Title: Host sex and genotype modify the gut microbiome response to helminth infection

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Abstract

The microbial community can be altered by direct/indirect interactions with parasites infecting host. Direct interactions can arise from physical/chemical contact with the parasite. Indirect interactions can involve parasite-induced changes in host immunity. If so, this would represent a case of genetic polymorphism in one species controlling an ecological interaction between other species. Here, we report a test of this expectation: we experimentally exposed *Gasterosteus aculeatus* to their naturally co-evolved parasite, *Schistocephalus solidus*. The host microbiome differed in response to parasite exposure, and between infected and uninfected fish. The microbial response to infection differed between host sexes, and also varied between variants at autosomal quantitative trait loci (QTL). These results indicate that host genotype regulates the indirect effect of infection on a vertebrate gut microbiome. Our results also raise the possibility that this sex-bias may be related to sex-specific microbial responses to the presence (or, absence) of helminthes. Therefore, helminth-based therapeutics as possible treatments for inflammatory bowel diseases might need to take account of these interactions, potentially requiring therapies tailored to host sex or genotype.

Keywords: gut microbiome, helminth infection, sex, genotype, *Gasterosteus aculeatus*

Introduction

Helminth infection is often associated with changes in the host's gut microbiome [1, 2], resulting in a complex multi-way ecological interaction between host, parasite, and diverse microbiota. For example, *Hymenolepis diminuta* infection reduces the abundance of Bacilli species in the gut of mammalian hosts, in both the lab and wild [3, 4]. However, other studies report no effects of infection on the microbiome: *Trichuris trichiura* and *Necator americanus* infections in humans do not alter the fecal microbiota [5, 6]. Sometimes the gut microbiota protects its host against helminth infection, such as the maternally transmitted bacterium *Spiroplasma* that protects *Drosophila neotestacea* against nematode parasitism [7], or *Bifdobacterium animalis* that protects mice against *Strongyloides venezuelensis* infection [8]. A recent study demonstrated that *T.muris* infection altered the gut microbiota in mice, and inhibited subsequent rounds of infection [9]. Conversely, *Lactobacillus* facilitates *H. polygyrus* and *T. muris* infections in mice [10, 11]. These opposing findings illustrate the inconsistent nature of helminth-microbiome interactions within their shared hosts. What biological variables explain such heterogeneous results? If we can identify the causes of these variable helminth-microbe associations, then we may be better able to treat dysbiosis and/or macroparasite infections [12-14].

The mechanisms underlying helminth-microbiome interactions are still being elucidated. Macroparasites can interact directly with the microbiome, for instance by secreting antibacterial peptides (e.g., the gastrointestinal nematode *Heligmosomoides polygyrus* secretes at least eight products with antibacterial effects [15]). Or, parasites can indirectly alter the gut microbiota via changes in host physiology, especially immune state. *H. polygyrus bakeri* infection induces colonic regulatory T cells in mice, which are widely regarded to arise in response to gut microbiota colonization [16], and protect mice from colitis [17]. Also, *H. polygyrus* can negatively regulate host intestinal mucosal IL-22 and IL-17, and decrease the host's expression of epithelial antimicrobial peptides [18]. But non-immune mechanisms also occur: helminth damage to the host gut epithelium can cause malnutrition that changes the microbiome [19].

Indirect interactions between the parasite and microbiota, acting through host traits, should presumably be contingent on host traits. Hosts often vary in their immune response to helminth infection, due to immunogenetic polymorphism [20, 21], energetic reserves [22], stress [23], and prior parasite exposure [24]. Males and females within a species also can respond differently to infections [25], because sex hormones modulate immune traits [26]. But, we lack evidence that

variation within host species changes the microbial response to parasitic infection. Here, we show that host sex and autosomal genotype alter the gut microbiota response to cestode infection. We experimentally exposed laboratory-bred threespine stickleback (*Gasterosteus aculeatus*) fish to their native cestode parasite (*Schistocephalus solidus*). We then assayed the gut microbial response to infection and host genotype, and non-additive interactions between infection and genotype. This host-parasite system is especially valuable for such studies because the microbiota have little opportunity for direct interaction with this cestode parasite. After the fish ingests infected copepods, the parasite exits the copepod and rapidly penetrates the intestinal wall (within hours) to establish a long-term infection in the peritoneal cavity, physically separated from the gut microbiota. So, cestode effects on the microbiota are likely to be indirect, mediated via host traits such as immune responses which are often polymorphic within, and divergent between, host species. In an initial exposure experiment (experiment 1), we show that cestode exposure suffices to alter the gut microbiota, even when the infection subsequently fail, but this effect varied among host full-sibling families. In a follow-up experiment (experiment 2) we show that infection presence/absence (controlling for exposure) does also alter the gut microbiota, but this effect varies between host sexes (which are genetically determined) and autosomal genotypes. This result demonstrates that genetic variation in a host species modifies the ecological interaction between helminth and microbiome communities.

Results

Effects of cestode exposure versus infection

Experiment 1 was designed to test if exposure alone, or infection presence, change microbial communities. This experimental design is illustrated in Additional file: Figure S1a and sample sizes of fish used in this experiment was listed Additional file: Table S1. The results show that cestode-exposed fish (infected or uninfected) had different microbial communities than the sham-exposed control fish (different unweighted PCoA1 axis scores), though this trend varied among host full-sib families (Fig. 1a and Additional file: Table S2; fish family $P<0.0001$, exposure $P=0.2589$, exposure*family $P=0.0363$). The significant exposure by family interaction occurs because one full-sib family (GG12) showed an atypical microbial response to exposure. Omitting this one family, the remaining families all showed a consistent microbiome response to parasite exposure (exposure $P=0.0378$, family $P=0.0579$, exposure*family $P=0.1675$). In

