Variation in local and systemic pro-inflammatory immune markers of wild wood mice after anthelminthic treatment

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Abstract

The immune system represents a host’s main defence against infection to parasites and pathogens. In the wild, a host’s response to immune challenge can vary due to physiological condition, demography (age, sex), and coinfection by other parasites or pathogens. These sources of variation, which are intrinsic to natural populations, can significantly impact the strength and type of immune responses elicited after parasite
exposure and infection. Importantly, but often neglected, a host’s immune response can also vary within the individual, across tissues and between local and systemic scales. Consequently, how a host responds at each scale may impact its susceptibility to concurrent and subsequent infections. Here we analysed how characteristics of hosts and their parasite infections drive variation in the pro-inflammatory immune response in wild wood mice (*Apodemus sylvaticus*) at both the local and systemic scale by experimentally manipulating within-host parasite communities through anthelminthic drug treatment. We measured concentrations of the pro-inflammatory cytokine TNF-α produced *in vitro* in response to a panel of TLR agonists at the local (mesenteric lymph nodes, MLN) and systemic (spleen) scales of individuals naturally infected with two gastrointestinal parasites, the nematode *Heligmosomoides polygyrus* and the protozoan *Eimeria hungaryensis*. Anthelminthic-treated mice had a 20-fold lower worm burden compared to control mice, as well as a 7-fold higher intensity of the non-drug targeted parasite *E. hungaryensis*. Anthelminthic treatment differentially impacted levels of TNF-α expression in males and females at the systemic and local scales, with treated males producing higher, and treated females lower, levels of TNF-α, compared to control mice. Also, TNF-α was affected by host age, at the local scale, with MLN cells of young, treated mice
producing higher levels of TNF-α than those of old, treated hosts. Using complementary, but distinct, measures of inflammation measured across within-host scales allowed us to better assess the wood mouse immune response to changes in parasite infection dynamics after anthelminthic treatment. This same approach could be used to understand helminth infections and responses to parasite control measures in other systems in order to gain a broader view of how variation impacts the immune response.

Introduction

Within-host parasite communities can be extremely diverse, with one individual being host to many parasite species simultaneously or sequentially over the course of its lifetime. This is especially true for individuals living in the wild, which face frequent and repeated challenges from both microparasites (e.g. viruses, bacteria, protozoa) and macroparasites (e.g. helminths, ectoparasites). The composition of these within-host parasite communities can have significant impacts on individual host health, parasite transmission dynamics and the epidemiology of each parasite species (Johnson et al. 2015; Lafferty et al. 2008; Pedersen and Fenton 2007; Poulin 1996; Rynkiewicz et al. 2015; Telfer et al. 2008). However, while we often consider the host individual as the
appropriate unit for investigating within-host processes, for parasites, a host represents a diverse ecosystem with many different habitats, each of which can be infected by different parasites (Griffiths et al. 2014; Restif and Graham 2015; Rynkiewicz et al. 2015). Therefore, a parasite’s preferred niche can be functionally determined by the properties of the tissues it infects and by the type of immune response it elicits (Graham et al. 2007; Lello and Hussell 2008; Pedersen and Fenton 2007). Correspondingly, the host immune system has evolved different mechanisms to respond to infection by a diverse set of parasites (Baucom and de Roode 2011; Hajnicka et al. 2005; Maizels et al. 2012; Parker et al. 2017). The host’s response to parasite infection can be measured at multiple sites or scales, including a local response at the site of infection which may focus on tissue repair or controlling initial parasite spread and damage, and a systemic response across the whole organism where circulating immune factors in blood and the spleen coordinate a more general response to infection. While local immune responses impact host susceptibility and infection success of parasites that infect the same location, systemic responses impact susceptibility to parasites coinfecting other tissues within the host (Munoz et al. 2005; Mwatha et al. 1998; Semenya et al. 2012; Zavala et al. 2018). Therefore, investigating parasite-host and parasite-parasite interactions at multiple within-host
scales will improve our ability to measure and predict parasite spread as well as quantify variation among hosts in how they respond to infection.

