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MHC, parasites and antler development in red deer: no support for the Hamilton & Zuk hypothesis

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Abstract

The Hamilton-Zuk hypothesis proposes that the genetic benefits of preferences for elaborated secondary sexual traits have their origins in the arms race between hosts and parasites, which maintains genetic variance in parasite resistance. Infection, in turn, can be reflected in the expression of costly sexual ornaments. However, the link between immune genes, infection and the expression of secondary sexual traits has rarely been investigated. Here, we explored whether the presence and identity of functional variants (supertypes) of the highly polymorphic major histocompatibility complex (MHC), which is responsible for the recognition of parasites, predict the load of lung and gut parasites and antler development in the red deer (*Cervus elaphus*). While we found MHC supertypes to be associated with infection by a number of parasite species, including debilitating lung nematodes, we did not find support for the Hamilton-Zuk hypothesis. On the contrary, we found that lung nematode load was positively associated with antler development. We also found that the supertypes that were associated with resistance to certain parasites at the same time cause susceptibility to others. Such trade-offs may undermine the potential genetic benefits of mate choice for resistant partners.

Introduction

Darwin (1871) explained that elaborated secondary sexual traits, such as deer antlers or peacocks' tails, which appear to impose survival costs on their bearers, may evolve as a result of their role in reproductive competition. Competition for mates causes sexual selection, which favours traits that can be used in fights (armaments) or to increase attractiveness to the opposite sex. While the role of sexual ornaments in increasing the sexual attractiveness of their bearers has been documented across many species (Andersson, 1994), the underlying evolutionary reasons for the emergence and maintenance of sexual preferences have remained controversial (reviewed in Kokko *et al.*, 2003; Andersson & Simmons, 2006; Prokop *et al.*, 2012). Fisher (1930) proposed that such preferences are selected for because the sons of choosy females achieve higher-than-average reproductive success (by being favoured by other choosy females) and thus pass on preference genes to future generations. However, once the genes for elaborated ornaments are fixed, these indirect benefits no longer apply and even the small costs of choosiness cause it to be selected against (Pomiankowski, 1988). This argument has increased the popularity of the indicator mechanism hypothesis, which posits that only high-quality individuals can afford sexual ornaments, and thus females may use these to gauge the quality of potential partners (Zahavi, 1975). In species in which females cannot derive direct benefits from mate choice, ornaments have been suggested to signal male genetic quality. Specifically, the 'good genes' hypothesis proposes that by choosing males with the biggest ornament, females benefit by increasing the fitness of their offspring. This hypothesis

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requires a mechanism for the maintenance of substantial genetic variance for fitness, and one such mechanism was proposed by Hamilton & Zuk (1982). They noted that host–parasite coevolution is a likely source of inexhaustible genetic variance for fitness and proposed that males that carry genes associated with better resistance to parasites are able to produce more elaborate sexual ornaments. Therefore, provided that host–parasite coadaptation cycles are long enough to cause between-generation correlation in resistance, females that choose ornamented males may produce offspring superior in parasite resistance (Hamilton & Zuk, 1982).

Because of their high degree of polymorphism and fundamental role in parasite resistance, genes of the major histocompatibility complex (MHC) appear to be obvious candidates for the 'good genes' of the Hamilton-Zuk mechanism. Proteins that are encoded by MHC genes bind parasite antigens and present them to T cells, thus initiating the adaptive immune response (Klein, 1986). The high polymorphism of these genes, typically comprising dozens of alleles, is thought to be maintained by selective pressures from parasites (reviewed in Bernatchez & Landry, 2003; Piertney & Oliver, 2006; Spurgin & Richardson, 2010). These pressures may result in negative frequency-dependent selection, which results from the ability of fast-evolving parasites to evade recognition by the most common host genotypes and prevents the loss or rare alleles (Bodmer, 1972; Potts & Wakeland, 1990; Borghans *et al.*, 2004). Another mechanism maintaining MHC polymorphism is heterozygote advantage, arising because MHC heterozygotes have the potential to recognize a wider range of parasites (Doherty & Zinkernagel, 1975). Thus, parasite resistance, and, consequently, ornament size, may be associated with either particular MHC alleles or MHC heterozygosity (or in the case of multiple loci, the number of MHC alleles). In the case of multiple MHC loci, the relationship may be nonlinear, with an intermediate number of alleles associated with optimal immune response. This is because the advantage of recognizing a wider range of parasites may trade off with the disadvantage of depleting the repertoire of T-cell receptors (Nowak *et al.*, 1992; Woelfing *et al.*, 2009).

Tests of the Hamilton-Zuk hypothesis have focused mainly on investigating the associations between ornament size and health predictors or parasite load. Many of these studies have supported the Hamilton-Zuk hypothesis (e.g. Milinski & Bakker, 1990 on sticklebacks, Costa & Macedo, 2005 on blue-black grassquits, Maan *et al.*, 2006 on cichlid fish, Martínez-Padilla *et al.*, 2007 on red grouse), but negative (e.g. Brønseth & Folstad, 1997 on sticklebacks, Votýpka *et al.*, 2003 on red-backed shrikes, Cramer & Cameron, 2007 on white-footed mice, Rantala *et al.*, 2007 on earwigs) or mixed (e.g. Müller & Ward, 1995; Ezenwa *et al.*, 2012) results have also been reported.

However, a full test of the Hamilton-Zuk hypothesis requires the demonstration of a link between the polymorphic genes associated with parasite resistance and the elaboration of the sexual ornament. To our knowledge, such studies have so far been performed in only two species (three-spined sticklebacks, Eizaguirre *et al.*, 2009; common yellowthroats, Dunn *et al.*, 2012). Eizaguirre *et al.* (2009) found a significant negative association between MHC diversity and parasite load, but they also reported a negative association between MHC diversity and male redness (but see Kalbe *et al.*, 2009). However, they found that males that carried an MHC F10 haplotype, which confers resistance to *Gyrodactylus* sp., achieved higher reproductive success, although the link to male epigamic traits was not demonstrated. In contrast, Dunn *et al.* (2012) found in common yellowthroats a positive association between the number of MHC II alleles and mask size (male ornament), but could not link those data with parasite load.

Here, we tested the predictions of the Hamilton-Zuk hypothesis in red deer (*Cervus elaphus*). This species, whose antlers were cited by Darwin in support of his sexual selection theory, is a model species in sexual and natural selection research. Antler mass has been demonstrated to be heritable and correlated with male reproductive success (Kruuk *et al.*, 2002). Furthermore, antler size positively influences the probability of holding a harem (Bartoš & Bahbouh, 2006). Antlers grow annually, imposing on their bearers enormous calcium demands (Landete-Castillejos *et al.*, 2007), which suggests that antlers should be an honest signal of male health. Additionally, the parasite fauna of red deer is well described (Demiaszkiewicz, 1987; Dróżdż *et al.*, 1997), and variation at most polymorphic MHC class II loci, which are responsible for the recognition of extracellular parasites, has been characterized (Swarbrick *et al.*, 1995; Swarbrick & Crawford, 1997). These factors make the red deer an excellent model for testing the Hamilton-Zuk hypothesis, yet the connection between antler development and parasite load has never been tested in this species. Other cervids have been examined in this respect (reindeer: Markusson & Folstad, 1997; white-tailed deer: Mulvey & Aho, 1993), but an association between parasite infection (fluke intensity) and antler size (number of points) was reported only in 1.5-year-old male white-tailed deer. Ditchkoff *et al.* (2001) reported a link between MHC alleles and antler development in white-tailed deer, but that study did not investigate the association between the MHC and parasite loads. Here, we investigated all facets of the Hamilton-Zuk hypothesis, including the links between the MHC, parasite load and antler development in red deer. In accordance with this hypothesis, we predicted that individuals that carried MHC class II DRB variants associated with reduced parasite load would carry more elaborated antlers. We also tested whether parasite load

and antler development in male red deer was associated with the total number of MHC DRB variants.

