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DISSERTATION

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„Costs and benefits of genetic mate choice“

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General Introduction

The main focus of my project was to investigate the relationship between the genetic make-up at the major histocompatibility complex (MHC) and different life-history traits in house sparrows (*Passer domesticus*) to test if mating with a "MHC compatible" and/or MHC heterozygote partner provides higher fertilization success and superior immunocompetence and survival in offspring. In many species females are highly selective when choosing a mate, because in general, they invest more into reproduction compared to the male (Andersson 1994, Kokko et al. 2003). Via mate choice females may gain direct benefits, like paternal care, but also indirect benefits in the form of e.g. "good genes" that could provide superior immunocompetence in the offspring (Birkhead 2000, Jennions and Petrie 2000, Tregenza and Wedell 2000, Kokko et al. 2003).

The MHC is important for an appropriate immune response against pathogens by encoding proteins that present foreign peptides to T-cells (Klein 1986). It has become a major study subject of evolutionary and ecological immunology across a large variety of taxa (Sommer 2005, Piertney and Oliver 2006, Spurgin and Richardson 2010). The highly polymorphic genes at MHC loci are believed to be under some form of balancing selection maintaining the high variation, such as pathogen-driven selection (Penn et al. 2002). Immunocompetence has already been shown to be associated with genes of the MHC where MHC heterozygosity can lead to a slower development, more effective clearance and reduced pathogenicity of numerous bacterial and viral infections or parasites in a multitude of vertebrate species. Specific MHC alleles can provide higher resistance/susceptibility or varying severity to numerous diseases (reviews by: Sommer 2005, Piertney and Oliver 2006).

MHC genes have already been shown to play an important role in mate choice in fish, birds and mammals (review in Piertney and Oliver, 2006, Kamiya et al. 2014). There are three different hypotheses of MHC-dependent mate choice. Females might prefer males with specific MHC alleles, heterozygote ones or males dissimilar from themselves. Males with specific MHC alleles can provide resistance to particular parasites (Penn and Potts 1999). Males with high allelic diversity of MHC alleles are able to resist a broader range of pathogens compared to homozygotes and so picking a heterozygote partner should be rewarded by increased immunocompetence in the partner, but also in the offspring (Brown 1997, Apanius et al. 1997, Penn 2002, Hughes 1992, Milinski 2006). Mate choice

for dissimilar males can be beneficial to prevent inbreeding and enhance heterozygosity in offspring (Brown 1997, Tregenza and Wedell 2000, Penn 2002), but with no MHC alleles shared, the risk of outbreeding depression increases and the disruption of co-adapted genes might lead to a severe fitness reduction (Thornhill 1993, Hendry et al. 2000).

Mate choice strategies might also influence fertilization success in pairs. It is quite common to find unhatched eggs in clutches of most bird species and this could be due to embryonic mortality, but also infertility (Koenig 1982). In Chapter I we investigated whether fertility success of mates might depend on male or female MHC composition. Fertilization success was measured as the maximum number of sperm counted on the perivitelline layer (PVL) of eggs (Birkhead et al. 1994) in a captive house sparrow population. Fertilization takes place in the female tract, where it is essential for females to separate poor from good quality sperm/mates (cryptic female choice). In this context female reproductive behavior, physiology and anatomy have evolved in response to previous male adaptations to control fertilization (Birkhead and Møller 1998).

During postcopulatory sexual selection, sperm interact with the female reproductive tract at the cellular level (Zeh and Zeh 1997). As a major role of the MHC in the immune system is to separate own tissue from foreign, it is likely that the extent at which sperm from different males is perceived as non-self depends on their genetic make-up. Consequently, an anti-sperm response could be a mechanism to ensure fertilization only by sperm with a fitting genotype (Birkhead et al. 1993). Sperm transport seems to be affected by the MHC genotype in mice, with more sperm of dissimilar males reaching the oviduct (Nicol and McLaren 1974). In fact, proteins that are coded for by MHC alleles are expressed on the membrane of sperm (Arnaiz-Villena and Festenstein 1976, Martin-Villa et al. 1999). There is evidence of an immune response against sperm that reduces fertility in humans, reptiles, fish and birds (Naz and Menge 1994, Wittzell et al. 1999, Skarstein et al. 2005, Birkhead and Brillard 2007).

One question that arises in line with this is, how important is the immune system for fertilization success, in particular the MHC of an individual male or female? If females with a superior immune system (many MHC alleles) are able to resist a wider range of pathogens, one might assume that they also initiate immune responses against a wider range of molecules expressed on the sperm surface of mating partners, especially when

they are genetically dissimilar from the female and hence detected as non-self. A possible way to reduce a drawback of possessing a very effective immune system on fertilization success could be that females choose a MHC similar mating partner. Therefore we predict that fertilization is highest in females with low MHC heterozygosity and pairs that are more similar at the MHC. Also, specific MHC alleles could have an influence on fertilization.

Apart from infertility, another reason for hatching failure of eggs is embryonic mortality. In Chapter II we tested if hatching failure, survival and growth in young nestlings depend on MHC heterozygosity, specific MHC alleles or MHC compatibility in parents. Many factors can influence embryonic mortality, survival and growth rates of young chicks, including genetic and environmental factors, such as stress, maternal condition, nutrition and microbial infections (Hemmings et al. 2012, Beissinger et al. 2005, Christensen 2001, Kempenaers et al. 1999, Monaghan and Nager 1997, Webb 1987, Møller 1994a).

In birds it is possible to make detailed observations of some of these factors since embryonic development is taking place outside the female. There are numerous studies on the negative effects of microorganisms on poultry egg development (Romanoff and Romanoff 1972, Board 1966, Bruce and Drysdale 1983), but little is known about their effect on embryo mortality in free-living birds (Bernard 1989) and on growth and mortality of young, especially altricial nestlings (Box 1967).

Embryonic mortality can also be result of genetic incompatibility between parents. Incompatibilities may result from in- or outbreeding (Lodge et al. 1971, Kempenaers et al. 1996, Christensen 2001, Sellier et al. 2005, Birkhead and Brillard 2007). In great reed warblers (*Acrocephalus arundinaceus*), hatching success of eggs was lower when pairs were genetically more similar (Bensch et al. 1994). In human and primate pairs who share MHC alleles, homozygote offspring are underrepresented, possibly because of *in utero* selection against homozygotes (Hedrick and Black 1997, Knapp et al. 1996, Komlos et al. 1977). We predict that hatching success and survival is highest with low MHC similarity in parents, high MHC heterozygosity in chicks and also that specific MHC alleles are advantageous or disadvantageous. We further expect that offspring with high MHC heterozygosity or specific MHC alleles may have advantages/disadvantages in terms of growth.

The aim of Chapter III was to test if the MHC is a suitable tool to measure and quantify immunocompetence and to demonstrate that mate choice for the right partner provides higher immunocompetence in offspring. Immunocompetence is an important determinant of fitness and indicates the ability of a host to prevent or control infection by pathogens (Altizer et al. 2003, Bernatchez and Landry 2003). It remains challenging to identify suitable methods to "measure" immunocompetence in non-model species because single immunological tests can only explain a small part of the immune defence and our knowledge about interactions within the immune system is still limited (Schmid-Hempel and Ebert 2003, Norris and Evans 2000, Keil et al. 2001).

The immune system can be divided into three main components: innate, cell-mediated and humoral immunity. To properly mirror immunocompetence, we conducted several immunological tests that correspond to the innate and the adaptive (cell-mediated and humoral) defence, one was done repeatedly in different life cycles. Also survival rate was monitored as an indicator of fitness.

Especially genes that are involved in the immune defence could provide important information and serve as tools for further investigations. In particular, genes of the MHC could be suitable, because they are present in almost all vertebrates and they play a central role in the immune system and pathogen resistance (Klein 1986). More specifically, they are responsible for cell-mediated immune responses that are mediated by T-lymphocytes that either regulate the function of B-lymphocytes and phagocytes or destroy infected host cells through interactions with antigens present on the surface of these cells (Roitt et al. 1998). We predict that one year old birds with intermediate to high MHC heterozygosity will have higher immunocompetence and also specific MHC alleles might be advantages or disadvantages.

In Chapter IV we investigated whether the expression of a sexually selected male ornament may constitute an indicator of health status and MHC based resistance against parasites. The black throat badge (or bib) of male house sparrows is a melanin-based ornament important in sexual selection. This "badge of status" serves to impress conspecific rivals or potential mating partners (Rohwer 1975, Møller 1987, Owens and Hartley 1991, Johnstone and Norris 1993, Liker and Barta 2001, Gonzalez et al. 2002, Nakagawa et al. 2007).

Female choice is thought to be responsible for the evolution of exaggerated ornaments, frequently observed in males. An excessively (notoriously) stressed example in this context is the male peacock tail (Petrie et al. 1991). Bigger or brighter ornaments could by itself be attractive (Weatherhead and Robertson 1979) leading to more attractive sons (Saino et al. 1997, Petrie et al. 1991). However a key issue in mate choice is whether ornaments are informative about the quality of the bearer e.g. health status reflected by immune system quality (Dufva and Allander 1995, Figuerola et al. 1999). A superior immune system for example may lead to viable offspring (Norris 1993, Møller 1994b) with increased disease resistance (Hamilton and Zuk 1982, Andersson 1986, Møller 1990, Clayton 1991).

Although a preference for brightly colored males has been reported in many species, the underlying purpose of this preference is not fully understood. Pale males, with respect to carotinoid-based ornaments for example, have been shown to suffer from parasitism and seem to be of low health status (review: Møller et al. 2000). In this context, Hamilton and Zuk (1982) proposed that plumage coloration has evolved as an honest signal of parasite and disease resistance and results in a negative relationship between parasite levels and ornament expression since only the healthiest individuals can afford the costs of developing a sexually selected trait. However, that an ornament may provide specific information about individual resistance towards a specific disease (parasite) as well as the underlying genetics has not been found so far. We identified a common threat, the avian malaria strain SGS1, and tested if specific MHC alleles and overall heterozygosity may provide resistance. Our prediction is that the black throat badge in house sparrows reflects parasite resistance and signals individual quality.

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Chapter I

Is more always better? The role of MHC heterozygosity and compatibility on fertilization success in house sparrows (*Passer domesticus*)

Manuscript by

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Is more always better? The role of MHC heterozygosity and compatibility on fertilization success in house sparrows (*Passer domesticus*)

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Abstract

Sexual conflict over fertilization is a major driving force for the evolution of defence mechanisms by creating a hostile environment in the female genital tract against male sperm. In this context little is known about the role of the immune system. Here we explore whether the immune system in particular the major histocompatibility complex (MHC), is important for fertilization success. Individuals that are heterozygote at MHC loci can bind more peptides compared to homozygotes, but when it comes to fertilization success, a weaker immune system with less MHC alleles in females might be beneficial for sperm. If females perceive sperm as antigens, it is likely that females with high numbers of different MHC alleles may also be very efficient in detecting and defending off more sperm. We used an aviary population of wild house sparrows (*Passer domesticus*) and removed the first clutch of all breeding individuals to count the number of spermatozoa on the inner and outer perivitelline layer of the egg to study the fertilization success. We sequenced alleles of MHC class I and analyzed individual heterozygosity (number of MHC alleles at the nucleotide level) and compatibility of pairs (percentage of shared alleles, genetic and functional distances). We found that females with low MHC heterozygosity, males with a specific allele and pairs whose alleles were more similar in the peptide binding region had the most sperm on their eggs and therefore the highest fertilization potential. Our data suggest that females with a strong immune system may suffer from lower fertilization success, which might be reduced by selecting sperm from more similar males.

