UNIWERSYTET JAGIELLOŃSKI
W KRAKOWIE

Wydział Biologii i Nauk o Ziemi

Instytut Nauk o Środowisku

Antler quality in red deer: a test of Hamilton and Zuk hypothesis

Mateusz Buczek

Rozprawa doktorska
wykonana pod opieką
prof. dra hab. Jacka Radwana
w Zespole Ekologii Molekularnej i Behawioralnej

Kraków 2015

The scholarship of the author was provided by the project “Launching interdisciplinary doctoral studies programme in ecology in English and increasing the didactic potential of the staff of the Institute of Environmental Sciences at the Jagiellonian University”, which was co-financed by the European Union under the European Social Fund.

This work was supported by founds, granted to Author, from Polish National Science Center (2011/01/N/NZ8/00187) and Jagiellonian University (DS/MND/WBINOZ/INOS/7/2012 and DS/MND/WBINOZ/INOS/7/2013).

Print: FHU SEZAM, Cracow
http://www.krakowksero.pl/

Author's address

Mateusz Buczek
Institute of Environmental Sciences
Jagiellonian University in Cracow

Supervisor

Prof. dr hab. Jacek Radwan
Institute of Environmental Sciences
Jagiellonian University in Cracow

Reviewers

Prof. dr hab. Jan Wójcik
Mammal Research Institute
Polish Academy of Sciences

Dr hab. Anetta Borkowska
Institute of Biology
University of Białystok

Table of contents

Summary

The evolution and maintenance of female preferences for exaggerated sexual ornaments in males of many species is still one of the unresolved problems in evolutionary biology. According to 'good genes' hypothesis, males in better condition can afford to develop bigger ornaments. If condition is heritable, females will increase fitness of their offspring by mating with more ornamented males. However, this mechanism should lead to depletion of genetic variance for condition.

In 1982 William D. Hamilton and Marlene Zuk proposed a solution to this paradox (termed lek paradox). They suggested that host-parasite co-evolution is a likely source of never-exhausted genetic variance for condition (and fitness). Males that carry genes associated with better resistance to parasites would produce more elaborate sexual ornaments and therefore females choosing males with bigger ornaments would produce more parasite-resistant offspring.

Despite over 30 years of research, the status of the hypothesis is still controversial. Here I present two tests of Hamilton and Zuk hypothesis using red deer (*Cervus elaphus*) as a model species. Hypothesis was tested using wild population and correlational approach, and captive populations of deer which allowed the use of experimental approach.

In the first study on wild red deer, I studied relationships between major histocompatibility complex (MHC) genes, lung and gastro-intestinal parasites and the antler size. I found significant associations between MHC functional variants and several parasite species. Different MHC variants gave either resistance or increased susceptibility to specific parasite species. Interestingly, MHC also showed antagonistically pleiotropic effects on parasite load, whereby one MHC variant gave resistance to one parasite species, but at the same time increased susceptibility to another parasite species. Furthermore, MHC-parasite association differed between populations.

In contrast to complex MHC-parasite associations, only one of 10 studied parasite classes (*Dictiocaulus* sp. larvae number) was significantly associated with the antler size. This relationship seems to have been mediated by MHC C2 supertype, conferring resistance to this parasite species. However, contrary to the predictions of Hamilton and Zuk hypothesis, the relationship was positive.

Working on wild populations gives the advantage of studying animals in their natural conditions, however, uncontrolled factors may mask the associations between parasite load and antler size thus hindering the interpretation of the results. That is why I also used experimental approach to test Hamilton and Zuk hypothesis.

Stags from a captive population, maintained in the experimental facility of the Institute of Animal Science in Prague, were randomly divided into two groups. Stags from the experimental group were administered with anthelmintic drug and males from the control group with control saline. Procedure was repeated each month from March till September, i.e. during the antler development period. However, even though the applied anthelmintic treatment significantly decreased infection level of gastro-intestine nematodes, it did not have a significant effect on body weight or antler size.

To conclude, both studies do not support predictions of the Hamilton and Zuk hypothesis. Host-parasite co-evolution does not appear to be the major force for evolution of sexual ornaments (i.e. antler size) in red deer.

1. 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 favors 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 favored 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 requires a mechanism for the maintenance of substantial genetic variance for fitness, and one such mechanism was proposed by Hamilton and 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, females that choose ornamented males may produce offspring superior in parasite resistance.

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 and 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 Bernatches & 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 non-linear, 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 and 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 and Zuk hypothesis (e.g., Milinski & Bakker 1990 on sticklebacks, Costa & Macedo 2005 on blue-black grassquits, Maan et al. 2006 on cichlid fish, Martinez-Padilla et al. 2007 on red grouse), but negative (e.g., Bronseth & Folstad 1997 on sticklebacks, Votypka et al. 2003 on red-backed shrikes, Cramer & Cameron, 2007 on white-footed mice, Rantala et al. 2007 on earwigs) or mixed (e.g., Muller & Ward 1995, Ezenwa et al. 2012) results have also been reported.

However, a full test of the Hamilton and Zuk hypothesis requires the demonstration of a link between the polymorphic genes associated with parasite resistance and the elaboration of the sexual ornament. To my 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. Additionally, most of the studies testing the Hamilton and Zuk hypothesis used the correlative approach, which is often the only option available for studying animals in their natural habitat. While such studies have an obvious advantage in providing natural context for the processes studied, uncontrolled, hidden factors may produces spurious associations or mask them. Thus, results of correlational studies must be interpreted with caution.

Additionally, most of the correlational field studies testing Hamilton and Zuk hypothesis have two common concerns. Firstly, they measure infection level and ornament size at the same time, but if parasites really have negative impact on ornament size, it can be expected that it will occur only during ornament development. So, by measuring parasite level and ornament size at the same time (e.g. rut time), it is assumed that current level of infection reflects parasite level during ornament development, which does not have to be necessarily true. Secondly, the parasite load is a combination of exposure and susceptibility. When the rates of exposure are unknown, as in the case of wild populations, considering the uninfected individuals as genetically resistant may be improper (Hawley & Altizer 2011). Therefore, the experimental approach, which enables control of confounding factors and infection level manipulation, is needed to complement results from correlative studies. However, so far a small number of studies, which used experimental approach, gave inconclusive results. In some studies experimental manipulation of parasite load revealed negative influence of parasites on ornament size (Moller 1990, Hill et al. 2004, Martinez-Padilla et al. 2007, Demuth et al. 2012), but in others experimental treatment did not influence ornament size (Folstad et al. 1996, Mougeot & Redpath 2004, Zirpoli et al. 2013).

Here, I tested the predictions of the Hamilton and Zuk hypothesis in the red deer (*Cervus elaphus*), using both correlative and experimental approaches. 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 to be correlated with male reproductive success (Kruuk et al. 2002). Furthermore,

antler size positively influences the probability of holding a harem (Bartos & 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 extra-cellular parasites, has been characterized (Swarbrick et al. 1995; Swabrick & Crawford 1997). These factors make the red deer an excellent model for testing the Hamilton and 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, all facets of the Hamilton and Zuk hypothesis were investigated in wild red deer populations. The study included the links between the MHC, parasite load, and antler development. In accordance with this hypothesis, it was predicted that individuals that carried MHC variants associated with reduced parasite load would carry more elaborated antlers. It was also tested whether parasite load and antler development in male red deer was associated with the total number of MHC DRB supertypes.

Additionally, the hypothesis was tested in the captive population of red deer by manipulation of parasite load during the period of antler growth. Randomly selected group of deer was treated with anthelminthic drug throughout the year, starting in spring (before antlers development). In accordance with Hamilton and Zuk hypothesis it was predicted that experimentally dewormed deer should be in better condition and therefore develop larger antlers than deer untreated with anthelmintic drug.

2. Methods

2.1. Field study

2.1.1. Data collection

Samples from 154 red deer were collected during three hunting seasons (2010-2012). Samples were collected in two regions of Poland, the Bieszczady mountains (2010-2012) in the southeast 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 feces 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. Feces 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.

2.1.2. Body mass, age and antler measurements

Foresters are legally obligated to collect data that characterize every shot red deer; therefore information about body mass, age, antler mass and antler measurements were possible to obtain 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 two 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 (Figure 1).

2.1.3. Parasite abundance estimates

The abomasum collected from each shot stag was brought to the lab 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. Then a sample of the sediment that represented 5% of the total volume was examined under a stereomicroscope and all found nematodes were transferred 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 used abomasum content sampling method analysis was repeated four times for 14 bulls and the repeatability (Lessels & Boag 1987) was estimated. Repeatability of measure of abomasum nematode load was very high ($R_i = 0.94$, $F = 65.33$, $df = 13$, $p < 0.001$).

To assess the parasite load of the gastrointestinal tract, faeces were analyzed 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. 3 g of each fecal sample was mixed with a saturated sucrose solution, poured into 15-ml tubes and centrifuged at 2500 rpm for 5 minutes. The tubes were filed with additional flotation solution and covered with coverslips. After 15 min each coverslip was placed on a microscope slide and parasite propagules were counted.

The Baermann method was used to assess the burden of lung nematodes. 5 g of each fecal sample was enclosed in surgical gauze, placed in a funnel filled with water and incubated at room temperature. After 24h 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.

Feces were analyzed 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 feces.

2.1.4. MHC genotyping

A 199-bp fragment of the 2nd exon of the red deer MHC II DRB gene was amplified using primers (CervL1 - AGTGTCATTTCYYCAAYGGGAC, CervE2endR – ACCTCGCCGCTGCACAGTGAAACT) designed 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'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG-NNNNNN-AGTGTCATTTCyyCAAyGGGAC-3',
reverse R – 5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG-NNNNNN-ACCTCGCCGCTGCACAGTGAAACT-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), 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). 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 – AATGATACGGCGACCACCGAGATCTACAC-TCGTCGGCAGCGTC, reverse P7MHC – CAAGCAGAAGACGGCATACGAGAT-GTCTCGTGGGCTCGG) 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 x 150-bp reads.

Reads from each pair were combined into a single sequence on the basis of read overlap using FLASH-1.2.6 (Magoc & 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 tags using jMHC software (Stuglik et al. 2011), available from http://code.google.com/p/jmhc/.