Materials and methods

Data collection

We obtained samples from 155 red deer collected during three hunting seasons (2010–2012). Samples were collected in two regions of Poland (Appendix S1), the Bieszczady mountains (2010–2012) in the south-east of the country and central Lakeland, north of Piła (2011–2012). Each year deer were shot by hunters between 28 August and 23 October, and data regarding body mass, age and antler size were gathered (see below). Additionally, the abomasum was excised and faeces were collected for parasitological analyses. The abomasum is the last part of a ruminant's stomach, located between omasum and intestine. Before being removed from the deer carcass, the abomasum was tied off at either end to prevent the loss of its contents. Organs prepared in this way were kept in sealed containers in 5% formalin. Faeces were collected from the rectum into plastic tubes and stored at 6°C. All samples were collected in cooperation with the Regional Directorate of State Forests in Krosno and Piła.

Body mass, age and antler measurements

Foresters are legally obligated to collect data that characterize every shot red deer; we were therefore able to obtain information about body mass, age, antler mass and antler measurements from Forest Inspectorates as part of an agreement with the Regional Directorate of State Forests. The standard procedure for the collection of these data is as follows. The carcass is weighed without the head and intestines and the age of each bull is estimated based on tooth abrasion. In red deer, all permanent teeth are present by the age of about 30 months, after which they are constantly exposed to wear. Based on the level of tooth damage, age can be estimated with an accuracy of 2 years (Bobek *et al.*, 1992). Finally, antlers are weighed and measured; antler mass includes the skull, but not the jaw. Other measurements are taken according to the standard CIC method (Jaczewski, 1981), which includes seven measurements that characterize antler size, number of points and the presence of crowns (Fig. 1).

Parasite abundance estimates

The abomasum collected from each shot stag was brought to the laboratory and opened, and its contents were emptied into a bucket filled with water; any remnants that stuck to the mucous membrane were scraped off and added to the bucket. The sedimentation and decantation methods were used several times until the fluid above the sediment was clear. We then took a sample of the sediment that represented 5% of the total volume and examined it under a stereomicroscope. We collected all nematodes that were present and transferred them to Petri dishes filled with a 5% glycerine solution (in 75% ethanol). After the fluid evaporated, the dishes were filled again with the glycerine solution. This procedure makes nematode bodies transparent, thus enabling the identification of species based on the male reproductive system. Females were pooled and counted. To check the precision of our method of sampling 5% of abomasum content, we repeated the analysis four times for 14 bulls and found that the repeatability (Lessells & Boag, 1987) of our measure of abomasum nematode load was very high ($R_i = 0.94$, $F = 65.33$, d.f. = 13, $P < 0.001$).

To assess the parasite load of the gastrointestinal tract, faeces were analysed using the flotation and Baermann methods. The flotation method is a standard parasitological method (Zajac & Conboy, 2006) that is based on the fact that less-dense material (e.g. eggs) floats to the top of a solution. We took 3 g of each faecal sample and mixed it with a saturated sucrose solution. Next, we poured the mixture into 15-mL tubes and centrifuged them at 2500 rpm for 5 min. The tubes were filled with additional flotation solution and covered with coverslips such that one side of the coverslip had contact with the solution. As a result, a thin solution layer, containing propagules, is stuck to the coverslip. After 15 min, the coverslip was placed on a microscope slide and parasite propagules were counted.

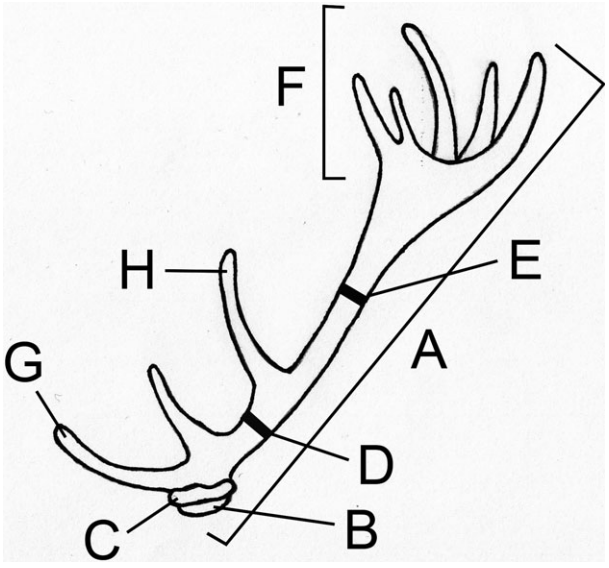

Fig. 1 Diagram showing red deer antler measurements: A, beam length; B, seal circumference; C, burr circumference; D, lower circumference; E, higher circumference; F, number of tops (called also royal or crown); G, brow tine; H, trez tine.

The Baermann method (Zajac & Conboy, 2006) was used to assess the burden of lung nematodes. We enclosed 5 g of each faecal sample in surgical gauze, placed it in a funnel filled with water and incubated it at room temperature. After 24 h, the tube at the end of the funnel was detached and approximately 2 mL of supernatant was removed. The remaining sediment was then examined for lung nematode larvae.

Faeces were analysed using the Baermann and flotation methods up to 7 and 16 days after collection, respectively. All parasite load data obtained by these methods were converted to dry weight of faeces.

MHC genotyping

We amplified a 199-bp fragment of the 2nd exon of the red deer MHC II DRB gene. For this purpose, we designed primers (CervL1 – AGTGTCATTTCYYCAAYG GGAC, CervE2endR – ACCTCGCCGCTGCACAGTGAA ACT), based on cervid sequences available in GenBank. Genotyping by sequencing was performed using the Illumina technology. Barcoded amplicons that contained Illumina adaptors were prepared in two consecutive PCR runs. The first PCR used two fusion primers (forward F – 5′-TCGTCGGCAGCGTCAGATG TGTATAAGAGACAG-NNNNNN-AGTGTCATTTCyyCAAy GGGAC-3′, reverse R – 5′-GTCTCGTGGGCTCGGAGATG TGTATAAGAGACAG-NNNNNN-ACCTCGCCGCTGCAC AGTGAAACT-3′) composed of three parts: the Illumina Read Sequencing Primer (respectively, 1 for F and 2 for R), a 6-bp tag to distinguish individuals (indicated with Ns), and an MHC-specific primer (CervL1 and CervE2endR, respectively). PCR amplification was performed in 15 μL reaction mixtures, which contained 10 to 75 ng of genomic DNA, 0.15 μM of each primer, 7.5 μL HotStarTaq Master Mix (QIAGEN, Germantown, MD, USA) and 3.75 μL H_2O. The PCR program consisted of 15-min initial denaturation at 95 °C followed by 33 cycles of: 30-s denaturation at 94 °C, 30-s annealing at 50 °C and 60-s extension at 72 °C. A final elongation step was run at 72 °C for 10 min. The concentrations of the PCR products were estimated through agarose gel electrophoresis. Amplicons were then pooled approximately equimolarly (Kloch *et al.*, 2010) and purified using the Clean-Up kit (A&A Biotechnology, Gdynia, Poland). Purified pools were used in a second PCR, in which the Illumina MiSeq adaptors (P5 and P7) were added to the amplified MHC fragments. The fusion primers (forward P5MHC – AATGATACGGC GACCACCGAGATCTACAC-TCGTCGGCAGCGTC, reverse P7MHC – CAAGCAGAAGACGGCATACGAGAT-GTCTC GTGGGCTCGG) used in the second PCR were composed of two parts: the Illumina adaptor (respectively, P5 and P7) and a fragment of the Illumina Read Sequencing Primer (respectively, 1 for P5 and 2 for P7). PCR amplification was done in a 20 μL volume and contained 1 μL of 100-fold-diluted purified amplicon pool from the first PCR, 0.10 μM of each primer, 10 μL HotStarTaq Master Mix (QIAGEN) and 7 μL H_2O. The PCR consisted of 15-min initial denaturation at 95 °C followed by 12 cycles of: 30-s denaturation at 94 °C, 30-s annealing at 50 °C and 60-s extension at 72 °C. A final elongation step was run at 72 °C for 10 min. Products of the second PCR were purified twice using the Clean-Up kit (A&A Biotechnology). The concentrations of the purified products of the second PCR were estimated using a Qubit® 2.0 Fluorometer. Amplicons were sequenced on an Illumina MiSeq apparatus using a v2 300-cycle kit which produced 2 × 150-bp reads.

