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Intermediate number of major histocompatibility complex class II*B* length variants relates to enlarged perivisceral fat deposits in the blunt-head cichlid *Tropheus moorii*

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Abstract

Studying the genetic basis of host–parasite interactions represents an outstanding opportunity to observe eco-evolutionary processes. Established candidates for such studies in vertebrates are immunogenes of the major histocompatibility complex (MHC). The MHC has been reported to reach high intra- and interindividual diversity, and a diverse MHC might be advantageous when facing infections from multiple parasites. However, other studies indicated that individuals with an intermediate number of MHC alleles are less infected with parasites or have other fitness advantages. In this study, we assessed the optimal number of MHC alleles in the blunt-head cichlid *Tropheus moorii* from Lake Tanganyika. We investigated the influence of the interindividual variation in number of MHC length variants on parasite infection and body condition, measured by the amount of perivisceral fat reserves. Surprisingly, there was no correlation between parasite infection and number of MHC length variants or perivisceral fat deposits. However, the individual number of MHC length variants significantly correlated with the amount of perivisceral fat deposits in males, suggesting that male individuals with an intermediate number of alleles might be able to use their fat reserves more efficiently.

Introduction

Investigating the genetic basis of variation in fitness traits and how contemporary processes feed back to long-lasting adaptive processes are major topics in evolutionary research (Lazzaro & Little, 2009; Spurgin & Richardson, 2010; Wilson *et al.*, 2010; Laine *et al.*, 2011). Infectious diseases are ubiquitous and constantly challenge the host's fitness or influence fitness-relevant traits (Windsor, 1998; Crompton & Nesheim, 2002; Allen & Maizels, 2011). There is often interindividual variation in immune resistance caused by processes related to population dynamics or spatio-temporal fluctuations of the parasites (Oliver *et al.*, 2009a, b; Spurgin & Richardson, 2010). Studying the genetic basis of pathogen recognition and defence is hence an outstanding opportunity to observe eco-evolutionary processes (Fussmann *et al.*, 2007).

Host species typically experience a wide range of pathogenic infections (Williams & Jones, 1994; Poulin & Morand, 2000). Probably as a result, they evolved defence strategies that differ greatly in mechanism and specificity. One strategy is physical and chemical barriers. Skin tissue prevents pathogens from invading the host's vital organs, and antimicrobial peptides can provide the host with additional protection (Arts & Kohler, 2009). When a pathogen eventually circum-

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vents the two first lines of defence, the host must be able to identify it and start a specific immune reaction. Some proteins encoded by the major histocompatibility complex (MHC) play a vital role in antigen recognition pathways by initiating the host's immune response or restarting it upon re-infection (Zinkernagel, 1979; Klein, 1986; Janeway *et al.*, 2011). Extracellular parasite-derived antigens are recognized by MHC class II proteins. These heterodimeric proteins are encoded by two gene families (subclass *A* and *B*, respectively), each encoding for one of the subunits. The antigen-binding sites are situated on the first peptide domain encoded by the second exon. MHC class II proteins typically show high specificity for certain antigens, hence providing protection against specific parasites (Apanius *et al.*, 1997; Milinski, 2006; Klein *et al.*, 2007).

Several studies have investigated how the exceptional polymorphism of MHC genes originated. At the macroevolutionary level, MHC diversity is the result of the complex interplay between gene duplication, recombination, mutation and selection (Klein *et al.*, 2007; Spurgin & Richardson, 2010; Spurgin *et al.*, 2011; Hablützel *et al.*, 2013). After the initial rounds of gene duplications, several loci may became lost in some taxa (e.g. in Atlantic cod, MHC class II genes got lost completely; Star *et al.*, 2011), resulting in a birth and death process of gene evolution (Nei *et al.*, 1997). In several taxa, repeated gene duplications led to high copy numbers of MHC genes (Málaga-Trillo *et al.*, 1998; Sato *et al.*, 1998, 2012; Michel *et al.*, 2009). An interesting observation in several of these taxa is the considerable interindividual variation in gene copy number. Under the hypothesis of heterozygous advantage, the possession of a diverse MHC is expected to increase the immunocompetence of their carrier (Penn *et al.*, 2002). In congruence with this hypothesis, several empirical studies found that heterozygous individuals perform best (Penn *et al.*, 2002; Lenz *et al.*, 2009; Oliver *et al.*, 2009b; Thoß *et al.*, 2011). Great reed warbler with specific MHC alleles or maximal MHC diversity is best protected against avian malaria (Westerdahl *et al.*, 2005). Another study on collared flycatchers found a negative association between functional MHC diversity and the probability of infection (Radwan *et al.*, 2012). However, other studies indicate that individuals with an intermediate number of variants are much more abundant than individuals with high MHC diversity. These individuals are less infected with parasites or have other fitness advantages (Wegner *et al.*, 2003a,b; Bonneaud *et al.*, 2004; Buchholz *et al.*, 2004; Madsen & Ujvari, 2006; Kloch *et al.*, 2010; Stiebens *et al.*, 2013). The cause of this effect is typically referred to as lymphic T-cell maturation (Nowak *et al.*, 1992; Woelfing *et al.*, 2009). During this process, T-cell clones with a strong affinity for self-peptides–MHC complexes are eliminated in the lymphic system to counteract autoimmune reactions. If the individual MHC allele number is too high, this negative selection on T cells reduces the number of functional T-cell clones below a critical value at which the individual is expected to suffer from reduced capacity to respond to parasite challenge (Nowak *et al.*, 1992; Woelfing *et al.*, 2009).

Considerable effort to study relationships between the number of MHC class II*B* variants and parasite infection, reproductive success and mate choice has been made in three-spined stickleback. In the wild, individuals with an intermediate number of MHC class II*B* variants suffered least from simultaneous infections with multiple parasite species (Wegner *et al.*, 2003a). This observation was confirmed with co-infection experiments with three parasite species in the laboratory (Wegner *et al.*, 2003b; Kurtz *et al.*, 2004). A semi-natural enclosure experiment revealed that stickleback females have an increased life-time reproductive fitness when they carry an intermediate number of MHC class II*B* variants (Kalbe *et al.*, 2009). Interestingly, stickleback females choose their mates to optimize the number of MHC class II*B* variants in their offspring (Reusch *et al.*, 2001; Aeschlimann *et al.*, 2003; Milinski *et al.*, 2005). In recent years, a handful of studies confirmed the 'optimality hypothesis' (Kloch *et al.*, 2010) in other organisms. Sparrows laid larger clutches of eggs (Bonneaud *et al.*, 2004), male turkeys had more conspicuous ornaments (Buchholz *et al.*, 2004), pythons suffered less from parasite infections and grew larger (Madsen & Ujvari, 2006), bank voles had fewer parasites (Kloch *et al.*, 2010), and loggerhead sea turtles grew larger (Stiebens *et al.*, 2013). But there is also a considerable number of studies that did not detect benefits of such intermediate individual MHC diversity (Westerdahl *et al.*, 2005; Radwan *et al.*, 2012; Sepil *et al.*, 2013), raising the question whether benefits from intermediate diversity depend on the species studied and the intensity of parasite infection (Milinski, 2006).

