**SIGNIFICANCE.**

***Duration of infection is a determinant of both individual health and public health*.** The number of days, weeks, months, or even years that an infection persists in a host has important health implications on multiple biological scales. For an infected individual, for example, the time it takes to clear an infectious agent can affect the likelihood that the infection will become lethal (e.g., for pneumonia [1]), the cumulative severity of symptoms such as anemia (e.g., during chronic malaria [2]), and the amount of tissue damage that must be repaired if the individual is to recover (e.g., from viral hepatitis [3] or gastrointestinal helminthiasis [4]). The work of physicians therefore tends to be easier when duration of infection is short.

Equally striking is the impact of infection duration on the population scale: the longer individuals are infected, the longer they tend to be infectious to others, whether by persistent coughing (e.g., for tuberculosis; [5]), spreading of fecal matter (e.g., by Typhoid Mary [6] and modern analogues [7]), or availability of transmissible propagules to biting vectors (e.g., for malaria [8]). Accordingly, infection duration and its inverse, clearance rate, are canonical parameters in models of dynamic epidemiology [9-11]. For example, the mean and variance of infection duration in a population predict epidemic outbreak risk (e.g., for norovirus [12]) and ease of control by public health interventions (e.g., for sexually-transmitted [13] and vector-borne [14] infections). The work of public health officials – in blocking spread of infections from one individual to the next – therefore tends to be easier when mean duration of infection is short and variance is low.

***Despite these profound individual- and population-scale impacts upon human health, the dynamic processes that determine infection duration are poorly understood.***  This is partly because duration arises from a complex interplay of host and parasite genetics with environmental context. For example, “resistant” host genotypes clear parasites more rapidly than “susceptible” genotypes do, often via powerful immune responses that mobilize appropriate effector mechanisms (e.g., secretion of mucus by goblet cells, accelerated epithelial turnover, and peristalsis to expel gastrointestinal nematodes [15]). Furthermore, parasite genotypes that immunosuppress (e.g., [16]) or metabolically co-opt (e.g., [17]) the host most vigorously often generate infections of longest duration.

Indeed, infection duration is known to vary among host genotypes (e.g., [18-21]), parasite genotypes (e.g., [22-24]), and to depend, in some cases, on the combination of host and parasite genotypes (e.g., GHxGP interactions for duration [25, 26]). Considerable effort has been expended to understand the molecular and cellular mechanisms behind the effects of host and parasite genetic backgrounds, yet **genetics (even GHxGP interactions) are not the only drivers of variation in infection duration**.

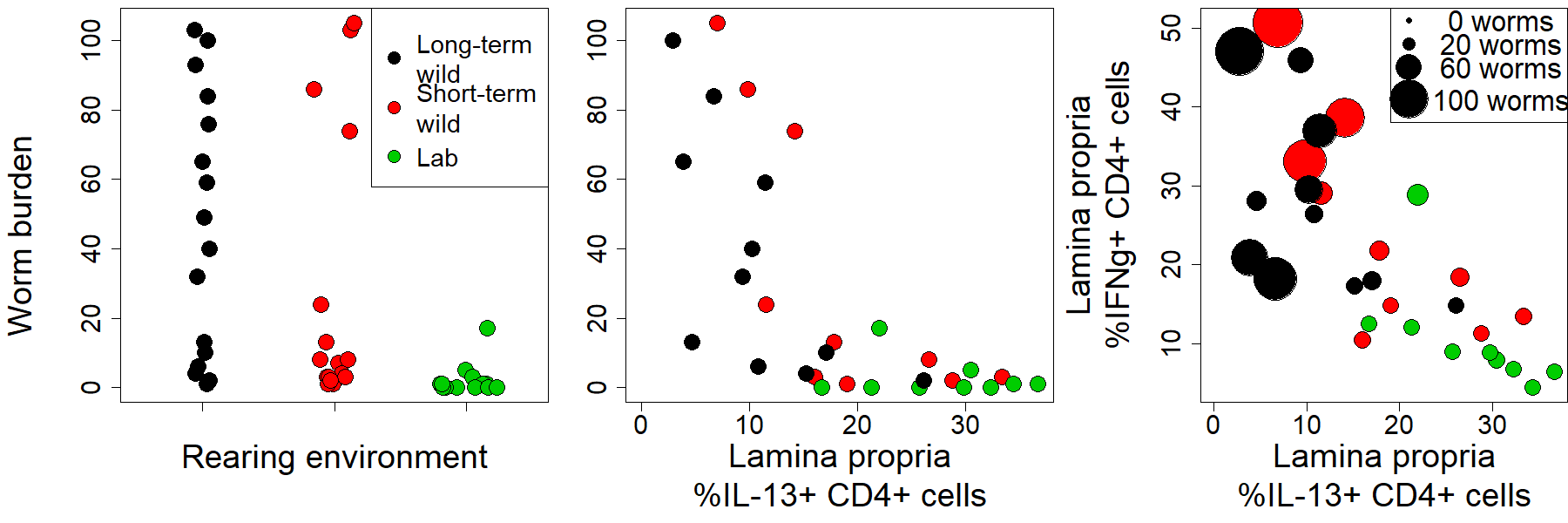
**Infection duration also varies strongly with environmental factors** such as inoculating dose [27, 28], rate of exposure [29, 30]) and resource availability [31, 32]. Perplexingly, the way in which duration changes with environmental factors often varies among host genotypes. For example, mouse strains (hereafter used to denote mouse genotypes)described as “resistant” due to their ability to clear a high dose of nematodes become chronically infected when exposed to a low dose, whereas other mouse strains are chronically infected regardless of dose [33-35]. Our own experiments on inbred mice infected with the nematode *Trichuris muris* show that infection durations observed under natural environmental conditions do not match those observed in the lab: **when inbred mouse strains are “rewilded” by moving them outdoors, infection is prolonged** (**Fig. 1**). Moreover, mice exposed to the natural environment only after receiving nematode infection (“short-term wild”) had more variable worm burdens than those that spent the entire infection in the lab or in the wild (**Fig. 1**; [36]).

More vexing still, infection duration often varies among individuals of a given genotype exposed to the same parasite dose, even in controlled experiments [18, 37, 38], and even with clonal isolates of parasites (such as rodent malaria [39] or streptococcus [40] infections). The variation in infection duration among individual hosts of the same genotype can be as large as the variation among host genotypes (e.g., [41]). This variation among individuals is typically ignored, treated as unwanted noise that potentially obscures differences among treatment means, rather than as an object of study in its own right. In contrast, **we suggest that seriously exploring such within-genotype variation in infection duration will help us to identify the processes that determine infection duration**.

a

b

c



**Figure 1.** **Parasite burden and infection duration depends on the immune response and varies among individuals.** The rearing environment (lab versus wild) has a large effect on worm burden (**panel a**), but within treatments, there is considerable individual variation in worm burden. Worm burden has a strong negative correlation with the fraction of CD4+ T-cells expressing Th2 cytokines, as expected (**panel b**). Expression of Th2 cytokines is negatively correlated with expression of Th1 cytokines, further indicating that chronicity is determined by the immune-parasite battle over Th1-Th2 cytokine expression (**panel c**).