147 contrast, we observed no difference between infected versus exposed-but-uninfected fish (Fig. 1b
148 and Additional file: Table S2). However, this initial experiment was had modest statistical power,
149 so in Experiment 2 we focused on evaluating only the effect of cestode presence/absence, among
150 fish who were all exposed to the cestode.
151
152 **Gut microbial community composition**
153 In the Experiment 2, we exposed 711 lab-raised adult F2 hybrid sticklebacks to *S.solidus*,
154 resulting in 256 successful infections (the experimental design is illustrated in Additional file:
155 Figure S1b and sample sizes of this experiment was listed in Additional file: Table S3). We
156 obtained 16S sequences 693 of these 711 fish (a few individuals' intestines were not retained),
157 yielding 11,586 Operational Taxonomic Units (OTUs). We retained an average of 11,066
158 sequence reads per fish (s.d.=13,394; median=7,575; range from 48 to 126,943). We excluded all
159 individual stickleback with fewer than 500 sequence reads (*N*=43 removed, 650 retained,
160 Additional file: Table S4). A summary of the gut microbial community composition is provided
161 in Dataset 1. The five most abundant Phyla in the sticklebacks' intestines were Firmicutes
162 (61.07% of reads on average), Proteobacteria (28.68%), Actinobacteria (5.13%), Bacteroidetes
163 (0.93%) and Planctomycetes (0.67%). The microbial Orders Bacillales (0.53% ~ 98.34%),
164 Burkholderiales (0.095% ~ 91.79%) and Actinomycetales (0.027% ~ 98.46%), and Family
165 Alcaligenaceae (0.025% ~ 98.27%) were detected in all individual stickleback. As previously
166 noted [27, 28], the stickleback gut microbiota differed dramatically among individuals
167 (Additional file: Figure S2), despite their similar ages and being reared on the same foods in the
168 same research facility (split between two adjacent rooms).
169
170 **Host sex, cross, and infection jointly affect the gut microbiome**
171 General linear model analyses of the 181 most common microbial Families (those present in at
172 least 20 fish) suggest that cestode infection and host genotype jointly affect the stickleback gut
173 microbiome. All models included room effect as a covariate to account for the large effect of
174 rearing room (Additional file: Figure S3). Host sex, cross, and mass each had significant main
175 effects on nearly a third of microbial Families (32.6%, 29.8%, and 27.1% respectively;
176 Additional file: Figure S4 and Dataset 2). Main effects of cestode infection were relatively

uncommon, however, affecting only 11.0% of microbial Families (still significantly more than expected from our 5% type I error rate; χ^2=12.7, *P*=0.0003).

Although relatively few microbial Families were associated with cestode presence/absence (consistent with the lower-powered Experiment 1), substantially more Families (50 of 181, or 27.6%) exhibit interactions between infection and other host traits. Specifically, 19.9% of microbial Families exhibited significant infection*cross interactions (contrasting ROB backcross, F2 intercross, or GOS backcross), and 17.1% of Families depend on infection*sex interactions. Fish mass had little effect on the cestode-microbe interaction: only 8.8% of microbial Families exhibited a mass*infection interaction, slightly more common than the 5% false positive rate (χ^2=4.8, *P*=0.027). Thus, the statistical main effect of infection (our focus in Experiment 1) underestimated the impacts of cestode presence, the majority of which were contingent on genetic characteristics of the host (sex, cross type). Comparable trends were observed if we examined other taxonomic ranks (e.g., 84 common microbial Orders, Fig. 2).

Examples of interactions between helminth infection and host genotype

A few illustrative examples of such interaction effects are plotted in Fig. 3 and Additional file: Figure S5. We observed a strong main effect of infection for some microbe Orders, such as Fusobacteriales (Fig. 3a and Additional file: Figure S5a, infection effect t=-2.65, *P*=0.0083), which were less abundant in infected sticklebacks' gut regardless of sex or genotype. As an example of a main effect of host sex, Lactobacillales were more abundant in male than in female stickleback regardless of infection status (Fig. 3b and Additional file: Figure S5b, sex effect t= 3.37, *P*<0.001).

Clostridiales illustrate the contingent effect of parasite infection (Fig. 3c and Additional file: Figure S5c), with a sex main effect (t=5.56, *P*<0.0001) and a sex*infection interaction (t=-7.46, *P*<0.0001). Clostridiales abundance was insensitive to infection in female hosts, but *S.solidus* infection strongly reduced Clostridiales abundance in male fish. Equivalently, one could say that Clostridiales abundance differed strongly between uninfected males and females, but did not differ between infected males and females. Rhodobacterales also show a sex*infection interaction Fig. 3d and Additional file: Figure S5d, increasing with infection in males but decreasing with infection in females. As a result, among uninfected fish Rhodobacterales were most abundant in females, whereas among infected fish this taxon was most abundant in males.

Host autosomal genotype also influenced the gut microbiota, as indicated by widespread differences between backcross versus F2 hybrids. Caulobacterales were significantly more common in F2 hybrids (Fig. 3e and Additional file: Figure S5e, cross main effect $F_{2,629}$=71.6, P<0.0001) than in either backcross. Other Orders were more common in one particular cross, or exhibited an additive trend (F2s intercrosses being intermediate between backcrosses). This autosomal effect altered the impact of cestode infection. Rhodobacterales were more abundant in hosts with greater ancestry from Roberts Lake, but only in the absence of the cestode (Fig. 3f and Additional file: Figure S5f). In contrast, in cestode-infected fish Rhodobacterales were uniformly common in all genotypes.

Discriminant function analyses of host cross and sex effects on microbiota

We next used multivariate analyses to evaluate the response of the overall gut microbiome community to infection and host genotype (summarized in Additional file: Table S5). LDA separating the four combinations of sex and infection confirmed that helminth infections alter the gut microbiome but sex modifies the effects of helminthiasis (Fig. 4a). The two leading LDA axes, respectively, exhibited a significant effect of sex ($F_{1,466}$=90.8, P<0.0001), and an effect of infection ($F_{1,466}$=4.64 , P=0.0317). For LDA axis 1 (LDA1), these variables also interacted ($F_{1,466}$=9.6, P=0.0021). Overall, cestode infection changed female gut microbiota composition more strongly than it changed male microbiota.

A separate discriminant function analysis of fish cross type and infection status (6 combinations) revealed several insights. The first LDA axis separated F2 hybrid from both reciprocal backcross populations (Fig. 4b), suggesting that there may be transgressive genetic effects on the microbiota (e.g., Fig. 3e). F2 hybrids were intermediate between the backcrosses along LDA2, consistent with additive genetic control of other aspects of the microbiota (Additional file: Figure S6). The third axis separated infected and uninfected fish, but in a highly genotype-dependent manner (Fig. 4b and Additional file: Figure S6). In F2 cross fish, LDA3 scores were insensitive to infection, whereas in both backcross populations the LDA3 scores changed strongly in response to infection. All three crosses had similar LDA3 scores when uninfected, but Gosling and Roberts backcross fishes' microbiomes diverged in response to infection.

QTL mapping of gut microbiota composition

Based on the effects of cross type, described above, we expected to be able to locate host loci that (i) explain variation in gut microbiota composition, and (ii) do so differently for infected versus uninfected fish. We used quantitative trait locus (QTL) mapping to test this expectation, and to identify chromosomal regions for future detailed mapping, and possible candidate gene identification and validation.

ddRADseq identified 234 SNPs with fixed differences between the Roberts and Gosling populations and sufficiently deep coverage within and among the F2 hybrid individuals, yielding approximately 10 markers per linkage group. Another paper will describe QTL mapping of infection outcomes and host immune traits (Weber et al, in preparation); here we focus only on mapping the gut microbiota. We located no significant QTL for any of the top 10 weighted PCoA axes, and no significant QTL for microbial diversity (at a rarefaction of 2000 or 4000 reads per fish). But, we did detect weak-effect QTL for an unweighted PCoA axis. Unweighted PCoA axis 5 exhibited three QTLs that narrowly exceeded our stringent threshold for significance (Additional file: Figure S7a-c). We had a stronger signal when we fused the many PCoA axes into a single metric using linear discriminant analysis (trained to distinguish the two backcrosses then applied to all samples). Using this first LDA axis we detected a single well-supported QTL on Chr9 (Additional file: Figure S7d-e). Note that because QTL mapping was done within each cross, using LDA to define an axis that distinguishes between crosses is not tautological. Chromosome 9 does not have any noteworthy effect on cestode infection success (infection QTL described in Weber et al, in preparation).