Our preliminary analyses 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). Therefore coverage of 200 reads was conservatively adopted as sufficient for provisional genotyping. After provisional genotyping using this threshold, up to seven alleles per individual were found. 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. Subsequently, as proposed by Sommer et al. (2013), a simulation approach was applied, 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, small indels, and PCR recombinants (chimeras) (Babik et al. 2009; Galan et al. 2010). To remove artifactual sequences from the dataset, two independent genotyping methods (Herdegen et al. 2014; Lighten et al. 2014) were applied. 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 'gray zone' in which both true alleles and artifacts co-occur, and proper genotyping. The gray zone is the region between two thresholds: typically, below the lower threshold, all variants can be classified as artifacts, such as a chimera of another, more common, variant within the same amplicon or a read containing substitution errors, while above the higher threshold, no sequence can be explained as an artifact. A clear definition of the gray 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 gray zone, first the maximum per amplicon frequency (MPAF) of each variant was calculated. Then, starting from a MPAF of 0.5% and proceeding upward, it was checked whether a variant was a putative artifact, perhaps a chimera or the result of a substitution error. All sequences with a per-amplicon frequency below 4.52% could be explained as artifacts, while all sequences with an MPAF above 10.83% were considered true alleles. Having identified the gray zone (MPAF of 4.52-10.83%), genotyping was performed. For each individual, all variants with frequencies above the gray zone were considered true alleles, and variants within the grey zone were considered true alleles only if they could not be explained as artifacts derived from true alleles.

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

2.1.5. Analysis of selection signatures in MHC and supertype definition

Two approaches were used to test for positive selection in MHC sequences. The average rates of synonymous (dS) and nonsynonymous (dN) substitutions were computed using MEGA5 for (1) all sites (Nei–Gojobori method with the Jukes–Cantor correction; standard errors were obtained through 1000 bootstrap replicates), (2) positions that code amino acids that form the antigen-binding sites (ABSs) in HLA DRB (Reche & Reinherz 2003), and (3) 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). 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).

The number of alleles detected (46) was too large to use them all as predictors in statistical analyses, so it was 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 two approaches were used. The first approach used ABSs defined on the basis of the crystallographic model of human DRB (Reche & Reinherz 2003). In the second approach clustering was performed based on positively selected sites (PSSs) that were identified by the Bayes empirical Bayes (BEB) procedure implemented in PAML (Yang 2007). 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 were considered to be MHC supertypes. Analyses were implemented in R (R Development Core Team, 2014) using the 'adegenet' package (Jombart 2008, Jombart et al. 2010).

2.1.6. Statistics

To describe the elaboration of red deer antlers a principal component analysis (PCA) was performed 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 et al. 1982), so it was necessary to include both predictors in models as covariates. However, since body mass is highly correlated with age ($r = 0.8$, $p < 0.001$, after log transformation), PCA was used 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, only the first principal component (hereafter called "body size"), which explained almost 90% of variation, was included.

To test main hypothesis, that parasites negatively influence antler size a series of general linear models (GLMs) with antler size as a dependent variable, were used. 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: *Spiculopteragia 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: *Ostertagia leptospicularis, O. kolchida, Ashworthius sidemi, Trichocephalus* sp., and *Eimeria* sp.), and total number of nematode species present. Data regarding *Nematodirus* sp., *S. mathevossiani*, and *O. 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. 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).

To test the association between MHC supertypes and parasites two series of nine generalized linear models were used. The models within a series differed in the response variable, i.e. the measure of infection, which was identical to those used in the GLMs for antler size described above. 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 quasipoisson distribution for total number of species. To meet the assumptions of normality, infection intensities were log-transformed. However, despite the data transformation, errors were not distributed normally for the measure of infection in two models: the model based on the total number of nematode species in the first model series and the model based on the infection intensity of *S. boehmi* in the second series. These models were therefore excluded from further analyses. In the first model series, the independent variables included body size, season, region, eight MHC clusters (cluster C7 was excluded from analysis because it was present only in five individuals), and interactions between region and clusters. The second model series contained: body size, season, region, number of MHC clusters (nC), and the interaction between the last two. The models were simplified based on AIC (or qAIC) values.

To test the association between MHC genotypes and antler size the same models as for testing parasite loads were used, but in the place of infection measure, the predictors were the presence of each of eight MHC supertypes or the total number of supertypes present. Again, all models were simplified based on AIC values.

Finally, two quadratic models (with $(nC)^2$) were analyzed to check if an optimal (mean), and not maximal, number of supertypes was associated with larger antler size or lower species richness (number of parasite species).

Additionally if any interaction term in any model was significant, additional analyses to facilitate the interpretation of these interactions (e.g., analyzing data separately for regions involved in an interaction), were performed. Similarly, 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.

The most comprehensive method to analyze the effect of MHC through parasites on antler development is path analysis. However, due to a large number of variables (eight MHC clusters, 10 infection measures), including all of them in the model would be inappropriate, as there would not be enough individuals for such large number of estimated parameters (Wolf et al. 2013). Instead, path analysis was used to elucidate complex interrelations between those variables which GLMs revealed to be significantly associated.

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

2.2. Experimental study

2.2.1. Population

Red deer males used in the experiment were from captive population maintained in the experimental facility of the Institute of Animal Science (Prague, Czech Republic). All animals at the facility are marked with an identification collar and kept in approximately 0.7-ha enclosure with shelters and water reservoirs. Food, which was always provided ad libitum, consisted predominantly of the pasture and is supplemented with hay and a mineral lick and occasionally also with potatoes, beets, apples, pears, barley and oats. Additionally, each year in March, all deer from the facility were administrated with the anthelmintic drug Ivomec (10 mg/ml Ivermectinum, Vétoquinolbiowet Sp.z.o.o., Gorzów WIkp., Poland).

2.2.2. Experimental design and sample collection

Experiment started in March 2012 using 25 red deer males. Stags were paired on the basis of body weight and then randomly assigned to two groups. Animals from one group received standard dose of Ivomec (0.02 ml/kg), while the stags from the second group received saline injection. The procedure was repeated every month until the rut. Next year (2013), experiment was repeated but with anthelmintic treatment being shifted between the groups. Stags from the second group were administrated with Ivomec and males from the first group with saline. Additionally, eight stags were eliminated from the population after first year, thus the experiment was replicated for 17 stags. Parasite load was monitored during the entire experiment. Faeces samples were collected monthly during drugs administration for subsequent parasitological analyses (starting from March 2012). First sample from each year was used to assess pre-treatment parasite load.

Antlers were measured twice a year (in late July and September), in a way recommended by Bartoš & Bahbouh (2006). All characteristics were measured accurately to 0.5 cm using the tape measure. The size of the antlers was estimated as the “total antler length” computed as the sum of the lengths of the beam and all tines divided by two (as proposed by Bubenik 1982).

Due to proximity to the rut (stags are more aggressive and difficult to handle), antler measurements from September were incomplete and therefore antler measurements from July were used in analyses. However, in July antler is almost fully developed and its future growth is negligible (Gaspar-Lopez et al. 2011).

All faeces samples and antler measurements were collected while the animal was fixed in a standard handling facility (squeeze chute). Parasite samples were analyzed as described above for field population (chapter 2.1.3.).

2.2.3. Statistics

To test if Ivomec treatment decreased infection level during antler development generalized linear model (GLMM) with repeated measures was used. Model contained treatment, group as a blocking factor; body weight, age and March infection level (before applying treatment) as covariates; and season and ID (nested in group) treated as random effects. Additionally, interactions between treatment and three variables (group, body weight and March infection level) were included in the model. If interaction was non-significant, it was removed from the model.

To test if Ivomec treatment influenced body weight or antler size (antler length) two GLMMs were analyzed. Both of them included treatment, group as a blocking factor; age and March infection level as covariates; and season and ID (nested in group) treated as random effects. Additionally, in the model testing antler size, body weight was included in a model as covariate.

Body weight and age of deer in the captive population were less correlated ($r < 0.80$) than in wild populations (see above chapter 2.1.6.) therefore both traits were included in models as separate variables.

All models were tested multiple times using different covariance structures (Littell et al. 2000). Models with the best fitting covariance structure were chosen based on Bayesian criterion (Schwarz 1978). In all models unstructured covariance structure fitted the data best.

Analyses were done on 17 individuals for which the data were available for both years. To meet the assumptions of normality and homoscedasticity, infection levels were log transformed in each model. All data were analyzed using SAS v. 9.3 (SAS Institute, Inc., Cary, NC, USA).

3. Results

3.1. Field study

3.1.1. Ornament measurements

Antler measurements (Figure 1) were collected from 152 bulls (92 and 60 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, it was used in subsequent analyses. Furthermore, antler mass was highly correlated with antler size ($r = 0.9$, $p > 0.001$), so it was decided to use only the latter to test the Hamilton and Zuk hypothesis.

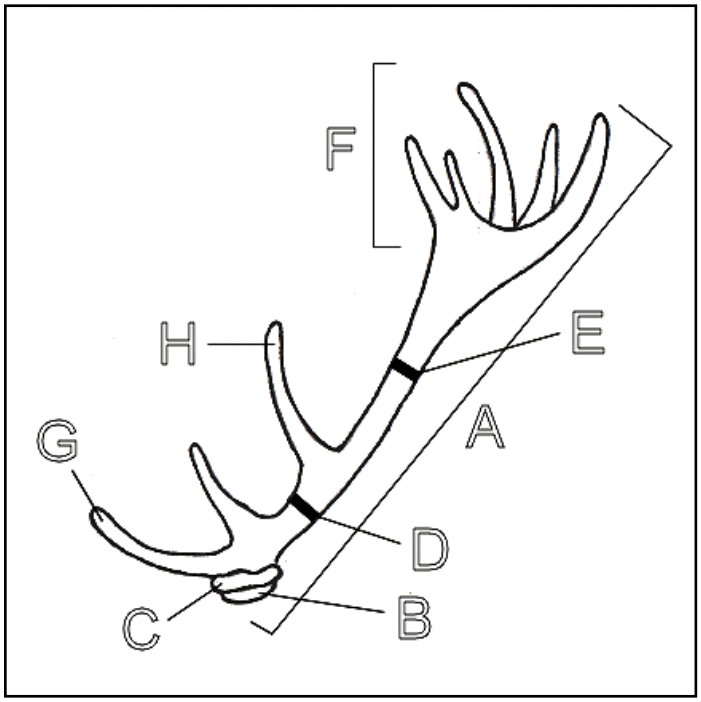


Figure 1. Diagram showing red deer antler measurements: A, beam length; B, seal circumference; C, burr circumference; D, lower circumference; E, higher circumference; F, number of tops (called also royal or crown); G, brow tine; H, trez tine.

3.1.2. Parasites

12 different parasite groups and species were found (Table 1). Using the flotation method eggs of three nematode groups (*Trichocephalus* sp., *Nematodirus* sp., Trichostrongylidae family) and *Eimeria* sp. oocysts were detected. The Baermann method revealed larvae of *Dictyocaulus* sp., *Elaphostrongylus cervi*, and *Varestrongylus 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, Ostertagia leptospicularis, O. kolchida, O. lyrata*, and *Ashworthius 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 feces.