Coinfection is very common in natural host populations, and in turn can lead to within-host parasite interactions (Abu-Raddad et al. 2006; Poulin 1996; 2001; Seabloom et al. 2015). These can be direct via competition for habitat, or indirect through competition for a nutritional resource or via the host’s immune system (Budischak et al. 2015; Graham 2008; Griffiths et al. 2015; Griffiths et al. 2014; Pedersen and Fenton 2007). An analysis of human coinfection networks showed that coinfecting parasites most commonly interact though shared host resources and a common infection site (Griffiths et al. 2014). However, there was also evidence of immune-mediated parasite interactions, where one parasite can negatively or positively impact the infection success or intensity of another through a shared immune response. When parasites interact via the host immune system, it is important to consider at which within-host scales the interaction is occurring (e.g. are these parasites infecting the same or different tissues) to assess their impact on host health or parasite transmission. For example, parasites that elicit a strong systemic immune response can potentially impact host susceptibility to other coinfecting parasite
species, even if they infect different within-host habitats. This type of interaction has been shown in wild African Buffalo, where reducing gastrointestinal nematode burdens using anthelmintic treatment significantly increased individual hosts’ odds of survival after bovine TB infection, due to altered systemic inflammation (Ezenwa and Jolles 2015). Consequently, the scale at which we measure metrics of the immune response will influence our conclusions about how individuals respond to infection, and how the variation in these responses may impact parasite coinfection and transmission dynamics.

How a host responds to infection can also vary depending on demographic/physiological characteristics such as age, sex, or reproductive condition, which can impact resource allocation to local and systemic immune responses (Buehler et al. 2009; French et al. 2007; Pedersen and Greives 2008; Vandegrift et al. 2008). Studies in Soay sheep and tree swallows, for example, have shown significant changes in a host’s immune response with increasing host age, with older hosts in most cases having higher levels of inflammatory markers (Babayan et al. 2018b; Cevenini et al. 2013; Nussey et al. 2012; Palacios et al. 2007). Further, there are key immunological differences between the sexes, with males often being less immuneresponsive than females. These differences are often attributed to steroid hormones, such as
testosterone, whose immunosuppressive effects can also vary across seasons (Demas and Nelson 1998; Lehmer et al. 2010; Pap et al. 2010). There are also immunological differences within the sexes, dependent on life-history stage. For example, pregnant females often have lower levels of inflammatory markers than non-reproductively active females (Chatterjee et al. 2014; Gorsich et al. 2014; Martin et al. 2008). These cases suggest the physiological trade-offs between sexually-selected traits and the immune response are an evolutionarily adaptive mechanism (Folstad and Karter 1992; McKean and Nunney 2005; Stoehr and Kokko 2006; Zuk 1996), emphasising the importance of studying the impacts of these trade-offs in evolutionarily- and ecologically-relevant contexts.

One key immunological process that can be measured at both the local and systemic within-host scales is inflammation, broadly defined as the recruitment of immune cells and signalling molecules to the site of an infection (Sompayrac 2012). Inflammation occurs early in an infection, involves both the innate and adaptive arms of the immune system, initiates tissue repair or containment, and/or clearance of an infectious agent. Inflammation is maintained by positive feedbacks, which are then disrupted or deactivated by subsequent regulatory or anti-inflammatory signals upon clearance of infection (Sears et al.
One key cytokine involved in controlling inflammation is tumour necrosis factor alpha (TNF-α), a pro-inflammatory cytokine which can activate both innate and adaptive immune responses (Bradley and Jackson 2008; Graham et al. 2007; Pasparakis et al. 1996; Tumanov et al. 2010; Vassalli 1992). In addition, it has been suggested that TNF-α levels indicate a host’s ability to respond to a broad range of parasites, such as worms, bacteria, viruses, or other microbial pathogens (Goff et al. 2002; Sjöwall et al. 2005; Smith and Ovington 1996; Zavala et al. 2018).

Here, we investigated the interactions between TNF-α as a marker of inflammation and parasite infection and coinfection in wild wood mice (Apodemus sylvaticus). Wood mice are commonly infected with a diverse community of parasites (Behnke et al. 2009; Bown et al. 2006; Knowles et al. 2013), including the gastrointestinal nematode Heligmosomoides polygyrus, which is closely related to H. bakeri, an established laboratory model for human nematode infections (e.g. Maizels et al. 2004; Behnke et al. 2005; Reynolds et al. 2012). H. bakeri is known to elicit strong immunomodulatory effects on the host’s immune system and skews the host response from a pro-inflammatory (Th1-type) immune profile to an anti-inflammatory (Th2/Treg-type) immune profile (Maizels et al. 2012; Maizels et al. 2009). Through the
use of anthelmintic treatment, we have shown that in the wild, *H. polygyrus* negatively interacts with another common gastrointestinal parasite of wood mice, the coccidian *Eimeria hungaryensis* (Clerc et al. 2019b; Fenton et al. 2014), and that this strong negative effect of *H. polygyrus* on *E. hungaryensis* infection burden is localised to the small intestine (Knowles et al. 2013). However, we currently lack an understanding how interactions between *H. polygyrus* and *E. hungaryensis* impact immune responses outside of the local site of infection. In addition, studies that experimentally investigate within-host variation in the immune response across different scales are rare, especially in wild populations. Researchers often generalise host immune reactivity from systemic measures, therefore there may be significant implications for understanding and predicting parasite community interactions at the host individual and population scales given the possible differences in immune activity between the local and systemic scales.