The lack of strong QTL for microbial ordination metrics led us to hypothesize that host control of the whole microbial community is highly polygenic. If host genetic variation acts on particular microbe taxa, it might act only weakly on PCoA scores and be correspondingly hard to map. So we next mapped microbial Orders separately, revealing numerous taxon-specific QTL. To illustrate, Fusobacterales exhibit two strong autosomal QTL, plus an association with the X chromosome (ChrX) indicating a sex effect (Fig. 5). Other examples are plotted in supplementary figures (Additional file: Figure S8), and their overlapping distributions in the genome are presented in Fig. 6. This summary reveals genomic ‘hotspots’ for QTL affecting the microbiota, on Chr1, Chr2, and Chr3. Many of these microbial Orders also mapped to the sex

chromosome (Chr19), consistent with the common main effect of sex. Similar results were obtained for Family level QTL mapping.

We next tested whether these QTL are contingent on cestode presence or absence, as expected from the interactive effect of infection and cross type, described above. We mapped QTL separately for infected fish and for uninfected fish, and then looked for loci with QTL in only one of these groups. We found numerous host loci that are only associated with microbial variation in uninfected fish, whereas the same locus is unrelated to the microbiota among infected fish (e.g., Additional file: Figure S9a-f). In fewer instances, we identified host QTL for microbiota in infected fish only (Additional file: Figure S9g-h). In several cases stratifying by cestode infection revealed QTL that would not have been detected otherwise. For example, Solirubrobacterales (Additional file: Figure S9e-f) have a QTL on Chr3 that is observed in all fish, but which in fact only acts on the uninfected fish (a larger sample size). When we ignored infection status, the same microbial taxon had a non-significant QTL on stickleback Chr16. The lack of Solirubrobacterales in infected fish was masking the effect of host genetic variation at that site.

Discussion

The interaction between hosts, parasites, and gut microbes represents a rich opportunity for experimental studies of multi-way ecological interactions. A growing literature suggests that helminth infection can alter the vertebrate gut microbiota [1]. Our experiment adds an important twist to this literature: the magnitude and direction of helminth-induced microbial changes depend on both the sex of the host, and the host's autosomal genotype. By examining F2 hybrids between two recently-diverged host populations, we were able to identify autosomal loci (QTL) that contribute to variation in microbiota composition, as well as variation in microbiota response to infection. The implication is that genetic variation within one species (the host) alters the ecological effect of another species (the cestode parasite) on a third party (the microbial community).

The inconvenient implication of this finding is that we may not readily generalize helminth-microbe effects beyond the genotypes (and sex) we study. This unwelcome news is tempered by the opportunity it presents: host genetic variation can therefore help us identify the mechanisms by which helminths alter the gut microbiota (or, vice versa). Doing so may allow us to develop a

more general predictive model that can account for such heterogeneity among study subjects, and ultimately explain why individual hosts differ in their microbial response to infection.

To date, many studies have evaluated effects of helminth infection on the gut microbiota of animals [1, 13]. Yet, we remain largely ignorant as to whether these helminth effects depend on other biological variables, whether environmental effects or host genotype. Here, we have provided evidence of such interactions: host genotype (sex and autosomal) and helminth infection synergistically alter the microbiota. One third of the common microbial Orders exhibited significant interactions between infection and host genotype (Fig. 2), whereas infection by itself affected only 7% of the Orders. For example, the abundances of some microbial Orders in female hosts were significantly different with those males when uninfected, but no difference between them when infected. Discriminant analysis suggested that females' microbiota were generally more sensitive to infection than males' were. This sex-dependent effect of infection is consistent with other recent papers on stickleback suggesting that sex modifies the gut microbiome's response to diet [27], and MHC genotype [29].

Host autosomal genotype also affected gut microbial composition, and modified microbes' responses to helminth infection. This inference is consistent with many other studies of vertebrates that have yielded many examples of host genetic control of microbiome composition [30, 31]. What makes the above results novel is that this host genetic variation also alters the microbiome's response to a third (parasitic) species. We coarsely localize these autosomal effects to a modest number of chromosomal loci (QTL). These QTL do not contain MHCIIb, which has previously been associated with natural variation in stickleback gut microbiota [29]. At present we lack the resolution fine-map down to specific candidate genes, but some intriguing possibilities exist. The QTL on Chr2, which affect multiple microbial Orders, contains transcription factor c-MAF, which activates the expression of IL-4 in Th2 cells and attenuates Th1 differentiation [32]. Interestingly, recently Xu et al. reported c-MAF-dependent regulatory T cells mediate immunological tolerance to intestinal microbiota [33]. In addition, the QTL on this chromosome contains reticulocalbin and synaptotagmin IX, which are associated with calcium ion binding; dietary calcium can affect the intestinal microbiota composition [34-36]. The QTL on Chr3 contains cadherins, which play a key role in intestinal homeostasis and barrier function [37]. Substantial future work is needed to fine-map candidate genes and experimentally validate their suspected effects on the gut microbiota, and on the cestode-microbiome interaction.

Interactions between host genotype and infection likely arise from host genetic variation in immune response to cestodes. The presence of helminths can interfere with TLR or downstream signaling pathways [38], which may have ancillary effects on the microbiota. Adaptive immune responses may be involved as well. Helminths can trigger a Th2 response that increases mucus production and epithelial cell turnover, altering the mucosal microbiota [39]. Any polymorphism in immune genes (e.g. TLRs) involved in these pathways may lead to an exaggerated (or, reduced) immune response to helminth infection, and thereby modify the effect of helminths on gut microbes. This logic applies equally to immune differences between males and females [40, 41].

In the specific case examined here, helminth effects on the microbiota are especially likely to involve indirect effects via host immunity, rather than a direct microbe-helminth interaction. This is because *S.solidus* is not an intestinal parasite. After being ingested, it quickly transits into the body cavity where it persists for months but cannot directly affect the microbiota. Prior studies confirm that stickleback vary in their ability to detect *S.solidus*, mount an effective immune response, and susceptibility to cestode immune suppression [42-44]. Polymorphism in these immune responses may cascade throughout the host body, imposing collateral effects on the microbiota.

The above results specifically apply to variation among cestode-exposed stickleback. But, our Experiment 1 suggests that stickleback gut microbiota depended most strongly on parasite exposure (regardless of outcome), rather than infection itself. Apparently there is a lasting effect of the transient presence of a tapeworm in the stickleback gut lumen, which outweighs the effect of tapeworm presence or absence in the peritoneum. If indeed brief parasite exposure sends lasting ripples through the microbial community, then the study of the wild microbiome will be still more difficult. We rarely if ever know our study animals' history of unsuccessful infections, so these ripples might generate substantial and untraceable variation among wild individuals. Yet infection success also alters the gut microbiome, as we then revealed in the follow-up Experiment 2.