	Parasite	n	median (range)	prevalence
A)	*S. boehmi*	110	19 (0-135)	97%
	S. mathevossiani	110	0 (0-3)	19%
	O. leptospicularis	110	3 (0-24)	76%
	O. kolchida	110	1 (0-12)	56%
	O. lyrata	110	0 (0-1)	1%
	A. 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% †
	E. cervi/V. 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.

3.1.3. MHC diversity and historical selection

179 red deer males (113 and 66 from Bieszczady and Piła regions respectively) were genotyped. After artifacts had been removed, the mean coverage was 557.0 reads per amplicon (SD = 221.4, range 59-1792). Coverage above 75 reads was achieved for 177 individuals. Both of the methods used to distinguish alleles from artifacts (artifact filtering, DOC) identified the same 46 putative alleles (available on Dryad) 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.

Simulations were conducted based on the obtained data, taking into account the variation in amplification efficiency among alleles (Sommer et al. 2013). From these simulations, only for one individual coverage may have been inadequate to identify all alleles (supplementary materials, S4). 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 it was 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 (sequences will be archived in Dryad), 5 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 non-synonymous substitution (dN) versus synonymous substitution (dS) across all sites (supplementary materials, S1). 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 (supplementary materials, S2). Additionally, the BEB procedure (implemented in PAML) identified four codons as evolving under positive selection and all of these overlapped with putative ABSs.

The clustering approaches based on ABS and PPS data yielded comparable results, indicating the optimal cluster number as 9 and 8, respectively. The results from the ABS approach were chosen to be used 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 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.

3.1.4. Antlers and parasites

The intensity of infection of two lung nematodes was positively associated with antler size, 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 2, Figure 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 2): bigger deer had bigger antlers. Because the lung nematodes significantly impacted antler development (Table 2), it was tested if the overall parasite burden of this fundamental organ, rather than a specific parasite species, affected antler size. To do this, an additional model, which contained total lung nematode burden (sum of concentrations of both lung parasites) as a predictor, was built. The simplification of this model revealed strong significant associations between antler size and both body size ($p < 0.0001$) and total lung nematode burden ($p = 0.0015$).

Table 2. 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	
S. 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	
E. cervi/V. sagittatus	body size	1.82	23.68	< 0.0001	*
	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	
A. 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	24.08	< 0.0001	*
Eimeria sp.	region	0.04	0.00	0.9577	
O. kolchida	season	- 0.46	0.59	0.4455	
O. 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

3.1.5. Parasites versus MHC

Associations between seven measures of infection (measures of intensity or prevalence for six parasites, plus Trichostrongylidae egg number) and at least one of the MHC supertypes, were found. All statistically significant results are shown in Table 3 (full results are presented in supplementary materials, S3). Additionally, for three parasites (*S. boehmi, O. kolchida, Trichocephalus* sp.) as well as for Trichostrongylidae egg number, the association between measure of infection and a particular MHC supertype differed significantly among regions (Table 3). Separate analyses for each region revealed three cases in which the association between the presence of a supertype and parasite load was statistically significant. In the Bieszczady mountains, deer that carried the C9 and/or C4 supertype had respectively significantly more *S. boehmi* nematodes ($n = 41$, $F_{1,36} = 5.35$, $p = 0.0266$) and/or were less likely to be infected by *O. kolchida* ($n = 41$, $Chisq = 6.42$, $p = 0.0113$), than stags without those supertypes. Similarly, deer from the Piła region that possessed the C1 supertype were characterized by a significantly smaller number of Trichostrongylidae eggs in the feces ($n = 55$, $F_{1,48} = 13.59$, $p = 0.0006$).

In second series of nine models, nC (number of supertypes) was excluded from all final models during the simplification procedure. The only exception to this was the model that tested the association of nC with *Eimeria* sp., for which the p-value approached, but fell just short of, the significance threshold ($n = 145$, $Chisq = 3.71$, $p = 0.0540$). The nC and $(nC)^2$ terms were also excluded during model simplification from the model that tested for an association between the optimal number of supertypes and parasite species richness.

Table 3. 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 supplementary materials (S3).

Dependent variable	n	Final model	coefficients	F/Chisq	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
S. 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
A. sidemi	97	region	- 3.82	28.08*	< 0.0001
		C4	1.82	6.05*	0.0139
		C9	- 1.78	8.11*	0.0044
O. leptospicularis	97	C5	2.46	7.31*	0.0069
O. 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

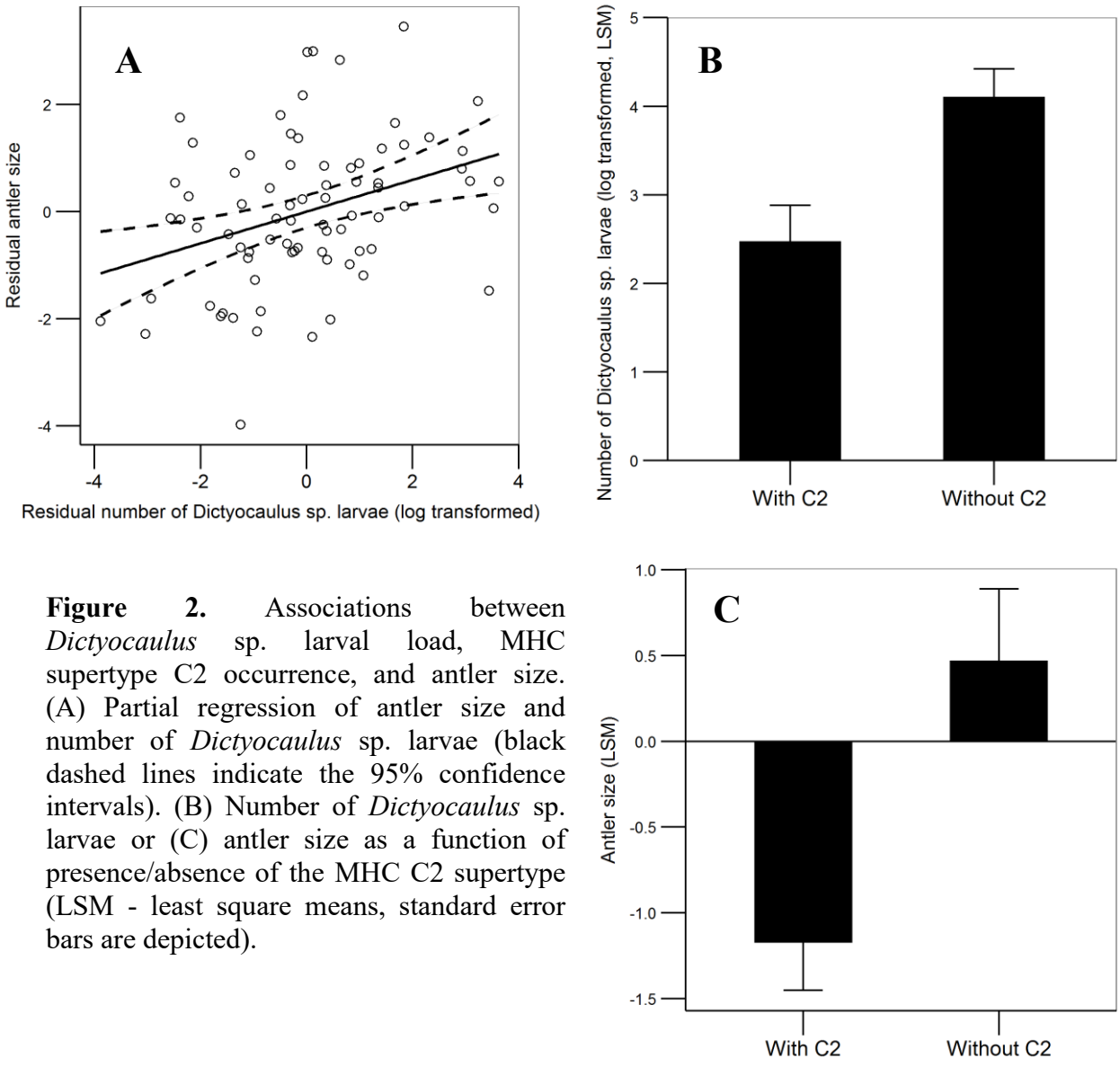


Figure 2. Associations between *Dictyocaulus* sp. larval load, MHC supertype C2 occurrence, and antler size. (A) Partial regression of antler size and number of *Dictyocaulus* sp. larvae (black dashed lines indicate the 95% confidence intervals). (B) Number of *Dictyocaulus* sp. larvae or (C) antler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

3.1.6. Competition between parasites

Increased resistance to *A. sidemi,* coupled with increased susceptibility to *O. kolchida* and (in Bieszczady) to *S. boehmi* could result from competition between parasites, such that resistance to *A. sidemi* opened niche to the other two parasites. To test for this, *A. sidemi* prevalence was included 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, Chisq = 7.03, p = 0.0080). Analysis of those putative competition relationships via path analysis was not possible, because despite data transformations, assumption of multivariate normality was not met.

3.1.7. MHC versus 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) (Table 3, Figure 2B). However, that deer that possessed this supertype had, on average, 2.15-times fewer *S. boehmi* nematodes, and 5.10-times fewer *Dictyocaulus* sp. larvae (Table 3, Figure 2C). Path analysis revealed that this relationship was driven mostly by significant C2 -> *Dictyocaulus* and *Dictyocaulus* -> antler associations. The direct C2 -> antler path was not significant (Figure 3, Table 4). Additionally, path analysis suggested the existence of indirect relationship between C6 supertype and antler size (through *Dictyocaulus* sp.)(Figure 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.

Additionally, the association between antler size and the C4 supertype differed significantly between regions (Table 3). In the Piła region, deer that had the C4 supertype were characterized by a larger average antler size (n = 51, $F_{1,45}$ = 5.41, p = 0.0246) than stags without it. In the Bieszczady mountains, instead, association between the presence of the C4 supertype and antler size was opposite and not significant (n = 24, $F_{1,18}$ = 0.60, p = 0.4486). Path analysis did not show any direct or indirect (through parasites) relationship between C4 and antler size in Piła region (supplementary materials, S5).

No significant association was found between antler size and the number of supertypes (nC or $(nC)^2$) carried by deer.