Reads from each pair were combined into a single sequence on the basis of read overlap using FLASH-1.2.6 (Magoč & Salzberg, 2011). Reads that contained the complete forward and reverse MHC primers and complete tags on both sides were extracted from the multifasta files and sorted according to the tags using jMHC software (Stuglik *et al.*, 2011), available from http://code.google.com/p/jmhc/.

Our preliminary analyses (10 individuals genotyped by sequencing using 454 technology) indicated the presence of three MHC DRB loci. This implied that the minimum number of reads that would be sufficient to detect six alleles with 99.9% confidence would be 62, assuming equal amplification of all alleles within an amplicon (Galan *et al.*, 2010), although this number could be higher if this assumption is violated (Sommer *et al.*, 2013). We therefore conservatively adopted a coverage of 200 reads as sufficient for provisional genotyping. After provisional genotyping using this threshold, we found up to seven alleles per individual. Again, using the approach of Galan *et al.* (2010), the minimum number of sequences per individual that would be required to detect seven alleles with 99.9% confidence, assuming equal amplification of all alleles, was 75. We subsequently applied a simulation approach, as proposed by Sommer *et al.* (2013), to investigate the effects of variation in amplification efficiency among alleles on the minimum number of reads required for reliable genotyping.

New generation sequencing methods (i.e. 454, Illumina) generate errors, which include substitutions and small indels; PCR recombinants (chimeras) are another source of artefacts (Babik *et al.*, 2009; Galan *et al.*, 2010). To remove artifactual sequences from our data set, we applied two independent genotyping methods (Herdegen *et al.*, 2014 and Lighten *et al.*, 2014). With the first method (e.g. Promerova *et al.*, 2012; Herdegen *et al.*, 2014), genotyping consisted of two steps: identification of the limits of the 'grey zone' in which both true alleles and artefacts co-occur, and proper genotyping. The grey zone is the region between two thresholds: typically, below the lower threshold, all variants can be classified as artefacts, such as a chimera of other, more common, variants within the same amplicon or a

read containing 1–2 bp substitution errors, whereas above the higher threshold, no sequence can be explained as an artefact. A clear definition of the grey zone facilitates genotyping because it limits the number of variants which must be manually verified through comparison to a more common variant within the same amplicon. To determine the limits of the grey zone, we first calculated the maximum per-amplicon frequency (MPAF) of each variant, and then checked, starting from a MPAF of 0.5% and proceeding upward, whether a variant was a putative artefact, perhaps a chimera or the result of a 1–2 bp substitution error. All sequences with a per-amplicon frequency below 4.52% could be explained as artefacts, whereas all sequences with an MPAF above 10.83% were considered true alleles. Having identified the grey zone (MPAF of 4.52–10.83%), we performed genotyping. For each individual, all variants with frequencies above the grey zone were considered true alleles, and variants within the grey zone were considered true alleles only if they could not be explained as artefacts derived from true alleles.

As an alternative, we applied the degree of change (DOC) method proposed by Lighten *et al.* (2014). In brief, the DOC method orders variants of each amplicon according to their frequency and sets the threshold between true sequences and artefacts at the point with the highest drop in frequency between a given variant and the next-most-common variant.

To check the repeatability of our genotyping, we used 10 individuals which were amplified and sequenced twice: first using 454 sequencing method (our preliminary analysis) and then with Illumina technology as described above.

Analysis of selection signatures in MHC and supertype definition

We used two approaches to test for positive selection in MHC sequences. The average rates of synonymous (dS) and nonsynonymous (dN) substitutions were computed using MEGA5 for (i) all sites (Nei–Gojobori method with the Jukes–Cantor correction; standard errors were obtained through 1000 bootstrap replicates), (ii) positions that code amino acids that form the antigen-binding sites (ABSs) in HLA DRB (Reche & Reinherz, 2003) and (iii) positions outside the ABSs. Another test was performed by fitting four models of codon evolution (nearly neutral: M1a & M7; positive selection: M2a & M8) in PAML (Yang, 1997). Each model allows the dN/dS ratio (ω) to vary among amino acid sites. Model M1a assumes two site classes with $0 < \omega_0 < 1$ and $\omega_1 = 1$ in proportions p_0 and $p_1 = 1\text{-}p_0$ respectively, and the alternative model M2a adds a proportion p_2 of sites with $\omega > 1$ (positive selection). Model M7 assumes continuous beta distribution for ω (in the interval $0 < \omega < 1$) for all sites, and the alternative model M8 adds an extra class of sites with positive selection ($\omega_s > 1$). If models M2a or M8 fit the data better than corresponding models M1a and M7, then positive selection is inferred. Comparing these two pairs of models is recommended by Young (2007) as being particularly effective in detecting codons under positive selection. The goodness of fit of the models was tested in pairs (M1a vs. M2a & M7 vs. M8) and evaluated using the Akaike information criterion (AIC; Posada & Buckley, 2004). Positively selected sites (PSS) were identified through the Bayes empirical Bayes (BEB) procedure implemented in PAML (Yang, 2007).

The number of alleles detected (46) was too large to allow us to use them all as predictors in statistical analyses, so we decided to concentrate on supertypes, which combine alleles into functionally similar clusters based on their physico-chemical properties (Sandberg *et al.*, 1998; Doytchinova & Flower, 2005; Schwensow *et al.*, 2007). In defining supertypes, the first step is to identify functionally important amino acids, for which we used two approaches. The first approach used ABSs defined on the basis of the crystallographic model of human DRB (Reche & Reinherz, 2003). In the second approach, we performed clustering based on PSS identified by the BEB procedure implemented in PAML. Then, each ABS (or PSS) amino acid was characterized by five physico-chemical descriptor variables (Sandberg *et al.*, 1998) and a discriminant analysis of principal components (DAPC) was performed to obtain clusters which we considered to be MHC supertypes. Analyses were implemented in R (R Core Team, 2014) using the 'adegenet' package (Jombart, 2008; Jombart *et al.*, 2010; Sepil *et al.*, 2012).