At present we do not know the fitness consequences of the altered microbiota that we document here. Some studies have reported detrimental helminth effects on the microbiota. Experimental *T. muris* infection in mice alters the microbiota composition leading to reduced availability of microbial metabolomics products needed by the host (vitamin D2/D3 derivatives,

many fatty acids, and amino acid synthesis intermediates) [45]. Conversely, helminth infection can be beneficial, such as reducing the risk of *H. pylori*-induced gastric lesions [46]. Our results suggest that these positive or negative effects of helminth-microbiota interactions will be contingent on the specific sex and genotype of the hosts considered.

The absence of helminths in wealthy counties has been postulated to contribute to the increasing prevalence of allergic and autoimmune diseases [47], which might be attributed, at least in part, to alterations to the intestinal microbiota [13]. Some of these auto-immune diseases are sex-biased in humans [48, 49]. Our results raise the possibility that this sex-bias may be related to sex-specific microbial responses to the presence (or, absence) of helminthes. Therefore, we propose that sex-specific environmental effects on the gut microbiota might contribute to understanding sex-specific inflammatory diseases and sex differences in dysbiosis. Recently, helminth-based therapeutics, e.g. infection with *T. suis*, have been tested as possible treatments for inflammatory bowel diseases [47]. If our results hold beyond stickleback, we predict that clinical therapeutic efficacy may differ between sexes, or between host genotypes. Consequently, such treatments might need to take account of these interactions, potentially requiring therapies tailored to host sex or genotype.

Materials and Methods

Stickleback breeding

Gosling Lake and Roberts Lake on Vancouver Island, British Columbia contain stickleback populations that differ in immune phenotypes, immune response to infection, parasite growth in the lab, and cestode prevalence in the wild (>70% and 0% respectively) [50, 51]. We generated pure Roberts (ROB), pure Gosling (GOS), and reciprocal F1 hybrid families (RG or GR), and raised these to maturity in the lab, at which time we interbred them to generate second generation (F2) hybrids. These F2 hybrids included intercross families (F1*F1 matings) and reciprocal backcross families (ROB*F1, F1*ROB, GOS*F1, F1*GOS). All fish were reared in freshwater conditions and housed with full-siblings (9-39 animals per family) in either 40-L or 10-L tanks.

Parasite Collection and Experimental Exposures

These fish crosses and infections were designed to obtain a quantitative trait locus (QTL) map of the genetic basis of host control of cestode growth and viability; those results will be reported

393 elsewhere (Weber et al, in preparation). Here, we focus on testing the hypothesis that host
394 genetic variation alters the impact of helminth infection on the vertebrate gut microbiota. We
395 conducted two sets of experimental infections (Additional file: Figure S1).
396 (Experiment 1) As a pilot study, we evaluated the effects of cestode exposure, and
397 successful infection, on the host gut microbiome. We experimentally exposed adult pure-bred
398 fish from six full-sib families from Gosling Lake, to *S.solidus*. Within each family, five
399 individuals were controls fed uninfected copepods (sham exposure), while the rest received
400 infected copepods, only some of whom were ultimately infected. This experimental design
401 yielded three categories of fish within each family: unexposed controls, exposed-but-uninfected
402 controls, and infected fish (*N*=30, Additional file: Figure S1a and Table S1). For these exposures
403 we followed a standard procedure described by Weber et al. [50]. Briefly, we dissected mature
404 *S.solidus* out of infected wild-caught fish from Gosling, and bred the tapeworms in culture media
405 in dark waterbath (mimicking the gut of piscivorous birds), to collect eggs. (Roberts Lake lacks
406 *S.solidus*, so this cannot be used as a source population.) We hatched the tapeworm eggs and fed
407 them to copepods (*Macrocyclops albidus*), then visually isolated infected copepods to feed in
408 controlled doses to the lab-raised stickleback. Forty-three days after parasite exposure, we
409 euthanized fish with MS-222 to check for infection success. We froze the entire intestine in
410 ethanol for subsequent microbiome analysis.
411 (Experiment 2) To evaluate the effect of host genotype on cestode-microbiome
412 interactions, we experimentally exposed adult F2 hybrid fish (intercross and backcross, *N*=711,
413 Additional file: Figure S1b and Table S3) to *S.solidus*, then assayed infection outcomes and
414 sequenced the gut microbiome. All fish from this experiment were exposed to *S.solidus*, but not
415 all were infected successfully, providing a contrast between fish with versus without the parasite,
416 controlling for initial exposure history. By excluding the unexposed (sham) treatment, we are
417 able to devote more statistical power to evaluating host genotype interactions with cestode
418 presence versus absence, without the substantial additional confounding variation associated
419 with parasite exposure (see Experiment 1 results).
420
421 **Amplification, sequencing and analysis of 16S rRNA amplicons**
422 We extracted DNA from the entire intestine (both content and mucosa) of stickleback (*N*=30
423 pure-bred GOS fish in Experiment 1; *N*=693 F2 hybrids in Experiment 2) using the MoBio

Powersoil DNA Isolation Kits, as described by Bolnick et al. [27, 28]. For the pure-bred GOS fish, we used the V4-V5 primers [52]. For the F2 fish, 16S rRNA amplicons were generated for the V4 hypervariable [27]. Sample-specific barcodes were used as described in Bolnick et al. [27]. PCR amplification was performed in triplicate for each sample in a reaction volume of 25 µL containing 1×Q5 High-Fidelity Master Mix, 5 pmol forward and reverse primers and 1µL template DNA. The PCR products for each sample were pooled and quantified with Picogreen double-stranded DNA reagent to facilitate pooling equimolar amounts of amplicons for sequencing. To test for contamination, negative controls (without samples) were set up for both the DNA extraction and 16S PCR amplification stages. The results indicated there was no detectable contamination because the PCRs yielded negligible DNA concentrations during Picogreen quantitation. Amplicon pools were paired-end sequenced on an IlluminaMiseq platform at GSAF (Genomic Sequencing and Analysis Facility) at the University of Texas at Austin.

The raw paired-end reads were demultiplexed, and subsequent sequence processing was performed using the mothur software package (v.1.39.1), following standard operating procedures (SOP) [53, 54] (https://www.mothur.org/wiki/MiSeq_SOP). Briefly, the sequences were trimmed for 16S rRNA gene primer sequences, and then assembled into contigs and aligned with 16S rRNA gene sequences from the ARB Silva v128 reference database [55]. Chimeric sequences were detected using VSEARCH within mothur in each sample and removed. The remaining sequences were classified by using Bayesian classifier with a training set (version 16) from the Ribosomal Database Project (http://rdp.cme.msu.edu) [56]. Operational Taxonomic Units (OTUs) were identified using the UCLUST algorithm based on 97% similarity. We rarefied the data to 4000 sequences per sample to calculate unweighted and weighted UniFrac distance and PCoA scores by phyloseq library in R [57].

