Intensity of infection with intracellular *Eimeria* spp. and pinworms is reduced in hybrid mice compared to parental subspecies

Abstract

Animal systems offering a range of hybrid genotypes between two parental species provide the opportunity to study the control of parasite burden by their host (resistance). In the European house mouse hybrid zone, the longstanding impression that hybrid mice are more highly parasitized and less fit than parentals persists despite the findings of recent studies. Working across a novel transect we assessed infections by intracellular protozoans, *Eimeria spp.*, and infections by extracellular macroparasites, pinworms. For *Eimeria* we found lower intensities in hybrid hosts than in parental mice but no evidence of lowered probability of infection or increased mortality in the centre of the hybrid zone. This means ecological factors are very unlikely to be responsible for the reduced load of infected hybrids. Focusing on parasite intensity (load in infected hosts) we also corroborated reduced pinworm loads reported for hybrid mice in previous studies. We conclude that intensity of diverse parasites, including the previously unstudied *Eimeria*, is reduced in hybrid mice compared to parental subspecies. We suggest caution in extrapolating this to differences in hybrid host fitness in the absence of, for example, evidence for a link between parasitemia and health.

Keywords: parasites, hybridization, resistance, eimeria

Introduction

The relevance of hybridization, producing individuals admixed between genetically distinct populations, is increasingly recognized by biologists. Mallet (2005) suggested that hybridization occurs in more than 10% of animal species and 25% of vascular plant species. Recently, the realization that humans are also a product of hybridization has raised interest further (Green et al., 2010). In a conservation context hybridization with introduced species can threaten autochthonous endangered animals (Simberloff, 1996). Parasites are omnipresent in natural systems and impact human and animal health (Schurer, Mosites, Li, Meschke, & Rabinowitz, 2016). It is therefore important for biologists to comprehend the interplay between parasites and hosts under hybridization.

The European house mouse hybrid zone (HMHZ), one of the first animal hybrid zones studied for differences in parasite loads (Sage, Heyneman, Lim, & Wilson, 1986), is a tension zone characterized by selection against hybrids replaced by immigrating less admixed mice (Barton & Hewitt, 1985). After ~500 000 years of (mostly) allopatric divergence two house mouse subspecies, *Mus musculus domesticus* and *Mus musculus musculus* (hereafter Mmd and Mmm), have come into secondary contact in Europe as a result of different colonization routes south and north of the Black Sea, respectively (Boursot, Auffray, Britton-Davidian, & Bonhomme, 1993; Duvaux, Belkhir, Boulesteix, & Boursot, 2011). The HMHZ is about 20 km wide and more than 2500 km long, running from Scandinavia to the coast of the Black Sea (Baird & Macholán, 2012; Boursot, Auffray, Britton-Davidian, & Bonhomme, 1993; Jones, Kooij, Solheim, & Searle, 2010; Macholán, Kryštufek, & Vohralík, 2003). This zone represents a semi-permeable barrier to

gene flow between the two taxa (Macholán et al., 2007; Macholán et al., 2011). The main selective forces acting against hybrids are thought to be endogenous rather than ecological (Baird & Macholán, 2012; Boursot, Auffray, Britton-Davidian, & Bonhomme, 1993), for example disruption of spermatogenesis in hybrids (Albrechtová et al., 2012; Turner, Schwahn, & Harr, 2012).

Parasites are traditionally seen as decreasing their hosts' fitness, and differences in resistance to parasites between hybrid and pure hosts were suggested to affect the dynamics of hybrid zones (Fritz, Moulia, & Newcombe, 1999). This traditional framework postulates differences in parasite loads in hybrids vs. parental hosts to result in effects on the strength of host species barriers. However, recent work in controlled conditions has shown that resistance (host capacity to reduce parasite burden) and tolerance (reduced impact of a given parasite burden on host health) are not necessarily correlated (Råberg, Sim, & Read, 2007).

Initial results on parasites obtained in the HMHZ and experimental studies seemed to indicate elevated parasite loads in hybrids. This has been interpreted as potentially leading to fitness reductions in hybrids, hampering hybridization and thus reinforcing species barriers (Moulia et al., 1991; Moulia, Le Brun, Dallas, Orth, & Renaud, 1993; Sage et al., 1986). Infection experiments using the protozoan *Sarcocystis muris* led to a similar conclusion (Derothe, Le Brun, Loubes, Perriat-Sanguinet, & Moulia, 2001). Other laboratory experiments, however, showed either no effect in inter-subspecies F1s on helminth load or even reduced load in inter-subspecies F1s compared to pure mouse strains (Derothe, Porcherie, Perriat-Sanguinet, Loubès, & Moulia, 2004; Moulia, Le Brun, Loubes, Marin, & Renaud, 1995). A more recent field study,

with high statistical power (more mice sampled and more suitable statistical approach) and improved sampling design found reduced helminth loads (especially the pinworms *Aspiculuris tetraptera* and *Syphacia obvelata* and the whipworm *Trichuris muris*) in hybrid mice (Baird et al., 2012). It should also be noted that the design of the field studies preceding the Baird et al. (2012) reappraisal usually suffered from low sample sizes and/or maintenance of mice under laboratory conditions before assessment of parasite burden, which may have allowed spurious signal to dominate the results. Nevertheless, even the basic direction of parasite load differences in hybrid mice compared to parental genotypes seems still controversial to some researchers.