***Here, we propose to use inoculating dose to “probe” within-host ecological dynamics across host strains and environments, thereby revealing the fundamental processes that drive variation in infection duration*.** By definition, infection duration is determined by the ecological dynamics of parasite biomass growth and mortality: the infection lasts until the immune response kills or expels the parasite, or, failing that, until the parasite dies. We will develop and test novel mathematical theory, to quantify the positive and negative feedbacks that regulate infection duration, in both controlled lab settings and under greater environmental realism. We will focus on the biology of gastrointestinal nematode parasites. Our emphasis on nematodes is pragmatic: nematode infection duration varies widely even in controlled conditions (e.g., some hosts take days, others take years to purge worms) and infection duration is strongly correlated with morbidity in human gastrointestinal nematode infections [42]. Our three Aims will address within-host processes that drive varied infection duration across host strains, among individuals within a strain, and as hosts move into a more natural environment, revealing general principles that likely apply to many infectious disease systems.

**INNOVATION.**

Our primary innovations are: A) to build a novel mathematical model of immune-driven “Allee effects” that generates testable hypotheses about the processes leading to variation in infection duration; B) to test the model using inoculating doses and naturalized microbiota as experimental probes of how environment alters duration; and C) to field-test the model in an unparalleled natural system. Our combined expertise in mathematics, immunoparasitology and fieldwork enables a powerful approach to that will identify the dynamic immunological mechanisms that generate variation in infection duration, and reveal how those processes differ between the lab and the wild.

In particular, we introduce the ecological concept of **Allee effects** to explain how within-host dynamics lead to variation in infection duration. Allee effects arise when positive feedback loops generate a positive relationship between *per-capita* growth rate and population density. **The key dynamical signatures of Allee effects are persistence thresholds** (e.g., [43, 44]): when density is below the threshold, the population declines to extinction; above it, the population persists. Near the threshold, subtle differences in system state can produce strikingly different persistence times. Positive feedback loops are likely to be ubiquitous within hosts (**Fig. 2a**). In particular, polarization of the T cell populations towards either a T-helper 1 (Th1) phenotype (which coordinates the immune response against intracellular pathogens) or a T-helper 2 (Th2) phenotype (which coordinates the immune response against extracellular parasites, like nematodes and thus promotes clearance) is driven by positive feedback between cytokine production and T cell activation, such that the per-cell growth rate of a T cell subpopulation increases as more T cells are activated [45], and by mutual inhibition between T-cell subpopulations. These positive feedback processes can be hijacked by the parasite when there is a positive relationship between parasite biomass and immunomodulation, such that the per-gram growth rate of the parasite may increase with biomass (e.g., due to escalating manipulation; [46]). Our preliminary results suggest that including such “clearance-promoting” and “chronicity-promoting” positive feedback mechanisms in mathematical models of the immune-parasite interaction generates Allee effects in parasite biomass growth, such that changing the initial dose of parasites or the initial state of the immune system can alter infection duration (**Fig. 2**).

To test the predictions of our theory using innovative experimental approaches, we will pair dose-response experiments in microbially naturalized mice in the lab with **experiments in** **our unique outdoor system that enables us to control host genetics and inoculating dose while titrating in further natural variation** likely to modulate within-host dynamics. Recent studies have highlighted the divergence between immune phenotypes of wild and laboratory mice [47, 48] and advocated naturalizing mice in various ways to improve mouse models of human immune function [49-52]. This logic likewise applies to classic work on enhanced susceptibility to nematodes among lab mice maintained in large indoor arenas [29, 30] as well as our own “rewilding” approach [36, 53-55]. Inspired by our data showing that rewilding mice for even a couple of weeks prolongs nematode infections (**Fig. 1**), we hypothesize that this increase in duration arises because rewilding pushes the system across a mathematical persistence threshold: a parasite Allee effect in action.

**APPROACH.**

Our approach combines the power of mathematical ecology with the tractability of mouse models in immunoparasitology, to illuminate causes of varied infection duration for gastrointestinal helminths. We use this approach to tackle three Aims:

**Aim 1:** Quantify the relative strength of chronicity- and clearance-promoting feedback loops across host strains and show that this explains the variable response to dose.

**Aim 2:** Experimentally manipulate rates of immune induction and effector impacts to alter the relative strength of feedback loops.

**Aim 3:** Titrate in full environmental naturalism, beyond gut microbes, in “rewilded” mice kept outdoors, to quantify effects upon immune feedbacks and thus duration of nematode infection.

Throughout the grant, we will titrate in a natural environmental context, via gavage of cecal material from inbred mice maintained outdoors in a germ-rich but nematode-free environment (in Aims 1 & 2), via trickle infections that match real transmission of nematodes better than bolus exposures (in Aim 2), and via dose-response experiments outdoors (in Aim 3). Before addressing these Aims in detail, we first argue that our mathematical approach will fill a knowledge gap in within-host dynamics, unify previous insights and resolve previously unexplained variation in infection duration. We then address unique suitability of our team to naturalize lab mice and blend mathematics and experiment, before explaining how we will use mathematics and experimental data on mouse-nematode interactions to address each of our Aims in turn.

***Do feedbacks in within-host ecology predictably determine infection duration?*** This is the core question motivating this proposal. And we have good reason to suspect that the answer is yes! For example, during experimental infections of fruit flies [56] and flour beetles [57], the duration of infection was acute in some insects, and chronic in others, despite stringent controls.Analyses of these datasets indicate that **variation in infection duration was caused by** **subtle differences in the initial rates of immune response induction and parasite replication**; such subtle dependence on initial conditions and early events is a hallmark of Allee effects and indicative of strong feedback mechanisms.

Working with **collaborators Professors Anieke van Leeuwen and Sarah Budischak**, we developed and parameterized a mathematical model of within-host ecology of gastrointestinal helminths that can generate thresholds in infection duration similar to those observed empirically [46]. Unlike all previous mathematical modeling approaches, **whether an infection is acute or chronic is an *emergent property* of the within-host ecological dynamics in our model** (instead of being pre-programmed into the math, as in the past (e.g., [58, 59])**.** Our result is due to an Allee effect in parasite biomass growth, driven by parasite manipulation of resources: when parasites wrest control of resources, they tip the system towards chronic infection; when they don’t, infection is acute.