Statistics

Ornament and body size

To describe the elaboration of red deer antlers, we performed a principal component analysis (PCA) based on the inside spread between beams and seven double (for left and right beam) measurements: main beam length, brow-tine length, trez-tine length, burr circumference, lower circumference, higher circumference and number of tines. Prior to the analysis, all measurements were standardized using Z-score scaling. The first principal component (hereafter called 'antler size') explained nearly 70% of the variance, so it was used as a measure of male ornamentation in all subsequent analyses.

Antler size is highly dependent on body mass and age (Clutton-Brock, 1982), so it was necessary to include both predictors in our models as covariates. However, as body mass is highly correlated with age ($r = 0.8$, $P < 0.001$, after log transformation), we used PCA to derive a single measure of these two variables in order to avoid collinearity. Both variables were log-transformed and standardized before analysis. In the models described below, we included only the first

principal component (hereafter called 'body size'), which explained almost 90% of variation.

Parasites vs. MHC

To test the association between MHC supertypes and parasites, we used two series of nine generalized linear models. The models within a series differed in the response variable, that is the measure of infection, which was identical to those used in the GLMs for antler size (*Spiculopteragia boehmi*, *Dictyocaulus* sp., Ec/Vs group, number of nematode eggs from Trichostrongylidae family, *Ostertagia leptospicularis, Ostertagia kolchida, Ashworthius sidemi, Trichocephalus* sp., *Eimeria* sp. and total number of nematode species). In the first model series, the independent variables included body size, season, region, seven MHC clusters (clusters C7 and C8 were excluded from analyses because they were absent or present in a very small number of individuals) and the interactions between region and clusters; and second model series contained: body size, season, region, number of MHC clusters (nC) and its quadratic term $(nC)^2$ (testing for optimal number of MHC supertypes), and the interaction between region and nC. Different error distributions were assumed for different infection measures: a normal distribution for infection intensity data (i.e. the number of parasites per individual), a binomial distribution for parasite prevalence and a quasi-poisson distribution for total number of species. To meet the assumptions of normality, infection intensities were log-transformed. However, despite the data transformation and fitting different distributions of response variables, assumptions could not be met in three models: the model based on the total number of nematode species in the first model series and two models based on the infection intensity of *S. boehmi* and Trichostrongylidae egg number in the second series. These models were therefore excluded from further analyses. We simplified models based on AIC (or qAIC) values.

Antlers and parasites

To test the prediction that parasites negatively influence antler size, we used a series of general linear models (GLMs) with antler size as a dependent variable. The series consisted of 10 models which differed in the measure of infection used as the predictor: infection intensity (four models, based on the number of individuals of: *S. boehmi* adults, *Dictyocaulus* sp. larvae, Ec/Vs group (*Elaphostrongylus cervi* and *Varestrongylus sagittatus* larvae); number of nematode eggs from Trichostrongylidae family), parasite prevalence (five models: *O. leptospicularis, O. kolchida, A. sidemi, Trichocephalus* sp. and *Eimeria* sp.), and total number of nematode species present. Data regarding *Nematodirus* sp., *Spiculopteragia mathevossiani* and *Ostertagia lyrata* were not included in the statistical analyses due to their low prevalence (<20%) in the sample. In addition to a measure of infection, each model in the series contained three additional independent variables: body size, region and season, and interactions between all the variables. To meet the assumptions (checked using diagnostic plots) of normality and/or homoscedasticity, infection intensities were log-transformed. Model simplification was based on AIC values. *P*-values were adjusted for multiple testing using the Benjamini–Hochberg correction method (Benjamini & Hochberg, 1995).

MHC vs. antler size

According to Hamilton & Zuk (1982) hypothesis, because of their association with parasite load, MHC variants should predict antler size. We first tested associations of MHC with antler size using general linear models, and then using path analysis, we analysed whether any such association is due to parasites investigated.

To test the association between MHC genotypes and antler size, we used models analogous to those used for testing associations between infection measures and MHC. So in the two models analysed, antler size was the dependent variable, and the predictors were as follows: body size, season, region, seven MHC clusters and interactions between region and clusters; and body size, season, region, nC, $(nC)^2$ and the interaction between region and nC. Again, all models were simplified based on AIC values.

As we tested a number of MHC supertypes, the probability of detecting a significant association with any one of them was increased. Therefore, we used the Benjamini–Hochberg correction method (Benjamini & Hochberg, 1995) to adjust *P*-values for tests of associations between the MHC supertypes and a given infection measure or antler size.

Additionally, if results suggested existence of competition between parasites, additional analyses were performed to test for that. Specifically, if parasite 'A' was suspected to have negative impact on parasite 'B', it was added as an independent variable to the model having parasite 'B' as a dependent variable to a full model including MHC.

Path analysis

The most comprehensive method to analyse the effect of MHC through parasites on antler development is path analysis, because it allows inferring significance of the effect of MHC on infection, and infection on antler size, in one model. Such model should also take into account other important explanatory variables and relationships between them. However, due to a large number of variables (eight MHC clusters, 10 infection measures), including all of them in one path analysis model would not be inappropriate, as we would not have enough individuals for such large number of estimated parameters (Wolf *et al.*, 2013). Instead, we used path analysis to elucidate complex interrelations between those variables which GLMs revealed to be significantly associated.

All data analyses were conducted in R (R Core Team, 2014).

Results

Ornament measurements

Antler measurements (Fig. 1) were collected from 155 bulls (92 and 63 from Bieszczady and Piła regions, respectively), but complete data regarding antler and parasite burden were obtained for 77 of these. The PCA performed on the data from these 77 individuals revealed that the first principal component (antler size) explained nearly 70% of variation and mainly described the total size of antlers, whereas PC2 explained almost 9% of total variation and mainly described antler shape, arranging antlers from short (main beans) and thin, but branchy, to long and thick, but less branchy. All other principal components were difficult to interpret. Because antler size explained most of the variation in antler development, we used this component in subsequent analyses. Furthermore, antler mass was highly correlated with antler size ($r = 0.9$, $P > 0.001$), so we decided to use only the latter to test the Hamilton-Zuk hypothesis.

Parasites

We found 12 different parasite groups and species (Table 1). Using the flotation method, we detected eggs of three nematode groups (*Trichocephalus* sp., *Nematodirus* sp. and Trichostrongylidae family) and *Eimeria* sp. oocysts. The Baermann method revealed larvae of *Dictyocaulus* sp., *E. cervi* and *V. sagittatus*, but the latter two species were pooled (Ec/Vs henceforth) because of difficulties in distinguishing them. Finally, a direct survey of abomasum contents revealed six nematode species of the *Trichostrongylidae* family: *Spiculopteragia boehmi, S. mathevossiani, O. leptospicularis, O. kolchida, O. lyrata* and *A. sidemi.*

No stag was totally parasite-free. The most common infections were caused by *S. boehmi*, *Dictyocaulus* sp. and the Ec/Vs group (found in 96.6%, 90.6% and 87.5% of samples, respectively, Table 1).

Table 1 Parasite loads in sexually mature red deer (*Cervus elaphus*) stags expressed as (A) number of nematodes found in 5% of abomasum content or (B) number of eggs (or larvae) per gram of dry faeces.