We used the production of the pro-inflammatory cytokine TNF-α in response to a panel of toll-like receptor (TLR) agonists in mouse mesenteric lymph node (MLN) cells as a proxy for the local inflammatory response surrounding the gastrointestinal tract, and in spleen cells as a proxy for the systemic inflammatory
response, in order to elucidate how each immune response may mediate within-host interactions between *H. polygyrus* and *E. hungaryensis*. Further, we used anthelmintic treatment to assess the effects of *H. polygyrus* removal/reduction on inflammation at the local vs systemic scales. We predicted that TNF-α production would differ between local and systemic sites due to different immune cell populations being involved and their proximity to the site of infection (Vassalli 1992). Because *H. polygyrus* modulates the host immune response towards an anti-inflammatory immune profile (Maizels et al. 2012; Wammes et al. 2016), we further hypothesised that anthelmintic-treated mice would have increased TNF-α production because of the reduced worm burden and subsequent reduced immunomodulatory effects. Due to the immunosuppressive effects of testosterone (Muehlenbein and Bribiescas 2005; Nunn et al. 2009; Saino et al. 1995; Zuk 1996), we also predicted that male mice would have lower TNF-α levels than females. Lastly, we expected older hosts to have higher concentrations of TNF-α, in line with the concept of immunosenescence (Cevenini et al. 2013). Even though TNF-α is not thought to be directly protective against *H. polygyrus*, it may be associated with the inflammatory immune response to *E. hungaryensis* infection (Maizels et al. 2012; Maizels et al. 2009; Pakandl et al. 2008; Smith et al. 1995).
Methods

Field sampling

We conducted two field experiments in wild wood mouse populations in Callendar Woods, Falkirk, Scotland (55.99° N, 3.77° W). We performed the experiment over two trapping sessions, each session lasting 8 weeks (Session 1: October to November 2014 and Session 2: June to July 2015). In 2014, the experiment consisted of two trapping grids in which 2 Sherman live traps (H. B. Sherman 2 × 2.5 × 6.5-inch folding trap, Tallahassee, FL, USA) were placed every 10 m in a 130 × 80 m grid (total 256 traps). In 2015, the large grid was split into two smaller grids that were separated by ~10 m, and an additional third grid was added ~50 m away from the other two grids. All three grids were 70 × 70 m in size and consisted of a total of 294 traps, leading to similar trapping effort in both years. Traps were baited with cotton bedding, mixed seeds, dried mealworms and a piece of carrot on the late afternoon before each trapping night. The following morning, traps were checked for the presence of animals and all animals were processed and released at the site of capture. For both of the trapping sessions, we trapped three consecutive nights per week for a total of 24 trap-nights per session (Home Office Project License 70/8543).
At first capture, each wood mouse received a unique passive induced transponder tag (PIT) injected subcutaneously in the scruff (AVID FriendChip), which allowed individual identification of mice at subsequent captures. Further, all captured mice at first capture were randomly selected to receive either a single oral dose of a combination of anthelmintic drugs: Ivermectin (which removes adult worms) at 100mg/kg and Pyrantel (which removes larval worms) at 9.4mg/kg (Babayan et al. 2018a; Clerc et al. 2019a; Wahid and Behnke 1993; Wahid et al. 1989); or an equal volume of water as a control. For each mouse at each capture, we recorded the mouse’s trap location, sex, reproductive status (females: perforated vagina, pregnant or lactating, males: non-reproductive, testes descended or scrotal), body weight and length. Faecal samples were collected from pre-sterilised traps each time an animal was captured, and then stored in 10% buffered formalin at 4°C until analysis (Dryden et al. 2005). *H. polygyrus* and *E. hungaryensis* infection (infected/uninfected) and burdens (number of eggs/oocysts per 1 gram of faeces OPG/EPG) were determined by counting parasite transmission stages (eggs/oocysts) after salt floatation using a microscope at either 10X or 40X magnification (see Knowles et al. 2013).
Mice were sacrificed approximately two-weeks after initial capture and treatment (anthelmintic or control), as previous work with wood mice has shown that anthelmintic drug treatments reduce *H. polygyrus* burdens for only about 10-16 days (Clerc et al. 2019a; Knowles et al. 2013). Spleen and mesenteric lymph nodes were collected and stored in sterile RPMI media containing 1% Pen/Strep antibiotics for use in the TNF-α assay. We also collected the digestive tract of each mouse, separated into small intestine, caecum and colon, to assess burdens of adult gastrointestinal parasites via dissection. The eyes of each mouse were also collected to use dried eye lens weight (grams) as a measurement of host age. This metric has been shown to be a reliable method for quantifying host age on a continuous scale, where older hosts have heavier eye lenses compared to younger hosts (Hardy et al. 1983; Morris 1972). Both eyes were stored in 10% Formalin at 4°C for at least 4 weeks. After separating the eye lenses from the surrounding tissue, they were dried at 56°C overnight and weighed in pairs to the nearest mg.