***Diagram, engineering drawing

Description automatically generatedPreliminary modelling reveals that changing the strength of clearance-promoting and chronicity-promoting feedbacks can qualitatively alter infection duration.*** Within-host ecology is characterized by both positive and negative feedback loops (**Fig. 2a**). Most obviously, there is a negative feedback loop between parasite biomass growth and the immune response: parasite biomass is reduced by an effective immune response; reduction of parasite biomass then reduces immune stimulation. For gastrointestinal nematodes, a Type 2 immune response promoted by T-helper (Th) 2 cells is effective, whereas neither a Th1 response nor a regulatory T cell (T-reg) response leads to clearance of worms [15]. Positive feedback loops are also ubiquitous within the immune system itself, and can act to drive clearance or chronicity. For example, cytokine production drives activation of T-helper cell populations that then secrete those same cytokines and suppress secretion of opposing cytokines (e.g., Th2 cells promoting Th2 while inhibiting a Th1 response, and vice versa [60, 61]). If a Th2 response is launched and propogated, these feedbacks are clearance-promoting; on the other hand, if the worm “gets the upper hand” via immunomodulation (e.g., by promoting either a T-regulatory cell response [62] or a Th1response, as *T. muris* does [34, 35, 63]), these same feedbacks become chronicity-promoting, allowing the parasite to grow more rapidly and gain further control, delaying clearance.

**Figure 2.** (a) Infection duration depends on the strength of negative and positive (chronicity-promoting and clearance-promoting) feedbacks. (b-d) When negative feedbacks are relatively strong, the parasite is always cleared rapidly, with higher doses leading to shorter durations due to the promotion of a Th2 response. (e-g) When clearance-promoting feedbacks are strong, low doses of parasites can become chronic by not provoking the Th2 positive feedback loop, whereas high doses are cleared. (h-j) When chronicity-promoting feedbacks are strong, high doses of parasites can become chronic by provoking the Th1 positive feedback loop, whereas low doses are cleared.

Mathematical theory provides testable insights into how these feedback processes affect the immune-parasite interaction. For example, **Fig. 2b-j** shows outcomes for a simple model, inspired by *T. muris*. The model includes all of the negative and positive feedbacks (**Fig. 2a)**, including self-promotion by Th1 and Th2 responses, Th1-Th2 cross-inhibition, and parasite induction of both Th1 (via immunomodulation) and Th2. We have found that models that include both negative- and positive-feedback mechanisms can always produce Allee effects, but **whether and how changing the initial state of the system (e.g., inoculating dose or the T-cell population) will affect duration depends on the relative strength of the feedbacks.**

If negative feedbacks dominate the dynamics of the system, then changes in the initial state of the system will have little effect on duration ([64, 65]), and Allee effects do not arise (**Fig 2b**). Increasing dose simply provokes a stronger Th2 response, leading to rapid clearance (**Fig. 2c**). However, if positive feedbacks dominate, then infection duration will exhibit the threshold behavior characteristic of Allee effects. In particular, if clearance-promoting feedback loops are stronger than chronicity-promoting loops (e.g., due to potent Th2 tendency of host strain or Th2-promoting environmental conditions) but the immune system is initially Th1-biased (e.g., due to prior Th1-promoting infection), then low doses produce a chronic infection but high doses are cleared (**Fig. 2e**, moving from gray to black); this is because low doses do not trigger a strong initial clearance-promoting Th2 response, and the initial bias towards Th1 keeps the Th2 response suppressed (**Fig. 2f-g**). However, if the immune system is initially Th2-biased (e.g., due to potent Th2 tendency of host strain or prior Th2-promoting infection), then infections are cleared rapidly, regardless of dose (not shown). However, the opposite response to dose is also possible. If chronicity-promoting feedback loops are stronger than clearance-promoting loops and the immune system is initially Th1-biased, then chronic infections occur regardless of dose (not shown). However, if the immune system is initially Th2-biased, then low doses are cleared, but high doses lead to a chronic infection (**Fig. 2h**); this is because low doses do not trigger a strong initial chronicity-promoting Th1 response, and the initial bias towards Th2 keeps the Th1 response suppressed (**Fig. 2i-j**). **We thus gain a key testable insight from this simple model: whether an infection is cleared or becomes chronic depends critically on the initial dynamics of the immune response**, and how those initial dynamics are affected by dose determines whether and how dose will affect duration. Additionally, our modelling results provide a mechanistic explanation for the results of previous experiments that have found that initial immune dynamics are critical determinants of infection duration [56, 57].

***Host-parasite battles over Th2ness drive variation in duration of trichurid nematode infections***. We now propose to test predictions of our model experimentally, in mice (*Mus musculus*) infected with whipworms (*Trichuris muris*). To support our experimental work, we will draw upon the trichurid immunology expertise of our **new** **collaborators Professors Kathryn Else and Richard Grencis** and the microbiological expertise of **collaborator Professor Ken Cadwell**, who is already a key collaborator on our rewilding work [54, 55] and continues enthusiastically in this role. *Trichuris muris* is a natural gastrointestinal nematode parasite of mice [66-68]. *Trichuris spp*. (whipworms) are transmitted via the fecal-oral route and inhabit the caeca of many mammals [69]. They burrow into the epithelium and, at high burdens, cause host wasting (e.g., *T. trichiura* in people [70]). As in many helminth infections, rapid clearance of *T. muris* requires the development of a Th2-polarized immune response, and chronicity is associated with dominance of other T-helper subsets, especially Th1 [34, 35, 63]. Th2 cells coordinate the activation of effector mechanisms such as mucins and antibodies that purge nematodes from the gut, whereas Th1 cells promote ineffective mechanisms such as phagocytosis [15]. The nematodes, unsurprisingly (given that Th1 promotes worm survival), secrete and excrete products that immunomodulate the host [71] into deploying Th1- rather than Th2-associated effectors (e.g., [72]), including a recently described, highly abundant protein (p43) that ablates a key Th2 effector cytokine, interleukin(IL)-13 *in vitro* and *in vivo* [63]. The *Mus-Trichuris* system thus has all of the ingredients necessary to test whether the mechanisms laid out in our mathematical model drive variation in infection duration.