Introduction

In many species females are highly selective when choosing a mate, because in general, they invest more into reproduction compared to the male (Andersson 1994, Kokko et al. 2003). In line with this a superior male immune system seems to be one important criterion in the mate choice process (Birkhead 2000, Jennions and Petrie 2000, Tregenza and Wedell 2000, Kokko et al. 2003). Immunocompetence is a good predictor of fitness (Altizer et al. 2003, Bernatchez and Landry 2003, Ziegler et al. 2005) and has been shown to be associated with genes of the major histocompatibilty complex (MHC) (Sommer 2005, Piertney and Oliver 2006, Eyto et al. 2007). The MHC region is highly polymorphic where every MHC molecule can bind a limited number of peptides and individuals that are heterozygote at the MHC are considered to have a selective advantage (Brown 1997, Hughes 1992, Milinski 2006).

MHC genes in fact, have already been shown to play an important role in mate choice in fish, birds and mammals. There are three different hypotheses of MHC-dependent mate choice. I) High allelic diversity and heterozygosity of MHC alleles will be favored because the MHC molecules of a heterozygote individual can bind a wider range of pathogens compared to homozygotes and so picking a heterozygote partner should be rewarded by increased immunocompetence in the partner, but also in the offspring. II) Genetic compatibility between partners (similarity or dissimilarity) at the MHC seems to be important. A dissimilar partner increases heterozygosity in the offspring, but also specific haplotypes perform better together in certain combinations. III) Specific rare or new alleles will be favored when facing co-evolving parasites (reviews in: Piertney and Oliver 2006, Kamiya et al. 2014).

However to understand mate choice processes it is essential to clarify the role of male and female immune system, in particular MHC, in the fertilization process. Fertilization takes place in the female tract, where it is essential for females to separate poor from good quality sperm/mates (cryptic female choice). In this context female reproductive behavior, physiology and anatomy have evolved in response to previous male adaptations to control fertilization (Birkhead and Møller 1998). During postcopulatory sexual selection, sperm interact with the female reproductive tract at the cellular level (Zeh and Zeh 1997). As a major role of the MHC in the immune system is to separate own tissue from foreign and since the immune system can distinguish between proteins that only differ by a single amino acid (Alberts et al. 1994), it is likely that the extent at which sperm from different

males is perceived as non-self depends on their genetic make-up. Consequently an anti-sperm response could be a mechanism to be involved in fertilization.

One question remains whether there is reasonable evidence about the mechanisms how an anti-sperm response could work in relation to fertilization success and control? As the whole process takes place in the female tract we could think of several possible mechanisms and locations where the immune system in particular MHC related processes may be involved. For instance one can identify two major defence lines, one in the infundibulum during the movement of the sperm to the egg, where the female immune system may regulate the recognition and consequently the defence of sperm. A second process may be related to the genetic compatibility of mating partners which may influence the interaction between sperm and ovum.

Sperm transport seems to be affected by the MHC genotype in mice, with more sperm of dissimilar males reaching the oviduct (Nicol and McLaren 1974). In fact, proteins that are coded for by MHC alleles are expressed on the membrane of sperm (Fellous and Dausset 1970, Arnaiz-Villena and Festenstein 1976, Martin-Villa et al. 1999). Consequently an anti-sperm response could be a mechanism to ensure fertilization only by sperm with a fitting genotype (Birkhead et al. 1993). There is evidence of an immune response against sperm that reduces fertility in humans, reptiles, fish and birds (Naz and Menge 1994, Wittzell et al. 1999, Skarstein et al. 2005, Birkhead and Brillard 2007).

One question that arises in line with this is, how important is the immune system for fertilization success, in particular the MHC of an individual male or female? If females with a superior immune system (many MHC alleles) are able to resist a wider range of pathogens, one might assume that they also initiate immune responses against a wider range of molecules expressed on the sperm surface of the mating partners, especially when the fertilization partners are genetically dissimilar from the female and hence detected as non-self. Thus one might predict that a too strong immune response in females, probably from being highly diverse at MHC, may have a negative effect on fertilization success. A possible way to reduce a drawback of possessing a very effective immune system on fertilization success could be that females choose a MHC similar mating partner, in particular at the peptide binding region (PBR) of MHC alleles (Schwensow et al. 2007, Sette and Sidney 1999). This would mean that the two classical hypotheses to explain MHC-based mate choice (choice for MHC heterozygote or

compatible mates) can apply within a species and females may use different mating tactics depending on their immune system.

Therefore we investigate whether fertility success of mates might depend on male or female MHC diversity, but also on compatibility (similarity) of MHC alleles in pairs, as well as a potential role of specific alleles, which e.g. has been found in fish, where one MHC allele was associated with increased fertilization (Skarstein et al. 2005). Based on our earlier formulated predictions we hypothesize that fertilization success should be highest for 1) females with low MHC diversity, 2) pairs with intermediate to high similarity (especially at the PBR) and 3) individuals with specific MHC alleles. To test our predictions on MHC and fertilization we conducted breeding experiments with a captive house sparrow population.

Methods

Breeding population and experimental design

In 2010, wild house sparrows (*Passer domesticus*) were captured in Austria and held in large outdoor aviaries (aviary size: 3.5 m × 3.5 m × 3 m) at the Konrad Lorenz Institute of Ethology, Vienna. House sparrows are widespread colony breeders with high sexual dimorphism. In 2013, 61 males and 58 females were put together in mixed flocks (3-5 breeding pairs/aviary) in 14 aviaries that were equipped with nest boxes and nesting material. All aviaries were equipped the same way with vegetation and several perches. Commercial food for granivorous passerines and water were provided *ad libitum*. Our aviary population allowed us to control for several environmental factors that might interfere with our results, like food availability, nest box quality and availability, predation and differences in microclimate as well as socio ecological factors including breeding density and sex ratio. Additionally, our set-up enabled us to remove sufficient numbers of clutches for sperm counts and to monitor future fertilization and hatching success in these pairs.

Birds were mixed on April 27th and data about the start of egg laying and clutch size was collected. We removed the first clutch of all breeding pairs (n=48) to count the number of sperm on the inner and outer perivitelline layer (PVL) of the egg. Additionally, we collected complete clutches from six pairs (>10%) a second time to compare repeatability

of sperm counts throughout the breeding season. The amount of sperm was measured for all eggs in all clutches since sperm number fluctuated highly within a clutch. The unincubated eggs were collected from the nest one day after the clutch was completed, stored at 4°C and opened within a week. Egg contents were preserved in formalin (5%) for detailed examination. For each egg, the germinal disk was collected to extract DNA and the PVL of each sample was cleaned and examined for the presence of sperm by staining their nuclei with the fluorescent Hoechst dye 33342 (Sigma-Aldrich).

We examined fertilization success by counting the number of sperm on the PVL of the egg which correlates closely to the sperm inseminated, stored and present near the site of fertilization (Wishart 1987, Birkhead et al. 1994, Birkhead and Fletcher 1994). This is a standard counting technique described in Birkhead et al. (1994). Sperm counts were carried out by K.M. and J.A. blindly for the predictions. For each clutch, we used the maximum number of sperm found in one egg since this measurement has also been used in other studies of fertilization success (e.g. Løvlie et al. 2013). Birds were allowed to breed again to determine hatching success (n=45). Since it is rather difficult to distinguish between infertility and early embryo mortality, especially if mortality occurred before any visible signs of embryo development were apparent (Birkhead et al. 2008), eggs were categorized simply as hatched or unhatched eggs. If possible, tissue from embryos was collected to extract DNA from it. Several body parameters were measured in adult birds (badge size in males, wing length, weight and tarsus in males and females) to control for assortative mating preferences. Blood was taken from the brachial vein in adults and nestlings during the breeding season for DNA. All animal experiments were in accordance to Austrian Law (Geschäftszahl: BMWF-68.205/0081-ll/3b/2012).

Molecular methods

Avian blood, germinal discs and tissue samples were stored in 95% ethanol and DNA was extracted using the DNeasy Blood & Tissue Kit (Qiagen).

MHC genotyping

MHC characterization was carried out using 454 amplicon sequencing. MHC class I exon 3 sequences that encode parts of the PBR in MHC molecules were amplified using individually tagged 454-adapted primers (according to Galan et al. 2010). The MHC-specific primer stretches used were forward primer (longfw2):

GTCTCCACACTGTACAGYGGC; and reverse primer (rv3): TGCGCTCCAGCTCCYTCTGCC) (Karlsson and Westerdahl 2013). These primers amplify 222-225bp long (primers not included) classical MHC alleles in house sparrows (Karlsson and Westerdahl 2013). Using Qiagen Multiplex MasterMix each 15µl PCR reaction contained 7.5µl of Hot Start Master Mix, 0.6µl (5µmol) each of the forward and reverse primer, 1µl of template (25ng genomic DNA) and 5.3µl dd H_2O. The PCR conditions were: 95°C/15 min, 35x(95°C/30 s, 65°C/1 min, 72°C/1 min), 60°C/5 min. PCR products were run on a 2% agarose gel and products with positive amplifications were purified using the MinElute PCR Purification Kit (Qiagen). Samples were pooled in eights and DNA was quantified with a Nanodrop instrument and adjusted accordingly before a second pooling for the 454 quadrants. The number of samples in the 454 quadrants was aimed at a sequence coverage of 300 reads per individual (according to Borg et al. 2011). 491 individuals were run in two different sequencing reactions (run 1 (R1) and run 2 (R2)) and these runs were filtered separately (see below). R1 contained 272 samples (243 individuals + 29 technical replicates (11.9%)) and R2 contained 286 samples (248 individuals + 38 technical replicates (15.3%)). 454 sequencing was done at Lund University Sequencing Facility (Faculty of Science), Sweden.

MHC - bioinformatics and data processing

After 454 sequencing, the data was extracted and assigned to samples using the program jMHC (Stuglik et al. 2011). To get rid of artefacts generated during the initial PCR, the emulsion PCR and the 454 sequencing reaction the raw data from the 454 run was filtered. Filtering steps were in accordance with Galan et al. 2010, Zagalska-Neubauer et al. 2010 and Sepil et al. 2012.

First, sequences in low abundances (<3 reads) were deleted. Identical sequences within individuals were detected and merged using the web-applications "seqeqseq" (http://mbio-serv2.mbioekol.lu.se/apps/seqeqseq.html) and "mergeMatrix" (http://mbio-serv2.mbioekol.lu.se/apps/mergeMatrix.html).