**Immunological methods**

To assess the ability of an individual mouse to respond to infection at both the systemic and local levels, we measured pro-inflammatory cytokine TNF-α production by spleen (systemic) and MLN (local) cells *in vitro*. Cells were stimulated with a
panel of toll-like receptor (TLR) agonists known to induce production of TNF-α (Akira et al. 2006) including in wild mice (Bradley and Jackson 2008). The agonists used were Zymosan (cell surface fungal ligand, TLR2), endosomal oligonucleotide ODN2006 (ODN, TLR9), cell surface Escherichia coli K12 lipopolysaccharide (LPS, TLR4), and Heat-killed Listeria monocytogenes (HKLM, TLR2; all acquired from InvivoGen, San Diego, CA, USA). TNF-α concentration was then measured by sandwich ELISA, a previously described, successful method in wild wood mice (Jackson et al. 2009).

Spleen and MLN samples were brought into the lab for cell culture on the same day as collection (tissues in media were kept on ice during transport until used in cell culture). Under sterile conditions, tissues were passed through 70µm nylon mesh into RPMI media containing heat-inactivated foetal calf serum (10% final volume) and 1% Pen/Strep antibiotics. Cell suspensions were spun down at 1200rpm, supernatant discarded, and resuspended in fresh media (5ml for spleen cells, 1ml for MLN cells). Before resuspension, spleen cells were incubated with Red Blood Cell (RBC) lysis buffer for 3 minutes, inactivated with 10ml of RPMI media. This suspension was spun down at 1200rpm, supernatant discarded, and cells resuspended with 5ml media. If all RBCs were not lysed, this previous step
was repeated a second time. Cell concentrations for each sample were then counted (Cellometer Auto T4 Bright Field Cell Counter, Nexcelom Bioscience) and resuspended to $1 \times 10^7$ cells/ml for spleen cell suspensions, $5 \times 10^6$ cells/ml for MLN cell suspensions. Different final cell concentrations were used for these two tissue types due to fewer cells available from MLN than spleens; this difference was accounted for in the statistical analyses by analysing results from each tissue type separately (see below).

Cells from the spleen and MLN were then plated in 96-round bottom well plates for stimulation. Final volumes for samples from each individual mouse varied and low volume samples from individual mice did not receive all agonists. Controls and TLR agonists were run in triplicate, with three wells of cells receiving either an equal volume of media or agonist suspension (100 µl of cell culture, 100 µl of control or agonist added to each well). Concentrations of each agonist were as follows: Zymosan: 20 µg/ml, ODN: 10 µg/ml, LPS: 6 µg/ml, HKLM: 6.0e7 µg/ml (from Jackson et al. 2009). Cell cultures were incubated for 24 hours at 37-degrees C (5% CO2). Subsequently, plates were spun at 1200 rpm for 2 minutes and supernatant harvested for use in TNF-α ELISA.
Concentrations of TNF-α produced during cell culture were measured using a mouse sandwich ELISA according to the manufacturer's instructions (DuoSet ELISA, Mouse TNF-α, R&D Systems). Pooled cell cultures from each mouse for each agonist were used in the ELISA to reduce variation among wells. Each individual mouse and agonist combination was run in triplicate. Plate absorbance was read at 450 nm, the peak wavelength for the plate’s indicator of cytokine concentration, and well absorbance converted to TNF-α concentration (pg/ml) based on the standard curve on each plate. As stated in Jackson et al. (2009), it is likely that affinity of A. sylvaticus to the TNF-α antibodies in these ELISA kits differs from Mus musculus, but the results should still accurately capture variation in this marker of inflammation among individuals of this species.