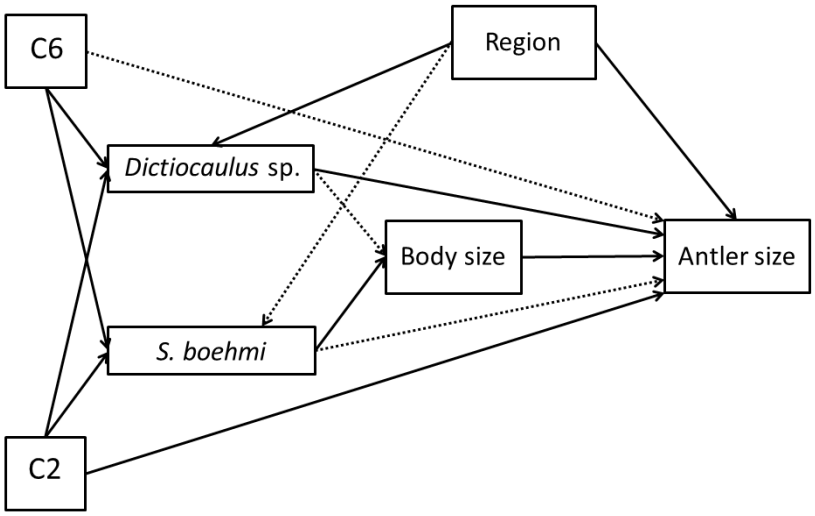


Figure 3. Path analysis diagram showing determinants of antler size, including all variables which could be potentially important, as determined by significant associations between them revealed by GLMs (see methods). Solid bars represent the paths retained in the final model.

Table 4. Final path analysis model testing relationships between: MHC superypes C2 and C6, *Dictiocaulus* sp. intensity, *S. boehmi* intenity, antler size, body size, region and season. Usignificant 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

3.2. Experimental study

3.2.1. Parasites

In analyses of faeces from the first sampling just before applying treatment procedure, no lung nematode larvae were detected. Therefore, no samples collected later were analyzed using Baermann method.

Using the floatation method, eggs of three nematode groups were detected: *Trichocephalus ovis*, *Nematodirus* sp. and *Trichostrongylidae* family (except *Nematodirus* sp.). Additionally, *Eimeria* sp. oocysts were found. However, *Nematodirus* sp. and *Eimeria* sp. were rarely detected (prevalence varied between months from 0% to 35%), even though individual egg/oocysts load could be substantial (>100). *Trichocephalus ovis* was more common, but only in season 2012 it was found in more than 50% of individuals. On the other hand *Trichostrongylidae* eggs were detected in almost all individuals and in large quantities (even > 1000). Thus, only this parasite group was used as a measure of efficiency of antihelminthic treatment in further analyses.

For three individuals in the second season, March feaces samples for parasitological analyses were missing.

3.2.2. Effects of anthelmintic treatment

According to the final model (after non-significant interactions were removed), anthelmintic treatment significantly decreased infection level during antler development ($F_{1,24}$= 23.91, p< 0.0001, Table 5, Figure 4). Additionally, infection level during experiment was negatively associated with body mass and positively with March infection level (Table 5). Other variables were not significant (Table 5).

Body weight and antler length were not affected by anthelmintic treatment (Table 5). Additionally, body weight was significantly associated with age and March infection level. Older stags were heavier and heavier deer were less infected before anthelmintic treatment was applied. In turn, as expected, older and heavier stags developed bigger antlers (Table 5).

Table 5. Results of three final general linear mixed models (GLMMs) with repeated measures and unstructured covariance structure testing for the effect of anthelmintic treatment on infection level during antler development, body weight and antler length, in red deer males. Analyses were done on 31 samples (30 in model with antler length, due to lack of data for one individual in second year) from 17 individuals.

	Infection level			Body weight			Antler length		
Effect	df	F	P	df	F	p	df	F	p
Treatment	1,24	23.91	< 0.0001	1,25	0.00	0.9919	1,23	2.14	0.1575
Group	1,24	4.16	0.0525	1,25	0.22	0.6453	1,23	0.21	0.6543
Age	1,24	3.10	0.0911	1,25	41.27	< 0.0001	1,23	11.03	0.0030
Body weight	1,24	7.42	0.0118	-	-	-	1,23	14.79	0.0008
IL_March	1,24	15.19	0.0007	1,25	15.35	0.0006	1,23	0.69	0.4153

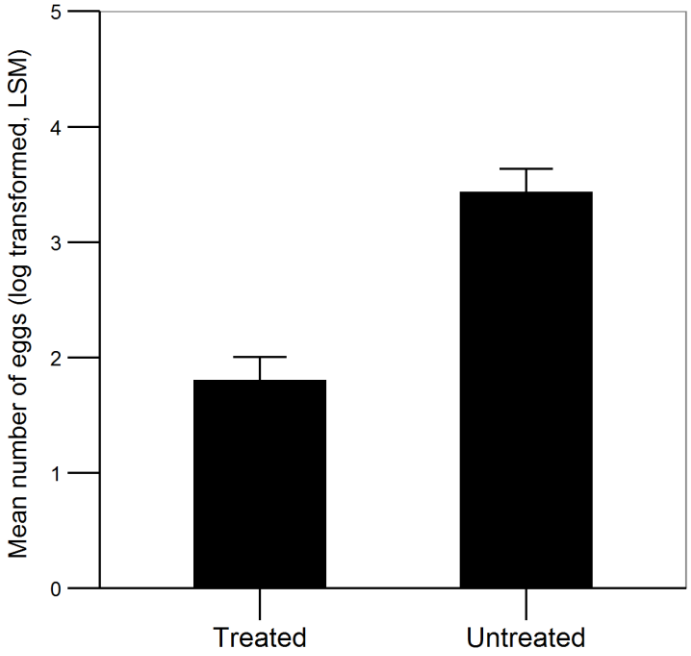


Figure 4. Effect of anthelmintic treatment on infection level (mean number of eggs from Trichostrongylidae family), in red deer males, during antler growth.

4. Discussion

In this study the Hamilton and Zuk hypothesis was tested using both correlation and experimental approaches. According to the hypothesis a negative relationship between parasite load and the size of sexual ornaments, as well as associations between MHC gene variants and antler development were predicted. However, results provided no support for this hypothesis, but revealed interesting and complex relationships between MHC genes, the measure of parasite infection, and sexual traits.

4.1. Field study

4.1.1. Antlers and parasites

From 10 studied infection measures only two (the infection intensity of the lung nematodes *Dictyocaulus* sp. and the Ec/Vs group) were significantly associated with antler size (Table 2). However, contrary to predictions, both of those relationships were positive. The finding that deer with bigger antlers carried more lung nematodes is intriguing given that *Dictyocaulus* sp. infections are much more severe than abomasum nematode infections and have major impacts on animal health (Mason 1994). According to the Hamilton and 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 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 no significant relationship between body mass and parasite infection was found.

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 and Zuk hypothesis be refuted, but also the entire good genes hypothesis.

Our data may reflect a tradeoff 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, obtained results shows that antler size in red deer is not an honest signal of health status and as such is not consistent with the Hamilton and Zuk hypothesis.

One caveat with gathered dataset 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). 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 the dataset, because it contained deer with poorly developed antlers which, being poor trophies, could otherwise be avoided. Therefore, I believe that the sample contained enough variation in antler size and parasite load to allow meaningful analyses. This is supported by the significant results reported above.

4.1.2. MHC supertypes and parasites

A number of MHC supertypes were associated with decreased (supertypes C2, C4, C9) or increased (supertypes C3, C4, C5, C6, C9) infection intensity and parasite prevalence (Table 3 & S3). Thus obtained 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 (Meyer-Lucht & Sommer 2005, Froeschke & Sommer 2005, Oliver et al. 2009, Kloch et al. 2010), birds (Bonneaud et al. 2006, Schou et al. 2006, Dunn et al. 2012, Sepil et al. 2013), and fish (Eizaguirre et al. 2009, Evans & Neef 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). Obtained results suggest the existence of at least two such relationships. First, supertype C9 was associated with resistance to *A. sidemi*, but at the same time was positively associated with *O. kolchida* prevalence. Similarly, deer with the C9 supertype in the Bieszczady region had increased resistance to *A. sidemi*, but at the same time, were more susceptible to *S. boehmi*. This trade-off can be due to specificity of antigen binding by MHC supertypes, or due 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.

The statistically significant interactions between MHC supertypes and region (Table 3) suggest that host–parasite interactions are population-specific. Similar results were reported by Hill (1998) for HLA-based resistance to malaria in humans, Bonneaud et al. (2006) for malaria in house sparrows, and more recently Kloch et al. (2010) and Biedrzycka et al. (2011) for rodent populations. This spatial variation in associations between MHC types and parasite infection, a likely result of dynamics of host-parasite coevolution, was suggested to be an additional mechanism helping to maintain high levels

of MHC polymorphism (Kloch et al. 2010). While significant associations between individual MHC supertypes and parasite load were found, neither linear nor non-linear relationship between infection and the number of MHC supertypes were present. The literature on this subject is mixed: some studies have reported positive (e.g., Radwan et al. 2012) or non-linear relationships (Wegner et al. 2003; Madsen & Ujvari 2006; Kloch et al. 2010) between individual MHC diversity and parasite load, but others have reported no significant effect (e.g., Dunn et al. 2012, Froeschke & Sommer 2012). However, while high individual MHC diversity may increase the chance of possessing a variant that confers resistance to a given parasite (Nowak et al. 1992; Woelfing et al. 2009), it will also increase the chances of possessing susceptible alleles (Kloch et al. 2013). Here, the lack of any association between infection and individual MHC diversity in red deer may be the result of co-occurrence of supertypes associated with increased susceptibility to infection and those conferring resistance.

4.1.3. MHC supertypes, parasites and antler size

Main prediction, derived from Hamilton and 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. Both 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 and Zuk hypothesis. Some possible explanations for the higher load of lung nematodes in stags with more developed antlers were presented above, 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), this explanation seems unlikely.

Apart from C2, GLM analysis revealed that supertype C4 was positively associated with antler size in the Piła region. The C4 supertype was, however, associated with both increased (Trichostrongylidae egg number, *A. sidemi*) and decreased (*O. kolchida* in Bieszczady region) parasite load, which makes interpretation in the light of Hamilton and Zuk hypothesis difficult. Furthermore, path analysis did not confirm the direct or indirect effect of C4 on antler size (S5).

Finally, path analysis revealed that infection with *S. boehmi*, being under significant influence of C2 and C4 supertypes, has a significant 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, it might reveal an information on MHC-based resistance to *S. boehmi* more efficiently. Nevertheless, given that this information revealed by antlers would contradict that about resistance to a more debilitating *Dictyocaulus* sp. (as discussed above) it is conclude that overall obtained results do not provide support for Hamilton and Zuk hypothesis.

