



Original article

Quantitative appraisal of murine filariasis confirms host strain differences but reveals that BALB/c females are more susceptible than males to *Litomosoides sigmodontis*

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Abstract

Litomosoides sigmodontis, a rodent filarial nematode, can infect inbred laboratory mice, with full development to patency in the BALB/c strain. Strains such as C57BL/6 are considered resistant, because although filarial development can occur, circulating microfilariae are never detected. This model system has, for the first time, allowed the power of murine immunology to be applied to fundamental questions regarding susceptibility to filarial nematode infection. As this is a relatively new model, many aspects of the biology remain to be discovered or more clearly defined. We undertook a major analysis of 85 experiments, to quantitatively assess differences in filarial survival and reproduction in male versus female and BALB/c versus C57BL/6 mice over the full course of infection. This large dataset provided hard statistical support for previous qualitative reviews, including observations that the resistant phenotype of C57BL/6 mice is detectable as early as 10 days postinfection (dpi). An unexpected finding, however, was that filarial survival was reduced in male BALB/c mice compared to their female counterparts. Worm recovery as well as the prevalence and density of microfilariae were higher in female compared with male BALB/c mice. Therefore, *L. sigmodontis* bucks the filarial trend of increased susceptibility in males. This could be partially explained by the different anatomical locations of adult *L. sigmodontis* versus lymphatic filariae. Interestingly, the effects of BALB/c sex upon microfilaremia were independent of worm number. In summary, this study has significantly refined our understanding of the host-*L. sigmodontis* relationship and, critically, has challenged the dogma that males are more susceptible to filarial infection.

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1. Introduction

Lymphatic filariasis affects some 120 million people worldwide [1]. To complement field studies of the clinical symptoms, parasitology, and immune responses associated with this disease, several animal model systems have been developed [2–7]. These model systems have provided valuable

insight into filarial biology and disease but have been limited by either the inability to utilise the extensive resources available to murine biologists or the failure of the parasite to undergo the full natural course of development in a permissive host. With the discovery by Bain and colleagues that the filarial nematode *Litomosoides sigmodontis*, a natural thoracic cavity parasite of the cotton rat (*Sigmodon hispidus*), can develop to patency in mice of the BALB genetic background [8], a major obstacle has been overcome. In BALB/c mice, *L. sigmodontis* infection is chronic and results in the circulation of the transmission-stage L1 larvae—microfilariae, or Mf—in the blood of approximately 50% of hosts [8]. C57BL/6 mice, on the other hand, are considered resistant to

Abbreviations used: dpi, days postinfection; Mf, microfilariae; Mf+, microfilaremic; Mf–, amicrofilaremic.

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chronic infection, as they clear adult parasites relatively rapidly and never permit blood microfilaremia [9].

In just the past 5 years, investigators have begun to realise the full promise of the *L. sigmodontis* model system. Infection of gene-knockout mice has revealed a complexity in the resistance to filarial parasites that was not previously appreciated. Although the importance of type 2 responses in the expulsion of gastro-intestinal nematodes has been repeatedly demonstrated [10–14], this had not been proven for nematodes (such as the filariae) that reside in the tissues. Our data now show that type 2 responses are indeed responsible for resistance in C57BL/6 mice but that BALB/c mice remain susceptible despite a potent type 2 response [9]. Recent data suggest that this is due to the ability of BALB/c mice to generate a strong regulatory response that overcomes type 2 effector function and permits survival of the adult parasite [15]. Hoerauf et al. have revealed even greater complexity by demonstrating that different stages of the parasite are controlled by different cytokines and that type 1 and 2 cytokines can synergise to kill the adult parasite [16,17].

In addition to deepening our understanding of susceptibility to filariasis, this model has direct application to human studies of filarial disease. For example, the biology of *L. sigmodontis* is sufficiently similar to *Onchocerca volvulus* [18] to allow it to be used for the study of this human parasite, which cannot develop in non-primates. With specific relevance to onchocerciasis, *L. sigmodontis* has been utilised to unravel the genetics of resistance to Mf [19], determine mechanisms of vaccine mediated immunity [20–23] and act as a test bed for new approaches to chemotherapy [24]. More recently, the model [15] has demonstrated remarkable parallels with reported immunological findings from human studies [25–30].