	Parasite	*n*	Median (range)	Prevalence, %
A)	*Spiculopteragia boehmi*	110	19 (0–135)	97
	Spiculopteragia mathevossiani	110	0 (0–3)	19
	Ostertagia leptospicularis	110	3 (0–24)	76
	Ostertagia kolchida	110	1 (0–12)	56
	Ostertagia lyrata	110	0 (0–1)	1
	*Ashworthius sidemi**	110	0 (0–261)	47
	Total abomasal load*	110	77 (0–448)	99
B)	Trichostrongylidae	126	46 (0–13795)	99†
	Trichocephalus sp.	126	0 (0–456)	29†
	Eimeria sp.	126	0 (0–348)	37†
	Nematodirus sp.	126	0 (0–5)	16†
	Elaphostrongylus cervi/ Varestrongylus sagittatus	93	55 (0–4555)	88‡
	Dictyocaulus sp.	93	27 (0–1687)	91‡

*Contain female nematode loads.
Prevalence determined in a sample of 154 (†) and 96 (‡) red deer stags.

MHC diversity and historical selection

Genotyping of 10 samples which were amplified and sequenced twice showed 100% consistency between the replicates. In the whole data set sequenced with Illumina, the mean coverage was 557.0 reads per amplicon (SD = 221.4, range 59–1792) after artefacts had been removed. Coverage above 75 reads was achieved for 177 individuals. Both of the methods that we used to distinguish alleles from artefacts (artefact filtering, DOC) identified the same 46 putative alleles (Appendix S2) and the same individual genotypes. The maximum number of alleles detected in one individual was seven, which suggest that the deer in this sample had at least four loci of MHC DRB.

We conducted simulations based on the obtained data which took into account the variation in amplification efficiency among alleles (Sommer *et al.*, 2013); from these, we identified only one individual in which coverage may have been inadequate to identify all alleles (Appendix S3). However, the difference between the number of reads observed and the number of reads needed according to the simulation was small (59 observed vs. 63 needed), so we decided to not remove this individual from the analysis.

The mean number of potentially functional alleles per individual was 4.06 (SD = 1.31, range 1–7). From 46 identified alleles, five were restricted to the Bieszczady population and 6 to the Piła population. The sequences were highly divergent, with variability at 76 of 199 (38.2%) nucleotides and 38 of 66 (57.6%) amino acid positions. No insertions/deletions or stop codons were detected.

Our results suggest that red deer MHC DRB genes have been evolving under positive selection, as indicated by the higher rate of nonsynonymous substitution (dN) vs. synonymous substitution (dS) across all sites (Appendix S4). However, the dN/dS ratio was significant only at ABSs ($Z = 2.076$, $P = 0.040$). Similar results were obtained from a comparison of the models of codon evolution. Both of the models of codon evolution that allowed for positive selection (M2a and M8) fit the data better than models without positive selection (Appendix S5). Additionally, the

BEB procedure (implemented in PAML) identified four codons as evolving under positive selection and all of these overlapped with putative ABSs (Appendix S6).

The clustering approaches based on ABS and PSS data yielded comparable results, indicating the optimal cluster number as 9 and 8, respectively. We chose to use the results from the ABS approach in subsequent analyses because both the BIC plot of this approach and the assignment of alleles to supertypes were much more consistent among runs; this was probably due to the greater number of sites contained in the ABS approach, which led to higher resolution in the results. The alleles contained in each of the nine supertypes are shown in Appendix S7. The average number of supertypes per stag was 3.25 (range 1–6). Most supertypes occurred at similar frequencies in both regions, except for C7, which was restricted to the Bieszczady population, and C8, restricted to the Piła population.

Parasites vs. MHC

After Benjamini–Hochberg correction, we found associations between five measures of infection (intensity or prevalence) and at least one of the MHC supertypes. All statistically significant results are shown in Table 2 (full results from final models are presented in Appendix S8).

In the second series of eight models, analysing the effect of the number of MHC supertypes (nC and $(nC)^2$) on infection, we found significant association only for *Eimeria* sp. Its prevalence was positively associated with $(nC)^2$ ($n = 145$, chi-square = 5.16, $P = 0.0231$), so individuals that possessed the average number of MHC supertypes were least likely to be infected.

Competition between parasites

Increased resistance to *A. sidemi,* coupled with increased susceptibility to *O. kolchida* and *S. boehmi*, could result

Table 2 Results of generalized linear models testing for the effects of MHC supertypes on nine infection measures and antler size in red deer males. Each model consisted of 11 independent variables: body size, region, season, presence of eight MHC supertypes (C1-C6, C8, & C9), as well as interactions between region and supertypes. Only statistically significant variables are presented. Complete results from final models are presented in Appendix S8.

Dependent variable	*n*	Final model	Coefficients	F/chi-square	*P*
Dictyocaulus sp.	86	Region	2.12	14.88	0.0002
		C2	−1.63	7.54	0.0075**
		C6	1.49	5.71	0.0193
Spiculopteragia boehmi	97	C2	−0.76	7.18	0.0088*
		C3	0.41	4.56	0.0355
		C6	0.96	9.09	0.0033*
		C9	1.03	11.94	0.0008*
		Region:C9	−0.96	6.61	0.0118
Trichostrongylidae	117	C4	0.82	4.48†	0.0366
		Region:C1	−1.84	5.58†	0.0200
		Region:C9	−1.36	4.00†	0.0481
Ashworthius sidemi	97	Region	−3.82	28.08†	< 0.0001
		C4	1.82	6.05†	0.0139*
		C9	−1.78	8.11†	0.0044*
Ostertagia leptospicularis	97	C5	2.46	7.31†	0.0069*
Ostertagia kolchida	97	C4	−2.78	7.47†	0.0063*
		C5	1.95	3.99†	0.0457
		C9	1.55	6.53†	0.0106*
		Region:C4	2.76	5.37†	0.0205
		Region:C5	−2.79	5.39†	0.0202
Trichocephalus sp.	145	Region	−1.46	4.16†	0.0413
		Region:C4	2.42	5.87†	0.0154
Antler size	75	Body size	−2.47	230.27	< 0.0001
		C2	−1.65	8.50	0.0048*
		Region:C4	1.96	5.02	0.0285

(†) results from Wald chi-square test; (−) negative coefficient for region indicates fewer parasites in Piła region; (*) significant or (**) marginally not significant ($P = 0.052$) after Benjamini–Hochberg correction.

from competition between parasites, such that resistance to *A. sidemi* opened niche to the other two parasites. To test for this, we included *A. sidemi* prevalence as a predictor in models with *O. kolchida* and *S. boehmi* as dependent variables. In the case of *O. kolchida*, *A. sidemi* was deleted from the model during simplification procedure, whereas positive association was found between *A. sidemi* and *S. boehmi* ($n = 97$, chi-square = 7.03, $P = 0.0080$). We could not analyse those putative competition relationships via path analysis, because despite data transformations, assumption of multivariate normality was not met.

Antlers and parasites

The intensity of infection of two lung nematodes (*Dictyocaulus* sp. and Ec/Vs group) was positively associated with antler size (Table 3), but only the association between *Dictyocaulus* sp. and antler size ($n = 77$, $F_{1,71} = 9.96$, $P = 0.0023$) was still significant after Benjamini–Hochberg correction (Table 3, Fig. 2a). In all other models (those which included infection intensity of *S. boehmi*; Trichostrongylidae nematodes egg count; total number of species; or prevalence of *A. sidemi*, *O. leptospicularis*, *O. kolchida*, *Trichocephalus* sp., or *Eimeria* sp.), infection did not predict antler size. In all final models, body size was highly associated with antler size (Table 3): bigger deer had bigger antlers. Because we found that lung nematodes had a significant impact on antler development (Table 3), we wanted to determine whether the overall parasite burden of this fundamental organ, rather than a specific parasite species, affected antler size. To do this, we built an additional model which contained total lung nematode burden (sum of concentrations of both lung parasites) as a predictor. The simplification of this model revealed strong positive associations between antler size and both body size ($n = 77$, $F_{1,71} = 229.00$, $P < 0.0001$)

Table 3 Results of general linear models testing for the effects of 10 infection measures on antler size in red deer males ($N = 77$). Each model contained four independent variables: body size, region (Piła or Bieszczady), season (2011 or 2012), infection measure, as well as interactions between the variables. Results from final reduced models are presented.