We now see that, despite working within the framework of the same hybrid zone, two different interpretations of parasite loads in hybrid mice have arisen. It should be noted that all the previous studies chose to focus on either helminth or protozoan parasite models. In vertebrates, the immune mechanisms of parasite control differs greatly between these two groups. Extracellular macroparasites like helminths trigger a T helper type 2 (Th2) -dominated response, and intracellular microparasites like protozoa trigger a T helper type 1 (Th1) -mediated response (Sher & Coffman, 1992). One way forward in such circumstances is to test hypotheses over replicates and "along different axes" of parasitism, and to consider simultaneously helminths and protozoans to address the generality of hybrid response. To distinguish between interpretations of parasite load we here asked if (1) parasite loads are higher or lower in hybrids compared to parentals, and (2) if these loads are consistent, or differ, between prevalent representative helminths and protozoa. We did so in a geographically new transect replicate of the HMHZ.

Pinworms (oxyurids) have been detected in mice in numerous field studies (see for example Behnke, 1975; Behnke, 1976; Kriska, 1993; Ressouche et al., 1998). They have been shown to be the most prevalent helminths infecting house mice in the HMHZ (Goüy de Bellocq, Ribas, & Baird, 2012). They are often considered to provoke mild symptoms on their hosts, even if in rare conditions (e.g. particularly high burden) they have been shown to affect the health of laboratory mice (Taffs, 1976). *Eimeria* spp. are often considered host-specific, with several thousand species parasitizing different vertebrates (Chapman et al., 2013; Haberkorn, 1970). These parasites infect the intestinal epithelial cells of vertebrates and induce symptoms such as weight loss and diarrhoea. For example, infecting the NMRI mouse laboratory strain with *Eimeria* oocysts isolated from mice captured in the HMHZ resulted in a weight loss up to 20% compared to control (Al-khlifeh et al., 2019). In the European HMHZ, three *Eimeria* species have been identified: *E. ferrisi*, *E. falciformis*, and *E. vermiformis* with prevalences of 16.1%, 4.2% and 1.1%, respectively (Jarquín-Díaz et al., 2019).

We assessed *Eimeria* infection in a novel transect of the HMHZ in Brandenburg, northeastern Germany, in which the hypothesis of hybrid resistance/susceptibility to parasite had never before been tested. We assessed the impact of host hybridization on intensity of this parasite. By focusing on parasite intensity (extent of parasite infection in only infected animals; Bush et al. 1997), we arguably exclude ecological factors for differences in load. We show that (1) parasite loads are consistently lower in hybrids compared to parental genotypes in the HMHZ and (2) that this pattern is similar for our intracellular and extracellular parasite models.

Material & Methods

Sampling

Our sampled individuals consist of 660 house mice trapped using live traps placed in farms or houses between 2014 and 2017. The study area ranges from 51.68 to 53.29 degrees of latitude (200 km) and from 12.52 to 14.32 degrees of longitude (140 km). Each year mice were trapped in September when it is possible to capture a high number of mice in this region. In addition, sampling at the same season every year reduces potential seasonal variation (Abu-Madi, Behnke, Lewis, & Gilbert, 2000; Haukisalmi, Henttonen, & Tenora, 1988). The locations for trapping were selected across a geographical range allowing both parental and hybrid/recombinant individuals to be captured. Mice were individually isolated in cages and then euthanized by isoflurane inhalation followed by cervical dislocation and dissection within 24 hours after capture (animal experiment permit No. 2347/35/2014). Tissue samples (muscle and spleen) were transported in liquid nitrogen and stored at -80°C for subsequent host genotyping. Digestive tracts were dissected and inspected for helminth parasites (see below). Ileum, caecum and colon tissues were frozen in liquid nitrogen and then stored separately at -80°C. Individual mice were measured (body length from nose to anus) and weighted.

Host genotyping

The admixture of mouse genomes across the HMHZ was estimated for each mouse as a value of the hybrid index (HI) calculated as a proportion of Mmm alleles in a set of 4-14 diagnostic markers (at least 10 loci in 92% of the mice). This set consists of one mitochondrial marker (*Bam*HI, a restriction site in the *Nd1* gene; Božíková et al., 2005; Munclinger, Božíková,

Šugerková, Piálek, & Macholán, 2002), one Y-linked marker (presence/absence of a short insertion in the *Zfy2* gene; Boissinot & Boursot, 1997; Nagamine et al., 1992), six X-linked markers (three B1 and B2 short interspersed nuclear elements in *Btk*, *Tsx* (Munclinger, Boursot, & Dod, 2003), and *Syap1* (Macholán et al., 2007), *X332*, *X347* and *X65* (Dufková, Macholán, & Piálek, 2011; Ďureje, Macholán, Baird, & Piálek, 2012)), and six autosomal markers (*Es1*, *H6pd*, *Idh1*, *Mpi*, *Np*, *Sod1*; Macholán et al., 2007). HIs ranged from 0 to 1, HI of 0 indicating a pure Mmd and HI of 1 a pure Mmm (Baird et al., 2012; Macholán et al., 2007). Histograms for the number of genotyped markers, as well as their distribution across the hybrid index indicate no bias in genotyping (**Supplementary Figure S1)**.

The expected centre of the HMHZ across the study area was estimated using the program Geneland v4.0.8 (with graphical resolution increased over defaults, the modified code is available at https://github.com/alicebalard/Geneland as a complete R-package), based on a subset of the six autosomal markers that were genotyped in all individuals with 6 diploid markers (N=598 mice). Geneland uses a Markov chain Monte Carlo (MCMC) approach to combine both geographical and genetic information (Guillot, Mortier, & Estoup, 2005). The number of clusters was set to 2, 10^6 MCMC iterations were performed and saved every 100th iterations (10^4 iterations saved). The first 200 iterations were discarded as burn-in and the resolution of the map was set to 2000 pixels for the x axis and 1400 for the y axes corresponding roughly to 1 pixel for 100m (Macholán et al., 2011).