Statistical Analysis
All analyses were performed in R (R Core Team 2018), version 3.5.1). Data on parasite infection and burden and in vitro inflammatory cytokine concentrations (pg/ml) were analysed using General Linear Mixed Models (GLMM) in the glmmADMB package. Initial data on parasite infection burden was determined measuring faecal egg/oocysts per gram faeces (EPG/OPG) of H. polygyrus or E. hungaryensis from samples collected at first capture, prior to the drug (or control) dosing (methods, see
Clerc et al. 2018)). Final measures of parasite infection were (i) final adult *H. polygyrus* burdens within the gut (number of adult worms) and (ii) final *E. hungaryensis* infection (presence (1)/absence (0) of oocysts in faeces). Analyses were performed using mean TNF-α concentrations for each individual and TLR agonist combination. Cytokine concentrations were log-transformed (log10(1+TNF-α concentration)) to improve normality (gaussian distribution in GLMM).

We measured the production of TNF-α from splenocytes as an indicator of the systemic immune response and mesenteric lymph node (MLN) cells as an indicator of the local immune response. There was variation in mean concentration of TNF-α produced *in vitro* in response to stimulation with different agonists, however, we analysed TNF-α concentrations from all control and stimulated wells together, as sample size did not permit analysing data from each agonist individually or inclusion of the agonist type as predictor variable in models. While this did not allow us to evaluate how a host may respond to a specific kind of infection (via the response to a single agonist), it did allow us to capture variation in TNF-α among individuals in response to anthelmintic treatment and simulate the complex innate immune stimuli that could be experienced by these mice (Jackson et al. 2009).
We included the following variables in each models as possible drivers of variation in the marker of inflammation (TNF-α production in response to the panel of antagonists): anthelmintic treatment (treated/control), total *H. polygyrus* adult worm burden, *E. hungaryensis* infection (0/1), host sex (M/F), reproductive condition as determined by presence of descended testes in males, and females that are visible pregnant or lactating (0/1), eye lens mass (proxy for age), and relevant biological interactions between treatment and other variables (Supplementary Table 1). We also separately analysed initial *H. polygyrus* and *E. hungaryensis* infection burdens (EPG/OPG from first capture) as possible predictors of final TNF-α production (Supplementary Table 1). We performed model reduction of all full models using maximum likelihood ratios to compare the full model to reduced models using the “anova” function in the glmmADMB package. Interactions followed by main effects were not retained in the final model if the maximum likelihood ratio test had a p-value >0.05, indicating that these terms did not significantly improve model fit. For TNF-α concentrations, random effects of mouse ID nested within ELISA plate, and TLR agonist were included in each model, to account for multiple measures for each individual mouse and variation in stimulation
between TLR agonists. A summary of the final GLMMs for parasite burdens and TNF-α concentrations can be found in Table 1.

Results

Impact of anthelmintic treatment and host characteristics on the gastrointestinal parasite community

A total of 148 wood mice were captured during the two replicate field experiments (2014 and 2015). The overall recapture rate (percentage of hosts recaptured at least once) was over 90% for this population; 60% of control hosts and 57% of drug-treated hosts were captured the target 12-18 days post-treatment. TNF-α concentrations were measured from 19 mice in Fall 2014 and 30 mice in Summer 2015 (for a summary of demographics and sample size, see Supplementary Table 2). Wood mice were commonly infected with gastrointestinal parasites: 86% (42/49) with the nematode *H. polygyrus*, while 57% (29/49) were infected with the coccidian protozoan *E. hungaryensis*; 14% (7/49) hosts were coinfectected when terminally sampled. There were no initial differences in *H. polygyrus* or *E. hungaryensis* infection intensity (EPG or OPG) between mice randomly allocated to anthelminthic treatment or control at first capture (first-capture parasite EPG/OPG: *H. polygyrus*: z = 0.47, p = 0.64; *E. hungaryensis*: z = -0.26, p = 0.796). Male mice, however, showed significantly higher initial *H. polygyrus* egg shedding than
females at first capture \((z = 3.17, p = 0.0015, \text{Figure 1a})\), but no such sex difference was found in initial \(E.\ hungaryensis\) oocyst shedding (Figure 1b).