Second, we only wanted to keep sequences with a suitable sequence depth. Since the maximum number of classical MHC alleles is eight in house sparrows we chose a threshold of 104 reads per sample, this gives a genotype score of 99.9% (m=8, Galan et al. 2010). Here we used the web-application "popMatrix" (http://mbio-

serv2.mbioekol.lu.se/apps/popMatrix.html) and filter 1. This web application has several filers, filters 1-4.

Third, true alleles are likely to occur more frequently than artifact alleles. Therefore we wanted to set a threshold that keeps true alleles but disregards artifact alleles. We used our replicates to set these thresholds (2% in R1 and R2) and all alleles that occurred in lower than 2% per sample were deleted using filter 3.

Forth, to verify MHC alleles they should occur in at least two independent PCRs. We worked with family data (189 adults produced about 300 chicks), these individuals were distributed across R1 and R2) and every allele is therefore likely to occur several times. Therefore we used the criteria that every allele should be found at least twice, here we used filter 4.

Fifth, the obtained sequences were examined in BioEdit (v7.2.0) and all sequences that did not meet the expected length of 222-225bp or displayed indels that were not multiples of three base pairs were deleted. The next step was to look for PCR recombinants (chimeras) and 1-bp substitutions. Sequences were examined starting with the lowest frequencies at the population level (Sepil et al. 2012). All sequences that occurred in a frequency below 2% at the population level were checked in all individuals for chimeras and 1-bp substitutions. 50% of the sequences between a frequency of ≥ 2% to 25% were checked but no chimeras or 1-bp substitutions were found. Therefore, we assume that sequences with a frequency of ≥ 2% are true alleles. Additionally, alleles were verified and controlled for with inheritance data of families (189 adults produced about 300 chicks).

The filtered MHC DNA sequences were translated into amino acid sequences. These sequences were translated according to the chemical binding properties of the amino acids in the PBR. The 16 amino acids from each sequence corresponding to the PBR in chicken (Wallny et al. 2006) were extracted and converted into five physicochemical descriptor variables: $z1$ (hydrophobicity), $z2$ (steric bulk), $z3$ (polarity), $z4$ and $z5$ (electronic effects) (Sandberg et al. 1998).

The genetic MHC distances between parents were calculated from a maximum-likelihood tree (2000 bootstraps) that was inferred for all unique translated MHC sequences (outgroup MHC class I from chicken, GenBank Acc nr AB159063) using the RAxML

software (ver 7.0.4) under the PROTMIX model and the JTT substitution matrix, with default settings (Supplementary Fig. 1). Pairwise overall MHC amino acid distances between individuals were then computed from this tree with the software Fast UniFrac (Hamady et al. 2010) available at http://unifrac.colorado.edu. Functional distances between MHC genotypes of parents (reflecting the difference in what pathogen antigens can be bound and detected between parents) were assessed by calculating the difference in chemical binding properties of the amino acids in the PBR. The unique set of functional PBR sequences (represented by the *z*-descriptors) was used to construct alternative maximum-likelihood trees with ContML in the PHYLIP-package (v. 3.69). The tree was rooted as before with the outgroup *Gallus gallus* (AB159063) (Supplementary Fig. 2). This tree represents clusters of functionally rather than evolutionary similar MHC sequences. Pairwise overall MHC functional distances between individuals were computed from this tree in the same way as for the amino acid distances (UniFrac).

MHC diversity was evaluated as: 1) the number of unique sequences/alleles per individual 2) the proportion of sequences/alleles shared in breeding pairs (=[100/number of shared sequences]/[total number of sequences male + female]) 3) genetic sequence distance in breeding pairs 4) functional distance in breeding pairs (distance measures according to Strandh et al. 2012) and 5) the occurrence of the most common sequences/alleles (> 10% at the population level) within a pair (0=no one has it, 1=only males have it, 2=only females have it, 3=both have it).

Parentage and genome-wide genetic variation

All individuals were typed at 13 highly polymorphic neutral microsatellite loci, for the assignment of biological parents and an average estimate of genome-wide individual heterozygosity in adults. Parentage analysis was conducted manually and the quality of loci (Hardy-Weinberg Equilibrium and Linkage Disequilibrium) was tested using the programs Cervus (v3.0.3) and Fstat (v2.9.3.2). Individuals were genotyped in two multiplex reactions according to the protocol of Dawson et al. (2012) (Supplementary Table 1). The loci used in this protocol are known to be distributed across at least seven different chromosomes and thus likely reflect the genome-wide level of neutral genetic variation (Dawson et al. 2012). Forward primers were fluorescently labeled using different dyes (YYE, FAM or AT550, Microsynth). The two multiplex PCRs were carried

out using a Multiplex Kit (Qiagen) in a final PCR reaction volume of 6µl containing 3µl of the Qiagen enzyme, 2µl of the primer mix and 1µl of the template (25ng genomic DNA). PCR cycling scheme and primer concentrations of the 2 multiplex PCRs were carried out according to Dawson et al. (2012). PCR products were diluted (1:20) and analyzed in an ABI genetic analyzer (Applied Biosystem 3130xl). Allele sizes were determined using GeneMapper (v3.0) with reference to an in-house DNA Size Standard (HMROX). From these 13 loci, Pdoµ6 had to be excluded due to amplification problems and Pdo40A was removed because it deviated from the Hardy-Weinberg Equilibrium (Supplementary Table 1).

Statistical analyses

Correlation of variables: The following variables were highly correlated and only one of them was used for further analysis: All sperm counts were transformed (square-root) and the maximum number of sperm/clutch correlated with the lowest and average number of sperm/clutch (linear regression: $p<0.001$ and $p<0.001$). The number of MHC alleles/individual correlated with the number of functional MHC alleles/individual in males and females (linear regression: $p<0.001$ and $p<0.001$). The proportion of MHC alleles shared in a breeding pair was transformed (arc-sin) and it highly correlated with the proportion of functional MHC alleles shared (linear regression: $p<0.001$).

Controlling for mate choice: We tested if sperm counts were related to partner attractiveness, which could potentially affect results about the influence of the MHC on fertility. We used male and female body size (tarsus and wing length, weight and badge size) as indicators of attractiveness. Females might store more sperm of an attractive partner to ensure a high probability of fertilization and males might invest more sperm for an attractive female. Attractiveness can also indicate good health, and healthy males might be able to produce larger ejaculates or copulate more frequently. We tested these relationships with a linear regression. Since individuals had only limited possibilities for mate choice (only a few individuals per aviary), the experiments were unlikely to be confounded by assortative mating. However, to make sure, we tested the following variables between males and females in a breeding pair: number of MHC alleles, microsatellite heterozygosity, tarsus length, wing length, weight and badge size (males) (linear regression). To assure that there is no correlation between the number of their MHC alleles and body parameters, we tested if the number of MHC alleles correlated

with the following measurements (linear regression): tarsus length, wing length, weight and badge size (males). We found no correlation between sperm counts and any measure of partner attractiveness ($p>0.05$ in all cases). We therefore conclude that our breeding population was not mating assortatively with respect to MHC alleles or different body parameters.

Aviary effect: We tested whether there was a random aviary effect of the 14 different breeding aviaries on the number of MHC alleles in males and females, number of shared MHC alleles between parents, and genetic and functional distances between mates (One-Way Anova). We did not find an aviary effect on these different MHC variables ($p>0.05$ in all cases).

Repeatability of sperm counts: To test if sperm counts are repeatable for a pair throughout the breeding season, six clutches were analyzed a second time and repeatability was tested with a linear regression.

Fertilization potential: We tested if the amount of sperm on the eggs reflects fertilization potential. Therefore we analyzed if the maximum amount of sperm (square-root transformed) in the first clutch correlated with the proportion of hatched chicks (arc-sin transformed) in the second clutch (linear regression).

Factors influencing the amount of sperm on the egg: We used linear models within the R package v.2.15.3 x32 (R Core Team 2013) to analyze the effect of different variables (number of MHC alleles in males and females, microsatellite heterozygosity in males and females (arc-sin transformed), genetic and functional MHC distance (arc-sin transformed), proportion of MHC alleles shared (=[100/number of alleles shared]/[number of alleles male + female]), arc-sin transformed), start of egg laying (week 1 to 7), number of eggs/clutch and the occurrence of the 11 most common MHC alleles (>10% at the population level) in a breeding pair as categorical variable: no one has the allele (0), only the male has it (1), only the female has it (2), both have it (3) on the amount of sperm found on the PVL. However, this approach raised concerns with over-fitting. In order to evaluate and compare different models, we used the Akaike Information Criterion corrected for small sample sizes (AIC_c, Burnham and Anderson 2002). In all cases there was not one clearly best model, so we used methods of model averaging and multimodel inference (Burnham and Anderson 2002). These methods allow inference over all models considered, but this was weighted according to model

support by the data. Additionally, these methods do not only estimate standard errors unconditionally for a single model, they also provide the probability for single variables being in the unknown "true" model (the so-called relative variable importance - RVI). Unlike variable selection based on p-values these techniques have a sound mathematical basis and are increasingly recommended (Symonds and Moussalli 2011). We conducted these calculations in R using the package MuMIn (Bartón 2013). Additionally, we inspected the residuals for normality visually using histograms and QQ-plots.

Before we included the 11 most common MHC alleles in our analysis, we had to do a pre-model selection with the rest of our variables. Only four variables with RVI values larger than 0.5 were kept in the analysis (number of MHC alleles in females, functional MHC distance, start of egg laying and number of eggs/clutch). As a next step we evaluated the relevance of the 11 alleles, while the other four variables were fixed. We only included one MHC allele in the final model that had an RVI value larger than 0.5 ('seq21013'). The final model consisted of 5 variables: number of MHC alleles in females, functional MHC distance, start of egg laying, number of eggs/clutch and occurrence of the MHC allele seq21013 in a breeding pair.

Results

MHC characterization

We had a total of 75894 sequence reads of 19200 unique sequences in 454 run 1 (R1) that had complete tags and primers. In run 2 (R2) we had a total of 125752 sequence reads of 34670 unique sequences. After filtering the data, a total of 85 different MHC alleles were found (Allele frequencies and GenBank Acc nr in Supplementary Table 2). In both datasets, there was an average of 4.7 alleles/individual (sd: 1.5 (R1) and 1.4 (R2)) ranging from 2-8 (R1) and 1-8 (R2) alleles/individual. Males and females had on average 4.4 alleles (sd males: 1.4, sd females: 1.4). Adults possessed on average 4.8 alleles (sd: 1.5), chicks had 4.4 (sd: 1.4). Of the technical replicates, 20 of 28 (R1) and 29 of 37 (R2) showed a complete match. We also found 134 of 142 (R1) and 164 of 180 (R2) alleles in both replicates.

Fertilization and sperm counts

Sperm on eggs: The first eggs were laid within a week of placing pairs together in aviaries (week 1-7; average: 2.2; sd: 1.2). The first clutch from 48 pairs was collected and opened (182 eggs; 1-5 eggs/clutch; average 3.8; sd: 1.0) and sperm on the PVL counted. Eggs contained from 0-291 spermatozoa (average: 49.3; sd: 32.2). Of the 48 pairs, 45 produced a second clutch (217 eggs; 1-6 eggs/clutch; average 4.8; sd: 1.0). Extra-pair paternity was not found in any clutch and we therefore assumed that the sperm counted on eggs belonged to the social father. Of the 48 pairs, seven males and one female also had a clutch with another partner. Since results are based on compatibility between different individuals, no clutches or individuals were removed.