In cichlid fishes, large intra-individual variation in the number of MHC class II*B* variants has been observed (Málaga-Trillo *et al.*, 1998; Sato *et al.*, 2012). Cichlids are prime models of adaptive radiation, and various studies have focused on cichlid MHC genes in the context of local adaptation and phylogenetic divergence (Klein *et al.*, 1993; Ono *et al.*, 1993; Blais *et al.*, 2007). Selective forces acting on cichlid MHC appear to be strong, indicated by rapid divergence of MHC gene pools in closely related species (Blais *et al.*, 2007). Positive and balancing selection seem to be the major driver of cichlid MHC diversity and might be responsible for high levels of individual heterozygosity (Blais *et al.*, 2007).

So far, 18 cichlid MHC class II*B* homology groups representing putative loci have been defined (Málaga-Trillo *et al.*, 1998; Hablützel *et al.*, 2013). The high levels of trans-species polymorphism at these loci (Klein *et al.*, 1993) indicate that they have been under positive or balancing selection for millions of years. Crossing

over between loci was observed in test crossings (Málaga-Trillo *et al.*, 1998), indicating that recombination can efficiently mix up loci on ecological time scales. Despite the high number of MHC loci found in cichlids (Klein *et al.*, 1993; Ono *et al.*, 1993; Blais *et al.*, 2007; Sato *et al.*, 2012; Hablützel *et al.*, 2013), each individual possesses only a small subset (Málaga-Trillo *et al.*, 1998). The 'optimality hypothesis' may provide a powerful theoretical explanation for this paradox, but empirical evidence is lacking so far.

In this study, we test the 'optimality hypothesis' in *Tropheus moorii* from Lake Tanganyika. This and other species of the genus *Tropheus* are famous for their diversity of allopatric colour morphs (Konings, 1998; Schupke, 2003). Many studies have focused on its ecology and population history (Takamura, 1983; Yanagisawa & Nishida, 1991; Baric *et al.*, 2003; Sturmbauer *et al.*, 2005; Salzburger *et al.*, 2006; Egger *et al.*, 2007; Koblmüller *et al.*, 2011). Recently, we investigated the spatial structure of parasite communities in *T. moorii* (Raeymaekers *et al.*, 2013). We found that allopatric *T. moorii* colour morphs were infected by contrasting parasite communities and that this pattern is stable, at least over a period of 2 years. In this study, we investigated the influence of the interindividual variation in the number of MHC length variants on parasite infection and body condition, measured by the amount of perivisceral fat reserves. Associations between body condition, parasite infection and MHC genotype may reveal valuable information on the fitness benefits of divergent immunogenetic adaptation in populations facing contrasting parasite communities.

Materials and methods

Sample collection and parasitological survey

The data for this study were collected in parallel with the study by Raeymaekers *et al.* (2013), who described the variation in ecto- and endoparasite communities among allopatric colour morphs of *T. moorii*. Fish were collected in August 2012 at six locations along the Zambian shoreline of Lake Tanganyika (Table 1). Thirty-six to forty individuals from each location (231 in total) were euthanized with an overdose of MS222, weighed to the nearest 0.1 g and measured to the nearest 0.1 mm (standard length). Individual fish were screened for infections with *Gyrodactylus* sp. (skin and fins), unidentified helminthic cysts (skin and fins), branchiurans (mouth cavity and gills), acanthocephalans (intestine), nematodes (intestine), digeneans (intestine) and *Urogyrus* sp. (urinary bladder). Gill arches from the right side were immediately stored in 100% EtOH and screened in the laboratory for infections with *Cichlidogyrus* sp., *Ergasilus* sp. and cysts. To measure parasite infection, we calculated an individual parasitation index (I_{PI}) according to Kalbe *et al.* (2002) based on both presence/absence and relative abundance of all ten parasite taxa. The I_{PI} was calculated for each sampling location separately to account for differences among populations. Perivisceral fat was extracted manually from the body cavity and weighed to the nearest 0.01 g. This fat tissue is scattered among the organs in the body cavity of fishes and can serve as a indication of body condition as the direct negative impact of parasites on perivisceral fat has been shown repeatedly (Barber & Svensson, 2003; Caffara *et al.*, 2007; Sitjà-Bobadilla & Palenzuela, 2012; Vanacker *et al.*, 2012). The sex of each specimen was determined by manual inspection of the gonads. A fin-clip was taken and stored in 100% EtOH for subsequent genetic analysis in the laboratory.

Assessment of intra-individual variation of number of MHC length variants

The intron 1 of cichlid MHC class II*B* genes is known to exhibit excessive variation in length due to frequent indels (Klein *et al.*, 1993). In contrast to intron 1, the second exon is typically invariable in length, although some alleles might have a deletion of a single codon (Hablützel *et al.*, 2013). When recombination occurs not too often, exon 2 sequence variants can, at least to a certain extent, be distinguished by the length of the intron 1 + exon 2 amplification product. Here, we took advantage of this property and genotyped *T. moorii* individuals by assessing MHC class II*B* length variants using fluorescently labelled primers.

Genomic DNA was extracted from fin clips of all *T. moorii* individuals using the NucleoSpin tissue kit (Macherey – Nagel) following the manufacturer's rec-

Table 1 Sampling location and sample size along with the number of major histocompatibility complex (MHC) class II*B* length variants in six *Tropheus moorii* populations. Only few length variants were specific for a single population.