Parasite load estimation

Mouse digestive tracts were dissected and inspected for helminth presence with a binocular microscope. Helminths were counted and stored in 70% ethanol for later identification by molecular analysis and, when more than one worm per host was present, in 3.5% formalin for later morphological comparison with species descriptions. As in this study we required high statistical power to test our hypotheses, we considered only the most prevalent helminths, the oxyurids *Syphacia obvelata* and *Aspiculuris tetraptera*. Histograms presenting the distribution of counts for other helminths can be found in **Supplementary Figure S2** and data is available in **Supplementary Table S3.**

DNA was extracted from ileum and caecum tissues and quantitative PCR (qPCR) was used for estimation of *Eimeria* spp. load. DNA extraction was performed using the innuPREP DNA Mini Kit (Analytik Jena AG, Jena, Germany) following the instructions of the manufacturer with additional mechanical tissue disruption with liquid nitrogen in a mortar. Both quality and quantity of isolated DNA were measured by spectrophotometry in a NanoDrop 2000c (Thermo Scientific, Waltham, USA). The presence of *Eimeria* spp. was tested using qPCR to detect intracellular stages of the parasite as well as a house mouse house-keeping gene as internal reference. Primers used for *Eimeria* spp. detection targeted a short mitochondrial *COI* region (Eim_COI_qX-F: TGTCTATTCACTTGGGCTATTGT; Eim_COI_qX-R: GGATCACCGTTAAATGAGGCA), while *Mus musculus* primers targeted the *CDC42* nuclear gene (Ms_gDNA_CDC42_F: CTCTCCTCCCCTCTGTCTTG; Ms_gDNA_CDC42_R: TCCTTTTGGGTTGAGTTTCC; Al-khlifeh et al., 2019; Jarquín-Díaz et al., 2019). These

qPCRs have been independently confirmed with respect to detection of experimental infection (Al-khlifeh et al., 2019) and with genotyping PCRs using different primers and markers (Jarquín-Díaz et al., 2019). Reactions were performed using 1X iTaqTM Universal SYBR® Green Supermix (Bio-Rad Laboratories GmbH, München, Germany), 400 nM of each primer and 50 ng of DNA template in 20 µL final volume. Cycling amplification was carried out in a Mastercycler® RealPlex 2 thermocycler (Eppendorf, Hamburg, Germany) with the following amplification program: 95°C initial denaturation (2 min) followed by 40 cycles of 95°C denaturation (15 s), 55°C annealing (15 s) and 68°C extension (20 s). Melting curve analyses were performed in order to detect primer dimer formation and unspecific amplification. ΔCt was calculated as difference of the threshold cycle (Ct) between mouse and *Eimeria* spp. values (corresponding to a log2 ratio between parasite and mouse DNA). This method was validated in an infection experiment of NMRI mice (Al-khlifeh et al., 2019). We considered ΔCt = -5 our limit of detection as at this limit it was possible to obtain genotyping data for all samples using independent PCR reactions (Ahmed et al., 2019; Jarquín-Díaz et al., 2019). Samples with a ΔCt lower than -5 were considered negative (unspecific signal due to amplification of non-target DNA). Samples with a ΔCt higher than -5 for at least one of the two intestinal tissues were considered positive, and in the case of detection in both tissues, the higher value was taken as a proxy of individual parasite load. This parasite load of the intestinal tissue stage is denoted as "$\Delta Ct_{Mouse-Eimeria}$" throughout the following. *Eimeria* identification at the species level was performed by means of two PCR markers (18S and COI) followed by a confirmation of morphology and tissue preference as described in Jarquín-Díaz et al., 2019 (column "eimeriaSpecies" of **Supplementary Table S3**).

General parasite assessment

As the distributions of parasite loads are expected to be highly skewed (Bliss & Fisher, 1953), the median (as an estimator for the mode) is more informative than the mean (Rózsa, Reiczigel, & Majoros, 2000). We therefore report the median of parasite load across all hosts (median abundance) and of parasite load of infected host (median intensity) for pinworms, and only median intensity for *Eimeria* spp. For qPCR some uninfected samples present technical noise due to unspecific amplification of non-target DNA. We therefore used a qPCR threshold validated by independent genotyping PCRs (see "Fig. 4" of Jarquín-Díaz et al., 2019) to establish the infection status of each sample (and we do not report abundance for *Eimeria,* see Jarquín-Díaz et al., 2019 for details). Prevalence (relative frequency of infected individuals amongst all tested individuals) confidence intervals were obtained with Sterne's exact method (Reiczigel, Földi, & Ozsvári, 2010; Sterne, 1954). Calculations were performed using the epiR package (Nunes et al., 2018) running within the R statistical computing environment (R Development Core Team, 2008).

Statistical design: testing hybrid resistance/susceptibility in a natural system

According to the SIR model of epdidemiology, individuals can be divided into susceptible (S), infected (I), and removed (R, dead or recovered). Animals captured in the field can show (1) absence, or (2) presence of a given parasite. Absence of a parasite in a given host can result from absence of exposure to the parasite, complete host resistance, recovery, or death (Krämer, Kretzschmar, & Krickeberg, 2010). On the other hand, quantitative parasite load depends on intrinsic host or parasite components or their interactions. We argue that when testing the

hypotheses of hybrid resistance or susceptibility in a natural system, a focus on the latter is beneficial. Therefore, we test a potential increase or decrease of parasite load in infected animals (intensity) towards the centre of the zone compared to its sides. We performed this analysis for our parasites of interest, but first verified that we could exclude differences in prevalence (probability of infection) across the hybrid index for each parasite. This leaves mortality as the only epidemiological factor (in the SIR model) to potentially influence both prevalence and intensity, we therefore additionally tested increased mortality by analyzing differences in (infected/uninfected) age categories across the hybrid index (see below: Statistical test for different mortality of hybrids).