Repeatability of sperm counts: Clutches from six pairs were collected a second time to compare repeatability of sperm counts throughout the breeding season. They were found to be highly repeatable ($p=0.035$, $R=0.972$) proving that this is an accurate method to determine fertilization success in pairs.

Fertilization potential: We tested whether the maximum number of sperm for a specific pair in the first clutch was correlated with the proportion of hatching chicks in their second clutch. Proportion of hatching chicks (in relation to laid eggs) was positively correlated with the amount of sperm found on the PVL (linear regression: $p=0.049$, Fig.1). These results show that the maximum amount of sperm per clutch is a good measure of fertilization potential in a pair.

Factors explaining the number of sperm in the egg: Using a linear model (Table 1) we found that the amount of sperm on the PVL was positively correlated with the number of eggs laid ($p<0.001$, RVI=1.00, Fig. 2a), but negatively correlated with the start of egg laying ($p<0.001$, RVI=1.00, Fig. 2b), the number of MHC alleles in females ($p=0.012$, RVI=0.91, Fig. 2c) and the functional MHC distance of pairs ($p=0.009$, RVI=0.92, Fig. 2d). For MHC distance, there might be an optimum between 0.6 and 0.8 (Fig. 2d). Also the occurrence of the MHC allele ‘seq21013’ negatively correlated with the amount of sperm on the PVL, especially when only males in a breeding pair possessed this allele ($p=0.004$, RVI=0.91, Fig. 2e). This allele was the second most common in this population with a frequency of 35.8%.

Discussion

We could demonstrate that fertility in pairs is influenced by the MHC genotype. More precisely, fertilization success decreases with female MHC heterozygosity. This is, furthermore to our knowledge the first evidence for a sex specific effect of the immune system related to the MHC region on fertility. Beside the negative effect of the number of female MHC alleles on actual fertilization success, we also found an effect of compatibility between mating partners with respect to the functional distance of MHC alleles. More similar couples seem to benefit in terms of an increase in fertilization success, but there might be an optimum level of similarity (see Fig.2d). Male MHC composition does not seem to be important except for one specific MHC allele. The exclusive occurrence of such a "sperminator" gene in males can be an important predictor of fertilization success. We did not find a relationship between male MHC heterozygosity and fertilization success. This is not surprising given that for male MHC we would only expect an indirect influence on fertilization success, since the benefits of having a higher diversity of different MHC alleles are more related to their own immune system and health (Penn and Potts 1999, Milinski 2006).

According to our results, a strong immune system in females constitutes a drawback in terms of fertilization success. More specifically in support of our first prediction, we found a negative correlation between the amount of sperm counted on the PVL and the number of MHC alleles in females. A general conclusion one might derive out of that result is that females with a high MHC heterozygosity may initiate an immune response against molecules that are expressed on the sperm surface. Although high MHC heterozygosity seems to negativly affect fertilization success in females, it might still be beneficial for the female in terms of higher immunocompetence (Hughes 1992, Milinski 2006) and consequently, this would also positively affect offspring survival by e.g. higher feeding rates.

Our results further suggest that the amount of sperm retained on the egg affect hatching and hence actual fertilization success. By showing that the maximum number of sperm on the PVL positively correlated with the proportion of viable chicks in the next clutch, as has been shown in other bird species (Wishart 1987, Birkhead and Fletcher 1998), we predict that this parameter is a good measure of fertilization potential.

From the theoretical poit of view males with intermediate to high MHC heterozygosity should be in general favored by females, because they provide a higher immunocompetence (Penn and Potts 1999, Milinski 2006, Piertney and Oliver 2006, Kamiya et al. 2014). Our results suggest that females might use different mating strategies depending on their own genetic make-up (MHC). As already mentioned a strong immune system in females constitutes a drawback in terms of reduced fertilization success but this negative effect could be mitigated or even eliminated by an alternative mating tactic. Females in particular with a strong immune system should reduce possible fertilization costs by incorporating a second aspect in their mate choice process, namely to mate with a genetically more similar male. Females with fewer alleles in contrast may more likely benefit from a male with a superior immune system to gain heterozygote offspring.

Such a female quality dependent mate choice strategy in relation to male MHC diversity has been in fact experimentally shown by Griggio et al. (2011). In this study female house sparrows with few MHC alleles showed a clear preference for males with a high number of MHC alleles, whereas females with many MHC alleles did not. Griggio et al. (2011) explained the lack of a preference with their reduced benefits to gain immunocompetent offspring. Our results now provide a more satisfactory explanation why immunocompetent females did not show a clear preference according to male MHC heterozygosity, namely they might suffer higher costs of reduced fertilization. Thus to understand how females use or incorporate the two strategies in their decision making process future mate choice experiments would need to include MHC similarity as an additional aspect.

Past studies usually concentrated only on similarity determined as the percentage of shared alleles (Bonneaud et al. 2006, Bichet et al. 2014, Løvlie et al. 2013), which might not necessarily be a suitable measure. Thus the incorporation of genetic and functional MHC distances in pairs is another novelty and improvement of this study. As far as we know, this is the first study that investigates the relationship between genetic and functional MHC distances and direct fertilization success. That only functional distance (and not overall genetic distance) was significant highlights the PBR as an important area in the evolution of compatibility. The postive selection is thought to be due to the different function and antigen binding of the PBR sites (Hughes 2002, Borg et al. 2011, Strandh et al. 2011). The key message here is that the immune system of females may not

initiate an immune response against molecules on sperm if they are functionally similar to those possessed by females. Thus further studies about fertilization success and mate choice should also include these different measures of compatibility, and not just the number of shared MHC alleles between a breeding pair. Also, fertility success should be a trade-off between inbreeding and outbreeding depression, where pairs with intermediate MHC distances should have the highest fertilization potential, but depending on the costs of in- or outbreeding in a given population, results might be hard to predict (Bichet et al. 2014).

Differential fertilization success according to genetic compatibility has been shown in various species including insects (Wilson et al. 1997, Clark et al. 1999) and mammals (Wedekind et al. 1996, Rülicke et al. 1998). In field crickets for example, the probability of fertilization by males was higher when mated to an unrelated female (Stockley 1999). In human and primate pairs who share MHC alleles, homozygotes are underrepresented, possibly because of *in utero* selection against homozygotes (Hedrick and Black 1997, Komlos et al. 1977, Knapp et al. 1996). In contrast, not sharing MHC alleles between parents increases the risk of outbreeding depression and any disruption of co-adapted genes might lead to a severe fitness reduction (Thornhill 1993, Hendry et al. 2000).

As already mentioned MHC alleles in males did not reveal to be a good predictor of fertilization success except one particular MHC allele ('seq21013'). When solely the male in a breeding pair possessed it, more sperm could be found at the PVL compared to pairs, where no one or only the female possessed this allele. However the underlying mechanisms are not known but this allele was the second most common in our population. To inherit it to offspring might be beneficial, probably because it provides resistance to a common pathogen or gives other benefits. Also Skarstein et al. (2005) found that one specific MHC allele was associated with increased fertilization success in fish.

Our results will have important implications to understand MHC-based mate choice. All three hypotheses mentioned to explain MHC-dependent mate choice, may not be mutually exclusive and even individuals within a species might integrate different aspects in their mating decision, namely high allelic diversity of MHC alleles, genetic compatibility in terms of MHC similarity between partners as well as the occurrence of specific alleles (review in Piertney and Oliver, 2006, Kamiya et al. 2014).

Conclusion

By counting the number of sperm on the egg membrane, we had the unique opportunity to measure the direct postcopulatory fertilization success. We showed that fertilization success in house sparrows is MHC-dependent and our results indicate that the number of MHC alleles in females, the compatibility of functional MHC alleles in pairs and the occurrence of a specific MHC allele in males were important predictors. Our results suggest that females should use different mating strategies depending on their own genetic make-up (MHC) to ensure fertilization of their eggs. Specifically, females with high MHC heterozygosity that may suffer from reduced fertilization success should choose a male, which is more similar at the PBR while females with low MHC heterozygosity should choose a male with higher MHC heterozygosity. Finally also the occurrence of a specific allele in males positively influenced fertilization success. These findings may have important implications that could explain individual based variation in mating strategies.

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Figures and Tables

Figure 1: Maximum number of sperm/clutch (square-root transformed) in first clutch in relation to the proportion of hatched chicks (arc-sin transformed) in second clutch

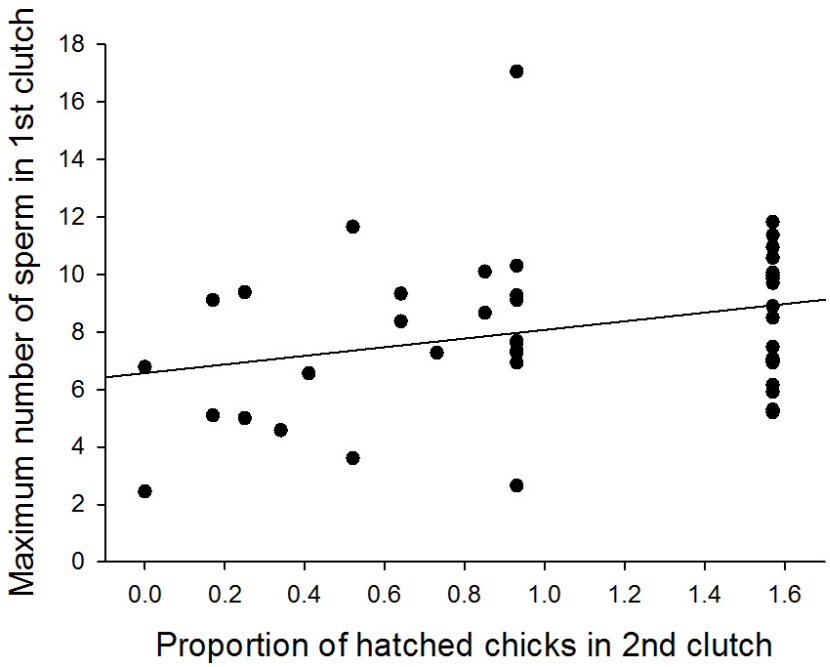


Figure 2a: Maximum number of sperm/clutch (square-root transformed) in relation to eggs laid (clutch size from 1 to 5)