Location	Latitude	Longitude	Sample size (males/females)	MHC length variants (unique)
Tumbi	08°42′10.7″ S	30°55′20.9″ E	36 (29/7)	25 (1)
Katoto	08°47′51.6″ S	31° 0111.8″ E	39 (26/13)	25 (0)
Mbita Island	08°44′57.1″ S	31°05′14.2″ E	40 (27/13)	28 (1)
Wonzye Point	08°43′07.6″ S	31°08′12.6″ E	38 (25/13)	30 (2)
Muzumwa	08°42′05.7″ S	31°11′59.8″ E	40 (17/23)	21 (0)
Toby's place	08°37′18.9″ S	31°11′59.9″ E	38 (19/19)	30 (0)
Total			231 (143/88)	39 (4)

ommendations. MHC class II*B* loci were amplified with the CiDxBF (5′-AGCCTCAGCACAGCAGGTA-3′) and CiDxBR (5′-GCACACTCACCTGATTTAGACA-3′) primer pair (of which the forward primer CiDxBF was fluorescently labelled), which spans intron 1 and exon 2 of this gene (Hablützel *et al.*, 2013). Length variants were called in an automated ABI 3130 sequencer (Applied Biosystems, Foster City, CA, USA). Detailed information about PCR and genotyping conditions can be found in the Supporting information.

Cichlid fishes are diploid organisms and can hence be homozygous or heterozygous for each MHC class II*B* length variant. With our method, we only noted the presence/absence of each MHC class II*B* length variant and did not take into account cases of homozygosity.

MHC sequencing

To test whether length variation of the intron 1 represents a good predictor of sequence identity of exon 2 (i.e. whether indels in intron 1 or recombinations between intron 1 and exon 2 do not occur too often), we sequenced 31 MHC class II*B* length variants from 30 individuals. PCRs were performed with an unlabelled CiDxB primer pair (Hablützel *et al.*, 2013). Multi-template PCR of MHC genes is known to be prone to sequencing artefacts (Lenz & Becker, 2008). We therefore followed the guidelines by Lenz & Becker (2008) for PCR amplification. Detailed information about PCR conditions and sequencing can be found in the Supporting information.

Additionally, we quantified the sequence length of 50 cloned MHC class II*B* length variants (GenBank Accession Numbers KF479265–KF479343) of which we previously had sequenced exon 2 (Hablützel *et al.*, 2013). These 50 sequences only contained independent replicates (i.e. sequences that were observed more than once in the same individual were excluded) and only sequences that were found in two or more independent PCRs (Hablützel *et al.*, 2013).

Based on the final dataset of 95 exon 2 sequences, we calculated a neighbournet graph using the program SplitsTree v.4.12.6 (Huson & Bryant, 2006). De novo-generated sequences were submitted to GenBank (Accession Numbers KJ921657–KJ921702).

Microsatellite genotyping

Individual MHC copy number might directly link to mean heterozygosity because fish with more closely related parents are also more likely to inherit identical MHC haplotypes. Individual genetic diversity may also correlate with fat content, as heterozygosity might influence fitness and parasite resistance (Coltman *et al.*, 1999; Hansson & Westerberg, 2002; Voegeli *et al.*, 2012). To test for a relationship between individual heterozygosity and MHC copy number variation, we genotyped a subset (20%) of the individual hosts for eleven neutral microsatellite loci. Because the individual fat–MHC relationship was present in large males but absent in females (see Results), we included all large males (i.e. male individuals with standard length of more than 8.2 cm) for microsatellite genotyping. The numbers of samples from each population were Tumbi: 5, Katoto: 10, Mbita Island: 13, Wonzye Point: 4, Muzumwa: 8 and Toby's Place: 8. Detailed information about PCRs and genotyping conditions can be found in the Supporting information.

Statistical analyses

To avoid problems of co-linearity between the linear and quadratic terms of number of MHC length variants, the values were standardized (mean = 0 and standard deviation = 1). As body length and body weight was highly correlated ($P < 0.0001$, adj-$R^2 = 0.93$, Fig. S1), only body length was included in the analyses to avoid problems with co-linearity. For the analysis of variance, we used type II sums of squares using the ANOVA function in the car package (Fox & Weisberg, 2011) in R v2.15.1 (R Development Core Team, 2011). The best models were chosen based on the Akaike's information criterion (AIC; Akaike, 1974). Stepwise selection was performed both forward and backward.

Parasite infection

I_{PI} was analysed with a general linear model with body length, sex, number of MHC length variants (linear and quadratic term), sampling location and fat deposits as explanatory variables. The quadratic term for number of MHC length variants was included as there is strong indication that the relationship with parasite infection might not be linear (Nowak *et al.*, 1992; Wegner *et al.*, 2003b; Woelfing *et al.*, 2009). To improve normality of the data and homogeneity of variances, I_{PI} values were square-root-transformed. The AIC-based model selection suggested to only include body length and location as explanatory variables.

Perivisceral fat deposits

Perivisceral fat deposits were analysed with a general linear model with body length, sex, MHC variant number (linear and quadratic term), sampling location and parasite infection as explanatory variables. Parasite infection was incorporated in the model either as I_{PI} or as the abundance of individual parasite taxa. To improve normality of the data and homogeneity of variances, fat weights were square-root-transformed. Based on AIC, the best model for the analysis with I_{PI} included body length, sex, and MHC variant number (including quadratic term). The best model for the analysis with the abundance of individual parasite taxa included body length, sex, MHC variant number

(including quadratic term), location, Acanthocephala, Nematoda and *Ergasilus* sp.

As there was a significant influence of sex on perivisceral fat deposits (see Results), the analysis was repeated for both males and females separately. Based on AIC, the best model with I_{PI} for male data included body length and MHC variant number (including quadratic term), whereas for females, body length and sampling location were selected. The alternative analysis with individual parasite taxa included body length, MHC variant number (including quadratic term), Acanthocephala, Nematoda, Digenea and gill cysts for males and body length, location and *Ergasilus* sp. infection for females.

To test for a relationship between individual multilocus heterozygosity and the amount of perivisceral fat deposits, we restricted the models to the 48 males that were genotyped for eleven neutral microsatellites. Individual multilocus heterozygosity (PHt, i.e. the proportion of heterozygous loci in an individual) was calculated using the Htest function in GENHET (Coulon, 2010) in R. AIC-based selection for the best perivisceral fat model never suggested to keep PHt in the analysis. We nevertheless tested for its significance by adding it to the best model which included body length, MHC variant number (including quadratic term), location, Nematoda, gill cysts and Branchiura. Finally, we applied a linear model to test for a relationship between MHC variant number and heterozygosity.