Health predictor	Final model	Coefficient	*F*	*P*
Spiculopteragia boehmi	Body size	3.13	21.67	< 0.0001*
	Region	1.91	1.50	0.2242
	Season	−0.53	0.79	0.3779
	Parasite intensity	0.38	1.63	0.2063
	Body size:region	0.68	2.52	0.1167
	Body size:parasite intensity	−0.44	5.46	0.0225
	Region:season	1.54	4.22	0.0437
	Region:parasite intensity	−0.68	1.95	0.1669
Dictyocaulus sp.	Body size	2.36	230.14	< 0.0001*
	Region	0.13	0.04	0.8455
	Season	−0.22	0.15	0.6989
	Parasite intensity	0.30	9.96	0.0023*
	Region:season	1.05	2.11	0.1505
Elaphostrongylus cervi/	Body size	1.82	23.68	< 0.0001*
Varestrongylus sagittatus	Region	−0.46	0.38	0.5414
	Season	−0.40	0.49	0.4872
	Parasite intensity	0.19	6.58	0.0124
	Body size:region	0.68	2.72	0.1035
	Region:season	1.55	4.6	0.0355
Ashworthius sidemi	Body size	2.44	225.29	< 0.0001*
	Region	0.44	0.38	0.5370
	Season	−0.32	0.30	0.5886
	Region:season	1.36	3.25	0.0757
Trichocephalus sp.	Body size	1.90	24.0796	< 0.0001*
Eimeria sp.	Region	0.04	0.00	0.9577
Ostertagia kolchida	Season	−0.46	0.59	0.4455
Ostertagia leptospicularis	Body size:region	0.66	2.38	0.1271
Trichostrongylidae	Region:season	1.47	3.81	0.0550
Number of species				

*Significant after Benjamini–Hochberg correction; (−) negative coefficient for region or season indicates fewer parasites in the Piła region or in 2012 (second season), respectively.

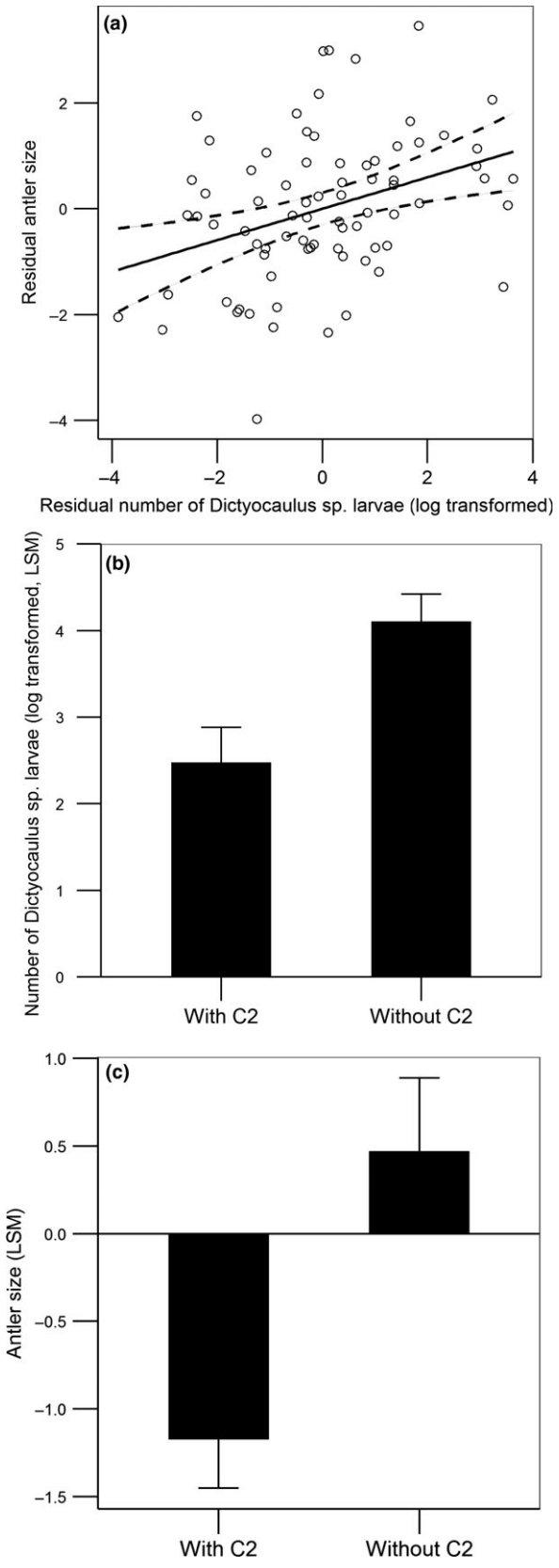

Fig. 2 Associations between *Dictyocaulus* sp. larval load, MHC supertype C2 occurrence and antler size. (a) Partial regression of antler size and number of *Dictyocaulus sp.* larvae (black dashed lines indicate the 95% confidence intervals). (b) Number of *Dictyocaulus* sp. larvae or (c) antler size as a function of the presence/absence of the MHC C2 supertype (LSM – least square means, standard error bars are depicted).

and total lung nematode burden ($n = 77$, $F_{1,71} = 10.90$, $P = 0.0015$).

MHC vs. antler size

Antler size was strongly negatively associated with the presence of the C2 supertype: $n = 75$, $F_{1,66} = 8.50$, $P = 0.0048$ (after B-H correction, $P = 0.0339$) (Table 2, Fig. 2b). However, deer that possessed this supertype had, on average, 2.15-times fewer *S. boehmi* nematodes, and 5.10-times fewer *Dictyocaulus* sp. larvae (Table 2, Fig. 2c). Path analysis revealed that this relationship was driven mostly by significant C2 -> *Dictyocaulus* and *Dictyocaulus* -> antler associations. The direct C2 -> antler path, which would indicate effect of C2 independently of its effect via *Dictyocaulus* sp. infection, was not significant (Fig. 3, Table 4). Additionally, path analysis suggested the existence of indirect relationship between C6 supertype and antler size (through *Dictyocaulus* sp.) (Fig. 3, Table 4), which was not detected by GLMs. The same analysis indicated that via the effect on *S. boehmi*, C2 and C6 were affecting body size, which in turn affected antler size. No significant association was found between antler size and the number of supertypes (nC or $(nC)^2$) carried by deer.

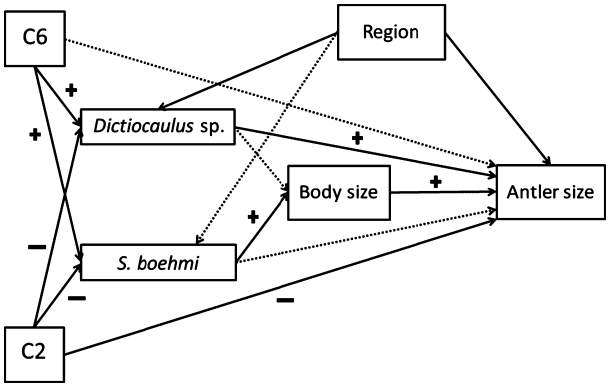

Fig. 3 Path analysis diagram showing determinants of antler size, including all variables which could be potentially important, as determined by significant associations between them revealed by GLMs (see methods). Solid bars represent the paths retained in the final path model (based on BIC, see methods); + and − symbols describe positive and negative relationships, respectively.