L. sigmodontis infection of inbred mice, along with complementary systems such as *B. pahangi* infection of rats [3], thus promises to be a critical tool in understanding filaria-host interactions and developing control strategies. As the *L. sigmodontis* model is relatively new, there has been only one review [31], which provided a qualitative overview of the life cycle. The time is ripe for a more quantitative assessment of this promising model system. Individual experiments with small groups of mice cannot reveal subtle but important quantitative influences (on survival kinetics, for example). For this purpose, an aggregate analysis of all available experiments was required.

In this study, we assessed the quantitative evidence for published, qualitative worm-killing curves in BALB/c versus C57BL/6 mice [31], as well as the reported ~50% prevalence of Mf positivity (Mf+) among chronically infected BALB/c mice [8]. We also looked for sex-related differences in filarial survival and transmissibility, as male and female vertebrate animals differ in exposure and/or susceptibility to many parasites [32,33]. Compiling 5 years of data on *L. sigmodontis* infections of wild-type mice, we thus tested for strain- and sex-related differences in worm survival (across the L4, young adult, and fecund adult filarial stages) and transmissibility (Mf+).

2. Materials and methods

2.1. Experimental designs and parasitological data

The life cycle of *L. sigmodontis* was maintained by cyclical passage between jirds (*Meriones unguiculatus*) and mites (*Ornithonyssus bacoti*), as described previously [34]. Our experiments focused upon *L. sigmodontis* infection of fully-susceptible BALB/c and relatively-resistant C57BL/6 mice (obtained from Harlan, UK, or an in-house breeding facility) that were 4–8 weeks of age at the time of infection. Statistical analysis confirmed that neither the source facility nor the age of mice affected their susceptibility to infection.

For all experiments in this analysis, 25 mite-derived L3 larvae were injected subcutaneously into each mouse. Experiments were then terminated during one of three 10-day windows after infection: during the worm's L4 stage (10–20 days postinfection, or dpi), young adult stage (35–45 dpi), or fecund adult stage (60–70 dpi). These categories correspond to the moult timings and thus worm life cycle stages of *L. sigmodontis* [31]. In experiments that focused upon the fecund adult stage, blood microfilaremia was detected by light microscopy, in thick smears of 10 μ l of tail blood taken on or after 58 dpi. Parasitological analysis of necropsied mice was performed at the end of each experiment. L4 or adult worms were removed from the thoracic cavity and counted as described previously [23]. The analysed dataset only included infected wild-type mice that had not received any other experimental treatment—e.g. the infected control groups from many of the experiments. In total, data on 697 mice from 85 experiments in our laboratory over the past 5 years were analysed. Extensive valuable data had been generated from these mice in the original analyses (now published or in preparation), and the analytical techniques applied in this study further extended the value of these experimental animals. Throughout all experiments, UK Home Office guidelines were strictly adhered to.

2.2. Statistical analysis of the number of L4 and adult filariae recovered

We were interested in whether the strain and sex of the murine host affected worm recovery at the L4, young adult, and fecund adult life cycle stages. We could not do a fully factorial strain-by-sex analysis because C57BL/6 females were under-represented in the dataset (our experiments included 14 C57BL/6 females, 221 C57BL/6 males, 255 BALB/c males, and 207 BALB/c females). Instead, the BALB/c males served as a reference group for independent analyses of strain and sex effects. Thus, the strain-effects analysis compared the worm counts of C57BL/6 males and BALB/c males, while the sex-effects analysis compared worm counts of female versus male BALB/c mice. In each analysis, we also were able to assess differences in worm survival across stages, as well as interactions of parasite stage with host strain or sex effects.

As is common in natural helminth infections [35], the distribution of worms among hosts was best described by a negative binomial function. In other words, the variance was much greater than the mean and so deviated from a normal as well as a random (Poisson) distribution [35,36]. (The negative binomial dispersion parameter was estimated by maximum likelihood and the goodness of fit formally assessed.) This was particularly true of C57BL/6 mice, due to the large number of zero values and thus the extremely left-skewed distribution. No transformation succeeded in making the data normal, so a negative binomial error distribution was used for most of the generalised linear analyses, though the sex-effects data (including only BALB/c mice) was better modelled with Poisson error. For intuitive ease, our results are graphically presented as raw means. The raw plotted means thus do not take the underlying distribution into account, but the formal analysis (and thus the reported χ^2 statistics and *P*-values) did. All statistical analyses (including those below) were conducted in the SAS System V8, with a *P*-value of 0.05 considered the cut-off for statistical significance.