Analyses of pure GOS fish experimentally exposed to *S.solidus* or a negative control

We sequenced the gut microbiota of GOS fish (Experiment 1) to obtain counts of microbe abundance. We used mixed model linear models to analyze effects of family and infection status on unweighted or weighted PCoA axis 1. Family was a random effect, with a random family*infection interaction. First, we contrasted unexposed (sham infections) versus exposed

fish (regardless of the outcome of exposure). Second, we compared uninfected fish (sham or failed infections) versus infected fish.

Analyses of F2 hybrid fish experimentally exposed to *S.solidus*

General Linear Model Analyses

Evaluating the results of Experiment 2, we used quasibinomial general linear models (GLMs), implemented in R, to examine the effects of infection and host genotype on microbial composition (the relative abundance of each commonly observed Order/Family, found in at least 20 fish [N = 84 Orders/181 Families]). For each taxon, we estimated a GLM in which the fraction of reads attributed to that taxon (out of all reads) was related to *S.solidus* presence/absence, and effects of host cross (Gosling backcross, F2 intercross, or Roberts backcross), host sex, and host mass. We included interactions of particular interest to us here: infection*cross, infection*sex, and infection*mass. As fish were reared in two rooms, we used a room effect as a covariate. We used a sequential Bonferroni correction to P-values when evaluating effects of particular microbial Orders/Families.

Discriminant Function Analyses

We next examined the whole-microbiome effects of infection, sex, and cross direction. We applied linear discriminant analysis (LDA) to the top 50 weighted/unweighted microbial PCoA axes (which cumulatively account for 99.99% of variance in microbial alpha diversity using phylogenetically weighted or unweighted presence-absence data). We first used LDA to distinguish four groups (combinations of sex and infection status). Second, we used LDA to distinguish six groups (factorial combinations of three host cross types and infection status). We used ANOVAs to test for effects of sex, cross, and infection status on each LDA axis. We also confirmed these statistical effects using a MANOVA applied directly to the top 50 PCoA axes to test for effects of sex, cross, infection, host mass, and interactions among these variables.

QTL mapping: ddRAD genotyping

We used a Promega Wizard SV 96-well plate kit to extract DNA from alcohol-preserved fin clips from all F2 hybrid fish (intercross and backcrosses), as well as all grandparents used to generate the crosses. We then genotyped the fish to obtain SNPs for quantitative trait locus (QTL)

mapping, using the ddRADseq protocol described in Peterson et al. [58], with bioinformatics steps to identify SNPs conducted as described in Stuart et al. [59]. We retained only SNPs exhibiting fixed differences between the Roberts and Gosling Lake grandparents (e.g., fully informative for QTL mapping). This conservative approach yielded 236 genetic markers for mapping, on average slightly more than 10 markers per linkage group.

QTL mapping: analyses

We mapped quantitative trait loci (QTL) for several microbiome measures: alpha diversity (using 2000 or 4000 read depth normalization), the top 10 weighted PCoA axes (or unweighted axes), and the relative abundance of the common microbial Orders (as described above for GLMs). We built linkage maps for each cross separately in R/qtl [60], and used the *scanone* function with 'hk' interval mapping (using rank-based nonparametric tests for microbial Order relative abundance). To account for the complex cross design (with backcrosses and F2 intercrosses), we built separate QTL maps within each of the three cross types, then summed their LOD scores. This yields one summary statistic measuring a locus' association with the focal trait, while accounting for between-cross differences in QTL effect size or marker linkage. We compared this summed LOD against null expectations obtained by 1,000 permutations of the focal phenotype across fish within each cross, each time redoing each cross' QTL map and summing cross null LOD scores. Conservatively, we consider an observed QTL significant when its summed LOD exceeded the 99.99% quantile from that marker's null values at that same marker. We double-checked each significant QTL with a GLM (as described above) testing for fish genotype effect (at the nearest genetic marker) on the focal microbiome variable.

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Authors' contributions

D.I.B., J.W., and N.C.S. designed the study; F.L., N.C.S., and L.M. performed the study; D.I.B., F.L., C.S., D.C., B.Z., and G.X.W. analyzed the data; D.I.B., and F.L wrote the paper.

Availability of data materials

Sequence data have been deposited in the Sequence Read Database (SRA) under project IDs SRP115642 (BioProject PRJNA398629) and SRP115678 (BioProject PRJNA398630) for experimental 1 and 2, respectively. All

other relevant data are available in this article and its Supplementary Information files, or from the corresponding authors upon request.

Competing interests

The authors declare no conflict of interest.

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Figure legends

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Fig. 2 Many microbial Orders (*N*=84, found in a minimum of 20 stickleback) exhibit significant effects of infection or fish characteristics. Here, we identify which microbial Orders exhibit various main and interaction effects. For each Order, a cell is colored if the relevant effect is significant at $P<0.05$. Note that there is a strong excess of significant results at this threshold above the null expectation of a 5% false positive rate (χ^2 tests $P<0.0001$ for all but the mass*infection interaction). Generalized linear model results for all common Orders (found in at least 20 fish) are summarized in File S2. Color denotes an effect direction. For cross, red denotes higher abundance with more Roberts Lake alleles (ROB backcross), blue denotes higher abundance in Gosling Lake alleles (GOS backcross). For mass, red denotes higher abundance in larger fish, blue in smaller fish. For sex, red denotes higher abundance in males, blue higher in females. For infection, red denotes higher abundance in infected than in uninfected fish, blue is the reverse. For the cross*infection interaction, red indicates instances where infection increases microbe abundance in ROB backcrosses while blue indicates infection increases abundance in GOS. For mass*infection, red indicates that infection increases microbe abundance more strongly in larger fish. For sex*infection, red denotes cases where infection increases microbe abundance most strongly in males, whereas blue implies infection increases microbe abundance mostly in females.

Fig. 3 Examples of how the gut microbiome composition depends on host sex, genotype (cross), and infection status, focusing on the relative abundance of microbial Orders. To visualize these effects we first adjusted for variation due to rearing location (room), by calculating residuals from a quasibinomial general linear model of the focal Order's read counts (out of the total reads per fish) regressed on rearing room. Here we plot the mean (and ±1 s.e. confidence intervals) for this residual abundance, for various groups of fish to illustrate infection and host effects. (a) Fusobacteriales exhibit a significant decrease in cestode-infected fish, both males (blue) and females (red). (b) Lactobacillales are more abundant in males than in females regardless of infection status. (c) Clostridiales abundance is reduced in infected males, but not infected females. (d) Rhodobacterales are more common in females for uninfected fish, but more common in males for infected fish. (e) Caulobacterales are more abundant in F2 hybrids than in either backcross, regardless of infection status (black = uninfected, red = infected). (f) Rhodobacterales are more abundant in fish with a greater fraction of Roberts lake ancestry, but only among uninfected fish; infection leads to a higher Rhodobacterales abundance that is similar across fish genotypes. Fig. S5 shows the same plots, but with all data points included to show the small effect size relative to high among-individual variation.