To my knowledge, only two previous studies (Eizaguirre et al. 2009, Dunn et al. 2012) have tested the Hamilton and 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 reported here, it appears that overall support for the Hamilton and Zuk hypothesis is mixed at best.

4.1.4. Comparison with other studies on ungulates

Focusing on cervids only, the results presented here are consistent with other studies, which found no (Markusson & Folstad 1997) or only weak support (Mulvay & Aho 1993, Ditchkoff et al. 2001) for Hamilton and Zuk hypothesis. Even though Ditchkoff et al. (2001) detected significant negative associations between abomasal nematode load and antler score, the effect was weak ($p= 0.05$) and would be not significant after using any correction for multiple comparisons (they tested six measures of infection). In turn, Mulvey & Aho (1993) found significant negative association between liver fluke intensity and antler size, but this included only one of four studied age groups. Considering that it was the youngest age group (1.5 year old), males that do not take part in male-male competition during the rut, the obtained results cannot be treated as a support for H&Z hypothesis. Additionally, it is worth noting that mentioned studies used different antler measurements (antler length, antler weight, number of points, antler score), which may indicate that none of antler characteristics reflect parasite load. It suggests that mechanism proposed by Hamilton and Zuk does not apply to these taxa.

Contrary to cervids, studies on other ungulates (bovine) in general support Hamilton and Zuk hypothesis. African buffalo (Ezenwa & Jolly 2008) and Grant's gazelle (Ezenwa et al. 2012) showed the negative association between parasite loads and horn size. Therefore, it seems that Hamilton and Zuk hypothesis may not be universal among animal taxa and host-parasite co-evolution is not the only force responsible for origin and maintenance of female preference towards male sexual traits.

4.2. Experimental study

4.2.1. Effects of anthelmintic treatment

The correlational approach to test Hamilton and Zuk hypothesis described above was complemented with experimental approach using captive population of red deer. However, even though anthelmintic treatment significantly decreased infection level of gastro-intestine nematodes, it did not have a significant effect on body weight or antler size. Interestingly, Folstad et al. (1996) obtained exactly the same results using semi-domesticated population of reindeer. Animals treated with anthelmintic drug had significantly fewer parasites, but it did not affect body weight or antler length. However, it should be mentioned that their study was done only using females. Since reindeer females develop smaller antlers than males (Melnycky et al. 2013) female antlers may be too small to constitute a handicap revealing parasite load.

There are several potential explanations for the lack of influence of anthelmintic treatment on the antler size. It is possible that other parasites, which were not analyzed (e.g. blood parasites) had stronger influence on deer condition, but anthelmintic treatment did not affect them. Or, conversely, parasites of major impact on condition were not present in this domesticated population. For instance, no lung nematodes, which are considered to have the greatest impact on deer health (Mason 1994), were detected. In both cases, association between parasite load and antler size would not be detected. However, the study on wild red deer populations (see above) did not found negative associations between any of the eight analyzed parasite species (including lung nematodes) and antler size, which is consistent with negative results obtained in the present study.

Finally, it is possible that the applied anthelmintic drug had an unknown side effect on deer. For example, a study on root vole (Kloch et al. 2013) showed that rodents treated with Ivomec survived worse than control individuals. The drug need not have had a toxic effect on the deer, it could have just affected other traits that influence antler size, such as testosterone level or calcium absorption.

Finally, in the population studied, deer were kept at stable density and were fed with the same food always provided ad libitum. It is possible that in such benign conditions, debilitating effects of parasites could be compensated e.g. by additional feeding. Future studies might test this hypothesis by e.g. imposing food limitation.

Nevertheless, the lack of the significant association between parasite load and the antler size show that parasites, without interaction with additional stressors, are not the major force influencing development of sexual ornament in red deer.

5. Conclusions

Although MHC supertypes predicted infection with several parasites, including debilitating lung nematodes, no clear support for the Hamilton and Zuk hypothesis was found. The positive association between lung nematode load and antler development revealed by the study of a natural population is clearly at odds with its predictions. Likewise, experimental manipulation of the infection did not influence antler development. These results suggest that tradeoffs between investment in immune response and sexual traits may make the latter a poor indicator of the presence of male genes that confer resistance to parasites. The results of this study also indicate tradeoffs 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 tradeoffs may undermine the potential benefits of inheriting particular MHC types, predicted by the Hamilton and Zuk hypothesis.

References

Andersson, M. 1994. *Sexual Selection*. Princeton University Press.

Andersson, M. & Simmons, L.W. 2006. Sexual selection and mate choice. *Trends Ecol. Evol.* **21**: 296–302.

Babik, W., Taberlet, P., Ejsmond, M.J. & Radwan, J. 2009. New generation sequencers as a tool for genotyping of highly polymorphic multilocus MHC system. *Mol. Ecol. Resour.* **9**: 713–9.

Bartoš, L. & Bahbouh, R. 2006. Antler size and fluctuating asymmetry in red deer (Cervus elaphus) stags and probability of becoming a harem holder in rut. *Biol. J. Linn. Soc.* **87**: 59–68.

Benjamini, Y. & Hochberg, Y. 1995. Controlling the false discovery rate: a practical and powerful approach to multiple testing. *J. Roy. Stat. Soc. Ser. B* **57**: 289 – 300.

Bernatchez, L. & Landry, C. 2003. MHC studies in nonmodel vertebrates: What have we learned about natural selection in 15 years? *J. Evol. Biol.* **16**: 363–377.

Biedrzycka, A., Kloch, A., Buczek, M. & Radwan, J. 2011. Major histocompatibility complex DRB genes and blood parasite loads in fragmented populations of the spotted suslik Spermophilus suslicus. *Mamm. Biol.* **76**: 672–677.

Bobek, B., Morow, K., Perzanowski, K. & Kosobucka, M. 1992. *Jeleń. The red deer (Cervus elaphus) - it's ecology and management.* Wydawnictwo Świat, Warszawa.

Bodmer, W. 1972. Evolutionary significance of the HLA-system. *Nature* **237**: 139–145.

Bonneaud, C., Pérez-Tris, J. & Federici, P. 2006. Major histocompatibility alleles associated with local resistance to malaria in a passerine. *Evolution (N. Y).* **60**: 383–389.

Borghans, J.A.M., Beltman, J.B. & De Boer, R.J. 2004. MHC polymorphism under host-pathogen coevolution. *Immunogenetics* **55**: 732–739.

Brønseth, T. & Folstad, I. 1997. The effect of parasites on courtship dance in threespine sticklebacks: more than meets the eye? *Can. J. Zool.* **75**: 589–594.

Bubenik, A.B. 1982. Proposal for standardized nomenclature for bony appendices in Pecora. In: *Antler Development in Cervidae*. Kingsville, Texas: Caesar Kleberg Wildlife Research Institute.

Clutton-Brock, T.H. 1984. Reproductive Effort and Terminal Investment in Iteroparous Animals. *Am. Nat.* **123**: 212.

Clutton-Brock, T.H. 1982. The Functions of Antlers. *Behaviour* **79**: 108–124.

Costa, F.J. V & Macedo, R.H. 2005. Coccidian oocyst parasitism in the blue-black grassquit: Influence on secondary sex ornaments and body condition. *Anim. Behav.* **70**: 1401–1409.

Cramer, M.J. & Cameron, G.N. 2007. Effects of bot fly, Cuterebra fontinella, parasitism on male aggression and female choice in Peromyscus leucopus. *Anim. Behav.* **74**: 1419–1427.

Darwin, C. 1871. *The descent of man and selection in relation to sex*. John Murray, London.

Demiaszkiewicz, A.W. 1987. Species composition and the extensiveness of invasion of deer by nematoda of the family Protostrongylidae at selected hunting grounds. *Wiadomości Parazytol.* **33**: 57–62.

Demuth, J.P., Naidu, A. & Mydlarz, L.D. 2012. Sex, war, and disease: the role of parasite infection on weapon development and mating success in a horned beetle (Gnatocerus cornutus). *PLoS One* **7**: e28690.

Ditchkoff, S. & Hoofer, S. 2005. MHC-DRB evolution provides insight into parasite resistance in white-tailed deer. *Southwest. Nat.* **50**: 57–64.

Ditchkoff, S.S., Lochmiller, R.L., Masters, R.E., Hoofer, S.R., An, R.O.A. V & Ussche, D.E.N.B. 2001. Major-histocompatibility-complex-associated variation in secondary sexual traits of white-tailed deer (Odocoileus virginianus): evidence for good-genes advertisement. **55**: 616–625.

Doherty, P.C. & Zinkernagel, R. 1975. Enhanced immunologic surveillance in mice heterozygous at the H-2 gene complex. *Nature* **256**:50–52.

Doytchinova, I.A. & Flower, D.R. 2005. In Silico Identification of Supertypes for Class II MHCs. *J. Immunol.* **174**: 7085–7095.

Dróżdż, J., Malczewski, A., Demiaszkiewicz, A.W. & Lachowicz, J. 1997. The helminthofauna of farmed deer (Cervidae) in Poland. *Acta Parasitol.* **42**: 225 – 229.

Dunn, P.O., Bollmer, J.L., Freeman-Gallant, C.R. & Whittingham, L.A. 2012. Mhc variation is related to a sexually selected ornament, Survival, and parasite resistance in common yellowthroats. *Evolution (N. Y).* **67**: 679–687.

Eizaguirre, C., Yeates, S.E., Lenz, T.L., Kalbe, M. & Milinski, M. 2009. MHC-based mate choice combines good genes and maintenance of MHC polymorphism. *Mol. Ecol.* **18**: 3316–3329.

Evans, M.L. & Neff, B.D. 2009. Major histocompatibility complex heterozygote advantage and widespread bacterial infections in populations of Chinook salmon (Oncorhynchus tshawytscha). *Mol. Ecol.* **18**: 4716–29.

Ezenwa, V. & Jolles, A. 2008. Horns honestly advertise parasite infection in male and female African buffalo. *Anim. Behav.* **75**: 2013–2021.

Ezenwa, V.O., Stefan Ekernas, L. & Creel, S. 2012. Unravelling complex associations between testosterone and parasite infection in the wild. *Funct. Ecol.* **26**: 123–133.

Fernandez-de-Mera, I.G., Vicente, J., Naranjo, V., Fierro, Y., Garde, J.J., de la Fuente, J., *et al.* 2009. Impact of major histocompatibility complex class II polymorphisms on Iberian red deer parasitism and life history traits. *Infect. Genet. Evol.* **9**: 1232–9.

Fisher, R.A. 1930. *The Genetical Theory of Natural Selection*.