The hybridization level on each individual was modelled as the degree to which new gene combinations are brought together compared to the pure subspecies. This was estimated from the hybrid index using the function for expected heterozygosity (Baird et al., 2012):

$$He = 2 \cdot HI \cdot (1 - HI) , \qquad (Eq.\ 1)$$

Statistical prediction of probability of infection by parasites along the hybrid zone

We considered the predicted probability of infection across the HI as equivalent to the prevalence and modelled a dichotomous response variable (uninfected = 0; infected = 1) by logistic regression. We performed two analyses, one testing for prevalence differences on both halves of the hybrid index separately and a second one with a unified “genetic distance to zone centre” (for individuals with HI between 0 and 0.5 the proxy is HI, for individuals with HI between 0.5 and 1 the proxy is 1 – HI). This means we do not blindly assume equality of prevalence at both ends of the hybrid index, but also maximize power to reject the null

hypothesis (esp. in case of a negative result in the separate analysis). Analyses were done in R with the function glm from the stats package (R Development Core Team, 2008) including host sex and interaction terms with the variable representing hybrid genetics.

Statistical test for different mortality of hybrids

Secondly, morbidity or mortality caused by hyperparasitism could impact both prevalence and intensity measures of parasite loads, as only the surviving, less parasitized mice could be captured. This, however, would also lead to differences in age distribution along the hybrid index. We used an age estimation based on weight (as in Behnke, 1976) as a proxy to test if hybrid mice were younger or older than that expected for intermediate between pure hybridizing taxa ("additivity"). Values of body weight are well described by the Normal distribution, parameterized by its standard deviation (allowed to vary freely during maximum likelihood searches) and its mean defined as:

$$ExpectedBodyWeight = (BW1 + (BW2 - BW1) \cdot HI) \cdot (1 - alpha \cdot He) , \quad (Eq.\ 2)$$

where BW1 is the expected body weight of pure Mmd, BW2 the expected body weight of pure Mmm. Alpha represent the hybridization effect, or deviation from additivity between the two parental genomes. We allowed difference between sex and taxons, fit the models using maximum likelihood (using the R package mle2; Bolker, 2017), either including or excluding the hybridization effect parameter (by setting HI = 0 in *ExpectedBodyWeight*), and we compared these two models using the G-test.

Statistical test of the host hybridization effect on parasite intensity

It has been shown that macroparasites tend to aggregate within their hosts, the majority of host carrying no or a low burden, and a minority a high one (Shaw & Dobson, 1995). We modelled this distribution of parasite burden in infected hosts as negative binomial. Following the approach of Baird et al. (2012), we tested if hybrid mice had higher or lower parasite burdens than that expected in case of additivity (if the relationship between host parasite load and hybrid index was linear).

The parasite load for a given HI was then estimated as follows:

$$ExpectedLoad = (L1 + (L2 - L1) \cdot HI) \cdot (1 - alpha \cdot He) , \qquad (Eq.\ 3)$$

where L1 is the parasite load of pure Mmd, L2 the parasite load of pure Mmm, and alpha the hybridization effect (deviation of parasite estimated load from the additive model). We considered four nested hypotheses increasing in complexity, and compared them with the G-test (likelihood ratio test) to consider a more complex hypothesis only when justified by a significant increase in likelihood. Expected parasite load is fixed to be identical for both subspecies and both host sexes in hypothesis H0. The more complex H1 allows load differences for the host sexes, H2 allows different loads between the subspecies at the extremes of the hybrid index, and H3 allows differences both between the subspecies and sexes.

Adequate distributions of values for each parasite and detection method considered were selected using log likelihood and AIC criteria and by comparing goodness-of-fits plots (density, CDF, Q-Q, P-P) (R packages MASS (Venables & Ripley, 2002) and fitdistrplus (Delignette-Muller & Dutang, 2015) (see **Supplementary Figure S4)**. The negative binomial distribution should perform well for macroparasite counts (Crofton, 1971; Shaw & Dobson, 1995), which was

confirmed for helminths in a previous transect (Baird et al., 2012). Values of ($\Delta Ct_{Mouse-Eimeria}$) were found to be well described by the Weibull distribution after being positively shifted.

The Negative Binomial distribution is parameterized by two arguments: its expectation (Expected Load, Eq. 3), and the inverse of its aggregation, which is allowed to vary across HI as:

$$Aggregation = (A1 + (A2 - A1) \cdot HI) + Z \cdot He\ , \qquad (Eq.\ 4)$$

Z being the deviation from the additive model, in proportion to *He*, which is maximal in the zone centre (Baird et al., 2012). The Weibull distribution is parametrized by its shape and scale parameters (allowed to vary freely during maximum likelihood search) linked by the formula:

$$Scale = ExpectedLoad / \Gamma(1 + 1/shape)\ , \qquad (Eq.\ 5)$$

Γ being the gamma function.

We fit the models using likelihood maximisation (using the R package mle2; Bolker, 2017). Parasite load was estimated either including or excluding the hybridization effect parameter (by setting HI = 0 in *ExpectedLoad*), and we compared these two models using the G-test. In the case of $\Delta Ct_{Mouse-Eimeria}$, the Weibull distribution requires positive values as input. Therefore, we estimated an extra "shift parameter" which was optimized by maximum likelihood.

Test of body condition differences between infected and non-infected mice across the hybrid zone

After the previous tests on hybrid resistance/susceptibility to parasites, we wanted to see if our field system could allow differences in tolerance to parasites to be tested. We thus tested whether

we could detect different body condition between infected and non-infected mice along the hybrid index. Residuals from ordinary least squares regression of body weight by body length were estimated for each individual, separately for males and females. Pregnant females were excluded from the analysis. Individuals with a positive residual were considered in better condition than individuals with a negative one, as this index correlates with variation in fat, water, and lean dry mass (Schulte-Hostedde, Zinner, Millar, & Hickling, 2005). We tested if hybrid mice had higher or lower residuals than that expected for intermediate between pure hybridizing taxa ("additivity"), and if the potential hybridization effect was different between infected and not infected mice, for *Eimeria* spp. as well as for pinworm infections. Differences between the subspecies were allowed.