Two pieces of empirical evidence buttress our hypothesis.The first is that **mouse strains have strikingly different dose-dependence in susceptibility to *T. muris*.** Given a high dose of eggs, “susceptible” mouse strains produce a Th1-polarized response and become chronically infected, whereas “resistant” strains produce a Th2-polarized response and clear the infection quickly ([33-35], reviewed in [67, 68]). **If the inoculating dose is reduced, now “resistant” strains become chronically infected, too.** This pattern cannot be explained solely by changes in the strength of processes that generate negative feedback. For example, it might seem possible that worm establishment, biomass growth, and fecundity are density-dependent (a negative feedback mechanism), such that all three might be increased in low-dose infections, leading to longer infection durations. However, this has been ruled out by experimental work [73]. Our theoretical results suggest that this puzzle is instead solved via the logic of **Fig. 2**, which shows that reducing dose leads to a chronic infection because the clearance-promoting Th2 positive feedback loop is never engaged, allowing the parasite to ‘fly beneath the radar’ of the immune system.

The second comes from our preliminary experimental work (depicted in **Fig. 1**) that suggests that moving mice from the lab to the field makes it easier to skew the system towards Th1ness. Indeed, mice with the highest, longest-lasting worm burdens had the most Th1 polarized immune profiles [36]. The farmlike environment of the mouse enclosures at Princeton’s research station alters a number of immunologically important factors for mice [74] that arguably make the impact upon nematode susceptibility unsurprising. For *T. muris* infections, for example, microbial diversity leads the nematodes to exhibit higher hatching rates than in sterile conditions [75], and graze upon microbial taxa within the colon that promote chronicity of infection [76]. Furthermore, these microbes are likely to promote Th1 and Th17 (among other immunological changes observed in naturalized mice [49-52]); **we thus expect that microbially natural environments may always benefit the worms and promote long duration of infection.**

**The close integration of experiments and mathematics that our team is poised to deliver is essential to reveal causes of varied duration of infection**. Our team is uniquely suited to this project. **PI Graham** is an ecological immunoparasitologist who uses experimental (e.g., [36]), observational (e.g., [77]) and clinical trial (e.g., [78]) study designs to elucidate genetic and environmental drivers of parasite (often nematode) dynamics within mammalian hosts. She also has a track record of using within-host theory on T-helper cell dynamics to investigate emergent effector decisions [45, 79]. **PI Cressler** is a mathematical ecologist with an excellent track record in infectious disease research (e.g., [31, 32, 80-82]) who has worked extensively to pair theory with experiment. Of particular relevance to this proposal is his work using mathematics to disentangle complex within-host dynamics across different host-parasite systems [31, 32]. The 2 PIs also have a track record of working together on theory to predict how optimal immune strategy varies according to the costs of immune defense and varied parasite virulence [83] and how infection duration emerges from within-host feedbacks [46]. We also have a track record of collaborating to ground the latter theory in the tractable experimental system proposed here (M. musculus infected by T. muris; [46, 74]). **Our collaborative team** includes an eco-physiologist (**Budischak**), two immunoparasitologists with unrivalled expertise on the experimental system (**Else** and **Grencis**, on the host genetics of susceptibility (e.g., [84]) and immunomodulation by the parasite (e.g., [63]), respectively) a mathematical ecologist (**van Leeuwen,** [46]), and a microbiologist (**Cadwell**), which will ensure we have the knowledge and support required to complete the project. Furthermore, we have **Ramya Smithaveni Barre**, a trained research assistant in Graham’s lab, who is ready to undertake the proposed experiments.

We propose to test our theoretical predictions fully in that empirical system, and we envision it as an iterative process: we will begin with experiments inspired by predictions of the initial mathematics; as we learn from our empirical findings, we will return to modify the mathematics to improve accuracy of the predictions, and so forth. Ultimately, **we will test whether rules governing infection duration are general across mouse strains and increasingly realistic environments**, by pursuing our 3 Aims, as follows.

**Aim 1. Leverage host genetic variation in response to varied parasite dose, to quantify the relative strengths of feedback loops that drive variation in infection duration.**

Drawing on our previous theoretical work [46] and pilot results (**Fig. 1**), we propose that variation in the relative strength of Th2- versus Th1-mediated feedbacks (**Fig. 2**) can explain the previously puzzling variation in infection duration in general, including dose- and environment-dependence. We specifically hypothesize that “resistant” host strains exhibit stronger Th2-escalation with increasing doses of *T. muris*, whereas “susceptible” strains fall prey to Th1 manipulation at low doses, and higher doses amplify the Th1 feedbacks. **The key challenge we address in Aim 1 is thus to identify and quantify the feedback mechanisms driving dose-dependence observed in susceptible and resistant mouse strains. This will give us novel insight into the *processes* that determine infection dynamics in this system.**

***Dose variation as an experimental probe in host strains with semi-naturalized microbiota.*** The goal of these experiments is to use dose manipulations, to quantify feedbacks between immune responses and parasite biomass growth, and to discover the processes governing duration. We will begin with several inbred mouse strains that are the focus of foundational immunological research on *T. muris* (C57BL/6 and BALB/c as “resistant” and B10.BR and AKR as “susceptible,” independent of their Major Histocompatibility Complex (MHC) genotype [67, 68]) – including divergent dose-dependencies. However, our experiments will be novel in several crucial ways.

An especially important aspect of our design is that we will microbially semi-naturalize the mice for all experiments. Our previous work [36, 53-55] and that of others [49-52] reviewed in [85] suggests that **the single most important bridge between lab mice and real adult mammals goes via microbial exposures**. For example, conferring antigenic experience by co-housing lab mice with “dirty roommates” [49] or providing lab mice with wild fecal transplants [52] or surrogate mothers [51] makes their immune cell distributions better resemble that of adult mammals (including humans) and dramatically alters their susceptibility to challenge infections in ways that mirror natural infections in wild mammalian hosts.

Two weeks prior to nematode inoculations, and again at the time of initial nematode infection, we will therefore orally gavage each mouse with a standardized slurry of cecal microbes (pooled from our frozen bank of over 200 helminth-negative mice that were kept outdoors for up to 3 months but tested negative for >30 mouse pathogens). We will swap bedding among cages each week to ensure within-experiment consistency of microbiota, and we will include rewilded fecal pellets each week (from our frozen bank from past rewilding) to sustain the microbiota. We have found that microbial transplants and coprophagy confer stable microbiota; e.g., we find that conventional microbiota does not block colonization by proteobacteria acquired outdoors [55]. We have also found that microbial exposure outdoors extends the duration of *T. muris* infection, even in the host strains and at doses associated with the most acute dynamics in conventional lab housing [36]. (**Fig. 1**) Thus, although dose-dependent susceptibility of C57BL/6, BALB/c, B10.BR and AKR mice to *T. muris* has been described in the lab [67, 68], **we** **expect our *GH x dose* data to reflect microbe-dependent shifts in immune responses and extended durations of infection compared to the *T. muris* laboratory norm** [68]**.**

A further design element concerns the array of doses of *T. muris*. Because we are interested in the feedbacks that may drive switchlike system-level behavior (toggling between Th1 and Th2 dominance), we must ultimately expose each host strain to fine-scale variation in dose. For our initial round of experiments, the doses of *T. muris* that we will deploy, via oral gavage (as in our previous work [36, 53, 74]), are boluses of: 20, 40, and 200 embryonated eggs per mouse. This relatively limited dose range will allow us to study all 4 host strains and both sexes, to establish immune and parasite dynamics of the ***GH x dose*** comparisons in the presence of diverse gut microbes.