We found that anthelmintic treatment significantly reduced \(H.\ polygyrus\) adult worm counts when hosts were captured two weeks post-treatment \((z = -5.34, p < 0.0001, \text{Figure 2a})\). While no mice were completely cleared of \(H.\ polygyrus\) worms, treated mice had a nearly 20-fold decrease in worm burdens compared to control mice. Anthelmintic treated mice also had significantly higher \(E.\ hungaryensis\) oocyst shedding (OPG, \(z = 2.04, p = 0.042, \text{Figure 2b}\)), with OPGs being 7-fold higher than control mice, which is consistent with our previous results (Clerc et al. 2019a; Clerc et al. 2019b; Knowles et al. 2013). Even though we had limited statistical power to test for the effect of reproductive status and sex on parasite burdens following anthelmintic treatment (only one non-reproductive male in the dataset, see Supplementary Table 2), we found a trend towards a positive effect of reproductive activity on worm burdens. The \(H.\ polygyrus\) burdens of reproductively active females were on average double that of non-reproductive females, while reproductively active males had 4 times more adult \(H.\ polygyrus\) worms in their gut compared to non-reproductive mice \((z = 1.76, p = 0.078, \text{Figure 3a})\), however sex as a main effect was not
retained in the final model. There were no statistically significant differences in *E. hungaryensis* oocyst between reproductive and non-reproductive mice (Figure 3b).

**TNF-α production**

Cell cultures stimulated with TLR agonists did produce more TNF-α than control cultures (z = 10.78, p < 0.0001; control: mean log₁₀(1+TNF-α concentration) 2.05 ± 0.170, stimulated: mean log₁₀(1+TNF-α concentration) 4.05 ± 0.099; Supplementary Figure 1)

TNF-α production was significantly different between systemic (spleen) and the local (MLN) scales, with systemic TNF-α levels being consistently higher than those measured locally (Spleen: 4.2 ± 0.14, MLN: 3.1 ± 0.12, z = 5.63, p < 0.0001; Figure 4), likely due to the difference in TNF-α producing cell concentrations between tissues. While there was a correlation between mean TNF-α concentrations measured from spleen and MLN cells, only 7% of the variation in cytokine concentrations in one tissue was explained by concentrations in the other (p = 0.068, adjusted R² = 0.072; Supplementary Figure 2). Therefore, we analysed local and systemic TNF-α production separately in our subsequent analyses. In addition, we found no significant difference in TNF-α concentrations between field experimental
replicates (spleen: $z = 0.74$, $p = 0.46$, MLN: $z = -0.01$, $p = 0.99$), so data were pooled from both years for analysis.

At the systemic level we found a significant effect of anthelmintic treatment on TNF-α production ($z = -2.55$, $p = 0.011$) and an interaction between treatment and host sex (sex*treatment, $z = 1.82$, $p = 0.069$, Figure 4b); systemic TNF-α in treated males were ~50% higher than those from control males. The opposite pattern appeared in females, where treated females showed ~50% lower systemic TNF-α concentrations than control females.

At the local scale, we also found a main effect of sex on local TNF-α production, with males producing ~25% more TNF-α than females ($z = 2.31$, $p = 0.021$; Figure 4a). At the local scale, we found a significant interaction between anthelmintic treatment and host age (age*treatment, $z = -2.72$, $p = 0.006$, Figure 5); lower production of local TNF-α was found in older, treated mice, while there was no effect of age on local TNF-α production in control mice. These interactions between host characteristics and anthelmintic treatment suggest that hosts are impacted differently by the reduction in H. polygyrus burden depending on their age or sex (see Clerc et al. 2019a). There was no relationship between pre-treatment H. polygyrus or E.
**Discussion**

In this study, we used targeted drug treatments to reduce *H. polygyrus* burdens in wild, demographically heterogeneous wood mice to determine how single and multiple infections with gastrointestinal parasites impacted the host immune response at two within-host scales. We found that anthelmintic treatment reduced burdens of the target parasite, *H. polygyrus*, by 20-fold 14 days after treatment (Figure 2a). This reduction in *H. polygyrus* burden in turn led to a 4-fold increase in oocyst shedding of the non-target parasite *E. hungaryensis* (Figure 2b). Further, using laboratory mouse reagents, we successfully measured levels of TNF-α production in spleen and MLN cell cultures, allowing us to use the levels of this important pro-inflammatory cytokine as a marker of inflammation and immune reactivity at different scales. We found that hosts responded differently to anthelmintic treatment depending on demographic factors (age and sex) and importantly, that the relationships between TNF-α production and infection status or demographics differed between the systemic (spleen) and local (mesenteric lymph node) scale. These results have implications for predicting the outcome of parasite coinfection, host health, and
parasite transmission dynamics. Perhaps more importantly, however, they increase our understanding of how host demography affects variation in immune markers, and the immune response more generally, depending on the scale at which they are measured.