Results

Parasite infection

Data on parasite prevalence, mean abundance and median infection intensities were presented in detail in Raeymaekers *et al.* (2013). An updated summary is presented in Table 2. In general, infection patterns greatly varied across parasite taxa and sampling location Fins and skin of *Tropheus* were infected with monogenean flatworms of the genus *Gyrodactylus* and unidentified helminthic cysts. *Gyrodactylus* was present at all locations and had its highest prevalence (20%) at Muzumwa. Cysts on fins were very rare, found only on two individuals from Katoto and Mbita Island, respectively. Similarly, we detected three individuals infected with a single fish louse (Branchiura) in the mouth cavity or on the gills in Mbita Island, Wonzye Point and Toby's place, respectively. The gills of more than half of the individuals at each location were infected with monogenean flatworms of the genus *Cichlidogyrus*, with median infection intensities up to nine individuals. Another gill parasite that regularly infected *Tropheus* was the copepod genus *Ergasilus*, although median infection intensity was low. In rare occasions, we encountered unidentified cysts on the gills of *Tropheus* individuals from Tumbi, Mbita Island, Wonzye Point and Muzumwa. In the intestine, acanthocephalans were the most prevalent taxon, infecting the majority of the

Table 2 Sampling location, sampling size and prevalence, mean abundance and median infection intensity for six *Tropheus moorii* populations along the Zambian shore of Lake Tanganyika.

Location	*n*	Acanthocephala	Nematoda	*Urogyrus*	Digenea	Cysts (gills)	Cysts (fins)	*Gyrodactylus*	*Cichlidogyrus*	*Ergasilus*	Branchiura
Prevalence (in %)											
Tumbi	36	55.56	0	11.11	0	11.11	0	5.56	66.67	30.56	0
Katoto	39	71.79	25.64	7.69	2.56	0	2.56	12.82	92.31	23.08	0
Mbita Island	40	95	5	10	0	7.5	2.5	2.5	92.5	45	2.5
Wonzye Point	38	84.21	15.79	2.63	0	2.63	0	5.26	89.47	26.32	2.63
Muzumwa	40	92.5	5	2.5	0	5	0	20	87.5	35	0
Toby's place	38	78.95	5.26	5.26	0	0	0	2.63	73.68	34.21	2.63
Mean abundance											
Tumbi	36	2.85	0	0.05	0	0.15	0	0.15	5.7	0.55	0
Katoto	39	4	0.49	0.13	0.03	0	0.03	0.26	11.72	0.38	0
Mbita Island	40	11.23	0.05	0.23	0	0.08	0.03	0.05	13.85	0.98	0.03
Wonzye Point	38	4.9	0.16	0.05	0	0.03	0	0.08	5.92	0.37	0.03
Muzumwa	40	6.15	0.05	0.03	0	0.13	0	0.48	10.48	0.45	0
Toby's place	38	6.47	0.05	0.13	0	0	0	0.03	3.66	0.42	0.03
Median infection intensity											
Tumbi	36	2	0	1	0	1	0	1.5	3	2	0
Katoto	39	4.5	1	1	1	0	1	2	8	1	0
Mbita Island	40	9.5	1	2	0	1	1	2	9	2	1
Wonzye Point	38	5	1	2	0	1	0	1.5	4.5	1	1
Muzumwa	40	5	1	1	0	2.5	0	2	9	1	0
Toby's place	38	6.5	1	2.5	0	0	0	1	3.5	1	1

individuals except in Tumbi, where only about half of the *Tropheus* were infected. Besides acanthocephalans, we also detected infections by intestinal nematodes. Their prevalence was much lower, median infection intensity was one worm, and they were even absent in Tumbi. Only one individual from Katoto was infected by a digenean. In the urinary bladder, we regularly encountered monogenean flatworms of the genus *Urogyrus* at all sampling locations and at low median infection intensities (1–2.5; Table 2).

MHC diversity

In total, we found 39 MHC class II*B* length variants, all of which occurred at least twice in the data set. The length ranged from 337 to 511 bp. Between 21 and 30 length variants were detected per population (Table 1). The individual number of MHC length variants varied among individuals and ranged from 1 to 7 (Fig. 1). The most frequent individual number of length variants was 4 (40.1% of the individuals across all populations). This general pattern was reflected in each population (see Fig. S1).

We generated sequences for 31 of the 39 length variants. These length variants accounted for 1101 of the 1132 genotyped length variants (97.26%) in the 232 *T. moorii* individuals. The other 8 length classes were extremely rare and occurred in only six or less *T. moorii* individuals. For 26 length variants, we obtained two or more sequences, accounting for 1057 of the 1132 genotyped length variants (93.37%). We found inconsistencies between the neighbournet graph (Fig. 2) and the intron length in only four cases [length variant 405, 435, 459 and 464, accounting for 179 (16.93%) of the 1057 genotyped length variants with two or more sequences]. This indicates that intron length variation provides a reasonable indication of exon sequence variation, although it likely underestimates the diversity (Fig. 1).

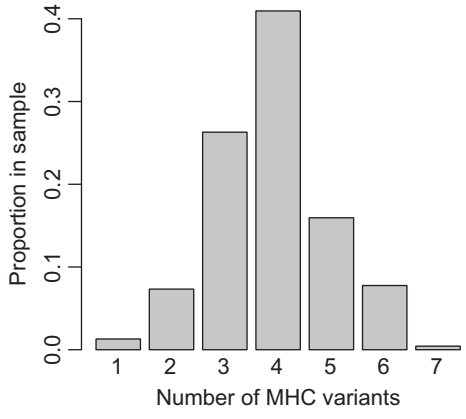

Fig. 1 Frequency distribution of the number of major histocompatibility complex (MHC) class II*B* length variants per individual host across both sexes and all populations.

One variant with a length of 384 bp was present in all individuals. Sequence analysis revealed identity of this length variant with the previously defined locus DBB5 (Sato *et al.*, 2012). This variant was excluded from the analysis because there are indications that it might have a nonclassical function (Fig. 2; Hablützel *et al.*, 2013).

Statistical analyses

Parasite infection

I_{PI} was correlated with body size, as larger individuals were infected by a higher number of parasites (Table 3). Consistent with earlier results (Raeymaekers *et al.*, 2013), infection patterns differed between the host populations, but not between sexes. There was no significant effect of number of MHC length variants or perivisceral fat deposits on I_{PI} (Table S1; linear term: $F_{1,221} = 0.41$, $P = 0.5225$, quadratic term: $F_{1,221} = 0.31$, $P = 0.5788$; Fig. 3; Table S1).