2.3. Statistical analysis of the odds of infected BALB/c mice becoming *Mf* positive

A logistic regression model was used to analyse the odds of blood microfilaremia for male versus female BALB/c mice. We specifically addressed whether sex effects were independent of any differences in adult worm counts between the two sexes. Mouse sex and worm counts were thus both included as predictors in the model, and their interaction was also tested for statistical significance.

2.4. Statistical analysis of microfilarial densities in *Mf*+ BALB/c mice

Like the worm counts, microfilarial densities were best described by a negative binomial distribution; *Mf* densities in people have also been found to follow a negative binomial distribution [36]. A generalised linear model with negative binomial error was thus used to analyse the *Mf* density data. We assessed whether male and female *Mf*+ BALB/c mice differed in mean density of blood *Mf*. Again, the formal analysis took the underlying distribution into account, although the raw plotted means do not.

3. Results

3.1. L4 and adult survival was determined by both the strain and sex of the host

To quantify filarial survival in different strains of inbred mice and to assess the evidence for sex-related differences in susceptibility to filariasis, we undertook an aggregate analysis of a large dataset on wild-type BALB/c and C57BL/6 mice. Each mouse had been infected with 25 L3 larvae of *L. sig-*

modontis and then necropsied for worm counts during one of three time frames postinfection: 10–20 dpi (when the filariae would be in the L4 stage), 35–45 dpi (the young adult stage), or 60–70 dpi (the fecund adult stage). We found that, even at the earliest worm life cycle stage that we assessed (L4), worm numbers were highest in BALB/c females, second-highest in BALB/c males, and lowest in C57BL/6 males (Fig. 1, the statistical details of which are explained below). These differences appeared early and persisted across all worm stages. Within a host type, worm recoveries declined over the course of infection, and filarial survival was particularly reduced in C57BL/6 mice. Our experiments included too few C57BL/6 females to enable a balanced strain-by-sex factorial analysis, but the limited data suggested that at the L4 stage, female C57BL/6 mice tended to be more permissive to infection than males (mean \pm S.E. = 6.8 ± 0.4 worms in five female C57BL/6 mice, versus 3.3 ± 0.4 worms in 74 male C57BL/6 mice; $\chi^2_1 = 3.3$; $P \sim 0.07$). However, by 60–70 dpi, C57BL/6 females were as resistant to infection as their male counterparts (mean \pm S.E. = 0.2 ± 0.2 worms in nine female C57BL/6 mice, versus 0.4 ± 0.1 worms in 91 male C57BL/6 mice).

Statistically, the strain-effects analysis (comparing BALB/c to C57BL/6 males and taking into account the negative binomial distribution of those data; $n = 476$ mice) indicated that, across all worm life cycle stages, BALB/c males had significantly higher worm counts than C57BL/6 males ($\chi^2_1 = 117.9$; $P < 0.0001$). In both strains, the number of worms declined across worm stages ($\chi^2_2 = 90.5$; $P < 0.0001$), but the decline was faster in C57BL/6 mice (strain-by-stage interaction $\chi^2_2 = 56.4$; $P < 0.0001$). In BALB/c males, the rate of worm

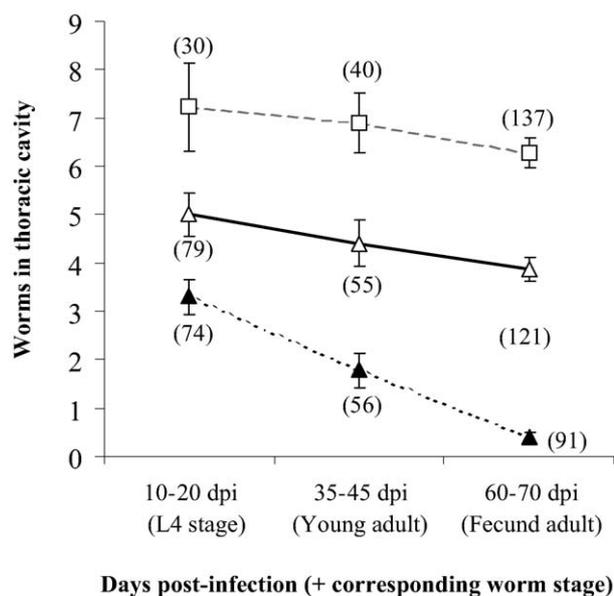


Fig. 1. The mean number of worms recovered in the thoracic cavity of BALB/c female (open squares and dashed line), BALB/c male (open triangles and solid line), or C57BL/6 male mice (filled triangles and dotted line), at three time points following infection with 25 L3 larvae of *L. sigmodontis*. Error bars represent S.E. of the mean. Number of mice per group is in parentheses.

killing was so slow that although recoveries of L4 and fecund adults differed, neither of those differed significantly from the intermediate young adult stage (Fig. 1).