Overall, we found that male mice had initial higher *H. polygyrus* infection burdens, as measured by egg shedding. Both local and systemic *in vitro* production of TNF-α also varied by host sex and anthelminthic treatment, where males had higher levels of this marker of inflammation than females. In addition, there was an interaction between host sex and drug treatment on systemic inflammation, where treated males showed higher systemic TNF-α production compared to control mice, while systemic TNF-α was lower in treated female mice. This was counter to our predictions, which were that males would demonstrate less immune activation than females, in line with the immunocompetence handicap hypothesis (Folstad and Karter 1992). However, a recent meta-analysis found that TNF-α was one of only a few immune markers significantly increased in male mammals compared to females (Kelly et al. 2018). Our results suggest that males may maintain higher production of inflammatory cytokines following removal of adult *H. polygyrus* worms, which are known to stimulate an anti-inflammatory immune response, potentially
leading to increased protection from subsequent infections (Lindstrom et al. 2004; Parker et al. 2017; Rynkiewicz et al. 2013). However, further research is needed to elucidate how this may impact costs of immunity, especially since we were unable to measure the immune reactivity before anthelmintic treatment, as the assay requires culling of animals.

We found that local TNF-α production varied with host age in response to anthelmintic treatment, with older, treated mice having lower TNF-α production in the MLN than younger treated mice, however, there was no effect of age on local TNF-α concentrations among untreated hosts (Figure 5). In contrast, we previously found that H. polygyrus-specific IgG1 antibody titres increased with host age, possibly in response to repeated exposure throughout a host’s lifetime (Clerc et al. 2019a). We predicted that older hosts would have higher levels of pro-inflammatory cytokines following anthelmintic treatment, as older hosts often have higher parasite burdens and/or richness compared to younger hosts (Poulin 1996), as well as often experience chronic elevation of inflammatory markers due to immunosenescence (Cevenini et al. 2013; Martin et al. 2006). While the latter has been shown in wild populations of Soay sheep (Nussey et al. 2009), the processes related to ageing have not been extensively studied in wild mice, and we do not know at
what age such effects would become detectable. Interestingly, we only saw variation in our marker of inflammation across different ages after anthelminthic treatment and subsequent *H. polygyrus* burden reduction. Chronic helminth infection may mask the effects of ageing on the wood mouse immune system, therefore making it difficult to see changes in immune strategy with age without removing this dominant gut parasite. The results presented here, in conjunction with those of Clerc et al. (2019a), show how measuring markers of host immunity in response to an experimental manipulation is a robust method for investigating the ecological and evolutionary implications of parasite infection.

The finite amount of resources available to an individual can lead to trade-offs between many important functions, such as growth, reproduction and the immune response. Often, these trade-offs are context-dependent, based on season, food availability or nutritional content, reproductive state, age or parasite coinfection (French et al. 2007; Nelson 2004; Pedersen and Greives 2008). Several studies have shown that reproduction can redirect host resources away from the immune system in both males and females, via hormonal changes or seasonal changes in physiology (Greenman et al. 2005; Nelson et al. 2002). In our
data, there was a slight trend towards reproductively active mice having higher final *H. polygyrus* burdens compared to non-reproductive mice, which also depended on anthelmintic treatment status (Figure 3). Males also showed increased levels of both local and systemic TNF-α production compared to females, which was again impacted by anthelminthic treatment (Figure 4). This is consistent with what has been observed in captive zebra finches given an experimental immune challenge, where males always showed higher induced inflammation compared to females, even though both sexes had lower general inflammation when breeding compared to a non-breeding state (Love et al. 2008). Male wood mice had significantly higher initial *H. polygyrus* burdens compared to females, and also shed fewer *E. hungaryensis* oocysts. Recently it has also been found that age, *H. polygyrus* burden before anthelmintic treatment, and *E. hungaryensis* co-infection had significant effects on *H. polygyrus* specific antibody responses in this population of wild wood mice (Clerc et al. 2019a; Clerc et al. 2018), so a combination of physiological- and parasite-driven challenges may be impacting the pro-inflammatory marker (TNF-α) measured here. Low sample sizes hindered our ability to analyse the effects of reproductive condition on local and systemic inflammation here, but this is clearly a physiological process that can have
significant impacts on allocation of within-host resources and immune response and is an avenue for future research.