Perivisceral fat deposits

I_{PI} was not retained in any of the models after model selection, and these models did not lead to different conclusions than the models with individual parasite taxa. To avoid redundancy, only the results of the latter analyses are presented (see Tables S2 and S3 for results of all full models). Analysis of the full dataset (including males as well as females) revealed that perivisceral fat deposits were positively correlated with body length and were slightly higher in females (Fig. 4). We found no significant effect of MHC variant number (Table 3; Fig. 5) and no significant variation in host perivisceral fat deposits among host populations (Table 3). Infection with acanthocephalan or *Ergasilus* sp. did not explain significant variation in fat deposits either, but a positive relationship was observed with nematode infection (Table 3; Fig. 6).

When males were analysed separately, the effect of body length and number of MHC genes (including quadratic term) was stronger than in the full dataset (Table 3; Fig. 5). The quadratic term of the individual number of MHC length variants significantly influenced the amount of perivisceral fat deposits (Table 3; Fig. 5), indicating a nonlinear relationship. The optimal number of MHC length variants was 3.78 based on the first-order derivative of the estimated quadratic relationship (Fig. 5). I_{PI} was not correlated with perivisceral fat deposits (Table S2; $F_{1,134} = 0.38$, $P = 0.5406$), but there was a positive relationship of perivisceral fat deposits with nematode infection (Table 3; Fig. 6). In females, no correlation between individual number of MHC length variants and perivisceral fat deposits could be detected (Table S2; linear term: $F_{1,78} = 1.13$, $P = 0.2904$, quadratic term of full model: $F_{1,78} < 0.01$,

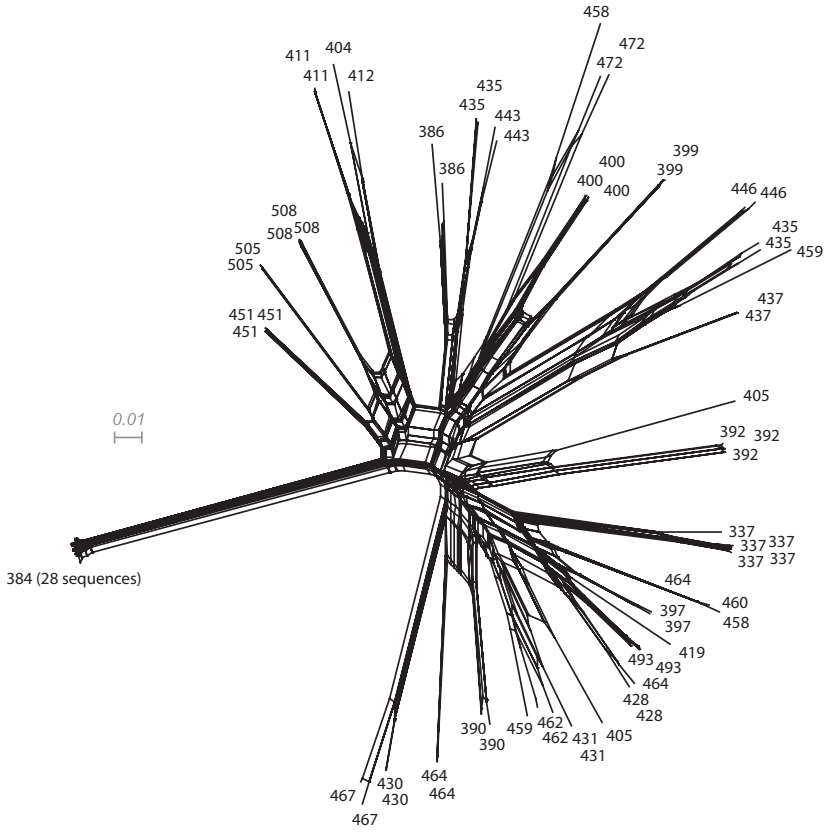

Fig. 2 Split network of 95 exon 2 sequences of major histocompatibility complex class II*B* genes. Numbers on tips represent the length of the amplified fragment (intron 1 + exon 2) in base pairs.

Table 3 General linear models after Akaike's information criterion-based model selection for the impact of host body length, sex, major histocompatibility complex (MHC) variant number, sampling location and perivisceral fat deposits on the host's parasite load (measured as I_{PI}; above) and the impact of host body length, sex, MHC variant number, sampling location and abundances of individual parasite taxa on the host's perivisceral fat deposits (below). Significant *P*-values (< 0.05) are indicated in bold.

	Effect	Estimate (SE)	Num d.f.	Den d.f.	*F*-value	*P*-value
Parasite infection						
All individuals	Body length	1.85 (0.32)	1	225	34.11	**< 0.0001**
	Location	–	5	225	8.02	**< 0.0001**
Fat deposits						
All individuals	Body length	5.58 (0.47)	1	219	142.67	**< 0.0001**
	Sex	–	1	219	11.92	**0.0006**
	n(MHC)	−0.10 (0.35)	1	219	0.07	0.7854
	$n(MHC)^2$	−0.41 (0.25)	1	219	2.57	0.1101
	Location	–	5	219	2.08	0.0690
	Acanthocephala	0.10 (0.06)	1	219	2.87	0.0919
	Nematoda	2.57 (0.70)	1	219	13.31	**0.0003**
	Ergasilus sp.	−0.64 (0.37)	1	219	2.89	0.0903
Males	Body length	6.53 (0.48)	1	136	181.72	**< 0.0001**
	n(MHC)	−0.14 (0.40)	1	136	0.13	0.7186
	$n(MHC)^2$	−1.00 (0.26)	1	136	14.7	**0.0002**
	Acanthocephala	0.09 (0.06)	1	136	2.02	0.1576
	Nematoda	2.37 (0.66)	1	136	13.04	**0.0004**
	Digenea	−8.02 (4.68)	1	136	2.94	0.0889
	Gill cysts	−3.10 (1.72)	1	136	3.26	0.0734
Females	Body length	3.69 (0.90)	1	80	16.74	**0.0001**
	Location	–	5	80	2.39	**0.0449**
	Ergasilus sp.	−1.53 (0.96)	1	80	2.56	0.1132