Fig. 4 Linear discriminant analysis of the top 50 unweighted PCoA axes of microbial composition reveals effects of host sex, host cross type, and infection status. (a) Results of LDA to separate all combinations of sex and infection status. LDA axis 1 (LDA1) separates infected (filled points) from uninfected (open points; $P<0.0001$). LDA2 separates males and females (blue and red points; $P<0.0001$), and exhibits a significant interaction effect ($P=0.002$). (b) Results of LDA to separate combinations of cross (blue = Gosling backcross, purple = F2, red = Roberts backcross) and infection status (open circles = uninfected, filled circles = infected). LDA1 (68% of variation) separates F2 hybrids from both backcrosses. LDA2 (16% of variation; not shown here) separates Roberts from Gosling backcross fish, with F2 hybrids being intermediate. LDA3 explains 7% of the microbial variation and is most strongly associated with infection status, but in a manner that depends on host cross: the three host crosses are on average almost identical along LDA3 when uninfected (open circles), but diverge when infected with F2s intermediate as expected from additive genetic control. Raw points are shown in faded colors, overlain by larger darker circles representing bivariate averages with ±1 s.e. error bars.

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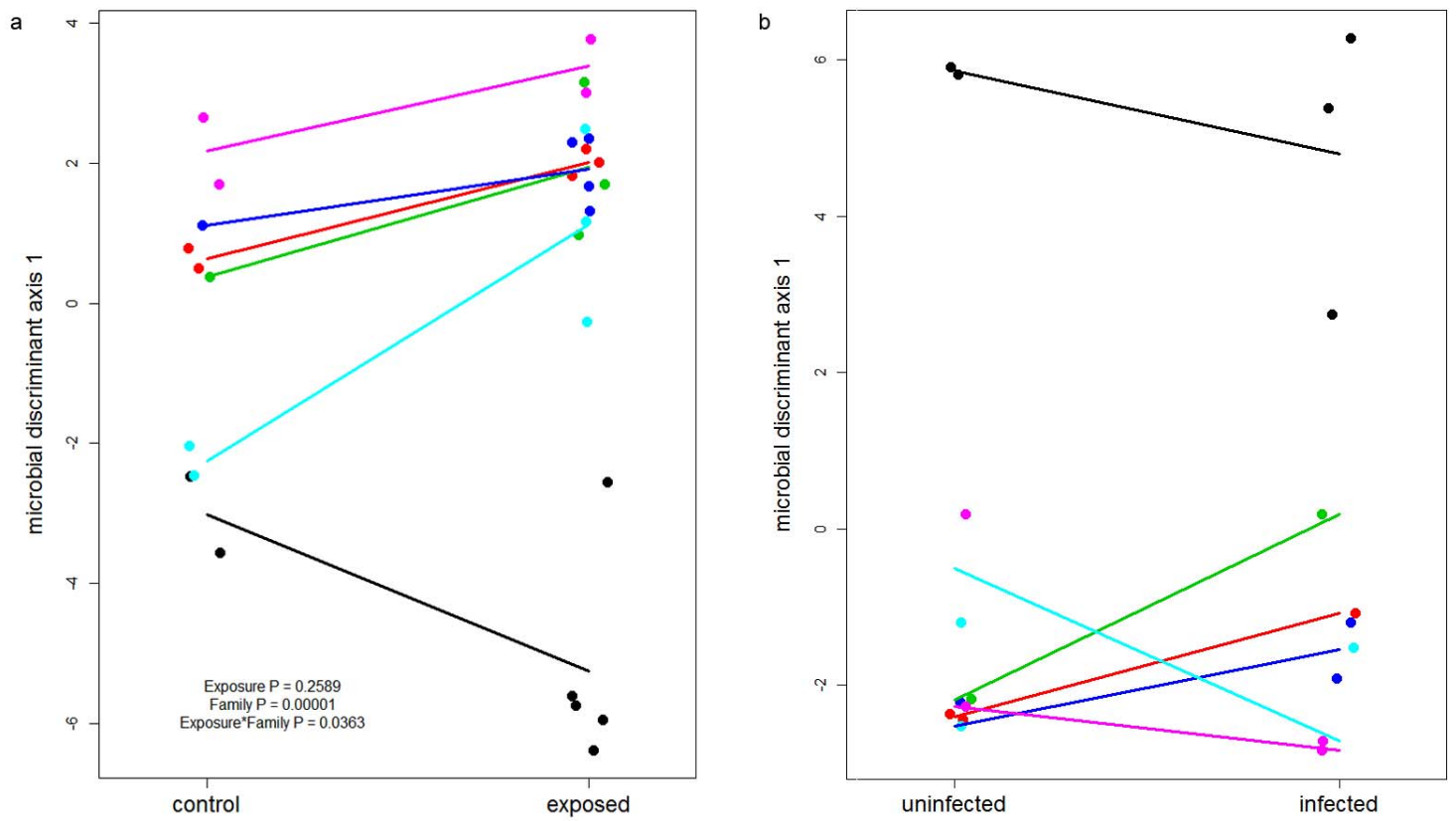