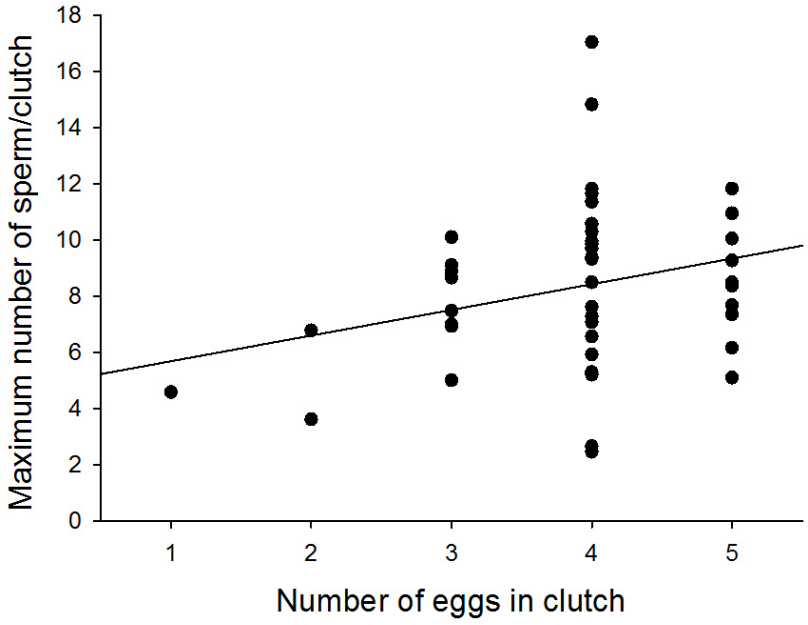


Figure 2b: Maximum number of sperm/clutch (square-root transformed) in relation to start of egg laying (week 1 to 7)

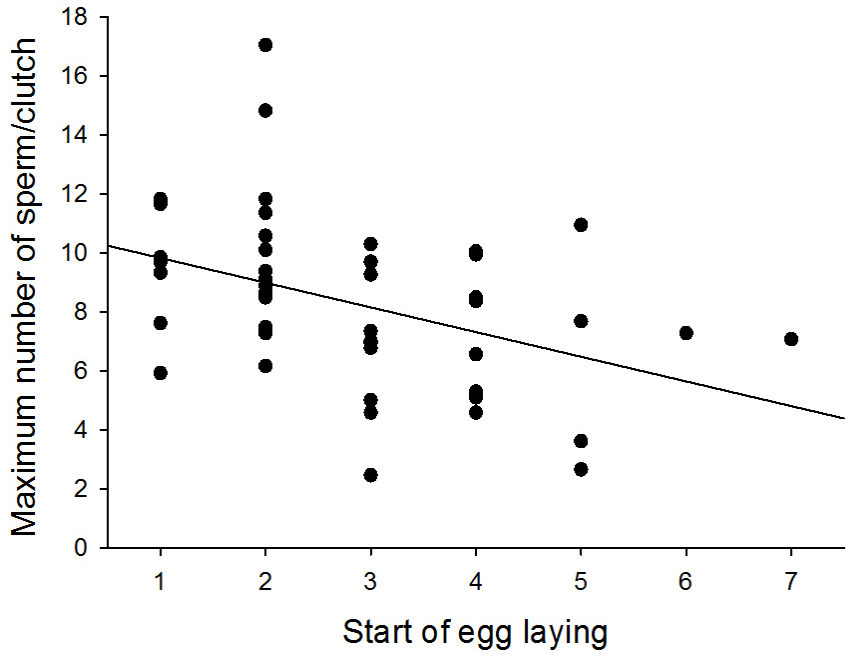


Figure 2c: Maximum number of sperm/clutch (square-root transformed) in relation to number of MHC alleles in females

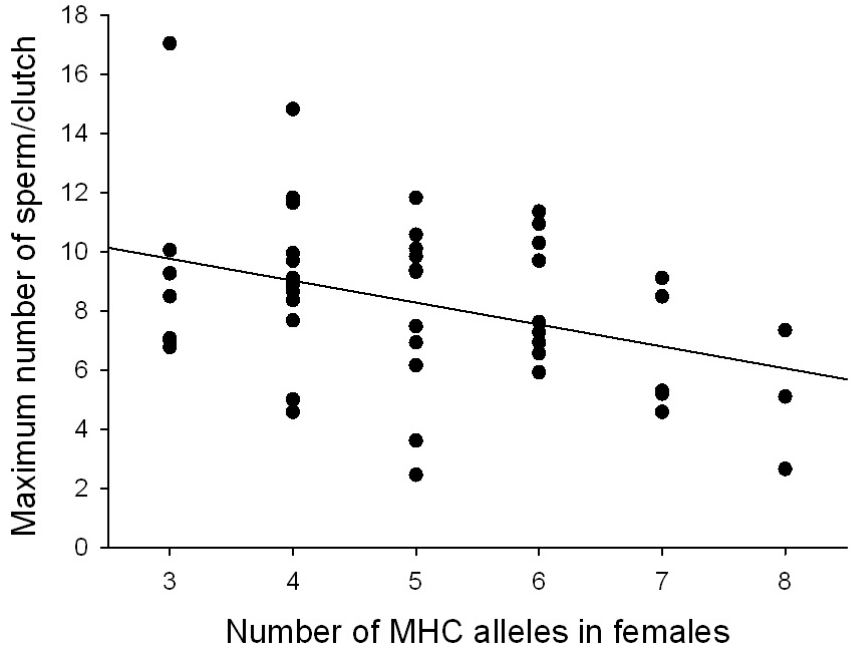


Figure 2d: Maximum number of sperm/clutch (square-root transformed) in relation to the functional MHC distance in pairs

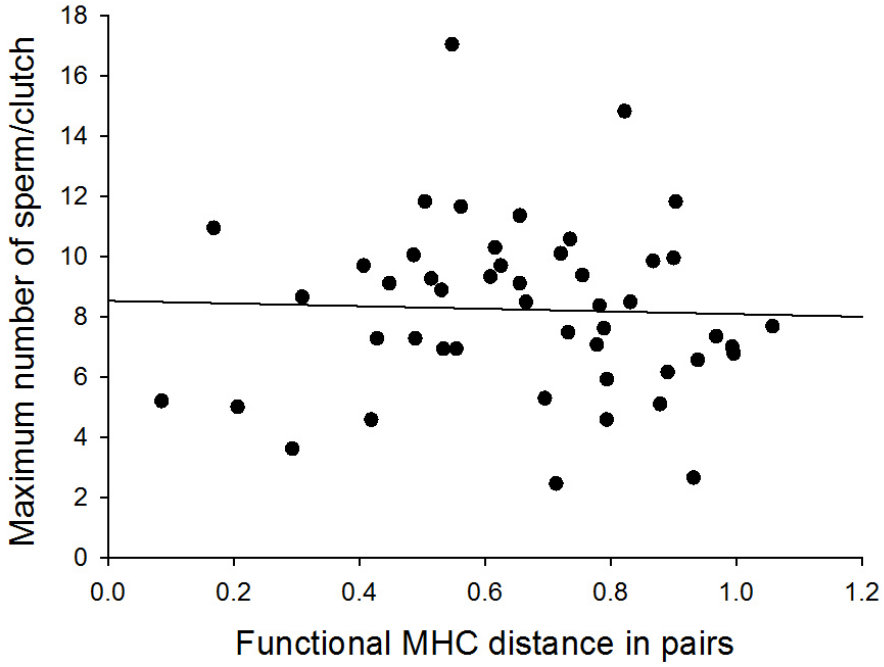


Figure 2e: Maximum number of sperm/clutch (square-root transformed) in relation to the occurrence of the MHC allele 'seq21013' in a breeding pair (mean with error bars)

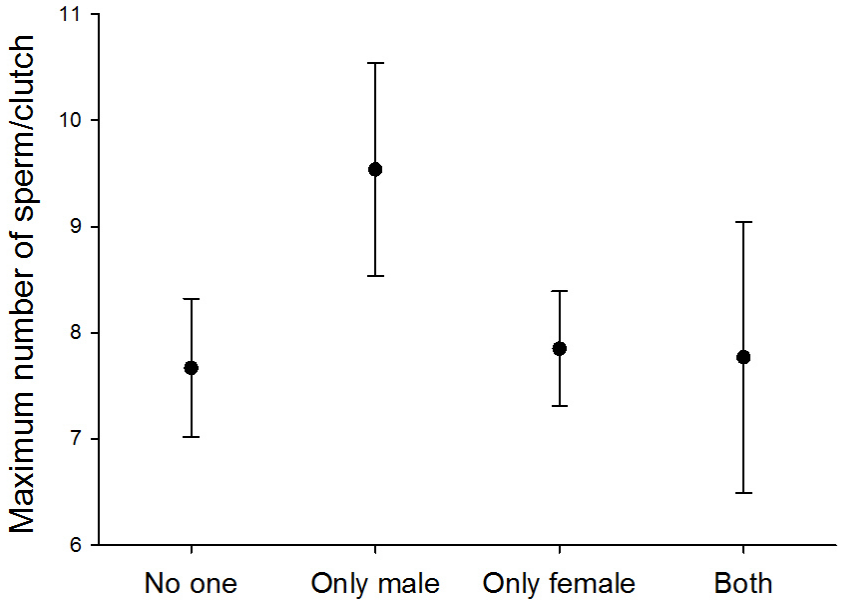


Table 1: Factors influencing sperm counts (linear model)

Model-averaged coefficients	Estimate	Adjusted SE	p value	RVI
(Intercept)	10.3854	2.1302	1.10e-06 ***	
Start egg laying	-0.9947	0.2320	1.81e-05 ***	1.00
Number of eggs	1.3614	0.3279	3.30e-05 ***	1.00
Functional MHC distance	-3.4592	1.3313	0.00936 **	0.92
Factor(seq21013) - only male	2.1603	0.7548	0.00421 **	0.91
Factor(seq 21013) - only female	-0.7253	0.9238	0.43241	
Factor(seq 21013) - both	0.4744	1.1498	0.67992	
MHC heterozygosity female	-0.6108	0.2441	0.01234 *	0.91

Supplementary Information

Supplementary Table 1: Parentage and genome-wide genetic variation

Primer set	PCR mix	Size range	Number of alleles/locus	Primer set reference
Ase 18	1	185-249	17	Richardson et al. (2000), see Griffith et al. (2007)
Pdoμ1	1	156-200	18	Neumann and Wetton (1996)
Pdoμ3	1	113-181	18	Neumann and Wetton (1996)
Pdoμ5	1	202-264	21	Griffith et al. (1999)
Pdoμ6*	1			Griffith et al. (1999)
Pdo9	1	362-424	18	Griffith et al. (2007)
Pdo10	1	102-152	14	Griffith et al. (2007)
Pdo16A	2	270-302	15	Dawson et al. (2012)
Pdo17	2	192-244	22	Dawson et al. (2012)
Pdo19	2	173-183	4	Dawson et al. (2012)
Pdo22	2	92-130	17	Dawson et al. (2012)
Pdo27	2	224-248	12	Dawson et al. (2012)
Pdo40A^{+}	2	298-328	15	Dawson et al. (2012)

* had to be excluded due to amplification problems

$^{+}$deviated from the Hardy-Weinberg Equilibrium

Supplementary Table 2: Allele frequencies and GenBank Acc nr of the MHC class I exon 3 alleles found