Values of residuals of body weight by body length regression are well described by the Normal distribution, parameterized by its standard deviation (allowed to vary freely during maximum likelihood searches) and its mean defined as:

$$ExpectedResidual = (R1 + (R2 - R1) \cdot HI) \cdot (1 - alpha \cdot He) , \quad (Eq. 6)$$

where R1 is the expected residual value of pure Mmd, R2 the expected residual value of pure Mmm, and alpha the hybridization effect. We fit the models using maximum likelihood (using the R package mle2; Bolker, 2017), either including or excluding the hybridization effect parameter (by setting HI = 0 in *ExpectedResiduals*), and we compared these two models using the G-test.

All graphics were produced using the R packages ggplot2 (Wickham, 2016) and ggmap (Kahle & Wickham, 2013), and compiled using the free software inkscape v.0.92 (https://inkscape.org).

Full R code used for this article can be found at:

https://github.com/alicebalard/Article_IntensityEimeriaHMHZ/tree/master/code

Results

Host genotyping and characterization of the HMHZ for a novel transect

We caught and genotyped a total of 650 mice (359 females, 291 males) over four sampling seasons (2014: *N*=86; 2015: *N*=156; 2016: *N*=167; 2017: *N*=241) at 149 localities. A median of 2 mice per locality were captured. A table of individual mouse data including hybrid indices, georeferences and parasite loads is available in **Supplementary Table S3**. On the probability map of the hybrid zone centre, shown in **Figure 1**, we see that the HMHZ runs across the former East Germany, making a broad arc around the city of Berlin, approaching within ca. 20 km of the bordering Oder River near Eberswalde.

Parasite prevalence and intensity

To investigate *Eimeria* infections we checked 384 mice sampled in 2016 and 2017 for the presence and intensity of tissue stages (**Figure 2a**). The estimated parasite prevalence was 18.2% (70/384) (Sterne's Exact method CI 95%: [14.5, 22.5]). To quantify the intensity of infection we determined the amount of *Eimeria* mitochondrial DNA per host nuclear DNA using $\Delta Ct_{Mouse-Eimeria}$. The median *Eimeria* intensity was -2.4 corresponding to 5.2 times less parasite mitochondrial DNA than host nuclear DNA.

Between 2014 and 2017, 585 mice were investigated for helminths (**Figure 3a**). Prevalence of pinworms in the transect was 52.5% (307/585) (Sterne's Exact method CI 95%: [48.4, 56.5])

with a median abundance of 1 pinworm per mouse and median intensity of 13 pinworms per infected mouse (maximum number of pinworms in one host: 489).

Overall prevalence of pinworms and *Eimeria* in our samples did not significantly differ between approximated age categories (using body weight as a proxy, as in Behnke, 1976) and between the sexes (Pearson's Chi-squared test, p-value > 0.05) (**Supplementary Table S5**).

Similar prevalence of parasites across the zone

In order to control for impact of ecological factors on prevalence, such as a host density trough at the zone centre, we tested if the probability of being infected was significantly lower for individuals at this zone centre. We performed this analysis (1) with a unified “genetic distance to zone centre”and (2) on both halves of the hybrid index separately . Logistic regression using a linear combination of the predictor variables “genetic distance to zone centre” and “Sex” (including interactions) didn’t show any statistically significant effect ($p > 0.05$) on the probability of infection when a unified “genetic distance to zone centre” (1) was used, neither for *Eimeria* spp. (**Figure 2b**) nor for pinworms (**Figure 3b**). Results were identical for specifically *Eimeria ferrisi* infected mice vs. non infected (see **Supplementary Figure S6a**). Similarly, we could not reject the hypothesis of constant prevalence by running the analyses on both halves of the hybrid scale separately (2), for both parasites ($p > 0.05$). We therefore could not find evidence of significantly more or less uninfected hosts in the centre hybrid zone, neither for *Eimeria* as a genus, nor the most prevalent species *E. ferrisi*, nor pinworms.

No evidence of hyper- or under-mortality of hybrids compared to parents

We tested the hybridization effect on body weight as proxy of age. Modelling the body weight across the hybrid zone showed an effect of taxon (G-test of model allowing taxon differences vs. no taxon differences (H1 vs. H0); p-value = 0.017, N = 456) and no effect of sex (G-test of models allowing sex differences vs. no sex differences (both H2 vs. H0 (p-value = 0.057), and H3 vs. H1 (p-value = 0.079), N = 456)). More notably, the model allowing taxon difference did not show a statistically significant hybridization effect (G-test; p-value = 0.214, N = 456; see **Supplementary Figure S7**). We therefore could not detect any decrease or increase of overall mortality in more admixed mice.

Eimeria spp. load is lower in infected hybrid vs pure Mmm and Mmd mice

To test more specifically the intrinsic host-parasite interplay of hybrids compared to pure mice, we considered only individuals infected by *Eimeria* spp. tissue stages (N = 70). Complex models involving differences between sexes and parental taxa did not fit the data significantly better than the null model (**Supplementary Table S8**). The fit involving the hybridization effect, however, showed significantly higher likelihood than the model without it (G-test; p-value = 0.02). Infected hybrids had significantly lower load of *Eimeria* spp. tissue stages than expected if the load was linear along the hybrid index, with a hybridization effect parameter alpha of 0.74 (**Figure 2d**, values of parameters of the fitted model given in **Table 1**). Considering only the more prevalent *Eimeria* species, *E. ferrisi,* infected mice (N=44), we found similar results: no significant improvement of the model when differences between sexes and parental taxa were included and significantly higher likelihood of the model with hybridization effect than the

367 model without it (G-test; p-value = 0.02, hybridization parameter = 0.73; see **Supplementary**
368 **Figure S6b**.