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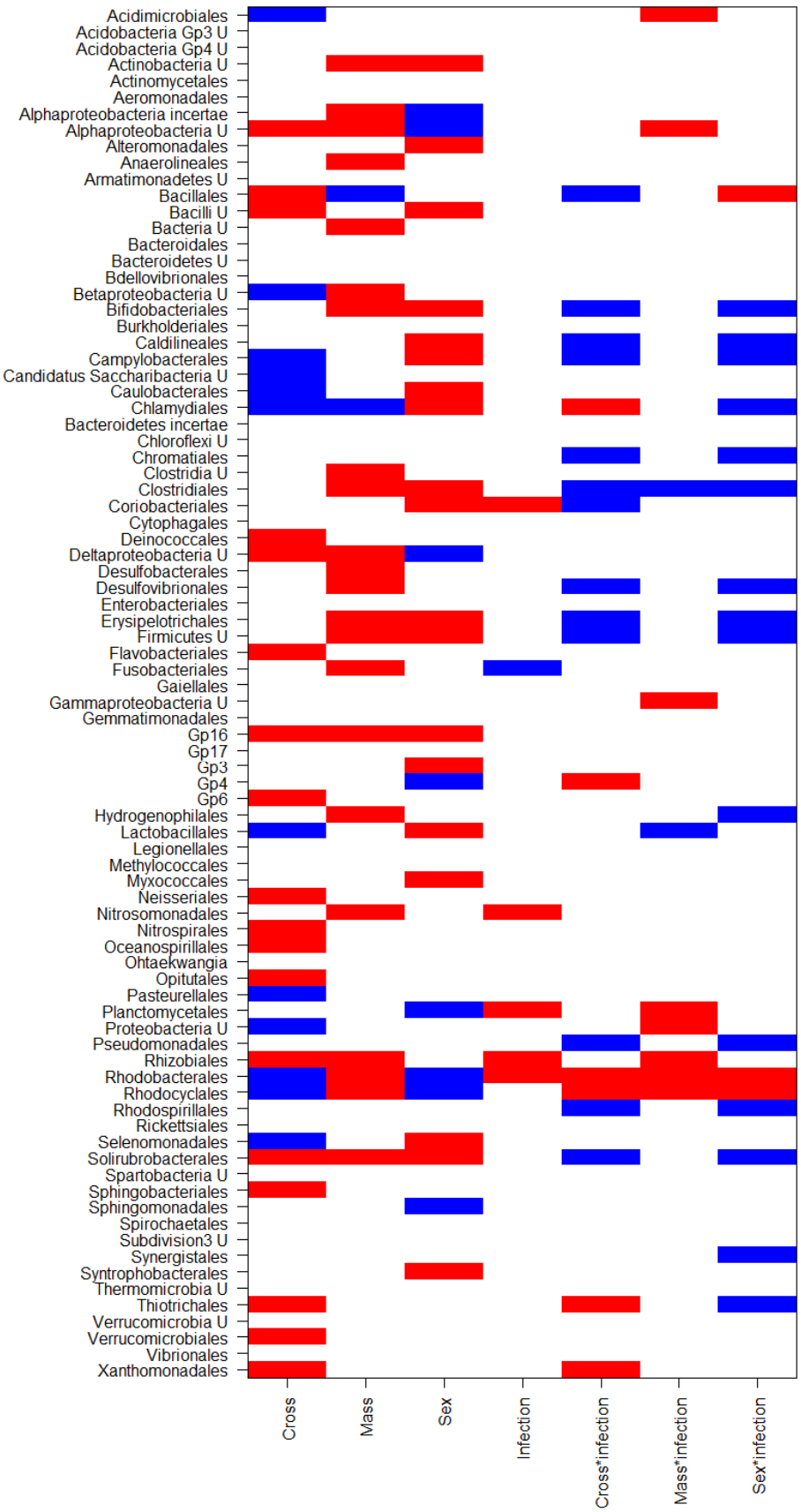

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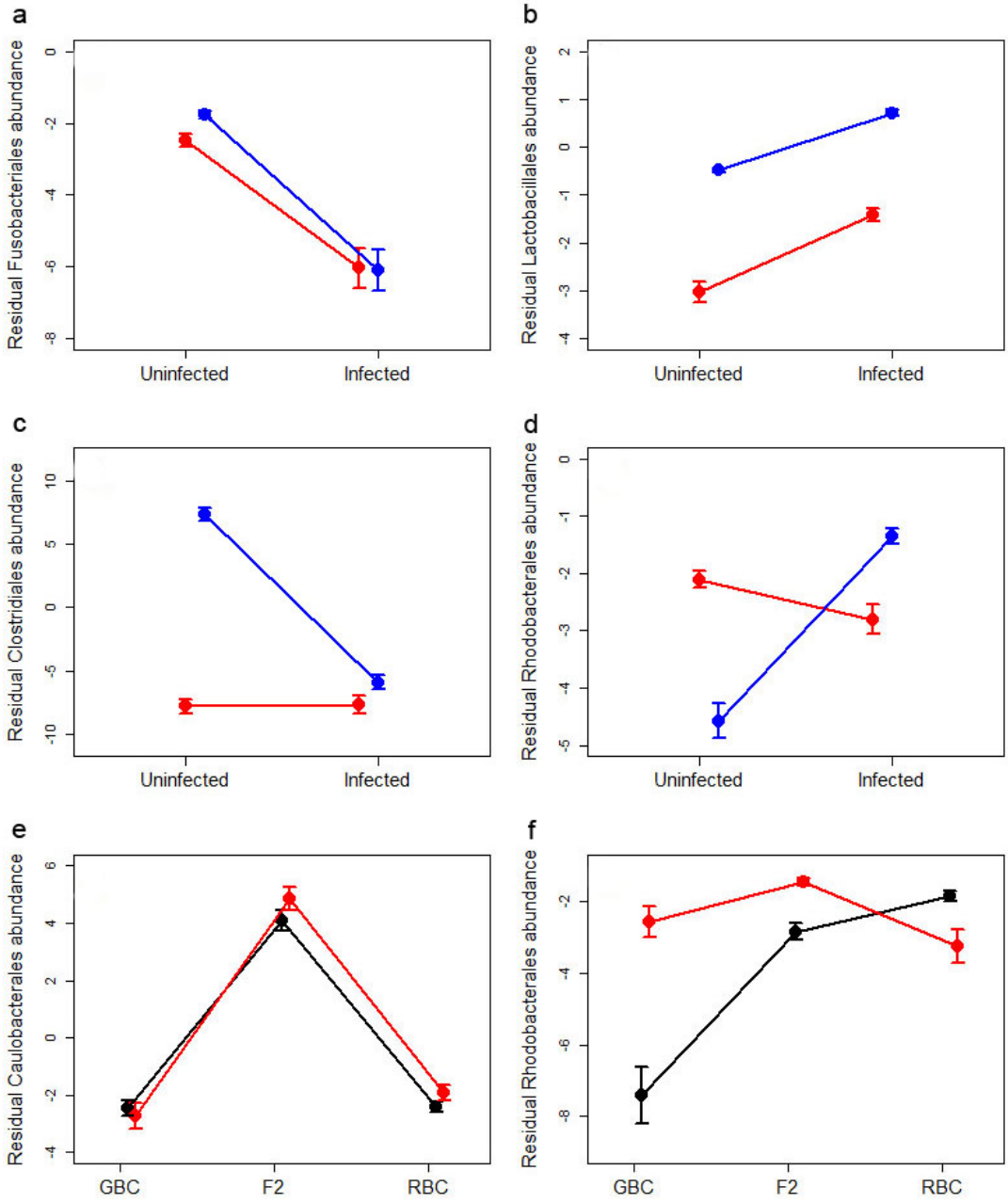