The sex-effects analysis (comparing male to female BALB/c mice and taking into account the Poisson distribution of those data; $n = 462$ mice) showed that, across all worm stages, female BALB/c mice had higher worm counts than males ($\chi^2_1 = 118.0$; $P < 0.0001$). Worm numbers declined as infection wore on ($\chi^2_2 = 17.2$; $P < 0.0001$), but males and females were killing worms at the same rate; i.e. the sex-by-stage interaction was not significant. All BALB/c mice killed worms so slowly that there was no difference in worm counts between the 35–45 and 60–70 dpi time frames ($P \sim 0.47$). As noted above, C57BL/6 females appeared to be as poor as BALB/c females at killing L3 larvae (prior to 10 dpi). However, by 60–70 dpi, strain effects on filarial survival were dominant, and C57BL/6 females, like the males, were virtually worm-free.

In sum, host sex affected early establishment of *L. sigmodontis* infection in BALB/c mice. Effects of host strain were also apparent at early time points but then additionally led to different kinetics of worm survival, with killing of filariae occurring at a higher rate in C57BL/6 compared to BALB/c hosts throughout the course of infection.

3.2. The presence and density of blood microfilaremia were sex-dependent in BALB/c mice

L. sigmodontis filariasis develops to patency only in mice of the BALB genetic background, and it has been reported that approximately 50% of BALB/c mice become Mf+ beyond 50 dpi [8], at the same dose and protocol as used in this study. With male and female mice together, the prevalence of Mf positivity across our experiments was indeed 52%. However, when this was broken down by host sex, only 27% of males were Mf+, whereas 68% of females were Mf+. This suggested an effect of host sex on the probability of developing blood microfilaremia.

To formally investigate whether Mf positivity was sex-dependent, we analysed the odds of male versus female BALB/c mice becoming Mf+, specifically taking into account the fact that worm numbers were higher in females than males. The analysis thus focused upon factors determining Mf presence/absence in experiments taken out to the fecund adult stage (60–70 dpi; $n = 222$ mice). Logistic regression indicated that increasing worm counts were associated with increased odds of Mf positivity: averaged across the entire range of counts, each additional adult worm raised the odds of becoming Mf+ by $\sim 14\%$ ($\chi^2_1 = 7.4$; $P < 0.01$). As shown above, female mice bore more worms than males, and that by itself might generate increased prevalence of Mf positivity among females. However, the predominant, highly significant result of the logistic regression was that females were over four times more prone to be Mf+ than males, independent of the number of adult worms borne ($\chi^2_1 = 21.4$; $P < 0.0001$; see odds ratios (OR) and associated confidence

Table 1
Odds Ratio (OR) and 95% confidence intervals (CI) for the development of blood microfilaremia in female versus male BALB/c mice: the effects of sex per se are independent of the effects of increased worm burden in females

Factors affecting odds of becoming Mf+	OR	(95% CI)	χ^2_1	P
Number of adult filariae present	1.14	(1.04–1.25)	7.4	<0.01
Mouse sex: F compared to M	4.19	(2.29–7.69)	21.4	<0.0001

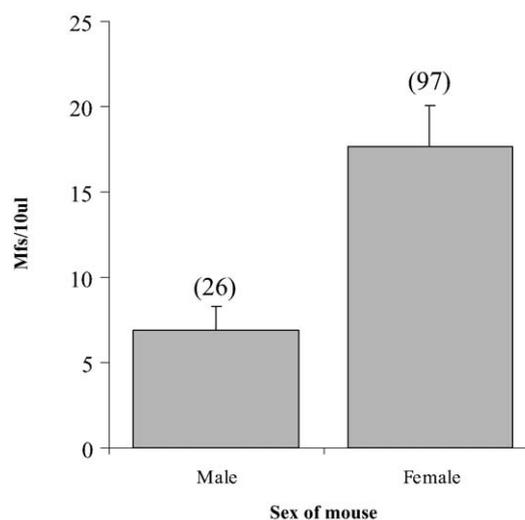


Fig. 2. The mean density of blood microfilariae (Mfs), days 60–70 postinfection, for male versus female Mf+ BALB/c mice. Error bars represent S.E. of the mean. The number of mice per group is in parentheses.

intervals (CI) in Table 1, with an OR > 1 associated with increased risk). So female hosts favoured survival of both adult worms and Mf. C57BL/6 mice never supported blood microfilaremia in our hands.