Folstad, I. & Karter, A. 1992. Parasites, bright males, and the immunocompetence handicap. *Am. Nat.* **139**: 603–622.

Folstad, I., Arneberg, P. & Karter, A. 1996. Antlers and parasites. *Oecologia* 556–558.

Froeschke, G. & Sommer, S. 2012. Insights into the complex associations between MHC class II DRB polymorphism and multiple gastrointestinal parasite infestations in the striped mouse. *PLoS One* **7**: e31820.

Froeschke, G. & Sommer, S. 2005. MHC class II DRB variability and parasite load in the striped mouse (Rhabdomys pumilio) in the Southern Kalahari. *Mol. Biol. Evol.* **22**: 1254–9.

Galan, M., Guivier, E., Caraux, G., Charbonnel, N. & Cosson, J.-F. 2010. A 454 multiplex sequencing method for rapid and reliable genotyping of highly polymorphic genes in large-scale studies. *BMC Genomics* **11**: 296.

Gaspar-López, E., Landete-Castillejos, T., Estevez, J.A., Ceacero, F., Gallego, L. & García, A.J. 2011. Seasonal variations in red deer (Cervus elaphus) hematology related to antler growth and biometrics measurements. *J. Exp. Zool. A. Ecol. Genet. Physiol.* **315**: 242–9.

Hamilton, W.D. & Zuk, M. 1982. Heritable true fitness and bright birds: a role for parasites? *Science* **218**: 384–387.

Hawley, D.M. & Altizer, S.M. 2011. Disease ecology meets ecological immunology: understanding the links between organismal immunity and infection dynamics in natural populations. *Funct. Ecol.* **25**: 48–60.

Herdegen, M., Babik, W. & Radwan, J. 2014. Selective pressures on MHC class II genes in the guppy (Poecilia reticulata) as inferred by hierarchical analysis of population structure. *J. Evol. Biol.* **27**: 2347–2359.

Hill, A. V. 1998. The immunogenetics of human infectious diseases. *Annu. Rev. Immunol.* **16**: 593–617.

Hill, G.E. 2004. The effect of mycoplasmosis on carotenoid plumage coloration in male house finches. *J. Exp. Biol.* **207**: 2095–2099.

Jaczewski, Z. 1981. *Poroże jeleniowatych*. PWRL, Warszawa.

Jombart, T. 2008. adegenet: a R package for the multivariate analysis of genetic markers. *Bioinformatics* **24**: 1403–5.

Jombart, T., Devillard, S. & Balloux, F. 2010. Discriminant analysis of principal components: a new method for the analysis of genetically structured populations. *BMC Genet.* **11**: 94. BioMed Central Ltd.

Kalbe, M., Eizaguirre, C., Dankert, I., Reusch, T.B.H., Sommerfeld, R.D., Wegner, K.M., *et al.* 2009. Lifetime reproductive success is maximized with optimal major histocompatibility complex diversity. *Proc. R. Soc. B Biol. Sci.* **276**: 925–934.

Klein, J. 1986. *Natural history of the histocompatibility complex*. Wiley, New York.

Kloch, A., Babik, W., Bajer, A., Siński, E. & Radwan, J. 2010. Effects of an MHC-DRB genotype and allele number on the load of gut parasites in the bank vole Myodes glareolus. *Mol. Ecol.* **19**: 255–65.

Kloch, A., Baran, K., Buczek, M., Konarzewski, M. & Radwan, J. 2013. MHC influences infection with parasites and winter survival in the root vole Microtus oeconomus. *Evol. Ecol.* **27**: 635–653.

Kokko, H., Brooks, R., Jennions, M.D. & Morley, J. 2003. The evolution of mate choice and mating biases. *Proc. R. Soc. B Biol. Sci.* **270**: 653–664.

Kruuk, L., Slate, J. & Pemberton, J. 2002. Antler size in red deer: heritability and selection but no evolution. *Evolution (N. Y).* **56**: 1683–1695.

Landete-Castillejos, T., Currey, J.D., Estevez, J. a., Gaspar-López, E., Garcia, a. & Gallego, L. 2007. Influence of physiological effort of growth and chemical composition on antler bone mechanical properties. *Bone* **41**: 794–803.

Lessells, C. & Boag, P. 1987. Unrepeatable repeatabilities: a common mistake. *Auk* **2**: 116–121.

Lighten, J., van Oosterhout, C., Paterson, I.G., McMullan, M. & Bentzen, P. 2014. Ultra-deep Illumina sequencing accurately identifies MHC class IIb alleles and provides evidence for copy number variation in the guppy (Poecilia reticulata). *Mol. Ecol. Resour.* **14**: 753–67.

Littell, R.C., Pendergast, J. & Natarajan, R. 2000. Modelling covariance structure in the analysis of repeated measures data. *Stat. Med.* **19**: 1793–819.

Loiseau, C., Zoorob, R., Garnier, S., Birard, J., Federici, P., Julliard, R., *et al.* 2008. Antagonistic effects of a Mhc class I allele on malaria-infected house sparrows. *Ecol. Lett.* **11**: 258–65.

Maan, M.E., Van Der Spoel, M., Jimenez, P.Q., Van Alphen, J.J.M. & Seehausen, O. 2006. Fitness correlates of male coloration in a Lake Victoria cichlid fish. *Behav. Ecol.* **17**: 691–699.

Madsen, T. & Ujvari, B. 2006. MHC class I variation associates with parasite resistance and longevity in tropical pythons. *J. Evol. Biol.* **19**: 1973–78.

Magoč, T. & Salzberg, S.L. 2011. FLASH: fast length adjustment of short reads to improve genome assemblies. *Bioinformatics* **27**: 2957–63.

Malo, A.F., Roldan, E.R.S., Garde, J.J., Soler, a J., Vicente, J., Gortazar, C., *et al.* 2009. What does testosterone do for red deer males? *Proc. R. Soc. B Biol. Sci.* **276**: 971–80.

Markusson, E. & Folstad, I. 1997. Reindeer antlers: visual indicators of individual quality? *Oecologia* 501–507.

Martínez-Padilla, J., Mougeot, F., Pérez-Rodríguez, L. & Bortolotti, G.R. 2007. Nematode parasites reduce carotenoid-based signalling in male red grouse. *Biol. Lett.* **3**: 161–164.

Mason, P. 1994. Parasites of deer in New Zealand. *New Zeal. J. Zool.* **21**: 39–47.

Melnycky, N. & Weladji, R. 2013. Scaling of antler size in reindeer (Rangifer tarandus): sexual dimorphism and variability in resource allocation. *J. Mamm.* **94**: 1371–1379.

Meyer-Lucht, Y. & Sommer, S. 2005. MHC diversity and the association to nematode parasitism in the yellow-necked mouse (Apodemus flavicollis). *Mol. Ecol.* **14**: 2233–43.

Milinski, M. & Bakker, T.C.M. 1990. Female sticklebacks use male coloration in mate choice and hence avoid parasitized males. *Nature* **344**: 330–333.

Moller, A. 1990. Effects of a haematophagous mite on the barn swallow (Hirundo rustica): a test of the Hamilton and Zuk hypothesis. *Evolution (N. Y).* **44**: 771–784.

Mougeot, F. & Redpath, S.M. 2004. Sexual ornamentation relates to immune function in male red grouse Lagopus lagopus scoticus Franc. *J. Avian Biol.* **35**: 425–434.

Müller, G. & Ward, P.I. 1995. Parasitism and heterozygosity influence the secondary sexual characters of the European minnow, *Phoxinus phoxinus* (L) (Cyprinidae). *Ethology* **100**: 309–319.

Mulvey, M. & Aho, J. 1993. Parasitism and mate competition: liver flukes in white-tailed deer. *Oikos* **66**: 187–192.

Nowak, M. 1992. The optimal number of major histocompatibility complex molecules in an individual. *Proc. Natl. Acad. Sci.* **89**: 10896–10899.

Oliver, M.K., Telfer, S. & Piertney, S.B. 2009. Major histocompatibility complex (MHC) heterozygote superiority to natural multi-parasite infections in the water vole (Arvicola terrestris). *Proc. R. Soc. B Biol. Sci.* **276**: 1119–28.

Paterson, S. 1998. Major histocompatibility complex variation associated with juvenile survival and parasite resistance in a large unmanaged ungulate population (Ovis aries L.). *Proc. Natl. Acad. Sci.* **95**: 3714–3719.

Piertney, S.B. & Oliver, M.K. 2006. The evolutionary ecology of the major histocompatibility complex. *Heredity (Edinb).* **96**: 7–21.

Pomiankowski, A. 1988. The evolution of female mating preferences for male genetic quality. In: *Oxf. Surv. Evol. Biol.*, pp. 136–184.

Posada, D. & Buckley, T.R. 2004. Model selection and model averaging in phylogenetics: advantages of akaike information criterion and bayesian approaches over likelihood ratio tests. *Syst. Biol.* **53**: 793–808.

Potts, W.K. & Wakeland, E.K. 1990. Evolution of diversity at the major histocompatibility complex. *Trends Ecol. Evol.* **5**: 181–187.

Prokop, Z.M., Michalczyk, Ł., Drobniak, S.M., Herdegen, M. & Radwan, J. 2012. Meta-analysis suggests choosy females get sexy sons more than "good genes". *Evolution (N. Y).* **66**: 2665–73.

Promerova, M., Babik, W., Bryja, J., Albrecht, T., Stuglik, M. & Radwan, J. 2012. Evaluation of two approaches to genotyping major histocompatibility complex class I in a passerine-CE-SSCP and 454 pyrosequencing. *Mol. Ecol. Resour. Ecol. Resour* **12**: 285–292.

R Development Core Team. 2014. R: A Language and Environment for Statistical Computing.

Radwan, J., Zagalska-Neubauer, M., Cichoń, M., Sendecka, J., Kulma, K., Gustafsson, L., *et al.* 2012. MHC diversity, malaria and lifetime reproductive success in collared flycatchers. *Mol. Ecol.* **21**: 2469–79.

Rantala, M.J., Roff, D. a. & Rantala, L.M. 2007. Forceps size and immune function in the earwig Forficula auricularia L. *Biol. J. Linn. Soc.* **90**: 509–516.

Reche, P. a. & Reinherz, E.L. 2003. Sequence Variability Analysis of Human Class I and Class II MHC Molecules: Functional and Structural Correlates of Amino Acid Polymorphisms. *J. Mol. Biol.* **331**: 623–641.

De Roode, J.C., Helinski, M.E.H., Anwar, M.A. & Read, A.F. 2005. Dynamics of multiple infection and within-host competition in genetically diverse malaria infections. *Am. Nat.* **166**: 531–42.