By measuring the same marker of inflammation at two different within-host scales, we found that reducing *H. polygyrus* burden through anthelmintic treatment affected the host immune response differently at the local vs systemic scales. Many studies have found that gastrointestinal helminths can have significant effects on a host’s immune profile at the systemic scale (spleen/blood) by shifting the immune response towards an anti-inflammatory Th2/Treg type profile (Allen and Maizels 2011; Maizels et al. 2009; McKay 2006). However, how a host responds to the parasite at the site of infection, in our case the gastrointestinal tract, is also important for understanding the interaction between host and parasite and possible effects on locally-coinfecting parasites. Local inflammation has been measured by proxy using the mesenteric lymph nodes surrounding the gut (Eberhardson et al. 2008), however measuring immune responsiveness in these tissues is difficult in rodents as it is a terminal procedure (Munoz et al. 2005). There was a correlation between the TNF-α concentrations produced from cells of each tissue within a host, but very little variation was explained ($R^2 = 0.07$), so it seems likely that the host characteristics we used in our analyses are more informative in
explaining the local and systemic production of this pro-inflammatory cytokine.

TNF-α production by spleen and MLN cells varied significantly due to different aspects of host demography as well as anthelminthic treatment, as was seen by the impacts of age and treatment on local TNF-α concentrations but not systemic concentrations. These complementary, but distinct, measures of the pro-inflammatory response measured at a local and systemic scale allow us to better assess the wood mouse immune response to anthelminthic treatment, and perhaps apply this same approach to understand helminth infections and responses to parasite control measures in other systems. These results also emphasise the importance of rigorously studying the immune system in wild, demographically diverse hosts, to truly understand the ecological and evolutionary context of how hosts respond to immune challenges. In conclusion, considering a hosts’ immune system as uniform when studying within-host parasite interactions does not reflect the reality of individuals living in natural environments. Further research into utilising the approach of measuring markers of immunity at multiple within-host scales will further aid in better understanding parasite-host interactions in the wild.
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**Figures and Tables**

**Table 1.** Summary of final GLMM models analysing parasite burdens and TNF-α production. Results are organised according to hypotheses presented and if the results support these hypotheses. Global (full) models, including main and random effects, are presented in Supplementary Table 1.

**Figure 1.** At first capture prior to anthelmintic treatment (a) male wood mice shed more *H. polygyrus* eggs per gram faeces (EPG; circles), (b) but there was no significant difference in *E. hungaryensis* shedding (oocysts per gram faeces; OPG; triangles).

**Figure 2.** a) Anthelmintic treatment significantly reduced the burden of adult *H. polygyrus* worms (circles) in treated wood mice. b) Anthelmintic treated mice also had a significant increase in the non-target coccidian parasite *E. hungaryensis* faecal OPG (triangles), which suggests evidence of a competition between these coinfesting gastrointestinal parasites.
**Figure 3.** a) Reproductively active (noted by “1”) mice had higher *H. polygyrus* worm burdens in their gut (circles) compared to non-reproductive mice (noted by “0”), and b) reproductive females had reduced *E. hungaryensis* EPG (triangles). There was no difference in *E. hungaryensis* egg shedding with reproductive status in male mice. All mice, control and treated, included here.

**Figure 4.** There was a significant effect of both host sex and anthelmintic treatment on TNF-α production at both local (a) and systemic (b) scales. There was a significant interaction between these variables impacting systemic TNF-α production (b): anthelminthic treated (day ~14), male mice (green) had higher in TNF-α production *in vitro* (measured by ELISA in pg/ml) at the systemic scale (spleen cell cultures), while treated females (purple) showed a decrease in this pro-inflammatory cytokine systemically (mean ± 1 SE shown).

**Figure 5.** Local TNF-α production (measured by ELISA in pg/ml) measured from MLN cells *in vivo* was reduced in older, treated mice (red), while we found no difference in TNF-α production across ages in control mice (blue) (shaded areas represent standard error of linear regressions).
Supplementary Table 1. Global (full) GLMMs for parasite burdens and TNF-α concentrations.

Supplementary Table 2. Sample sizes of individual wood mice in the treated and control groups, divided by sex and reproductive condition.

Supplemental Figure 1. Cells stimulated with toll-like receptor (TLR) agonists (blue) produced significantly higher TNF-α compared to cells that were not stimulated (controls; yellow), supporting that these wood mouse cells did respond to in vivo stimulation methods. Concentrations of each agonist (µl/ml) used were based on the methods described in Jackson et al. (2009).

Supplemental Figure 2. Correlation between TNF-α concentrations (pg/ml) in splenocyte and mesenteric lymph node (MLN) cells. This correlation was not statistically significant and little variation was explained by a linear regression (p = 0.068, adjusted R² = 0.072).

References