Finally, in many 60–70 dpi experiments, we quantified the density of Mf (/10 μ l) in all Mf+ individuals. Taking into account the negative binomial distribution of these data, analysis for sex effects revealed that female mice had higher Mf densities than males did ($\chi^2_1 = 12.8$; $P < 0.0005$; $n = 123$ mice; Fig. 2). Female BALB/c hosts thus supported more transmission-stage filariae, so filariasis might be more transmissible from the female mouse.

4. Discussion

Our analysis revealed a strong effect of both mouse strain and sex on the survival and transmissibility of *L. sigmodontis*. Differences in *L. sigmodontis* worm burdens in C57BL/6 versus BALB/c mice at various time points postinfection had been described previously—for example, at 40 or 60 dpi [9]—but this study is the first formal, quantitative demonstration of the strain-difference kinetics proposed by [31], which were necessarily based on a limited dataset available at the time. Several investigators have observed differential worm counts at early time points (during the L4 stage, at 10 dpi [37] or 20 dpi [9]), but these had never reached statistical significance and until now had remained unverified. This

early effect of host type on worm establishment might be explained by differences between C57BL/6 and BALB/c mice in the very early, localised immune response to infection [37] and is consistent with data suggesting that early immunological events are responsible for dose-dependent differences in filarial survival [38]. In any case, our data confirm that worm counts are higher in BALB/c compared to C57BL/6 mice throughout the course of infection. This study also supports the notion [31] that survival of adult filariae essentially falls to nil in C57BL/6 hosts after 40 dpi.

The match between the qualitative review [31] and our quantitative kinetics is not perfect, however. Contrary to the suggestion of a steep decline in filarial survival in BALB/c mice from 50 dpi [31], our data indicate that survival in BALB/c hosts was stable through 70 dpi. There was in fact no difference in worm counts between the 35–45 and 60–70 dpi sampling windows in these mice. Due to limited data past 70 dpi, we were unable to assess worm survival in BALB/c hosts beyond that point. In our experience, however, worm counts decline by day 80 pi in male BALB/c mice (with a mean of 1.7 ± 0.3 worms per mouse; $n = 37$ mice; data not shown), whereas in females, worms can survive longer than 90 days (our own unpublished observations, substantiated by data from Babayan et al. [38]). These observations are in agreement with our finding that beyond the effects of mouse strain upon filarial parasitology, there are strong effects of host sex.

Sexual dimorphism in susceptibility is usually associated with higher prevalence and intensity of infection among males [32]. We found the reverse: the *L. sigmodontis* burden was higher in female than in male BALB/c mice. Even on the relatively-resistant C57BL/6 genetic background, we found a trend towards a greater number of L4 worms in females, and the majority of the difference in filarial survival in male versus female BALB/c mice was established prior to the L4 stage of the worm. Later in infection, we found that the odds of becoming Mf+ were over four times higher in BALB/c females than in males. Importantly, this was independent of the higher worm counts in female hosts. Female BALB/c mice were therefore dually prone to Mf+ filariasis, and overall, the prevalence of Mf+ among females was 68%, whereas it was only 27% among males. It thus appears that the reported ~50% prevalence of Mf+ [8] applies only when the two sexes of BALB/c mice are combined. However, our finding that Mf density was higher in Mf+ females than in males accords well with the previous report [8]. In any case, the effects of host sex on microfilaremia in BALB/c mice appear strong. We know that *L. sigmodontis* Mf are controlled by type 2 cytokines (IL-4 and IL-5) [17,39], to a greater extent than those same cytokines are able to control adult worm burden [17]. It is interesting that females are more prone to Mf+ *L. sigmodontis* infection, given the alleged type 2 bias of females (as explored in [33]). However, IL-10 is essential for Mf persistence [19]; perhaps this is where females excel. Consistent with this possibility, estrogen has been implicated in increased IL-10 levels in BALB/c mice [40].