The response of duration to dose across this range will be sufficient to help identify whether the system is better characterized by **Fig.** **2b-d**: low variation in duration leading to clearance, indicative of strong negative feedbacks between parasite biomass and Th2 immunity; **Fig. 2e-g**: shift from long duration to short duration with increasing dose, indicative of strong clearance-promoting (Th2-mediated) feedbacks; or **Fig. 2h-j**: shift from short duration to long duration with increasing dose, indicative of strong chronicity-promoting (Th1-mediated) feedbacks. While we expect strong positive feedbacks based on the foregoing discussion, **more critical is to identify the *processes that are driving system dynamics****.* If we do find dramatic shifts in infection duration with dose for a particular subset of host strains/sexes, we will carry out follow-up experiments, using broader dose ranges such as 10, 20, 40, 100, 200, and 400 embryonated eggs per mouse (encompassing the full range considered relevant for C57BL/6 mice in conventional SPF housing [34, 63]). We note that we manipulate dose (number of inoculating eggs) rather than parasite strain (e.g., [86-88], comparing **E**dinburgh, **J**apan, and **S**obreda isolates) so that we could exert greater experimental control over the extent of immune activation, since the theory suggests that the initial dynamics of the immune response are critical to determining infection duration.

We will use 24 adult mice per ***GH x dose*** combination per experiment (**with 2 host strains, 1 sex and 3 dose levels per experiment, culled at 4 different time points**). We will conduct 2 independent experiments per strain-by-dose combination; this gives 12 mice per strain/sex/dose/timepoint across 2 experimental blocks. This accords with sample sizes identified in power calculations, given the magnitude of differences among strains and within-strain variance in immune response induction in our past work. Each experiment will also include uninfected but microbially semi-naturalized controls to capture baseline immunophenotypic variation among strains and cohorts of mice.

***Collection of rich immunoparasitological data (common to all experiments in the proposal).*** We will quantify duration and dynamics of whipworm burden in terms of the number, developmental stage (e.g., larval stage L3 vs L4 vs adult), and biomass (as in our prior work [36, 74] as well as estimating the ATP content [89] of nematodes collected from the cecum at four serial cull timepoints per experiment (2, 3, 5 and 7 weeks post-infection) to reveal how worm survival, development and biomass correlate with different aspects of immunity. **We will culture isolated nematodes (as in [63]) to collect E/S products and purify/quantify production of the immunomodulatory (IL-13-blocking) molecule p43; we will then test whether, as we expect, larger and later-stage worms are capable of greater immunomodulation.** We will also collect fecal egg counts from all mice; infection is expected to become patent around 4 weeks post-infection [67, 68]. **In one experiment for each *GH-by-dose* treatment combination, we will also include a separate group of mice that will be followed until fecal egg counts drop to zero** (in case it takes until worms die of old age at ~14 weeks, for example; [67, 68]). We will characterize and quantify gut bacterial microbiota with 16srDNA sequencing of fecal pellets collected weekly and at experimental endpoints, as we’ve done before [53]. We will quantify health and nutritional plane via weekly changes in host body weight, serum albumin, and total protein [90], as well as endpoint measures of epithelial damage by histopathology [16] and body composition via both leptin and carcass weight (as we did in [74]).

Our modeling results indicate that both the initial state of the immune system (absolute numbers of T cells and any bias towards Th1 or Th2) and the early dynamics of the immune response are key measurements that could reveal the mechanism underlying duration variation (**Fig. 2**). Longitudinal immune data are therefore essential. Starting with baseline measurements 2 weeks prior to nematode infection, we will quantify immune dynamics weekly in terms of fecal concentrations of resistance-associated mucins and REsistin-Like Molecule (RELM)-ß [91] and susceptibility-associated calprotectin and lipocalin 2 [92], and serum antibody profiles, in which IgG2a:IgG1 ratio approximates the Th1:Th2 bias [93, 94]. We will also carry out weekly flow cytometric analysis of Peripheral Blood Mononuclear Cell (PBMC) to count total T cell densities and per-cell expression of transcription factors Tbet vs GATA-3 (to quantify Th1 & Th2 master regulator expression, respectively) and IFN- vs IL-4/IL-13 (to quantify Th1 & Th2 cytokine expression, respectively), alongside standard markers of T cell phenotype (e.g., CD3, CD4, CD8). In addition, key endpoint measurements will entail phenotyping of mesenteric lymph node (MLN) and lamina propria cells (as we measured in [36]), including T cells expressing Tbet, GATA-3, IFN-, IL-4 and IL-13, restimulation of MLN cells with *T. muris* antigen and subsequent production of a 12-plex panel of cytokines. We will also analyze PBMC via flow cytometry and antibodies via ELISA to verify patterns observed longitudinally in smaller sample volumes from the same animals. Given the larger number of cells available for flow cytometry at experimental endpoints, we will also include markers of proliferative and gut-homing potential (e.g., Ki67 & CCR6, respectively) in our panel.

***Hypothesis tests.*** We hypothesize that when we measure dynamics of parasite expulsion versus biomass in relation to Th1 and Th2 transcription factors and cytokines across these strains and doses, **we will discover whether linear or tipping point escalations (likely differing by host strain) determine duration**. However, because we expect greatest growth of parasite biomass once hosts have tipped to Th1, we also expect a considerable contribution of biomass-dependent parasite feedbacks to sustaining the Th1 milieu.