369 **Pinworm load is lower in infected hybrid vs. pure Mmm and Mmd mice**

370 We tested pinworm intensity (*N* = 307) in infected hybrids comparing them to infected 'pure
371 parental' mice in our Brandenburg transect, excluding potential ecological confounders in the
372 same way. The model allowing differences between the parental taxa and sexes (H3) was found
373 to fit our observations significantly better than the less complex models (**Supplementary Table**
374 **S8**). For both sexes, the fit including the hybridization effect showed significantly higher
375 likelihood than the model without it (G-test; p-value = 0.04 for females, p-value < 0.001 for
376 males). Infected hybrids had significantly lower pinworm load than expected if the load was
377 linear across the hybrid index, with the hybridization effect parameter alpha 0.91 (females) and
378 1.46 (males) (**Figure 3d**, values of parameters of the fitted model given in **Table 1**).

379 **Comparison of pinworms loads with previous reports**

380 To compare the strength of the hybridization effect between our Brandenburg transect and the
381 Czech-Bavarian portion of the HMHZ we applied the H1 model (differences between the taxa
382 but not between the host sexes) to our pinworm abundance data, once with freely varying alpha
383 (fit 1), and once with alpha set to 1.39 as in Baird et al. (2012) (fit 2). Within fit 1, alpha was
384 found significant (G-test; p-value < 0.001). The comparison between the model with freely
385 varying alpha (fit 1) and that using fixed alpha (fit 2) showed no significant likelihood difference
386 (G-test; p-value = 0.11). Therefore, we can conclude that pinworm load differences found in

hybrids in this study are consistent with the results obtained in the previously studied Czech-Bavarian transect (Baird et al., 2012).

No evidence of body condition differences between infected and non-infected mice along the hybrid zone

To test whether infections have a different effect in hybrids vs. parental mice we assessed body condition, which could be a better proxy for host health than parasite load. Modelling of the residuals from ordinary least squares regression of body weight by body length across the hybrid zone (**Figure 4a**) did not show a statistically significant hybridization effect (G-test; p-value > 0.05 in both parasite datasets considered). When infected and non-infected individuals were considered separately, neither *Eimeria* spp. infected individuals (G-test; p-value = 0.58) nor *Eimeria* spp. non-infected individuals (G-test; p-value = 0.90) showed a hybridization effect in body condition index (**Figure 4b**). The same was true for pinworm infected individuals (G-test; p-value = 0.44) and pinworm non-infected individuals (G-test; p-value = 0.96; **Figure 4c**).

Discussion

We found lower intensities of the intracellular parasites *Eimeria* spp. and intestinal parasite pinworms in hybrid than in parental subspecies hosts in a previously unstudied transect of the European HMHZ. Lower intensity in hybrids is unlikely to be explained by ecological differences across the HMHZ, as we did not find the probability of infection to be similarly reduced in hybrid hosts, and no overall increase or decrease or mortality towards the zone centre.

406 House mouse hybrids in the European HMHZ are not first-generation crossings, but rather
407 genetically complex “late generation” recombinants. This means that each of their genomes
408 presents a complex admixture of both Mmm and Mmd tracts (Macholán et al., 2007). There is no
409 clear cut-off between hybrids and parental individuals. Therefore, individuals in such systems
410 should not be considered in categories, but rather on a continuous scale of “hybridicity” (a hybrid
411 index) when analyzing parasite infections or any other trait (Baird et al., 2012). We followed the
412 statistical analysis of Baird et al. (2012) and explicitly modelled the effect of hybridization on
413 parasite intensity by approximating the number of new combinations of genes brought together
414 in a hybrid genotype by its expected heterozygosity (He). In other words we used He to derive
415 non-linear predictions for hybridization effect based on the observed individual hybrid indices.
416 To increase reproducibility, we make our analysis available in an R package (Balard &
417 Heitlinger, 2019). The package allows statistical modelling with distributions additional to the
418 original negative binomial distribution for (worm) count data (Baird et al., 2012). This allowed
419 us to model the intensity of *Eimeria* infections as measured by a recently established quantitative
420 PCR (Ahmed et al., 2019; Al-khlifeh et al., 2019; Jarquín-Díaz et al., 2019).

421 To our knowledge no studies have previously tested the effect of mouse hybridization on
422 parasites other than helminths in a field setting of the HMHZ. To understand parasite processes
423 in host hybrid zones, it is necessary to test different types for parasites. Our parasite models
424 present differences that are likely to involve different resistance mechanisms in their hosts (and
425 also different impact on host health and immune systems, with intracellular parasites triggering
426 mainly Th1 vs. extracellular parasites triggering mainly Th2 responses (Jankovic, Sher & Yap,
427 2001; Maizels & Holland, 1998)). Yet the pattern of reduced load in hybrid hosts is the same for

428 the two parasites. These findings confirm that reduction in parasite intensity is either an effect
429 intrinsic to the host individuals (e.g. enhanced immune reactions leading to increased resistance),
430 or, if dependent on the parasite and/or parasite-host interplay, can be generalized over very
431 different parasites.