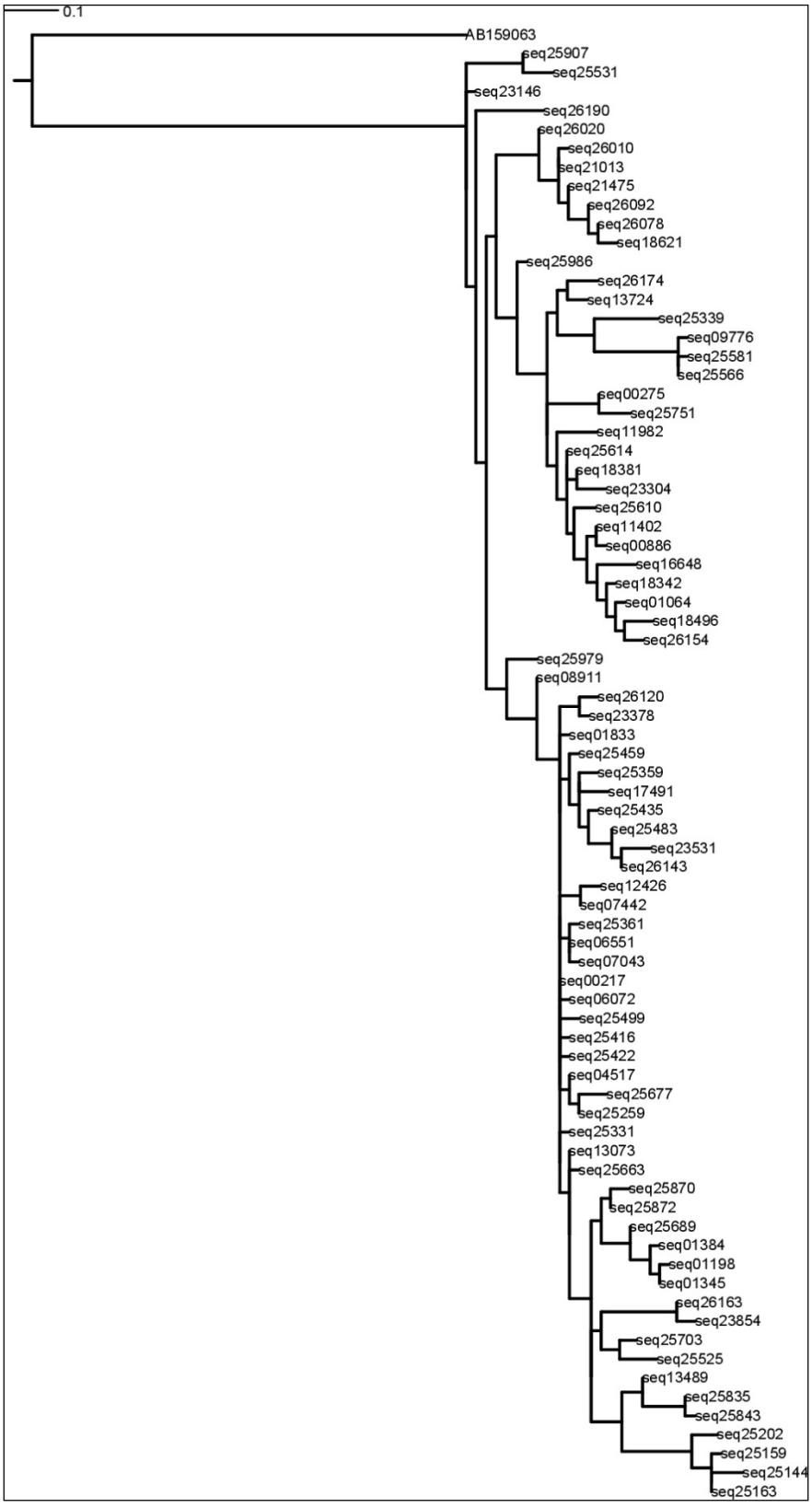
Sequence identity from the program "seqeqseq"	Genbank Acc nr (NCBI BLAST)	Frequencies (%)
seq00217	Pado-UA_238	58.90
seq21013	Pado-UA_239	35.79

seq25566	Pado-UA_251	29.45
seq25663	Pado-UA_245	21.27
seq25614	Pado-UA_338	15.75
seq25401	Pado-UA_299	14.52
seq25986	Pado-UA_244	14.31
seq00275	Pado-UA_261	12.88
seq25835	Pado-UA_343	12.47
seq25361	Pado-UA_246	11.45
seq26078	Pado-UA_248	10.43
seq25163	Pado-UA*322_gb|JN609643.1|*	9.61
seq26163	Pado-UA_257	8.79
seq25202	Pado-UA_297	8.38
seq26190	Pado-UA_267	8.18
seq25435	Pado-UA_329	8.18
seq07442	Pado-UA_256	7.57
seq25764	Pado-UA_342	7.36
seq08183	gb|KC585634.1|*	7.16
seq25459	Pado-UA_330	6.54
seq25907	Pado-UA_258	6.54
seq25422	Pado-UA_328	6.34
seq25483	Pado-UA_331	6.34
seq24170	Pado-UA_294	5.93
seq04612	Pado-UA_269	5.93
seq25499	Pado-UA_332	5.52
seq26010	Pado-UA_346	5.52
seq00886	Pado-UA_262	5.32
seq25331	Pado-UA_298	5.32
seq26020	Pado-UA_347	5.11
seq26092	Pado-UA_349	5.11
seq25144	Pado-UA_295	4.70
seq25872	Pado-UA*319_gb|JN609642.1|*	4.50
seq18381	Pado-UA_284	4.09
seq25416	Pado-UA_254	3.89
seq07562	Pado-UA_253	3.68
seq25677	Pado-UA_339	3.48
seq25689	Pado-UA_340	3.48
seq13724	Pado-UA_281	3.27
seq26130	Pado-UA_350	3.27
seq04517	Pado-UA_268	3.07
seq22920	Pado-UA_289	2.86
seq06551	Pado-UA_272	2.66
seq25531	Pado-UA_334	2.66
seq25581	Pado-UA_250	2.66
seq18342	Pado-UA_283	2.45

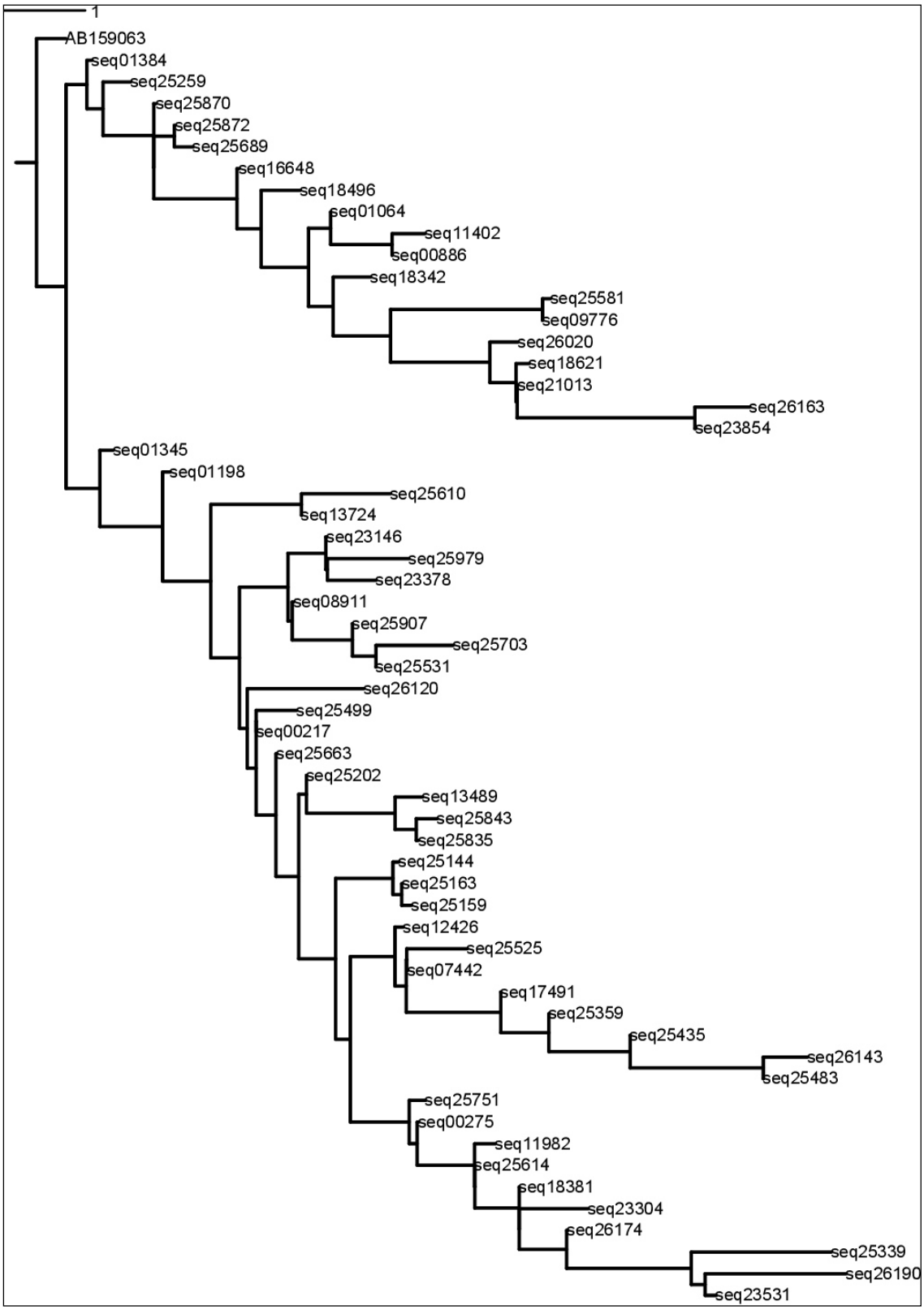
seq26154	Pado-UA_352	2.45
seq23531	Pado-UA_292	2.25
seq12426	Pado-UA_279	2.04
seq18845	Pado-UA_287	2.04
seq01833	Pado-UA_266	2.04
seq25339	Pado-UA*315_gb|JN609647.1|*	2.04
seq25159	Pado-UA_296	1.84
seq25703	Pado-UA_247	1.84
seq25751	Pado-UA_341	1.84
seq23146	Pado-UA_252	1.64
seq23304	Pado-UA_290	1.64
seq11402	Pado-UA_277	1.64
seq26143	Pado-UA_351	1.64
seq13073	Pado-UA_280	1.43
seq11982	Pado-UA_278	1.43
seq25525	Pado-UA_333	1.43
seq25610	Pado-UA_337	1.23
seq18496	Pado-UA_285	1.02
seq06555	Pado-UA_273	1.02
seq01345	Pado-UA_264	1.02
seq06072	Pado-UA_271	1.02
seq01064	Pado-UA_263	1.02
seq25843	Pado-UA_344	1.02
seq16648	Pado-UA_255	0.82
seq18621	Pado-UA_286	0.82
seq09776	Pado-UA_276	0.82
seq13489	Pado-UA_336	0.82
seq04799	Pado-UA_270	0.82
seq01198	Pado-UA*317*	0.82
seq25359	Pado-UA_335	0.82
seq23854	Pado-UA_293	0.61
seq08911	Pado-UA_275	0.61
seq17491	Pado-UA_282	0.61
seq23378	Pado-UA_291	0.61
seq07043	Pado-UA_274	0.41
seq01384	Pado-UA_265	0.41
seq25870	Pado-UA_345	0.41
seq28555	Pado-UA_348	0.41
seq21475	Pado-UA_288	0.20