**Whereas the general model analysis (Fig. 2) motivates our hypotheses and experiments, we can go beyond the general by using the empirical data to quantify the specific strengths of negative and positive feedback mechanisms in each mouse strain** by estimating the parameters of our mechanistic

mathematical model. Building on existing theory for Th1-Th2 interactions (e.g., [45, 60, 61, 95]), we consider the model of immune-parasite interaction given in Box 1. **Note that negative feedback processes in this model come primarily through the parasite biomass growth terms, whereas positive feedback processes dominate the immune terms.**

Although the specific functional forms in model (1) have been validated by other authors, we can also estimate functional shapes by fitting flexible splines (e.g., [96]) to the data across doses. This will be especially important for functions that are less well-resolved, such as parasite biomass growth rates and parasite immune induction. We will use iterated filtering [97, 98] to fit the dynamical system specified by model (1) to the experimental data, especially the measures of T cell abundance and Th1:Th2 balance and the parasite biomass data (measured directly from the serial culls and estimated indirectly from fecal egg count data).

|  |  |
| --- | --- |
|  | (1) |
| **Box 1**: The parameter captures “baseline” expression of Th*-i* immunity. The term captures the induction of Th-*i*  immunity by *T. muris*. The term captures self-promotion of Th-*i* immunity by, for example, production of cytokines (e.g., IL-13, IFN-). The term captures cross-inhibition of Th-*i* immunity by Th-*j* cells. Existing theory-data syntheses give reasonable starting estimates for many of the parameters of these self-promotion and cross-inhibition terms [45]. Parasite biomass growth rate is density-dependent , and parasites are killed by effector cells activated by Th2 cells at a rate proportional to . | |

Fitting is a well-studied problem [97-99], and existing software packages (e.g., pomp [100]) provide considerable flexibility for estimating the parameters of dynamical systems from noisy, incomplete data. Model fit will be assessed using post hoc comparison of simulated immune and parasite dynamics to those observed in the data (e.g., following [99]). **By comparing parameter estimates across mouse strains, we will test our hypothesis that variation in infection duration is driven by variation in the parameters that determine the strength of positive and negative feedbacks.**

***Expected outcomes and potential pitfalls.*** The work proposed under this Aim is relatively low risk and high reward. We anticipate that the primary challenges would arise from the complexity of our *in vivo* experiments. However, we have experience with all of the protocols required [36, 46, 74], including prior gavage of microbes [55] and immunological dose-response work [101, 102]. We therefore do not anticipate difficulty in completing the experiments. If we find it necessary to maximize gut microbiota diversity, we will use litters of germ-free mice that receive cecal slurry via oral gavage, following [52]. In any case, we expect the experimental data *per se* to represent an important advance: a nuanced understanding of how rates of immune response induction and duration of infection depend upon microbially semi-naturalized host strains and doses will be of broad interest in immunology.

The theory development is an important advance as well. Current mathematical approaches to studying within-host interactions have not advanced our understanding of the determinants of infection chronicity, despite offering other insights [14, 58, 59, 103]. The theory-data integration we propose here will provide novel information about the magnitudes of key immunological and parasitological processes in this system, but will also develop methodologies that can be used to analyze infection duration across host-parasite systems in general, especially those prone to generating highly variable chronicity (like TB [5]). **A particularly compelling conceptual motivation for studying the role of Allee effects in driving parasite persistence is the key role of Allee effects for understanding and deriving management strategies for invasive species** [44]. For example, mathematical models that incorporated positive feedback between pine beetle exploitation and pine defense were able to accurately predict invasion thresholds across environments [43], and inform management strategies focused on shifting establishment thresholds [44, 104]. Understanding Allee effects in host-parasite systems may similarly inform treatment strategies.

A potential pitfall is if the above model is unable to reproduce observed infection dynamics (diagnosed as a poor fit of the model to data). Decades of experimental work indicates that we will certainly observe variation in infection duration. The most likely reason for the model’s inability to fit the data well would be a lack of immunological detail, for example on the dynamics of cytokines or transcription factors. Fortunately, as noted above, other authors have considered more detailed models that can be brought to bear on the data. If this were the case, we can extend the model to include additional variables, such as the dynamics of immune cell activation and proliferation in response to cytokines [60, 61, 95, 105], the dynamics of cytokine expression by Antigen-Presenting Cells (APCs) and T helper cells [106, 107], and the dynamics of immune cell polarization and master regulator expression [95, 108]}. We can extend the model above to include some of these other variables to gain deeper insights into the mechanisms underlying the observed infection durations. **Importantly, even if we do not find strong evidence for tipping point behavior, either empirically or theoretically, our theory-data integration will still provide valuable insights into the processes that *do* drive variation in infection duration.**

**Aim 2: Test hypothesized drivers of infection duration by experimentally manipulating the rates of induction and effector impacts of within-host feedbacks.**

Our preliminary mathematical results indicate that subtle variation in baseline and early induced immune responses interact with feedback processes to drive quantitative variation in infection duration (and corresponding qualitative variation; i.e., acute vs chronic infections) (**Fig. 2d,g**). Under Aim 2, our work will therefore focus on manipulating rates of induction and effector impacts of immune responses, with a combination of trickle infections and select knockout strains of mice.

***Experimental manipulation of feedbacks to alter dose-dependence of duration in hosts with semi-naturalized microbiota.***  We will manipulate feedbacks directly by trickling in parasites at a low rate, and by breaking connections among T-helper feedbacks, effectors and parasite biomass. These manipulations allow us to expand our understanding in several ways. First, trickle dosing allows us to manipulate the rates [29, 30] of Th1 and Th2 induction by *T. muris* [34, 63], which theory suggests critically determine duration (**Fig. 2**). Second, trickle dosing also increases realism, since helminthiases in natural populations are much more likely to be due to consistent low exposure, rather than bolus “all-at-once” infections. Finally, manipulation of immune feedbacks independent of parasite dose (using Th2 signaling or effector knockouts or treating with immunomanipulative parasite extracts) allows us to experimentally decouple host and parasite agency in response induction. **We note that, in each experiment, we will provision all mice with rewilded gut microbes and collect data at the timepoints described in Aim 1.**

Our trickle infections will entail regimens of either 20 or 40 eggs/inoculum on each of days 0, 2, 4, 6 and 8; this cumulates to a total of 100 or 200 eggs (and the former is consistent with trickle infection regimens that challenge C57BL/6 mice in conventional laboratory conditions [34, 63]). We estimate that we will need 24 adult mice per *GH x regimen* combination per experiment (with 2 host strains, 1 sex and 4 dose regimens (2 trickle doses and 2 cumulative-dose matched boluses per experiment), culled at 4 different time points). We will conduct 2 independent experiments per combination resulting in 12 mice per strain/sex/regimen/timepoint across 2 experimental blocks.

Our remaining experiments under Aim 2 will break the feedbacks loops by disrupting their effector function in mice on the C57BL/6 genetic background. We will use IL-13-/- mice to disable Th2 signaling and thereby slow expulsion [67, 68]. We will use Muc5ac-/- because they permit potent Th2 responses but are deficient in the key mucin required for *T. muris* expulsion [91]. **Thus we hypothesize that the negative feedback of immunity upon parasites will be broken, fostering growth of parasite biomass and accelerating the positive feedback of parasites in enhancing a Th1 response.** Resulting duration of infection should be long indeed; it will be fascinating to compare duration in the 2 knockouts with wild types. We will use boluses of 20, 40, and 200 embryonated eggs/mouse. We will use 24 adult mice per *GH x dose* combination per experiment (with 3 host strains, 1 sex and 4 dose regimens (2 trickle doses and 2 cumulative-dose matched boluses per experiment), culled at 4 different time points). We will conduct 2 independent experiments per combination resulting in 12 mice per strain/sex/regimen/timepoint across 2 experimental blocks.