432 Adding more evidence to the original observations of reduced parasite loads for previously
433 investigated parasites, we also found reduced pinworms loads in hybrids of our novel transect of
434 the HMHZ. We found differences between the Brandenburg and Czech-Bavarian transects in
435 pinworm infection such as distinct loads between males and females and lower prevalence
436 (52.5%) and abundance (18.7) in the former compared to the latter (no significant difference
437 between sexes; prevalence 70.9%, abundance 39.18; Baird et al., 2012). Power to detect distinct
438 male/female load is expected to be reduced by lower prevalence and abundance. Despite the fact
439 that these studies took place in distant geographical locations of the HMHZ likely to present
440 different ecological conditions, the direction and strength of the hybridization effect were very
441 similar in the two study areas. This similarity reinforces our confidence that reduced parasite
442 load in mouse hybrids is a general phenomenon, intrinsic to the individual host or host-parasite
443 interplay rather than a by-product of ecology.

444 A novel aspect of our work compared to previous studies of parasitism in the HMHZ is the
445 separate study of parasite prevalence and intensity. This approach should not only reduce
446 problems in statistical inference caused by false negative measurements (so called zero-inflation)
447 but also allows us to address two different questions separately: (i) Is the *probability* of infection

448 different for hybrids and pure subspecies? and (ii): Is there a difference in parasite *intensity*
449 between infected hybrid and infected pure individuals?

450 An illustrative example of an ecological factor that could potentially lead to parasite load
451 differences is the density of hosts. Densities of mouse populations in the HMHZ centre may be
452 lower than outside (either due to selection against hybrids or because the HMHZ as a tension
453 zone tends to be trapped in "density troughs" sensu Hewitt 1975). Host density is expected to be
454 positively correlated with pathogen transmission (Anderson & May, 1979) and as a result
455 prevalence may be higher in more dense populations (Morand & Guégan, 2000; Hakkarainen et
456 al., 2007). This is, however, not a general law as host density and *Eimeria* spp. prevalence are,
457 for example, negatively correlated in bank voles (Winternitz, Yabsley, & Altizer, 2012).
458 Independent of the direction of the effect, correlation between abundance and prevalence could
459 be confounded with intrinsic effects of hybrid hosts.

460 Our analysis of prevalence (presence/absence in a logistic regression), did not however show any
461 significant decrease of this probability of infection towards the centre of the zone, for neither
462 *Eimeria* spp. nor pinworms. We argue here that, in conjunction with higher intensities, this
463 distinguishes intrinsic hybrid effects from potential ecological confounders.

464 Animals tolerant of low-pathogenic parasites might not suffer fitness reduction during high
465 parasitemia. This could be the case, for example, if the parasite is beneficial for the host's
466 interaction with other parasites (Heitlinger, Ferreira, Thierer, Hofer, & East, 2017) or if immune
467 responses against the parasite are costly relative to the harm it causes (Råberg, Sim, & Read,
468 2007). In addition, according to the "Old Friend" (or "Hygiene") hypothesis, the constant

presence of helminths in natural populations has led to the evolution of a background basal release of regulatory cytokines (Rook, 2009) which might in turn impact the outcome of more pathogenic infections. Even for relatively pathogenic parasites, such as *Eimeria*, differences in resistance could be uncoupled from health effects by differences in tolerance (Råberg et al., 2007). For these reasons parasite load in itself should not be blindly considered as a proxy for host health and certainly not for host fitness comparisons across hybrid zones (see Baird & Goüy de Bellocq, 2019). We here used body condition as a proxy for the health component of host fitness. We, however, did not find evidence for differences in body condition between hybrids and pure mice upon infection. We conclude that we do not have evidence that lower parasitemia in hybrids increases their health.

Intensity of a particular parasite infection is not necessarily correlated with reduced health and a fitness decrease. For example, the fitness of sterile hybrids (always zero) is invariant to infection intensity. Moreover a hybrid host could be robust due to heterosis (though it may still be sterile). Even if we had found increased health of hybrids, this would not be interpretable as leading to a higher total hybrid fitness, as the parasite mediated health fitness component is only one (likely minor) component of overall fitness. It has been shown for example that male mice in the HMHZ centre have reduced fertility compared to parental individuals (Albrechtová et al., 2012; Turner et al., 2012). If reduced parasite intensity is host driven (and not a result of host-parasite interactions) one could conclude that some physiological systems (e.g. reproductive) may be more dependent on “co-adapted complexes”, while others – such as the immune system – benefit from diversity. This latter would be hybrid vigour in the narrow sense (Baird et al., 2012), but

would still not necessarily lead to any effects on host species barriers (Baird & Goüy de Bellocq, 2019).

We can in future ask whether host (immunity and resistance), parasite (infectivity and virulence), or their interactions are underlying reduced parasite intensity in hybrid house mice. *Eimeria* spp. are suitable pathogens to perform experimental and field studies in this endeavour. An experimental setup investigating resistance (inverse of parasite intensity) and tolerance (impact on host health measured by weight loss) during an infection in mice of pure subspecies and crosses between them could address this question in more detail.

A prime candidate locus for mediating a positive effect of hybridization on the immune system (hybrid vigour) is the major histocompatibility complex (MHC). In mice two genes of the MHC showed different levels of polymorphism as well as population structure with many alleles inferred to be shared between the subspecies by maintenance of ancestral polymorphism (Čížková, Goüy de Bellocq, Baird, Piálek, & Bryja, 2011). Additionally, the small demes of house mice can function as reservoirs of MHC alleles, contributing to the diversity of this system across demes and populations (Linnenbrink, Teschke, Montero, Vallier, & Tautz, 2018). The genetic structure of the MHC and especially polymorphism shared across subspecies should make these loci good candidates to investigate for mechanisms behind hybrid vigour, among a number of other loci including Toll-like receptors (Skevaki, Pararas, Kostelidou, Tsakris, & Routsias, 2015). Previous work on toll-like receptor 4 already suggests different evolutionary patterns between the house mouse subspecies (Fornuskova, Bryja, Vinkler, Macholán, & Piálek,

510 2014). For host parasite interactions major candidate loci are immunity related GTPases on the
511 host side and rhoptry kinases in coccidia (Lilue, Müller, Steinfeldt, & Howard, 2013).

