2019; Telfer et al., 2010).

Ethics statement

All experimental procedures were approved by the University of Antwerp Ethical Committee for Animal Experimentation (2016-63), adhered to the EEC Council Directive 2010/63/EU, and followed the Animal Ethics guidelines of the Research Policy of the Sokoine University of Agriculture.

Availability of data and materials

The biological material and data used in the current study are available from the corresponding author on reasonable request. The generated sequences were deposited in GenBank with the following accession numbers: OL982744, OL982745, OL982748 & OL984911. The data-analysis is available in the R-markdown file.

CRediT authorship contribution statement

Bram Vanden Broecke: Conceptualization, Data curation, Formal analysis, Methodology, Writing – original draft, Writing – review & editing. **Pamela Jones June Tafompa:** Methodology, Writing – review & editing. **Baraka Edson Mwamundela:** Methodology, Writing – review & editing. **Lisse Bernaerts:** Writing – review & editing. **Alexis Ribas:** Methodology, Supervision, Writing – review & editing. **Ladslaus L. Mnyone:** Methodology, Supervision, Writing – review & editing. **Herwig Leirs:** Funding acquisition, Supervision, Writing – review & editing. **Joachim Mariën:** Conceptualization, Data curation, Supervision, Writing – original draft, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.actatropica.2023.106939.

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