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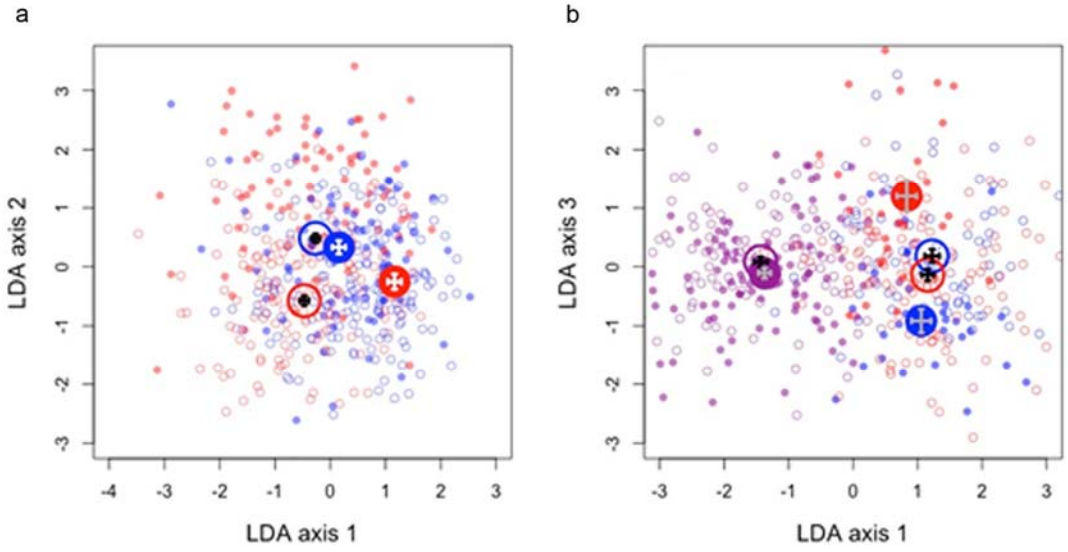

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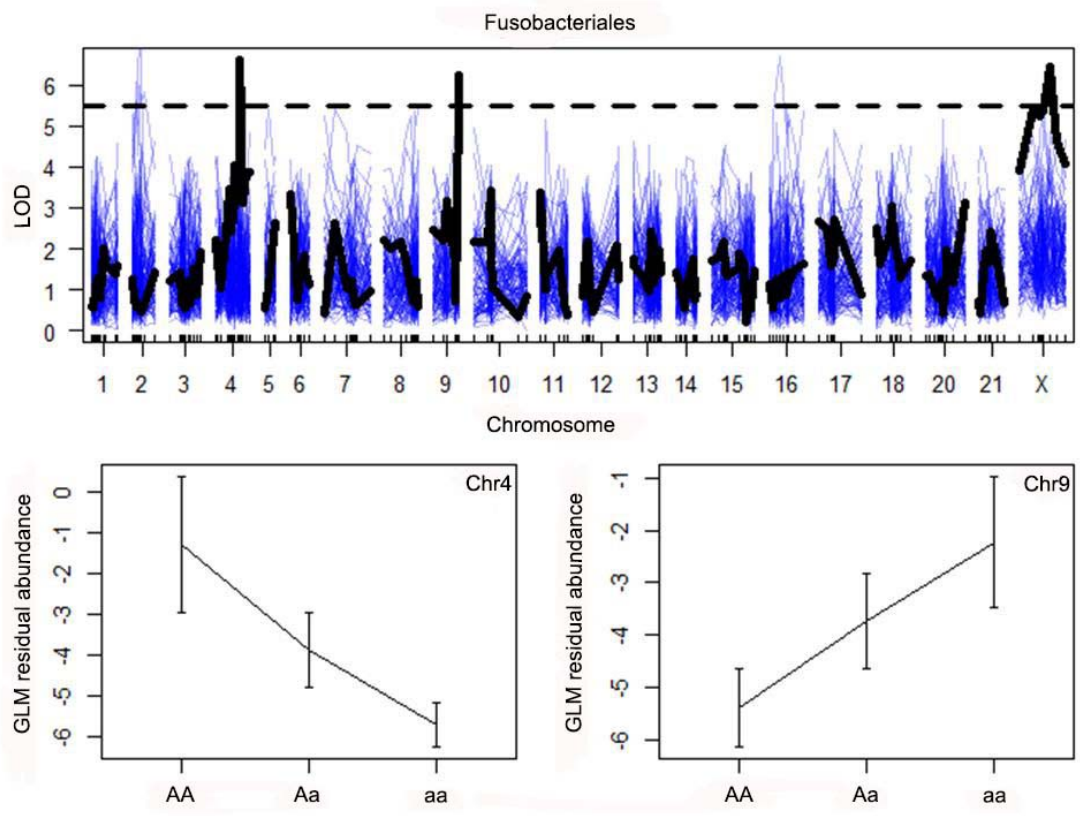

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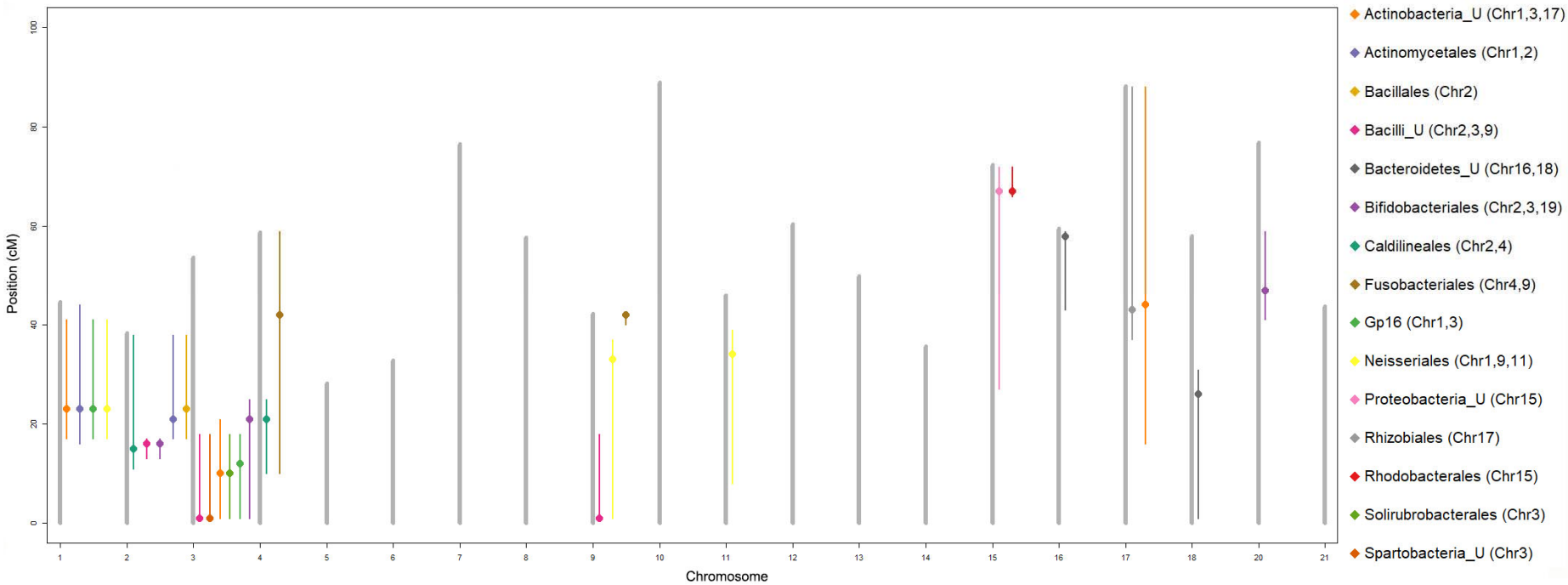

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