Finally, we will use the *T. muris* excretory/secretory molecule p43, which binds to IL-13, interfering with the development of an appropriate effector response [63]. Following the protocol under development by **collaborator** **Grencis**, we will use purified p43 to suppress Th2 effector mechanisms, decoupled from the biomass of nematodes. Interestingly, trickle infected male C57BL/6 mice do not immunologically target p43 so become more prone to chronic infection[63]. We will map the generality of this with trickle and bolus infections in p43-treated C57BL/6 and AKR mice of both sexes, using 24 adult mice per *GH x regimen* (with 2 host strains, 1 sex and 4 dose regimens (2 trickle doses and 2 cumulative-dose matched boluses per experiment, culled at 4 different time points). We will conduct 2 independent experiments per combination resulting in 12 mice per strain/sex/regimen/timepoint across 2 experimental blocks.

***Hypothesis tests.*** For each host strain, we hypothesize that we can shift the interaction towards acute infection by strengthening Th2 feedbacks, and shift the interaction towards chronic infection by strengthening the Th1 feedbacks or by decoupling the worm-clearing effector mechanisms (such as mucins [15]) from an induced Th2 response. Finally, we hypothesize that parasite biomass-driven immune feedbacks will manifest as acceleration of Th1ness above and beyond what these manipulations confer.

These hypotheses manifest as differences in either initial conditions or parameter values in our mathematical model. Trickle infections change the initial conditions of the model and introduce discontinuities into the dynamics (because parasite biomass “jumps”). The effect of these differences depends on the dynamical regime the model is in: trickle infections can be more likely to be chronic because the lower initial parasite biomass does not provoke a strong Th2 response, allowing the immune dynamics to become “trapped” at a lower immune activation. IL13-/- mice will have a lower value of the parameter in model (1), weakening the strength of clearance-promoting feedbacks; Muc5ac-/- mice will have a lower value of the parameter in model (1), weakening the strength of the negative feedback between parasite biomass and the Th2 response. Manipulations of p43 alter the value of in model 1, altering the strength of the chronicity-promoting feedback loop. Following the same procedure for model fitting as in Aim 1, we will test the predictions of the mathematical model by fitting the data from these experiments to the model and estimating model parameters.

***Expected outcomes and potential pitfalls.*** The work under this Aim is relatively low risk and high reward. We do have experience with timing and dosing such manipulations for sustained effect (reviewed in [109]) and the rest of the experimental procedures are familiar from past work (as noted under Aim 1). The experimental work *per se* would be high reward in that parasite burden and multivariate immunological data following manipulation of rates and effector impacts of induced immune responses are unknown. We do intend to give trickle infections to wild type host strains, while we will give bolus infections to knockouts. However, we may ultimately challenge knockouts with trickle infections as well, depending on early results, in order to decouple time and signal dependent effects on dose-dependence.

These two experiments will allow us to overcome key challenges with theory-data integration in Aim 1. In particular, the early dynamics of the immune response depend on the early dynamics of parasite biomass growth (**Fig. 2**), which we cannot observe either directly or indirectly (because culls occur later in infection and egg shedding only happens after reaching adulthood). **By timing immune measurements to occur on days following trickle dosing, we will gain important insight into the *initial rates* of clearance- or chronicity-promoting feedbacks by parasite biomass.** This will both test the model predictions and help us identify the shapes of key functions in the model.

Direct manipulation of the molecules that generate the feedback processes provides complementary insights. One of the major challenges for parameterizing mechanistic models of antagonistic interactions (the essential step that allows us to *quantify* the feedbacks) is that it is difficult to understand which partner is driving the interaction. For example, Th2 stimulation is driven by parasite biomass, but acts to suppress the growth of that biomass, so an observation of low Th2 stimulation and low parasite biomass could be due to intrinsically slow growth of parasite biomass or a highly effective immune response preventing parasite biomass growth. So, by manipulating Muc5ac, we can more clearly observe the interaction between parasite biomass and Th2 induction (quantifying clearance-promoting feedbacks) because we have separated the effect of the parasite on induction from the effect of induction on the parasite. We gain similar mechanistic insight by manipulating other cytokines and parasite immunomodulatory molecules, allowing us to more fully develop the model to gain an understanding of the processes driving infection dynamics.

However, if we find we are still unable to fit the data well, we can simplify our complex dynamical model to a discrete dynamic model [110], which represents the immune system as network of interacting nodes, each of which can take only two states (ON or OFF). This approach is useful when quantitative data are insufficient to characterize the functional relationships between variables [111]. Such models have been successfully applied to model host-macroparasite interactions [112, 113], suggesting that they are a viable alternative pathway to our system as well.

**Aim 3: Embed strain x dose interactions in a more realistc natural environment by rewilding mice and quantifying effects upon immune feedbacks and duration of infection fuller environmental naturalism in “rewilded” mice kept outdoors, to quantify effects upon immune feedbacks and thus duration of nematode infection.**

Under Aim 3, we will take an even greater step towards environmental realism, to “field test” our predictions. Naturalizing lab mice with “dirty roommates” [49], serial infections [50], or fecal [52] or *in utero* [51] exposures to the microbes borne by wild mice under otherwise-controlled conditions has shown that lab mice exposed to natural microbes rapidly exhibit shifts in immune phenotype: e.g., lab mice whose immune systems have benefited from these antigenic experiences show increased resistance against microparasites like influenza [52] and more human-like inflammatory reactions [51]. Thus, the importance of incorporating environmental realism into immunological experiments is increasingly appreciated [47, 48]. Those of us who have taken lab mice into mesocosms, whether indoors [29, 30] or outdoors [36, 53-55] have found that mice in a more natural environment exhibit greater duration of nematode infection than mice in vivaria. These observations suggest that naturalizing mouse models can make chronicity of their nematode infections better model human helminthiases.

An ecological perspective further suggests that the environment is much more than just the microbes! Indeed, although we’ll bring semi-natural microbial diversity and trickle infections into the mouse model under Aims 1 & 2, **there is no substitute for full realism, including natural abiotic conditions (e.g., New Jersey summer temperature & humidity) and behaviors (e.g., digging burrows)**. We also note that fully natural conditions can bring important microbial surprises: e.g., fungi have proven important for promoting neutrophilia in rewilded mice, but have also failed to establish via fecal transplant [55]. We will therefore open the door to a wider array of environmental variables under Aim 3.