512 Hybridization has played a significant role during and after the divergence of house mouse
513 subspecies as well as during the formation of “classical inbred strains” (Yang et al., 2011).
514 Improving our understanding of parasite process across the HMHZ provides valuable
515 information on the house mouse as the (non-human) model species with the most thoroughly
516 understood immune system. A transfer of knowledge from this model might further
517 understanding of the interplay between parasites and hybridizing species, our own as well as
518 species relevant for conservation.

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Tables

Table 1. Parametrisation of fitted models. Parameters estimated by maximum likelihood for each dataset. Alpha is the hybridization effect (deviation of parasite estimated load from the additive model) given with its significance p-value. If sexes are separated, corresponding parameters for each sex are given with symbols ♀ and ♂. Nested hypotheses are as follow. H0: same expected load for the subspecies and between sexes; H1: same expected load across sexes, but can differ across subspecies; H2: same expected load across subspecies, but can differ between the sexes; H3: expected load can differ both across subspecies and between sexes. *Mus musculus domesticus* and *Mus musculus musculus* are named hereafter Mmd and Mmm.

***Eimeria* intensity**	Hyp.	Alpha (p-value)	Load in ΔCt for both parental subspecies	Shape
present study, *Eimeria* sp.	H0	0.74 (0.02)	-0.70	2.33
present study, *Eimeria ferrisi*	H0	0.74 (0.02)	-0.70	2.33

Pinworm intensity	Hyp.	Alpha (p-value)	Load in count Mmd	Load in count Mmm	Aggregation Mmd	Aggregation Mmm	Z parameter
present study	H3	♀ 0.91 (0.04) ♂ 1.46 (<0.001)	♀ 35.57 ♂ 30.38	♀ 68.67 ♂ 51.86	♀ 1.45 ♂ 2.10	♀ 2.00 ♂ 1.33	♀ -1.04 ♂ -1.23
present study (data from Baird et al. 2012)	H1	1.21 (<0.001)	94.37	46.81	1.88	1.34	-0.13

Figure legends

Figure 1. Geographic range of house mouse subspecies in the European house mouse hybrid zone. Spatial organization of the HMHZ was inferred using all individuals with 6 autosomal markers available (N=598 mice) (*Es1*, *H6pd*, *Idh1*, *Mpi*, *Np*, *Sod1*). *Mus musculus domesticus* is found west of the hybrid zone (blue), *Mus musculus musculus* east of it (red). The numbers at the level contours indicate posterior probabilities of population membership for each mouse subspecies. White dots represent each mouse included in the study.

Figure 2. Probability of infection is constant and intensity of *Eimeria* infection is reduced in hybrids. Individual mice tested for detection and quantification of *Eimeria* spp. tissue stages (a) and mice tested positive (c) are displayed on a map (point color indicates mice genotype, on a gradient ranging from blue (pure Mmd) to red (pure Mmm); increasing number of mice sampled at one locality is displayed as decrease in transparency). The predicted probability of infection does not differ in more admixed mice (b) for males (green) and females (orange)(average overall observed probability of infection (prevalence) for males and females considered together: grey dotted line). *Eimeria* intensity (white dots = individual mice) is reduced at intermediate values of the hybrid index (d), represented as a gradient ranging from 0 (pure Mmd, in blue) to 1 (pure Mmm, in red). The optimized fit is represented by a solid line, the 95%CI of the fit as all parameters are allowed to vary in their 95%CI, is plotted as a grey ribbon. The 95%CI of the hybridization parameter alpha, as all parameters are fixed to their fitted value while alpha is allowed to vary in its 95%CI, is plotted as dashed lines.

Figure 3. Probability of infection is constant and intensity of pinworm infection is reduced in hybrids. Individual mice tested for detection and quantification of pinworms (a) and mice

tested positive (c) are displayed on a map (point color indicates mice genotype, on a gradient ranging from blue (pure Mmd) to red (pure Mmm); increased number of mice sampled at one point displayed as decrease in transparency). The predicted probability of infection does not differ in more admixed mice (b) for males (green) and females (orange)(average overall observed probability of infection (prevalence) for males and females considered together: grey dotted line). Pinworm intensity (white dots = individual mice) is reduced at intermediate values of the hybrid index (d), represented as a gradient ranging from 0 (pure Mmd, in blue) to 1 (pure Mmm, in red), for males (green) and females (orange). The optimized fit is represented by a solid line, the 95%CI of the fit as all parameters are allowed to vary in their 95%CI, is plotted as a grey ribbon. The 95%CI of the hybridization parameter alpha, while all parameters are fixed to their fitted value and alpha is allowed to vary in its 95%CI, is plotted as dashed lines.

Figure 4. Body condition does not significantly differ between hybrids and pure mice upon infection. We modelled the residuals from ordinary least squares regression of body weight by body length along the hybrid zone. The fit and residuals for female and male mice is given in (a). The hybrid index is represented as a gradient ranging from 0 (pure Mmd, in blue) to 1 (pure Mmm, in red). ”Body condition” residuals along the hybrid index (for *Eimeria* spp. (b) and pinworms (c)) show no difference for infected mice (light green) and non-infected mice (grey). The optimized fit is represented by a solid line, the 95%CI of the fit as all parameters are allowed to vary in their 95%CI, is plotted as a grey ribbon. The 95%CI of the hybridization parameter alpha, as all parameters are fixed to their fitted value while alpha is allowed to vary in its 95%CI, is plotted as dashed lines.