Table 4 Final path analysis model testing relationships between: MHC superypes C2 and C6, *Dictyocaulus* sp. intensity, *Spiculopteragia boehmi* intensity, antler size, body size, region and season. Insignificant paths were removed from the model based on Bayesian information criterion (BIC).

Path	Estimate	SE	*Z*	*P*
Antler size←region	0.98	0.35	2.82	0.0048
Dictiocaulus sp.←region	1.19	0.41	2.94	0.0033
Dictiocaulus sp.←C2	−1.51	0.38	−3.99	0.0001
Antler size←C2	−0.59	0.34	−1.75	0.0808
Dictiocaulus sp.←C6	1.33	0.39	3.41	0.0007
S. boehmi←C2	−0.55	0.21	−2.63	0.0085
S. boehmi←C6	0.69	0.22	3.2	0.0014
Antler size←*Dictiocaulus* sp.	0.29	0.09	3.28	0.0010
Body size←*S. boehmi*	0.36	0.15	2.44	0.0147
Antler size←body size	2.39	0.12	20.15	< 0.0001

Discussion

In this study, we tested the Hamilton-Zuk hypothesis, according to which we predicted a negative relationship between parasite load and the size of sexual ornaments, as well as associations between MHC gene variants and antler development. Our results provided no support for this hypothesis, but revealed interesting and complex relationships between MHC genes, the measure of parasite infection and sexual traits.

MHC supertypes and parasites

We found that several MHC supertypes were associated with either decreased (supertypes C2, C4, C9) or increased (supertypes C4, C5, C6, C9) infection intensity and parasite prevalence (Table 4 & Appendix S8). However, it should be mentioned that in case of prevalence, positive association between parasite (*A. sidemi*, *O. kolchida* and *O. leptospicularis*) and supertype (C4, C9 and C5, respectively) does not necessary imply that this particular supertype increases the infection susceptibility. It is possible that in some cases we are dealing with quantitative disease resistance (QDR), such that the apparently susceptible supertype is in fact responsible for suppression of the infection to the chronic level (Westerdahl *et al.* 2012). This predicts that such apparently susceptible alleles should have lower intensity of infection compared with other infected individuals (Westerdahl *et al.* 2012). Unfortunately, we were not able to test this because despite the data transformation, the assumptions of normal distribution and homoscedasticity of errors could not be met.

Overall, our results add to a growing body of evidence that documents both negative and positive associations between the presence of particular MHC molecules and infection; previous work in this field includes studies on ruminants (Paterson *et al.*, 1998; Ditchkoff *et al.*, 2005; Untalan *et al.*, 2007; Fernandez-de-Mera *et al.*, 2009), rodents (Froeschke & Sommer, 2005; Meyer-Lucht & Sommer, 2005; Oliver *et al.*, 2009; Kloch *et al.*, 2010), birds (Bonneaud *et al.*, 2006; Schou *et al.*, 2007; Dunn *et al.*, 2012; Sepil *et al.*, 2013) and fish (Eizaguirre *et al.*, 2009; Evans & Neff, 2009).

It would be particularly likely to find both positive and negative relationships between infection and MHC variants if a given MHC variant had antagonistic effects on infection with different parasites (De Roode *et al.*, 2005; Loiseau *et al.*, 2008; Froeschke & Sommer, 2012). Our results suggest the existence of at least two such relationships. First, we observed that supertype C9 was associated with resistance to *A. sidemi*, but at the same time was positively associated with *O. kolchida* prevalence. Similarly, in deer with the C9 supertype, we observed increased resistance to *A. sidemi*, but at the same time, increased susceptibility to *S. boehmi*. This trade-off can be due to specificity of antigen binding by MHC supertypes, or due to competition between parasites co-existing in the same environment, namely deer abomasa. Under the latter scenario, by conferring a quantitative degree of resistance to *A. sidemi*, C9 would reduce the nematode's within-host growth rate and consequently reduce its competition with *O. kolchida* or *S. bohemi*. However, additional analyses did not support the competition hypothesis. *A. sidemi* was not a significant predictor of *O. kolchida* prevalence and was positively associated with *S. boehmi* infection level.

Whereas we found a number of significant associations between individual MHC supertypes and parasite load, we found only one (nonlinear) relationship between infection and the MHC supertype diversity. Individuals that possessed the average number of MHC supertypes were less likely to be infected by *Eimeria* sp., compared with individuals carrying a small or a large number of MHC supertypes. This result is in line with the optimality hypothesis (Nowak *et al.*, 1992) according to which advantage of carrying an optimal number of MHC variants is a result of a trade-off between the ability to identify a large number of antigens and impairment of immune response due to the loss of self-reacting lymphocytes during the negative thymic selection. Nonlinear relationships between the number of MHC alleles or supertypes and parasite load were earlier reported in sticklebacks (Wegner *et al.*, 2003), pythons (Madsen & Ujvari, 2006) and bank voles (Kloch *et al.*, 2010). However, other studies reported positive linear associations (Radwan *et al.*, 2012) or no significant effect (e.g. Froeschke & Sommer, 2012; Dunn *et al.*, 2012).

Antlers and parasites

Of the 10 infection measures that we used in our models, only two: *Dictyocaulus* sp. and the Ec/Vs group (one,

after B-H correction) were significantly associated with antler size (Table 3). However, contrary to our predictions, both of those relationships were positive.

Parasites of gastrointestinal tract (nematodes and *Eimeria* sp.) had no significant effect on antler size which likely reflects their limited impact on host health. Gastrointestinal nematodes (all nematodes detected in abomasum, plus *Trichocephalus* sp. and *Nematodirus* sp.) cause damage and inflammation of the abomasum and intestinal lining, and infected individuals can lose weight and have signs of anaemia (in case of blood feeding species: *A. sidemi* and *Ostertargia* sp.), but the infection can cause death of young individuals only at high parasite load (Ramisz *et al.*, 2001). Similarly, *Eimeria* sp., gastrointestinal protozoan, can cause chronic disease called coccidiosis, but rarely leads to animal death (Ramisz *et al.*, 2001).

On the other hand, lung nematodes from genus *Dictyocaulus* and *Varestrongylus* inhabit the bronchi and trachea and cause respiratory disease. Parasites mechanically damage the mucosa, leading to the inflammation that spreads to other tissues, which causes coughing and respiratory problems. Death occurs at high parasite load due to suffocation as a result of impairment of gas exchange or bronchial clogging (Demiaszkiewicz & Przybysz, 2002). In case of *E. cervi*, only its larvae are present in lungs, whereas adults inhabit muscles and nervous system. Nematodes which settled in muscle have small effect on animal health, but those located in nervous system often cause death.