***Leveraging the real-world environmental context to hone and field-test predictions.*** Our experiments under Aim 3 will entail dose-response studies of mice experiencing additional environmental variables and temporal fluctuations in the field. Each May-September for the full duration of the project, we will therefore complete dose-response experiments outdoors in C57BL/6 and B10.BR mice (these are predation-resistant non-albino strains described as “resistant” and “susceptible” to *T. muris*, respectively, in vivarium conditions [67, 68]). We will study one sex and one strain at a time (to facilitate management outdoors). Some groups of mice will receive nematode doses prior to moving outdoors, while others will receive nematodes after 2 weeks outdoors (allowing us to explore intriguing patterns in our past work (Fig. 1; [36]) . Our doses of *T. muris* will begin with boluses of 20 or 200 embryonated eggs per rewilded mouse and littermate controls maintained in the vivarium but at elevated temperature and humidity to match abiotic conditions outdoors [36, 53]. In years 4 and 5 of the project, our rewilding experiments will also begin to incorporate IL-13-/- and Muc5ac-/- mice on the C57BL/6 genetic background; following on from what we’ve learned under Aim 2 and allowing us to test whether such dramatic phenotypes in lab are as muted by rewilding as was Th2 transcription deficiency of STAT6-/- in our past work [36]. **We note that we will collect the data types described under Aim 1, BUT that logistics of trapping and mesocosm use demand that we sample mice bi-weekly instead of weekly, and that we will not go beyond 5 weeks of infection**, to not allow infections go patent outdoors**.** Thus individual mice will be longitudinally sampled at weeks -2, 0 (when infections will be delivered via gavage), 2, 4 and 5; endpoint sampling of subsets of mice will occur at weeks 2, 4 and 5 post-infection.

Our sample sizes outdoors will be larger than in lab experiments but still tractable (in line with our past work that was sufficiently powered in each treatment group). We will use 36 adult mice per ***dose regimen*** per experiment (**with 1 host strain, 1 sex, 2 dose levels and 2 bolus timings per experiment, culled at 3 different time points**). We will conduct 2 independent experiments per strain-by-dose combination; this gives 24 mice per strain/sex/dose regimen/timepoint across 2 experimental blocks.

**We are especially keen to observe whether outdoor living shifts all host strains towards greater chronicity of infection (and potentially towards greater parasite domination of immune signaling). We hypothesize that infection duration will indeed be enhanced across all host\*dose combinations with Th1 enhanced outdoors.**  We further hypothesize that we will be able to explain those shifts via alterations to the relative magnitude of Th1 feedbacks and parasite biomass-driven Th1 feedback in the more natural environment. It will be especially fascinating to learn whether full naturalization prolongs nematode infections beyond what our microbially gavaged mice will experience.

**Chart, line chart, histogram

Description automatically generated*Hypothesis tests*.** We hypothesize that rewilding leads to chronic infections by shifting the immune response towards Th1ness, as seen in our preliminary data (Fig. 1b,c). The simple model suggests multiple possible mechanisms for the observed shift in duration (**Fig. 4**). One possibility is that rewilding reduces the values of the parameters governing clearance-promoting feedbacks (such as and ), thereby allowing parasite persistence due to a lowered immune response (**Fig. 4b**, black line). Another is that rewilding increases the background level of Th1 stimulation (the parameter ), thereby altering the initial Th1ness of the immune system, making immunomodulation easier (**Fig. 4c**, solid gray line). Following the same procedure for model fitting as in Aim 1, we will test the predictions of the mathematical model by fitting the data from these experiments to the model and estimating model parameters.

**Figure 4.** Hypotheses for shifts to chronicity in rewilded mice. Chronic infections can be achieved if rewilding causes an initial bias towards Th1 due to a higher baseline level of exposure to Th1-promoting micobes. This makes immuno-modulation easier and leads to a high Th1 response that suppresses the Th2 response. Alternatively, rewilding could weaken clearance-promoting feedback loops by weakening the stimulation of the Th2 response by parasites or by reducing positive Th2 self-stimulation. This leads to a general weakening of the immune response that prevents it from being able to clear the parasite.

***Expected outcomes and potential pitfalls.*** The experimental work is the most challenging of the 3 Aims, yet we have 5 years of experience running rewilding experiments that will promote our success. Great rewards accompany the greater risk: we will offer new biological insights into mammalian immune function in a natural environment. This is an essential component of this research program. Mechanistic immunology has largely been developed by working in highly controlled laboratory environments. It is critical to know how “wild immunology” differs from lab immunology: how does rewilding alter the underlying feedback processes that drive immune responses? Is it possible to explain the dramatic alterations of infection duration observed in rewilding (**Fig. 1**) using immunological insights developed from decades of laboratory experiments, or do new mechanisms need to be invoked?

That is**, in Aim 3 we will determine whether moving outdoors simply changes parameter values, or instead whether entirely new mechanistic detail must be added to the model to capture infection dynamics outdoors.** In particular, microbes found in nature, but not in the lab, may open new channels of immune crosstalk that are not found in the lab [47, 48]. We hypothesize that the more natural the environment, the greater duration of infection. Thus mice outdoors will exhibit even greater infection duration compared to microbially naturalized mice that never left lab. To address this, we will use the model fitting process described in Aim 2 on the data from rewilded mice. We will use the lab-parameterized model as a starting point, and compare that model to additional models that include immune mechanisms that appear to be important based on observed differences in immunological measures from rewilded mice and lab-reared siblings.

Moreover, the question of how complex a model of immunity needs to be in order to accurately capture immune dynamics is an open one in theoretical and computational immunology [103, 114]. Indeed, we may find that different functional forms will predict infection duration in lab and field, such that our central hypotheses about Allee effects within mouse guts are unsupported in rewilded mice. By iterating between theory and data, we will still have a rich understanding of the ecology of within-host interactions that shapes the dynamics of helminth infections. While theoretical immunology has been successful in developing detailed mathematical models for host-microparasite interactions (e.g., HIV [115], malaria [96], dengue [116] & tuberculosis[117]), there has been considerably less work on modeling the within-host ecology of macroparasites [112, 118]. By systematically building in realistic complexity across the three Aims here, we will have a novel perspective on this question. This process will help to identify a general approach to determining the critical level of complexity needed to predict infection dynamics in the real world.

**OUTLOOK**

Gnotobiotics and other reductionist directions that a future proposal could pursue, depending upon our findings here..

On within host dynamics per se: Gnotobiotic experiments (state of the art facility at UNL) to dissect microbes responsible for especially pronounced effects on immune response induction and lengthening infection duration like in Amanda’s cancer papers

So what if we get really fancy and propose a small number of inducible K/O? mice who are normal until we stop them signaling with a given cytokine (at least in a dominant cell type).

On external environmental drivers: opportunity to exercise, natural rhythms in temperature as well as light