*already published genbank sequences

Supplementary Figure 1: Maximum-likelihood tree for all unique translated MHC sequences (outgroup: MHC class I from chicken. GenBank Acc nr AB159063)



Supplementary Figure 2: Alternative maximum-likelihood tree that represents clusters of functionally similar MHC sequences (outgroup: MHC class I from chicken. GenBank Acc nr AB159063)



Chapter II

Major histocompatibility complex genes partly explain hatching success and early nestling growth in house sparrows

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Major histocompatibility complex genes partly explain hatching success and early nestling growth in house sparrows

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Abstract

It is quite common to find unhatched eggs in clutches of most bird species. In house sparrows (*Passer domesticus*), mortality of eggs and nestlings can exceed 50%. Many factors can influence mortality and growth, including exposure to variable temperatures, humidity, food supply, clutch size, genetic incompatibilities between parents and infections. Individual variation in resistance or susceptibility to infections could partly depend on the genetic set-up of the immune system, where the major histocompatibility complex (MHC) plays a central role. The highly polymorphic MHC genetic region is important for an appropriate adaptive immune response against pathogens by encoding proteins that present "self" and "non-self" peptides to T-cells. The aim of this study was to investigate if MHC partly explains hatching success and nestling survival. More specifically, we studied if nestling hatching success, survival and growth on day 1-12 depends on MHC heterozygosity or specific MHC alleles. To control for environmental factors, our study was carried out on an aviary population of house sparrows. We found that one specific MHC allele was associated with both hatching failure and smaller size in six days old chicks. These results indicate that specific MHC alleles or other genes linked to them might be responsible for a lower resistance/higher susceptibility to specific pathogens that are most severe during incubation and in the first days in the nest.

Introduction

Many factors can influence embryonic mortality, survival and growth rates of young, including genetic and environmental factors, such as stress, maternal condition, pollutants, nutrition and microbial infections (Hemmings et al. 2012, Beissinger et al. 2005, Christensen 2001, Kempenaers et al. 1999, Monaghan and Nager 1997, Webb 1987, Fry 1995, Price 1980, Møller 1994). In birds it is possible to make detailed observations of some of these factors since embryonic development is taking place outside the female. It is quite common to find unhatched eggs in clutches of most bird species and this could be due to infertility or embryonic mortality (Koenig 1982). There are numerous studies on the negative effects of microorganisms on poultry egg development (Romanoff and Romanoff 1972, Board 1966, Bruce and Drysdale 1983), but little is known about their effect on embryo mortality in free-living birds (Bernard 1989) and on growth and mortality of young, especially altricial nestlings (Box 1967).

The major histocompatibility complex (MHC) is a highly polymorphic gene region that encodes proteins that present foreign peptides to T-cells which are important for an appropriate adaptive immune response against pathogens (Klein 1986). The amino acids in the particularly polymorphic peptide-binding regions (PBR) of MHC molecules determine what antigens can bind and are therefore crucial for the function of each allele (Doytchinova and Flower 2005, Schwensow et al. 2007).

Heterozygosity level at the MHC loci and specific MHC alleles might influence survival and growth. Individuals that are heterozygote at the MHC loci possess more MHC alleles that are able to resist a broader range of pathogens compared to homozygotes (Brown 1997, Apanius et al. 1997, Penn 2002, Hughes 1992, Milinski 2006). Also specific MHC alleles have been shown to provide higher resistance/susceptibility to numerous diseases. According to the "frequency-dependent selection", especially rare MHC alleles are beneficial in the host population (Takahata and Nei 1990). In sheep (*Ovis aries*) common MHC alleles were associated with lower lamb or yearling survival, while a rare MHC allele was associated with higher survival of yearlings (Paterson et al. 1998). Also in rodents (*Rhabdomys pumilio)* and a lemur species (*Microcebus murinus*), infected individuals more frequently possessed the most common MHC allele, while individuals with a rare MHC allele had lower parasite loads (Froeschke and Sommer 2005, Schad et al. 2005).

Embryonic mortality is sometimes a result of genetic incompatibility between parents. Incompatibilities may result from in- or outbreeding (e.g. Lodge et al. 1971, Etches 1996, Kempenaers et al. 1996, Brillard et al. 1998, Christensen 2001, Sellier et al. 2005, Birkhead and Brillard 2007). In great reed warblers (*Acrocephalus arundinaceus*), hatching success of eggs was lower when pairs were genetically more similar (Bensch et al. 1994). Potentially, MHC incompatibility between parents leads to embryonic mortality. In human and primate pairs who share MHC alleles, homozygote offspring are underrepresented, possibly because of *in utero* selection against homozygotes (Hedrick and Black 1997, Knapp et al. 1996, Komlos et al. 1977).

The overall aim of this study was to investigate if hatching success, survival and growth in young chicks during the nestling stage depends on MHC heterozygosity or specific MHC alleles. We predict that hatching success and survival is highest with low MHC similarity in parents, high MHC heterozygosity and also that specific MHC alleles are advantageous or disadvantageous. We further expect that offspring with high MHC heterozygosity or specific MHC alleles may have advantages/disadvantages in terms of growth. To test this, we used an aviary population of wild house sparrows (*Passer domesticus*). Our aviary population allowed us to control for several environmental factors and we also had the possibility to collect unhatched eggs and dead chicks to obtain DNA samples.

Methods

In 2010, we captured wild house sparrows (*Passer domesticus*) in Vienna, Austria. These birds were housed in large outdoor aviaries (10 birds/aviary; aviary size: 3.5m × 3.5m × 3m) at the Konrad Lorenz Institute of Ethology, Vienna. In April 2012, 62 males and 73 females of these birds were put together in mixed flocks (4-5 breeding pairs/aviary) in 15 aviaries that were equipped with nest boxes and nesting material. All aviaries were equipped the same way with vegetation, perches, commercial food for granivorous passerines (ad libitum) and water. Our aviary population of wild house sparrows allowed us to control for several environmental factors that might interfere with our results, like food availability, nest box quality and availability, predation and differences in microclimate as well as socio ecological factors including breeding density and sex ratio.

Data about the start of egg laying, number of clutches/female and clutch size was collected. We analyzed 293 offspring (107 females and 186 males also including embryos), that derived from 59 different families (average: 5 chicks/family) and were born in different clutches/female (offspring from clutch 1 (140), 2 (75), 3 (66) and 4 (12)). Survival status was assessed at three different time points, when chicks were 1, 6 and 12 days old. Survival on day 1 reflects hatching success and hatched chicks were tested against unhatched embryos. In cases where we were not sure about a natural cause of death (e.g. chicks thrown out of the nest box), we excluded them from the survival analysis. Tarsus length and body mass were measured when chicks were 6 and 12 days old. We used body weight as a quality indicator because it is assumed to reflect a juvenile's fat reserve that is also linked to survival (Lack 1966). Tarsus length was used as a size indicator, it has been suggested that body size confers an advantage to physically compete for resources against siblings (Garnett 1981). Data from several passerine studies showed positive correlations between weight, body size and survival at the time of fledging (review: Maness and Anderson 2013).

We controlled nest boxes and aviaries every day and sampled eggs/embryos/chicks that were thrown out or died in the nest boxes. Five days after the first chick in a clutch hatched, we opened the unhatched eggs (hatching is normally synchronized within one to three days for each clutch) and took tissue samples of embryos. Blood samples (50 µl per bird in total) were collected from the brachial vein in adults and nestlings during the breeding season to extract DNA from it to assess paternity and MHC diversity. The breeding season and all measurements were performed prior to MHC screening and therefore blindly with respect to individuals' genotypes. All animal experiments were in accordance to the Austrian Law (Geschäftszahl: BMWF-68.205/0081-ll/3b/2012).

Molecular Methods

DNA extraction

Avian blood and tissue samples were stored in 95% ethanol. The ethanol was evaporated and DNA was extracted according to the manufacturer's instructions using the DNeasy Blood & Tissue Kit (Qiagen).

MHC genotyping

MHC characterization was carried out using 454 amplicon sequencing. MHC class I exon 3 sequences that encode parts of the peptide binding region in MHC molecules were amplified using individually tagged 454-adapted primers (according to Galan et al. 2010). The MHC-specific primer stretches used were forward primer (longfw2): GTCTCCACACTGTACAGYGGC; and reverse primer (rv3): TGCGCTCCAGCTCCYTCTGCC) (Karlsson and Westerdahl 2013). These primers amplify 222-225bp long (primers not included) classical MHC alleles in house sparrows (Karlsson and Westerdahl 2013). Using Qiagen Multiplex MasterMix each 15µl PCR reaction contained 7.5µl of Hot Start Master Mix, 0.6µl (5µmol) each of the forward and reverse primer, 1µl of template (25ng genomic DNA) and 5.3µl dd H_2O. The PCR conditions were: 95°C/15 min, 35x(95°C/30 s, 65°C/1 min, 72°C/1 min), 60°C/5 min. PCR products were run on a 2% agarose gel and products with positive amplifications were purified using the MinElute PCR Purification Kit (Qiagen). Samples were pooled in eights and DNA was quantified with a Nanodrop instrument and adjusted accordingly before a second pooling for the 454 quadrants. The number of samples in the 454 quadrants was aimed at a sequence coverage of 300 reads per individual (according to Borg et al. 2011). 491 individuals were run in two different sequencing reactions (run 1 (R1) and run 2 (R2)) and these runs were filtered separately (see below). R1 contained 272 samples (243 individuals + 29 technical replicates (11.9%)) and R2 contained 286 samples (248 individuals + 38 technical replicates (15.3%)). 454 sequencing was done at Lund University Sequencing Facility (Faculty of Science), Sweden.

MHC - bioinformatics and data processing

After 454 sequencing, the data was extracted and assigned to samples using the program jMHC (Stuglik et al. 2011). To get rid of artefacts generated during the initial PCR, the emulsion PCR and the 454 sequencing reaction the raw data from the 454 run was filtered. Filtering steps were in accordance with Galan et al. 2010, Zagalska-Neubauer et al. 2010 and Sepil et al. 2013.

First, sequences in low abundances (<3 reads) were deleted. Identical sequences within individuals were detected and merged using the web-applications "seqeqseq" (http://mbio-serv2.mbioekol.lu.se/apps/seqeqseq.html) and "mergeMatrix" (http://mbio-serv2.mbioekol.lu.se/apps/mergeMatrix.html).

Second, we only wanted to keep sequences with a suitable sequence depth. Since the maximum number of classical MHC alleles is eight in house sparrows we chose a threshold of 104 reads per sample, this gives a genotype score of 99.9% (m=8, Galan et al. 2010). Here we used the web-application "popMatrix" (http://mbio-serv2.mbioekol.lu.se/apps/popMatrix.html) and filter 1. This web application has several filers, filters 1-4.