Sandberg, M. & Eriksson, L. 1998. New chemical descriptors relevant for the design of biologically active peptides. A multivariate characterization of 87 amino acids. *J. Med. Chem.* **2623**: 2481–2491.

Schou, T.W., Permin, a, Juul-Madsen, H.R., Sørensen, P., Labouriau, R., Nguyên, T.L.H., *et al.* 2007. Gastrointestinal helminths in indigenous and exotic chickens in Vietnam: association of the intensity of infection with the Major Histocompatibility Complex. *Parasitology* **134**: 561–73.

Schwarz, G. 1978. Estimating the dimension of a model. *Ann. Stat.* **6**: 461–464.

Schwensow, N., Fietz, J., Dausmann, K.H. & Sommer, S. 2007. Neutral versus adaptive genetic variation in parasite resistance: importance of major histocompatibility complex supertypes in a free-ranging primate. *Heredity (Edinb).* **99**: 265–77.

Sepil, I., Lachish, S., Hinks, A.E. & Sheldon, B.C. 2013. Mhc supertypes confer both qualitative and quantitative resistance to avian malaria infections in a wild bird population. *Proc. R. Soc. B Biol. Sci.* **280**: 20130134.

Sheldon, B. & Verhulst, S. 1996. Ecological immunology: costly parasite defences and trade-offs in evolutionary ecology. *Trends Ecol. Evol.* **11**: 317–321.

Sommer, S., Courtiol, A. & Mazzoni, C.J. 2013. MHC genotyping of non-model organisms using next-generation sequencing: a new methodology to deal with artefacts and allelic dropout. *BMC Genomics* **14**: 542. BMC Genomics.

Spurgin, L.G. & Richardson, D.S. 2010. How pathogens drive genetic diversity: MHC, mechanisms and misunderstandings. *Proc. Biol. Sci.* **277**: 979–988.

Stuglik, M.T., Radwan, J. & Babik, W. 2011. jMHC: software assistant for multilocus genotyping of gene families using next-generation amplicon sequencing. *Mol. Ecol. Resour.* **11**: 739–42.

Swarbrick, P.A. & Crawford, A. 1997. The red deer (Cervus elaphus) contains two expressed major histocompatibility complex class II DQB genes. *Anim. Genet.* **28**: 49–51.

Swarbrick, P.A., Schwaiger, F.W., Epplen, J.T., Buchan, G.S., Griffin, J.F.T. & Crawford, A.M. 1995. Cloning and sequencing of expressed DRB genes of the red deer (Cervus elaphus) Mhc. *Immunogenetics* **42**: 1–9.

Untalan, P.M., Pruett, J.H. & Steelman, C.D. 2007. Association of the bovine leukocyte antigen major histocompatibility complex class II DRB3*4401 allele with host resistance to the Lone Star tick, Amblyomma americanum. *Vet. Parasitol.* **145**: 190–5.

Votýpka, J., Šimek, J. & Tryjanowski, P. 2003. Blood parasites, reproduction and sexual selection in the red-backed shrike (Lanius collurio). *Ann. Zool. Fennici* **40**: 431–439.

Wegner, K.M., Kalbe, M., Kurtz, J., Reusch, T.B.H. & Milinsici, M. 2003. Parasite Selection for. *Science.* **301**: 1343.

Woelfing, B., Traulsen, A., Milinski, M. & Boehm, T. 2009. Does intra-individual major histocompatibility complex diversity keep a golden mean? *Philos. Trans. R. Soc. Lond. B. Biol. Sci.* **364**: 117–28.

Wolf, E.J., Harrington, K.M., Clark, S.L. & Miller, M.W. 2013. Sample Size Requirements for Structural Equation Models: An Evaluation of Power, Bias, and Solution Propriety. *Educ. Psychol. Meas.* **76**: 913–934.

Yang, Z. 2007. PAML 4: phylogenetic analysis by maximum likelihood. *Mol. Biol. Evol.* **24**: 1586–91.

Yang, Z. 1997. PAML: a program package for phylogenetic analysis by maximum likelihood. *Bioinformatics* **13**: 555–556.

Zahavi, A. 1975. Mate selection-a selection for a handicap. *J. Theor. Biol.* **53**: 205–214.

Zajac, A.M. & Conboy, G.. 2006. *Veterinary Clinical Parasitology*. Wiley.

Zirpoli, J.A., Black, J.M. & Gabriel, P.O. 2013. Parasites and plumage in Steller's jays: an experimental field test of the parasite-mediated handicap hypothesis. *Ethol. Ecol. Evol.* **25**: 103–116.

Zuk, M. & McKean, K.A. 1996. Sex differences in parasite infections: Patterns and processes. *Int. J. Parasitol.* **26**: 1009–1024.

Supplementary materials

S1

Synonymous and nonsynonymous substitution rates

	dN	dS	p	Z
All	0.127 (0.023)	0.076 (0.021)	0.081	1.761
ABS	0.384 (0.082)	0.167 (0.071)	0.040	2.076
no-ABS	0.067 (0.017)	0.054 (0.022)	0.638	0.472

The average rates of nonsynonymous substitution per nonsynonymous site (dN) and synonymous substitution per synonymous site (dS) computed according to the Nei-Gojobori method with Jukes–Cantor correction; in parentheses, standard errors obtained through 1000 bootstrap replicates, and the results of the Z test of neutrality.

S2

Evaluation of the goodness of fit for different models of codon evolution

Model	free pars	lnL	AIC	ΔAIC
M1a	2	-1836.18	3676.36	79.58
M2a	4	-1790.03	3588.05	best
M7	2	-1836.93	3677.87	81.09
M8	4	-1794.39	3596.78	best

ΔAIC – the difference between the value of the Akaike Information Criterion (AIC) of a given model and the best model.

S3

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-C7, C9), as well as interactions between region and supertypes.

Intensity	N	Final model	F	p
E. cervi/V. sagittatus	86	season	2.81	0.0976
		region	7.60	0.0072
Dictyocaulus sp.	86	body size	3.84	0.0535
		region	14.88	0.0002
		C2	7.54	0.0075
		C3	3.37	0.0701
		C4	3.87	0.0527
		C6	5.71	0.0193
		region:C3	2.28	0.1355
S. boehmi	97	region	0.07	0.7929
		C2	7.18	0.0088
		C3	4.56	0.0355
		C6	9.09	0.0033
		C9	11.94	0.0008
		region:C9	6.61	0.0118
Trichostrongylidae	117	body size	2.55	0.1133
		region	1.04	0.3110
		C1	0.65	0.4224
		C3	2.27	0.1349
		C4	4.48	0.0366
		C6	2.82	0.0960
		C9	1.45	0.2307
		region:C1	5.58	0.0200
		region:C3	3.85	0.0524
		region:C9	4.00	0.0481
Antler size	75	body size	230.27	< 0.0001
		region	2.32	0.1324
		C2	8.50	0.0048
		C4	0.75	0.3893
		C6	2.88	0.0942
		C9	0.85	0.3585
		region:C4	5.02	0.0285
		region:C9	3.19	0.0787

Prevalence	n	Final model	Chisq	p
A.sidemi	97	region	28.08	0.0001
		C4	6.05	0.0139
		C5	1.53	0.2154
		C9	8.11	0.0044
		region:C5	2.62	0.1056
O. leptospicularis	97	region	0.38	0.5372
		C5	7.30	0.0069
		region:C5	3.62	0.0572
O. kolchida	97	body size	2.49	0.1147
		region	0.87	0.3503
		C4	7.47	0.0063
		C5	3.99	0.0457
		C6	2.30	0.1293
		C9	6.53	0.0106
		region:C4	5.37	0.0205
		region:C5	5.39	0.0202
Trichocephalus sp.	145	body size	3.78	0.0518
		region	4.16	0.0413
		C4	2.95	0.0859
		C5	0.67	0.4119
		region:C4	5.87	0.0154
		region:C5	3.25	0.0713
Eimeria sp.	145	region	2.57	0.1086
		C5	3.08	0.0793

S4

Number of reads obtained per individual, number of detected alleles, and minimum number of reads required for reliable genotyping.

ID	Reads	Alleles	MinNrReads
15	1175	4	38
17	537	4	41
18	637	6	65
20	590	4	42
22	654	4	40
30	377	4	41
34	419	2	17
37	564	5	56
67	170	2	15
77	557	6	64
81	637	5	57
84	710	4	41
85	855	3	28
86	558	1	2
89	717	2	16
94	833	4	42
103	707	4	39
105	193	5	53
107	469	4	42
109	577	6	66
110	808	4	41
111	635	5	56
113	560	4	46
125	739	3	27
B001	118	3	29
B003	545	5	57
B008	730	4	41
B009	601	4	38
B017	692	5	54
B020	354	1	2
B021	879	5	54
B023	378	4	42
B029	59	4	41
B033	453	4	40
B034	551	3	27
B035	603	4	41
B038	745	6	70

B040	630	4	41
B042	716	7	87
B043	645	4	41
B044	672	6	66
B045	719	2	15
B046	598	6	66
B047	646	4	41
B049	645	4	40
B052	789	3	27
B054	389	3	27
B055	458	3	26
B056	348	3	32
B057	531	3	27
B058	341	3	27
B059	299	3	27
B060	456	3	27
B061	492	4	39
B062	583	5	56
B063	439	7	87
B064	374	4	41
B075	422	3	27
B076	329	3	27
B077	430	4	40
B078	589	5	57
B079	373	5	58
B080	452	3	27
B081	534	4	46
B083	529	4	46
B084	480	4	41
B086	339	3	27
B087	307	4	41
B088	422	3	27
B089	423	4	38
B090	453	4	57
C041	671	2	16
C042	438	1	2
C043	469	3	27
C044	700	3	28
C045	260	2	16
C046	774	6	62
C047	626	4	41
C048	611	7	87
C049	643	3	29
C050	307	4	41