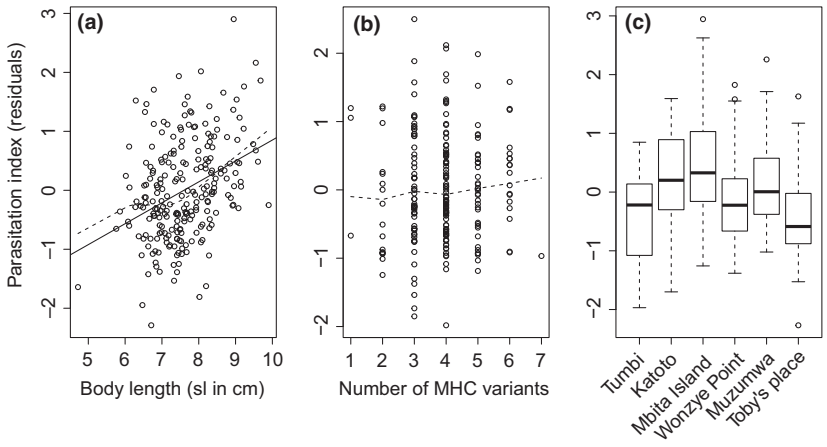

Fig. 3 Correlation of residual parasite infection (I_{PI}) with (a) host body length (the function of the linear regression line is $y = -12.65 + 1.67x$), (b) individual number of major histocompatibility complex (MHC) length variants and (c) variation of parasite infection (I_{PI}) across host populations. The solid lines are linear regression lines; the dashed lines represent LOWESS smoothers.

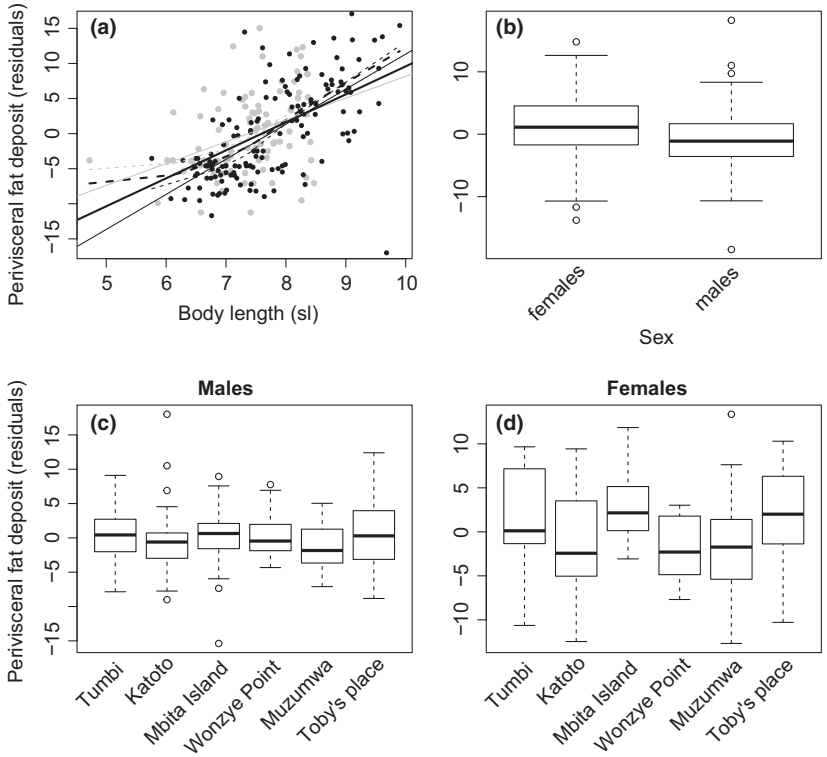

Fig. 4 (a) Correlation of residual perivisceral fat deposits with host body length (sl; the function of the linear regression line for both sexes, male and female host infection is $y = -30.31 + 3.98x$, $y = -38.66 + 5.00x$ and $y = -22.93 + 3.11x$, respectively). (b) Comparison of residual perivisceral fat deposits between sexes and (c + d) variation of residual perivisceral fat deposits of males (c) and females (d) across populations. The solid lines are linear regression lines; the dashed lines represent LOWESS smoothers. Grey dots and lines represent female host data, black dots and thin lines male host data, and black bold lines smoothers and regressions of both sexes combined.

$P = 0.9354$; Fig. 5), but there was a strong effect of body length, and the amount of perivisceral fat significantly varied among populations (Table 3; Fig. 4).

The results of the analysis including only the males genotyped for eleven microsatellite loci gave similar results as the analysis including all male individuals (Table 4). However, there was a significant negative effect of gill cysts on perivisceral fat deposits. Most notably, individual multilocus heterozygosity had no significant effect on fat deposits (Table S2; $F_{1,37} = 0.20$, $P = 0.6578$). There was also no significant correlation between multilocus heterozygosity and MHC variant number ($r^2 = -0.02$, $P = 0.9742$).

Discussion

Variation in perivisceral fat reserves

Male *T. moorii* individuals with an intermediate MHC class II*B* diversity were in better physiological condition (measured as the relative amount of perivisceral fat deposits) than individuals with few or many MHC class

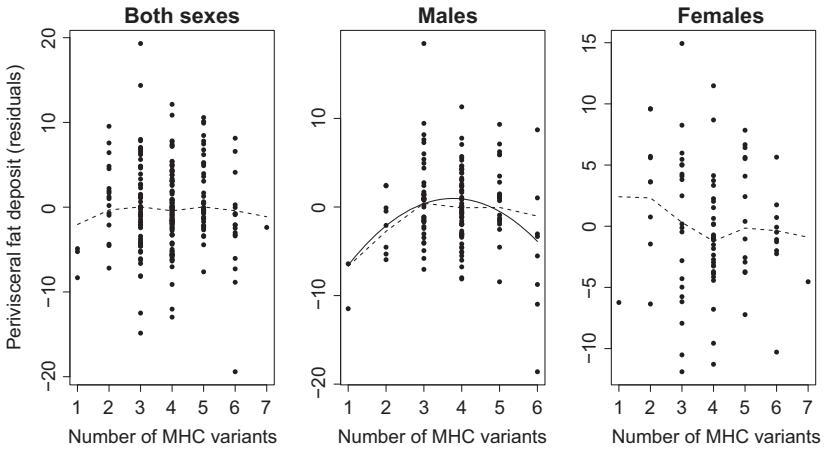

Fig. 5 Correlation of residual perivisceral fat deposits with individual number of major histocompatibility complex (MHC) length variants (the function of the linear regression line for male perivisceral fat deposits is $y = -12.99 + 7.40x - 0.98x^2$). The solid line is a regression line; the dashed lines represent LOWESS smoothers.