Therefore, given that infections with lung nematodes are much more severe than infections with abomasum nematodes and that *Dictyocaulus* sp. is considered to be the biggest threat to the health and survival of farmed red deer (Mason, 1994), our finding that deer with bigger antlers carried more lung nematodes is intriguing. According to the Hamilton-Zuk hypothesis, such infection, particularly by such debilitating parasites, should be likely to have a negative impact on sexually selected traits. However, a similar relationship between ornament size and disease intensity was reported in a study by Ezenwa *et al.* (2012), who found a positive relationship between horn size and the abundance of a nematode larva morphotype (Morph B) in Grant's gazelle. However, the same study reported a negative relationship between horn size and the presence of a gut nematode (*Trichuris*). Thus, the direction of the relationship may vary depending on the identity of the parasite.

There are several explanations for the positive pattern we observed here. It could be that there is some tolerable level of parasite load that is dependent on male condition. Perhaps bulls in the best condition are able to cope with bigger parasite loads and allocate all saved resources in antler growth. In this case, antler size could be a good predictor of condition. However, if we use body mass as a proxy for condition, this hypothesis is not supported as we did not find any significant relationship between body mass and parasite infection.

It is also possible that the most heavily infected males had decreased chances to survive to the next season and thus terminally invested in sexual ornaments. Such cases of terminal investment (Clutton-Brock, 1984) represent 'cheating' and in this case antler size would not be an honest signal of health and condition. Under this scenario, not only would the Hamilton-Zuk hypothesis be refuted, but also the entire good genes hypothesis.

Our data may reflect a trade-off between testosterone and immune function (Folstad & Karter, 1992; Sheldon & Verhulst, 1996; Zuk & McKean, 1996). A high level of testosterone is necessary to develop male sexual ornaments, but this hormone is also a potent inhibitor of the immune system. If variation in male quality is relatively smaller than variation in testosterone-dependent investment in antlers, such a trade-off would result in a negative relationship between antler elaboration and immune function. It may be simply not possible to develop big antlers and at the same time be free of parasites (Malo *et al.*, 2009).

Finally, it should be stressed that the parasite load is a combination of exposure and susceptibility. If the deer with bigger antlers are more exposed to parasites (e.g. because exposure is increased in harems), they may carry higher parasite loads even if they are more resistant. Irrespective of the mechanism involved, our research shows that antler size in red deer is not an honest signal of health status and as such is not consistent with the Hamilton-Zuk hypothesis.

One caveat with our data set is that bulls were shot according to the so-called selective shooting policy of Polish hunting law. This means that in each age class (except the highest), hunters were targeting deer with the worst antlers (e.g. deformed, with an odd number of tins, without crowns) or in visibly poor condition. However, such assessments are often difficult to make by eye in the field, and in common opinion of hunters, deer are shot to a large extent randomly. In fact, selective shooting might have had a positive effect on variation of our data set, because it contained deer with poorly developed antlers which, being poor trophies, could otherwise be avoided. Therefore, we believe that our sample contained enough variation in antler size and parasite load to allow meaningful analyses. This is supported by the significant results we report.

MHC supertypes, parasites and antler size

Our main prediction, derived from Hamilton & Zuk (1982) hypothesis, was that resistance to parasites (i) would be associated with the presence of particular MHC supertypes and (ii) would predict antler development. We found a strong negative association between antler size and the presence of the C2 supertype, and

path analysis revealed that this association is mediated by a significant effect of C2 supertype on the intensity of infection by *Dictyocaulus* sp. However, the presence of this parasite was actually associated with larger, not smaller, antlers, an observation contrary to the predictions of the Hamilton-Zuk hypothesis. We presented above some possible explanations for the higher load of lung nematodes in stags with more developed antlers, but another possibility is that supertype C2, while increasing resistance to *Dictyocaulus* sp., is associated with susceptibility to another pathogen which was not investigated here. As discussed above, such a pleiotropic effect may not be uncommon (De Roode *et al.*, 2005; Loiseau *et al.*, 2008; Froeschke & Sommer, 2012). Under this scenario, deer that carried the C2 supertype would be strongly infected with this unknown parasite, which would result in the development of smaller antlers. However, given the serious fitness consequences of lung nematodes (Mason, 1994), we find this explanation unlikely.

Finally, path analysis revealed that infection with *S. boehmi*, being under significant influence of C2 and C6 supertypes (negative and positive, respectively), has a significant positive effect on body size, which in turn is a significant determinant of antler size. As indicator trait, antler size could be expected to be impacted by parasites independently of body size, which was not the case. However, if antler size is easier for females to assess than male body size, its size might reveal an information on MHC-based susceptibility to *S. boehmi* more efficiently. Nevertheless, such information would contradict predictions of Hamilton-Zuk hypothesis, similar to that about susceptibility to a more debilitating *Dictyocaulus* sp revealed by larger antlers (as discussed above). Thus, we conclude that overall our results do not provide support for Hamilton-Zuk hypothesis.

To our knowledge, only two previous studies (Eizaguirre *et al.*, 2009; Dunn *et al.*, 2012) have tested the Hamilton-Zuk hypothesis in wild populations by evaluating the relationships among immune genes, parasite loads and sexual ornaments. Eizaguirre *et al.* (2009) reported significant negative associations between MHC diversity and total parasite load, as well as between MHC diversity and male redness (sexual ornament), in three-spined sticklebacks. However, the authors argued that the negative association between MHC diversity and redness was caused by measuring breeding coloration at the end of the reproduction season, when redness is no longer a good predictor of male quality (Kalbe *et al.*, 2009). In this respect, red deer antlers are a much better sexual ornament for study, because after the velvet is rubbed off, antlers do not change in size.

Additionally, the same study also reported a significant association between a specific MHC haplotype (F10) and the monogenean parasite *Gyrodactylus* sp., but it could not be linked with the male sticklebacks' ornament (red belly). Similarly, Dunn *et al.* (2012) found an association between a specific MHC variant (allele 82) and *Plasmodium* infection in common yellowthroats, but they found no relationship with mask size (male ornament). However, that study did find an association between mask size and the total number of MHC II alleles. Taking into account this previous work as well as the new results we report here, it appears that overall support from studies involving MHC genes for the Hamilton-Zuk hypothesis is at best mixed.

Conclusions

Although MHC supertypes predicted infection with several parasites, including debilitating lung nematodes, we found no clear support for the Hamilton-Zuk hypothesis. The positive association revealed here between lung nematode load and antler development is clearly at odds with our predictions. This result suggests that trade-offs between investment in immune response and sexual traits may make the latter a poor indicator of the presence of genes that confer resistance to parasites. Our data also indicate trade-offs that involve associations between the MHC and parasite resistance. Supertypes that enable resistance to one species of parasites may at the same time cause susceptibility to others. Such trade-offs may undermine the potential benefits of inheriting particular MHC types.

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Author contributions

JR, HO and MB designed research; MB collected samples; MB and AWD performed parasitological analyses; MB and JR performed molecular analyses; MB analysed data; and MB and JR wrote the paper.

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Supporting information

Additional Supporting Information may be found online in the supporting information tab for this article:
Appendix S1 Map showing the location of studied populations.
Appendix S2 Sequences of 46 alleles of MHC DRB exon 2.
Appendix S3 Numbers of reads per amplicon.
Appendix S4 Synonymous and nonsynonymous substitution rates in red deer MHC DRB exon 2.
Appendix S5 Evaluation of the goodness of fit for different models of codon evolution.
Appendix S6 Putative ABS positions of MHC DRB exon 2.
Appendix S7 MHC alleles assignment to functional supertypes.
Appendix S8 Full results of GLMs testing for the effects of MHC supertypes on nine infection measures and antler size.

Data deposited at Dryad: doi: 10.5061/dryad.nk25m

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