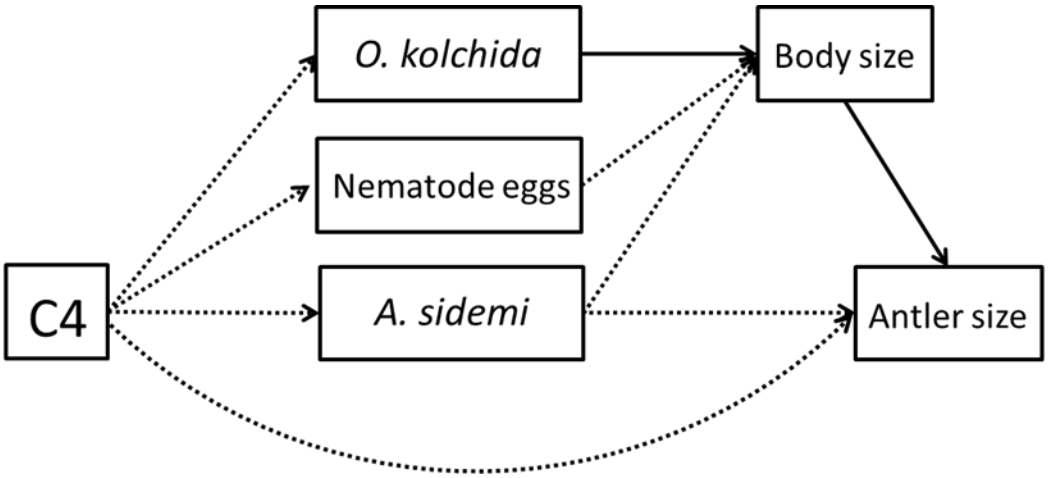
C051	543	5	58
C052	862	7	83
C055	590	5	53
C056	799	4	41
C057	583	3	27
C058	519	6	74
C062	429	4	40
C063	361	2	16
C065	502	3	27
C066	370	3	27
C067	423	4	43
C068	270	3	27
C069	483	3	27
C070	508	4	40
C076	59	6	63
C077	385	4	42
C091	599	4	41
C092	404	5	57
C093	487	4	57
C095	556	3	31
C096	439	2	15
C097	647	4	41
C100	392	4	59
C102	104	3	29
C104	550	6	68
C107	578	4	41
C111	643	5	58
C113	450	3	27
C114	520	6	68
C117	506	3	27
C119	507	4	40
C120	450	6	64
D001	409	1	2
D002	440	5	54
D004	329	5	54
D005	920	5	55
D006	1066	4	40
D007	1141	5	54
D008	1792	2	15
D009	1214	6	68
D010	937	4	59
D011	1207	4	59
D012	492	5	58
D013	270	5	54

D014	555	4	43
D015	355	4	38
D016	600	5	52
D017	310	3	31
D018	460	5	57
D020	328	2	17
D021	647	4	39
D022	305	4	41
D023	657	5	54
D024	483	3	28
D025	502	4	42
D027	598	4	41
D030	259	4	39
D031	476	5	55
D032	584	4	41
D033	669	5	54
D034	442	5	55
D035	466	2	15
D036	604	7	84
D037	383	3	27
D038	782	6	96
D039	574	3	28
D040	606	1	2
D041	813	2	16
D042	502	5	57
D044	566	5	56
D045	763	3	28
D046	644	3	27
D047	943	5	80
D048	514	4	42
D049	368	5	57
D050	822	6	68
D052	475	6	105
D053	449	5	60
D054	461	5	72
D055	500	3	28
D057	377	3	29
D058	756	4	41
D059	439	3	27
D060	899	4	41
D061	343	4	57
D062	638	6	66
D063	584	3	27
D064	239	7	86

D065	858	4	40
D067	526	4	43
D068	669	2	16
D069	809	7	84
D070	293	3	28
D072	591	5	53
D091	672	4	41
D092	580	5	54
D094	777	6	66
D095	715	6	68

Reads – coverage per amplicon after removal of artifacts; MinNrReads – simulated minimum number of reads required to achieve reliable genotyping, taking into account variation in amplification efficiency between alleles (Sommer et al. 2013).

S5



Path analysis diagram showing determinants of antler size in Piła region, including all variables which could be potentially important, as determined by significant associations between them revealed by GLMs (see chapter 2.1.6.). Solid bars represent the paths retained in the final model.

Final path analysis model testing relationships between: MHC superype C4, *O. kolchida* prevalence, *A. sidemi* prevalence Nematode eggs intenity, antler size and body size in Piła region. Usignificant paths were removed from the model based on Bayesian Information Criterion (BIC).

Path	Estimate	SE	z	p
body size←O.kolchida	0.49	0.31	1.56	0.1179
antler size←body size	2.60	0.20	13.08	<0.0001

Streszczenie

Utrzymywanie się preferencji samic w stosunku do ornamentów płciowych u samców wielu gatunków jest jednym z ciągle nierozwiązanych problemów biologii ewolucyjnej. Do tej pory z kilku zaproponowanych hipotez tłumaczących to zjawisko najbardziej popularne są te łączące wielkość ornamentu płciowego samca z jego jakością genetyczną. Zgodnie z tymi hipotezami osobniki posiadające tak zwane „dobre geny" są w lepszej kondycji i mogą sobie pozwolić na wykształcenie większego ornamentu. Z kolei samice, przez wybór do rozrodu samców o większych ornamentach, zwiększają dostosowanie swojego potomstwa. Jednak można oczekiwać, że proces ten w szybkim tempie doprowadziłby do wyczerpania zmienności w genach dostosowania i wielkości wytwarzanych przez samce ornamentów, a wówczas ocena jakości samca, przez samice, za pomocą ornamentu byłaby się niemożliwa.

W 1982 roku William D. Hamilton i Marlene Zuk zaproponowali rozwiązanie tego paradoksu. Sugerowali oni, że zmienność w genach dostosowania nigdy nie ulega wyczerpaniu dzięki niekończącemu się wyścigowi zbrojeń między pasożytami a żywicielem. Samce posiadające lepsze geny odporności nie chorują lub tracą mniej energii na zwalczanie infekcji i zaoszczędzone w ten sposób zasoby mogą przeznaczyć na wyprodukowanie większego ornamentu. Z kolei samice kojarząc się z samcami o większych ornamentach zwiększą odporność chorobową swojego potomstwa.

Pomimo przeszło 30 lat badań status hipotezy ciągle nie jest rozstrzygnięty. W niniejszej pracy prezentuję wyniki badań, testujących przewidywania hipotezy Hamiltona i Zuk w populacjach jelenia szlachetnego (*Cervus elaphus*). Testowanie hipotezy przeprowadzono zarówno za pomocą podejścia korelacyjnego w środowisku naturalnym, jak i w sposób eksperymentalny, w pełni kontrolowanych (hodowlanych) warunkach.

W pierwszej części pracy przedstawiam wyniki badań terenowych, których celem było przetestowanie hipotezy Hamiltona i Zuk w najbardziej kompleksowy sposób, czyli poprzez jednoczesne badanie genów odporności, poziomu zainfekowania i wielkości ornamentu płciowego. Badania zostały przeprowadzonych na dwóch wolnożyjących populacjach jelenia szlachetnego. W obu populacjach analizowałem związki między genami głównego kompleksu zgodności tkankowej (MHC – ang. Major Histocompatibility Complex), pasożytami płucnymi i jelitowo-żołądkowymi oraz wielkością poroża.

Wykryłem kilka istotnych statystycznie zależności między funkcjonalnymi wariantami MHC i różnymi grupami pasożytów. Niektóre warianty MHC dawały odporność, a inne zwiększały podatność na konkretny gatunek pasożyta. Wykryłem też efekty plejotropowe polegające na tym, że niektóre warianty MHC zwiększały odporność na jednego lub kilka różnych pasożytów, a jednocześnie zwiększały podatność na inne pasożyty. Dodatkowo związki między MHC a pasożytami różniły się między badanymi populacjami, czyli wykazywały interakcję genotyp x środowisko.

W przeciwieństwie do skomplikowanych relacji między MHC a pasożytami, poziom infekcji tylko jednego z badanych pasożytów (*Dictiocaulus* sp.) był skorelowany z wielkością poroża. Co więcej, związek ten był pozytywny i nawet wykrycie wariantu MHC (C2) nadającego odporność na tego pasożyta nie zmienia faktu, ze wynik ten jest przeciwny do przewidywań hipotezy Hamiltona i Zuk.

Praca na dzikich populacjach ma tą zaletę, że badamy zwierzęta bezpośrednio w ich naturalnym środowisku. Jednocześnie to naturalne środowisko jest źródłem wielu niedających się kontrolować czynników, które mogą maskować badane związki między pasożytami a porożem, a przez to utrudnić interpretację wyników. Dlatego dodatkowo przetestowałem hipotezę Hamiltona i Zuk również w warunkach kontrolowanych, manipulując poziomem infekcji.

Samce z eksperymentalnej hodowli utrzymywanej w Instytucie Nauk o Zwierzętach w Pradze przydzieliłem losowo do dwóch grup. Jeleniom z jednej grupy podawano lek przeciwko robakom (tasiemcom i nicieniom), natomiast osobniki z drugiej grupy otrzymywały zastrzyk z soli fizjologicznej. Zabieg ten był powtarzany co miesiąc przez cały okres wzrostu poroża, czyli od marca do września. Jelenie, którym aplikowano lek miały istotnie mniej pasożytów jelitowo-żołądkowych niż osobniki, którym podawano placebo. Jednak nie wpłynęło to w żaden sposób na masę ciała i wielkość poroża badanych jeleni.

Podsumowując, zarówno wyniki badań populacji naturalnej, jak i eksperymentalne badania populacji utrzymywanej w zagrodzie, nie są zgodne z przewidywaniami hipotezy Hamiltona i Zuk. Ko-ewolucja między pasożytami a żywicielami nie wydaje się więc główną siła odpowiedzialną za ewolucję ornamentów płciowych u jelenia szlachetnego.

Acknowledgements

This thesis would not be possible without the help and support of many people, to whom I am grateful and would like to thank here.

First I would like to thank my supervisor, Prof. Jacek Radwan, for the idea of the project, assistance in managing it, and resolving problems that arose during study. His experience and knowledge were invaluable during the data analysis and text writing.

Secondly I would like to thank Prof. Henryk Okarma for contact with Regional Directorates of State Forests and help in organizing fieldwork; and Prof. Aleksander W. Demiaszkiewicz for training me in parasite identification and help with more complicated cases.

I would like to thank all workers of Bircza and Lipka Forestry involved in sample collection, especially Józef Czwerynko and his sons, and Janusz Bujacz.

Experimental study would not be possible if not for Prof. Ludek Bartos and Dr Adam Dusek from Charles University, who enabled me to work with red deer population maintained by Institute of Animal Science.

I am grateful to my friends, Dr Kasia Bojarska and Isabel Elguero for invaluable help during the first, hard year of fieldwork. In molecular laboratory I could always count on Kasia Dudek and Dr Wiesław Babik for assistance and during struggle with data analysis I could always rely on advices and tips from Dr Zosia Prokop, Dr Edyta Sadowska, Ania Skrzynecka and Ewa Prawdzik. And finally, this thesis would not be so well written without the help from Tereza Horvathova, who mercilessly pointed out all the weaknesses of the text.