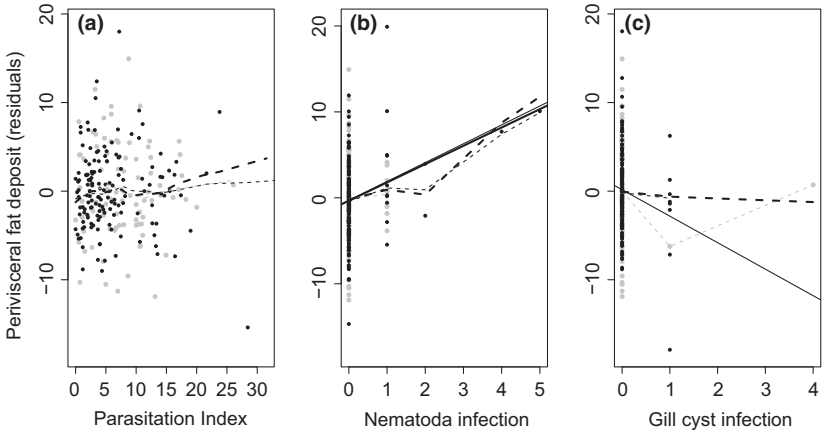

Fig. 6 Correlation of residual perivisceral fat deposits with (a) parasite infection (I_{PI}), (b) Nematoda infection (the function of the linear regression line for both sexes and male host infection is $y = -0.29 + 2.19x$ and $y = -0.34 + 2.13x$, respectively) and (c) gill cyst infection in males (the function of the linear regression line is $y = 0.17 - 2.99x$). The solid lines are linear regression lines; the dashed lines represent LOWESS smoothers. Grey dots and lines represent female host data, black dots and thin lines male host data, and black bold lines smoothers and regressions of both sexes combined.

Table 4 General linear model for the impact of host body length, major histocompatibility complex (MHC) variant number, sampling location and abundances of individual parasite taxa on perivisceral fat deposits in 48 *Tropheus moorii* males with known multilocus heterozygosity for 11 neutral microsatellite markers (PHt). PHt was not retained in the model after Akaike's information criterion-based model selection. Significant *P*-values (< 0.05) are indicated in bold.

Fat deposits	Effect	Estimate (SE)	Num d.f.	Den d.f.	*F*-value	*P*-value
Males	Body length	8.07 (1.88)	1	36	17.94	**0.0001**
	n(MHC)	−0.66 (0.76)	1	36	0.52	0.4768
	$n(MHC)^2$	−1.42 (0.53)	1	36	6.90	**0.0127**
	Location	–	5	36	1.95	0.1105
	Nematoda	1.71 (0.83)	1	36	3.83	0.0583
	Gill cysts	−8.04 (2.96)	1	36	8.61	**0.0059**
	Branchiura	5.57 (5.49)	1	36	1.60	0.2149

II*B* length variants. This pattern was apparently absent in females. Neither MHC variant number nor physiological condition was explained by individual multilocus heterozygosity. Because MHC genetic diversity plays a key role in immune defence (Janeway *et al.*, 2005), variation in host condition is likely linked to variation in immunogenetically mediated suppression of parasite infections.

Nonlinear relationships between individual MHC copy number variation and parasite infection or fitness traits have been reported in other vertebrates such as three-spined stickleback, house sparrow, water python, bank vole and loggerhead sea turtle (Wegner *et al.*, 2003a, b; Bonneaud *et al.*, 2004; Buchholz *et al.*, 2004; Madsen & Ujvari, 2006; Kloch *et al.*, 2010; Stiebens *et al.*, 2013). The accumulating evidence for superior

performance of individuals with intermediate rather than maximized MHC diversity in a wide range of taxa, encompassing fish, reptiles, birds and mammals, suggests that this observation is common. The current wide application of high-throughput sequencing methods likely will further increase this taxon list (Kloch *et al.*, 2010; Stiebens *et al.*, 2013). On the other hand, a number of studies suggest that individuals might also benefit from maximized MHC heterozygosity (Penn *et al.*, 2002; Lenz *et al.*, 2009; Oliver *et al.*, 2009a,b; Thß *et al.*, 2011). Whether or not there is an optimum in the individual MHC diversity, and at how many alleles this optimum lies may depend on several factors. One of these factors might be the diversity of the parasite community, but evidence is limited to very few taxa (Milinski, 2006).

Researchers have used a wide range of methods to genotype the highly variable MHC genes, ranging from single-strand conformation polymorphism (SSCP) analysis (Wegner *et al.*, 2003a) to denaturating gradient gel electrophoresis (DGGE; Forsberg *et al.*, 2007) and restriction fragment length polymorphism (RFLP) analysis (Madsen & Ujvari, 2006) to 454 amplicon sequencing (Kloch *et al.*, 2010; Stiebens *et al.*, 2013). Some of these methods (especially DGGE and RFLP analysis) might underestimate actual MHC diversity, whereas 454 sequencing might overestimate actual MHC diversity due to PCR artefacts (Lenz & Becker, 2008; Sommer *et al.*, 2013). In our study, we distinguished MHC length variants by length variation of intron 1. When validating our genotyping method by cloning and Sanger sequencing, we found that it potentially underestimated actual MHC diversity. It hence appears likely that the actual optimal MHC variant number for *Tropheus*, now estimated at 3.78, is in fact higher. Unfortunately, it remains uncertain to what extent over- or underestimating MHC variant number could affect the power of testing the 'optimality hypothesis'. But the differences between males (optimum) and females (no optimum) indicate that our genotyping method is capable to detect biologically relevant differences.

Another point that cannot be fully addressed at the moment is the occurrence of three base pair deletions in the second exon of some cichlid MHC class II*B* length variants (results not shown). The presence of these deletions seems to be relatively common and is not restricted to *T. moorii* and its close relatives (Hablützel *et al.*, 2013). They are not associated with a stop codon, and it is currently unknown whether these deletions might alter the functionality of MHC length variants. We are therefore uncertain whether these deletions lead to a bias in the estimation of the number of functional MHC length variants.