This pattern of increased susceptibility in BALB/c females is in contrast to reports on diverse host-parasite systems, where, despite many exceptions [33], males tend to be more susceptible to infection by parasites [32], including filariae. For example, both Indian [41] and Cameroonian [42] studies have shown that human males are more prone to Mf+ *Wuchereria bancrofti* infection. In Papua New Guinea, *W. bancrofti* Mf densities do not differ by sex overall [36] but are reduced among reproductive-age females, indicating an interaction between age-related and sex- or pregnancy-related effects [43]. In Burkina Faso and Liberia, geographical differences in transmission interact with sex-related differences in *O. volvulus* filariasis, such that higher Mf densities among men are only seen in low-transmission areas [44]. The effects of host sex on filarial parasitology in nature may indeed interact with other effects, and immunological or hormonal differences might be difficult to separate from sex-related differences in exposure. But worldwide, reduced filariasis among women is probably not due to exposure differences alone [45]. Consistent with this assertion, controlled experimental infections of cats and rodents have shown that males are more susceptible than females to filariasis [3,5,46–48]. Hormones are often implicated as the cause of this sexual dimorphism in parasitism, though immunosuppressive testosterone is not sufficient to explain the pattern entirely [32,49,50].

Why resistance to *L. sigmodontis* should be different from other filariae is not immediately clear. Tissue tropism, though, could help to explain our findings. Whether due to male-specific lymphatic architecture or mere gravity, the scrotum is an important intra-host niche for lymphatic filariae. For example, a strong scrotal tropism of *W. bancrofti* has been documented in men [51]. In rats, the number of *B. pahangi* worms in the testes can equal that of the rest of the body put together, and the scrotum is a preferred site for worm survival and reproduction [3]. Castration studies that abolish male bias [48] remove both the potentially-immunosuppressive effects of testosterone and the special niche. These two factors together might help to explain increased prevalence of lymphatic filariasis in males.

By contrast, the intra-host niche of *L. sigmodontis*—the thoracic cavity—is equally available in male and female BALB/c mice. Thus, although this model is not a natural host-parasite combination it has the potential to unmask the effects of sex-related hormonal and immunological differences. In other words, future studies in *L. sigmodontis* will avoid the confounding sex-related factors of exposure and intra-host niche, to reveal how hormones and cytokines influence susceptibility to filariasis. This is particularly true because, although the BALB/c-*L. sigmodontis* model does not fully replicate human filariasis in terms of pathology or anatomical location, it provides a remarkably good model of human immune responses to filariasis. In addition to the characteristic Th2 response, as infection progresses and patency is achieved, mice exhibit T cell hypo-responsiveness, T regulatory cell activity and reduced expression of the effector cytokines IL-5 and IFN- γ [15] directly parallel to that observed in human infection [25–30].

The analyses presented here were rendered extremely powerful by sample sizes that are not attainable in the context of a single experiment, but this study can nonetheless inform future experimental design. Major analyses were essential to accurately estimate the magnitude of strain- or sex-related differences in the kinetics of worm killing, as well as to enable a powerful test of the odds of Mf positivity for female versus male mice. However, power tests [52] based upon the variance in worm recovery from 40 to 60 dpi in individual experiments indicate that a difference of two worms (i.e. a difference comparable to the strain and sex effects on worm counts reported here) can be detected with as few as 5–6 mice per group. At earlier time points, however, when BALB/c and C57BL/6 are more similar in worm burden, 10 mice per group would be necessary. When *L. sigmodontis* worm recovery is used as an index of treatment effects, the time point of interest should influence the sample size used.

This study opens the way for a more comprehensive understanding of the relationship of filarial parasites with their mammalian hosts. The relative contributions of hormones, innate defences, and adaptive defences to the observed strain- and sex-related differences in *L. sigmodontis* parasitology remain largely unknown. In addition, a complete picture of worm demography would include growth [37,38], which we have not assessed here. These are important directions for future research, which will be facilitated by the detailed and accurate profile of *L. sigmodontis* infection in susceptible and resistant mouse strains provided here. Further, Morales-Montor et al. [33] recently challenged the paradigm of ‘female host supremacy’ in infectious disease, by showing that females are not always less susceptible than males. Critically, our findings show that even within the filariae, the paradigm fails.

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