Rather surprisingly, the number of MHC class II*B* length variants in *T. moorii* did not influence the individual parasitation index. Neither was there any relationship of parasite infection (I_{PI}) on host body condition (perivisceral fat deposits). In fish, the direct impact of parasites on the loss of perivisceral fat has been shown repeatedly. The taxonomic range of these parasites is broad, including unicellular flagellates (Sitjà-Bobadilla & Palenzuela, 2012) and Myxozoa (Caffara *et al.*, 2007) as well as multicellular macroparasites (Barber & Svensson, 2003; Vanacker *et al.*, 2012). The lack of correlations between MHC class II*B* length variant number and parasite infection might have several reasons. First, parasite species differ in virulence and some species might have a substantially higher influence on the fitness of their host than others. Given the lack of information on virulence of the investigated parasite taxa on *T. moorii* or on other Tanganyika cichlids, it is currently not possible to properly account for this effect. Nevertheless, limited virulence might play a role, as some of the most abundant parasite taxa have been suggested to be nonpathogenic under natural conditions in other African cichlids (e.g. *Cichlidogyrus* species: Paperna, 1996). Second, the observed parasite species might be challenged by other host defence mechanisms such as intensified mucus production or antimicrobial chemicals, whereas antigen recognition by MHC II*B* genes may play only a minor role, if any. Third, our screening method was imperfect as it focused on large macroparasites. Virulent extracellular unicellular parasites might be an important target of MHC class II*B* genes and were not considered in this study.

Interestingly, our analyses revealed a positive relationship between nematode infections and perivisceral fat deposits in *T. moorii* hosts. First, this might be an effect of tropical transmission of nematode infections via intermediate hosts (Paperna, 1996), as fish with higher feeding rates (and consequently higher fat deposits) may experience increased parasite infection. Second, Råberg *et al.* (2007, 2009) pointed out that host and parasite can co-evolve in two different scenarios. They can engage in evolutionary arms races by antagonistic co-evolution of host resistance and parasite virulence. Another possibility is that the parasite reduces its virulence and the host simultaneously increases its tolerance against parasite infections, potentially leading to a stable co-existence of the two species. The lack of a negative response of host perivisceral fat reserves on parasite infection might be an indication of host tolerance. Indications of tolerance for metazoan parasite infection in natural vertebrate populations are scarce (but see Blanchet *et al.*, 2010). A positive correlation with parasite infection might come as a surprise, but empirical studies have shown that the impact of parasites on their vertebrate host might be weak or nonexistent (Stjernman *et al.*, 2008). Immune reactions are costly, and we speculate that hosts tolerating a moderate amount of parasite infection might outperform hosts with a more sensitive immune system (Stjernman *et al.*, 2008).

Differences between sexes

In *T. moorii* males, perivisceral fat reserves are influenced by individual MHC genotype. This was not the case for females, raising the question which behavioural, ecological or physiological difference might determine this contrasting pattern. *Tropheus* cichlids are territorial fishes, with each individual defending a small territory in the littoral zone of rocky outcrops (Takamura, 1983; Sturmbauer *et al.*, 2008). To mature the eggs in their ovary, females leave their own feeding territories and forage under the protection of a territory male, which typically occupy the best feeding grounds (Yanagisawa & Nishida, 1991). Males feed less during the courting period and invest more time in territory defence. Hence, both males and females have reproductive cycles throughout which energy investment and feeding rate are fluctuating in a different manner and at a different rate (Yanagisawa & Nishida, 1991). It appears that the reproductive cycle in females is shorter than in males as females change their territories much more frequently (Yanagisawa & Nishida, 1991). A possible explanation for the lack of connection between perivisceral fat reserves and reproductive activity in females is therefore that fat reserves may fluctuate too fast and adumbrate potential effects of the MHC genotype. Considering fluctuations of fat reserves in function of the reproductive cycle, it seems that male individuals with the best genes might be able to use their fat reserves more efficiently and are likely to recover faster from a physically exhaustive mating period. Further research is needed to reveal whether there is a direct association of lifetime reproductive success and optimal MHC diversity in *Tropheus* cichlids (Kalbe *et al.*, 2009).

Conclusion

The MHC of vertebrates provides an illustrative example of how contemporary processes such as selection and drift may feed back on long-lasting adaptive processes of gene or genome evolution. At the molecular level, both mutations and recombination steer the evolution of MHC class II *B* gene clusters in (cichlid) fishes (Wegner, 2008; Hablützel *et al.*, 2013). Spatial or temporal variation in the relative frequencies of MHC class II*B* alleles can be the result of the combined action of parasite-mediated selection (Eizaguirre *et al.*, 2012), mate choice (Reusch *et al.*, 2001), and selectively neutral processes such as drift (Radwan *et al.*, 2010) and the Mendelian inheritance of tightly linked loci (Wegner *et al.*, 2004). Here, we showed that in the cichlid *T. moorii*, individual MHC class II*B* diversity significantly influences the body condition. It appears plausible that the cichlid MHC evolves in analogy to the stickleback MHC, where spatiotemporal variation in parasite selection pressures and recombination of haplotypes sharing one or more alleles have been proposed to maintain variation in individual MHC copy number (Wegner *et al.*, 2004). Whether or not there is a link between intermediate MHC variant number, parasite infection and mate choice in cichlids remains to be investigated.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Appendix S1 Supporting information.

Figure S1 Frequency distribution of number of MHC class II*B* length variants per individual host across both sexes in each population.

Figure S2 Pairwise scatter plot for body length (sl), body weight and perivisceral fat deposits in *Tropheus moorii*.

Table S1 ANOVA table including parameter estimates and standard error (SE) of a general linear model for the impact of host body length, sex, MHC variant number, sampling location and perivisceral fat deposits on the host's parasite load (measured as I_{PI}).

Table S2 ANOVA table including parameter estimates and standard error (SE) of general linear models for the impact of host body length, sex, MHC variant number, sampling location and the host's parasite load (measured as I_{PI}) on the host's perivisceral fat deposits.

Table S3 ANOVA table including parameter estimates and standard error (SE) of general linear models for the impact of host body length, sex, MHC variant number, sampling location and abundances of individual parasite taxa on the host's perivisceral fat deposits.

Table S4 General linear models after AIC-based model selection for the impact of host body length, sex, MHC variant number, sampling location and abundances of individual parasite taxa on the host's perivisceral fat deposits.

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