Third, true alleles are likely to occur more frequently than artifact alleles. Therefore we wanted to set a threshold that keeps true alleles but disregards artifact alleles. We used our replicates to set these thresholds (2% in R1 and R2) and all alleles that occurred in lower than 2% per sample were deleted using filter 3.

Forth, to verify MHC alleles they should occur in at least two independent PCRs. We worked with family data (189 adults produced about 300 chicks), these individuals were distributed across R1 and R2) and every allele is therefore likely to occur several times. Therefore we used the criteria that every allele should be found at least twice, here we used filter 4.

Fifth, the obtained sequences were examined in BioEdit (v7.2.0) and all sequences that did not meet the expected length of 222-225bp or displayed indels that were not multiples of three base pairs were deleted. The next step was to look for PCR recombinants (chimeras) and 1-bp substitutions. Sequences were examined starting with the lowest frequencies at the population level (Sepil et al. 2013). All sequences that occurred in a frequency below 2% at the population level were checked in all individuals for chimeras and 1-bp substitutions. 50% of the sequences between a frequency of ≥ 2% to 25% were checked but no chimeras or 1-bp substitutions were found. Therefore, we assume that sequences with a frequency of ≥ 2% are true alleles. Additionally, alleles were verified and controlled for with inheritance data of families (189 adults produced about 300 chicks).

The filtered MHC DNA sequences were translated into amino acid sequences. These sequences were translated according to the chemical binding properties of the amino acids in the PBR. The 16 amino acids from each sequence corresponding to the PBR in chicken (Wallny et al. 2006) were extracted and converted into five physicochemical descriptor variables: $z1$ (hydrophobicity), $z2$ (steric bulk), $z3$ (polarity), $z4$ and $z5$ (electronic effects) (Sandberg et al. 1998).

The genetic MHC distances between parents were calculated from a maximum-likelihood tree (2000 bootstraps) that was inferred for all unique translated MHC sequences (outgroup MHC class I from chicken, GenBank Acc nr AB159063) using the RAxML software (ver 7.0.4) under the PROTMIX model and the JTT substitution matrix, with default settings. Pairwise overall MHC amino acid distances between individuals were then computed from this tree with the software Fast UniFrac (Hamady et al. 2010) available at http://unifrac.colorado.edu. Functional distances between MHC genotypes of parents (reflecting the difference in what pathogen antigens can be bound and detected between parents) were assessed by calculating the difference in chemical binding properties of the amino acids in the PBR. The unique set of functional PBR sequences (represented by the *z*-descriptors) was used to construct alternative maximum-likelihood trees with ContML in the PHYLIP-package (v. 3.69). The tree was rooted as before with the outgroup *Gallus gallus* (AB159063). This tree represents clusters of functionally rather than evolutionary similar MHC sequences. Pairwise overall MHC functional distances between individuals were computed from this tree in the same way as for the amino acid distances (UniFrac).

MHC diversity was evaluated as: 1) the number of unique functional MHC alleles per individual 2) the proportion of functional alleles shared in parents (= [100/number of shared alleles]/[total number of alleles male + female]) 3) genetic sequence distance in parents 4) functional distance in parents (distance measures according to Strandh et al. 2012) and 5) the occurrence of the most common functional MHC alleles (> 10% at the population level) per individual.

Genome-wide genetic variation

For the assignment of genome-wide individual heterozygosity and paternity, all individuals were typed at 13 highly polymorphic neutral microsatellite loci and one sexing loci (P2D/P8). The quality of loci (Hardy-Weinberg Equilibrium and Linkage Disequilibrium) was tested using the programs Cervus (v3.0.3) and Fstat (v2.9.3.2). Individuals were genotyped in two multiplex reactions according to the protocol of Dawson et al. (2012) (Supplementary Table 1). The loci used in this protocol are known to be distributed across at least seven different chromosomes and thus likely reflect the genome-wide level of neutral genetic variation (Dawson et al. 2012). Forward primers were fluorescently labeled using different dyes (YYE, FAM or AT550, Microsynth). The

two multiplex PCRs were carried out using a Multiplex Kit (Qiagen) in a final PCR reaction volume of 6 µl containing 3µl of the Qiagen enzyme, 2µl of the primer mix and 1µl of the template (25 ng genomic DNA). PCR cycling scheme and primer concentrations of the 2 multiplex PCRs were carried out according to Dawson et al. (2012). PCR products were diluted (1:20) and analyzed on an ABI genetic analyzer (Applied Biosystem 3130xl). Allele sizes were determined using GeneMapper (v3.0) with reference to an in-house DNA Size Standard (HMROX). We amplified 13 loci and one sexing loci (Supplementary Table 1). However, we excluded loci Pdoµ6 due to amplification problems and Pdo9 and Ase18 because they deviated from the Hardy-Weinberg Equilibrium, this left 10 highly polymorphic neutral microsatellite loci and one sexing loci in the final analysis.

Statistical analysis

We used linear and logistic mixed models within the R package v.3.0.3 x64 (R Core Team 2013) to analyze the effects on growth (weight and tarsus) in 6 and 12 days old chicks and survival of chicks that were 1, 6 and 12 days old. In order to evaluate and compare different models, we used the Akaike Information Criterion corrected for small sample sizes (AIC_c, (Burnham and Anderson 2002)). In all cases there was not one clearly best model, so we used methods of model averaging and multimodel inference (Burnham and Anderson 2002). These methods allow inference over all models considered, but this was weighted according to model support by the data. Additionally, these methods do not only estimate standard errors unconditionally for a single model, they also provide the probability for single variables being in the unknown "true" model (the so-called relative variable importance - RVI). Unlike variable selection based on p-values these techniques have a sound mathematical basis and are increasingly recommended (Symonds and Moussalli 2011). We conducted these calculations in R using the package MuMIn (Barton 2013). Additionally, we inspected the residuals for normality visually using histograms and QQ-plots. Since early development could have an important influence on growth and survival of chicks, the day they were born (day numbers in 2012) as well as the clutch number (we used birds that originated from clutch 1-4 of specific females) were recorded. Since breeding was highly synchronized, these two parameters were correlated ($p<0.001$) and only clutch number was used. Before we included the 10 most common functional MHC alleles (frequency on the population level >10%) in our analysis, we had to do a pre-model selection with the rest of our variables. Only variables

with RVI values >0.5 were kept in the analysis. As a next step we evaluated the relevance of the 10 functional alleles, while the remaining variables were fixed. We only included functional MHC alleles in the final model that had RVI values larger than 0.5. In the results section, only variables that have RVI values >0.7 and p-values <0.05 are discussed.

Influence of MHC on hatching success and survival: We used a logistic mixed model to analyze if any of our variables (number of functional MHC alleles, microsatellite heterozygosity, clutch number, clutch size, sex, the presence/absence data of the ten most common functional MHC alleles and family as random effect correlated with survival (0) or death (1) in chicks. Additionally, we included the following variables that corresponded to MHC similarity in parents: Proportion of functional MHC alleles shared in breeding pairs (arc-sin transformed), genetic and functional distance (arc-sin transformed) in breeding pairs. We included these variables because incompatibilities between mates could also cause embryonic mortality (Hedrick and Black 1997, Komlos et al. 1977, Knapp et al. 1996, Hendry et al. 2000).

Influence of MHC on growth: We used linear mixed models to analyze if any of our variables (number of functional MHC alleles, microsatellite heterozygosity, clutch number, clutch size, sex, the presence/absence data of the ten most common functional MHC alleles and family origin as a random effect) correlated with tarsus length and body mass.

Results

MHC characterization

We had a total of 75894 sequence reads of 19200 unique sequences in 454 run 1 (R1) that had complete tags and primers. In run 2 (R2) we had a total of 125752 sequence reads of 34670 unique sequences. After filtering the data, a total of 85 different MHC alleles were found that could be translated into 59 unique functional MHC alleles, based on their chemical properties (Sandberg et al. 1998). There was an average of 4.2 functional MHC alleles/individual (ranging from 1-8). Males had on average 4.2 alleles (sd: 1.4), females had 4.3 (sd: 1.4). Adults possessed on average 4.3 alleles (sd: 1.5), chicks had 4.2 (sd:

1.3). In adults and chicks, there were 10 functional MHC alleles that occurred in frequencies larger than 10% at the population level, these were included in the analysis (Supplementary Fig. 1).

Influence of MHC on hatching success and survival

DAY 1: Out of 293 eggs, 31 embryos did not hatch. Hatching success was significantly correlated with clutch number (p=0.005, RVI=0.98) and tended to be associated with the functional MHC allele 'seq25259' (p=0.037, RVI=0.77, Fig. 1). The more clutches a specific pair produced, the higher was the probability of unhatched eggs in these later clutches. Also, individuals that did possess the functional MHC allele 'seq25259' were less likely to hatch. The proportion of functional MHC alleles shared in breeding pairs, genetic sequence distance and functional distance in breeding pairs, MHC and microsatellite heterozygosity, clutch size, sex and the presence/absence data of the other nine most common functional MHC alleles exerted a minor effect (RVI values <0.7 and p-values >0.05).

DAY 6: Out of 251 hatched chicks, 38 died before day 6. We excluded some chicks from the analysis that did not die naturally (killed by adults). Survival was related to both clutch size (p=0.002, RVI=0.99) and sex (p=0.007, RVI=0.98). Individuals that were born in larger clutches and male nestlings were less likely to survive. The proportion of functional MHC alleles shared in breeding pairs, genetic sequence distance and functional distance in breeding pairs, MHC and microsatellite heterozygosity, clutch number and the presence/absence data of the ten most common functional MHC alleles exerted a minor effect (RVI values <0.7 and p-values >0.05).

DAY 12: Out of 213 nestlings that were alive on day 6, 25 died before day 12. Survival was related to clutch number (p<0.001, RVI=0.99). This means that not only embryos (day 1) but also older nestlings were less likely to survive in later clutches. The proportion of functional MHC alleles shared in breeding pairs, genetic sequence distance and functional distance in breeding pairs, MHC and microsatellite heterozygosity, clutch size, sex and the presence/absence data of the ten most common functional MHC alleles exerted a minor effect (RVI values <0.7 and p-values >0.05).

Influence of MHC on growth

DAY 6: In nestlings that were 6 days old (N=213), body mass was related to clutch number (p=0.012, RVI=0.90), clutch size (p=0.025, RVI=0.83) and the MHC allele 'seq25259' (p=0.029, RVI=0.79, Fig. 2). Nestlings that were born in later clutches, in clutches with more eggs or that possessed the functional MHC allele 'seq25259' had lower weight. Tarsus size on day 6 was similarly related to clutch number (p<0.001, RVI=1.00) and the MHC allele 'seq25259' (p=0.037, RVI=0.75, Fig. 3), but not to clutch size. Nestlings that were born in later clutches of a specific pair or possessed the MHC allele 'seq25259' had a shorter tarsus. The MHC and microsatellite heterozygosity, sex and the presence/absence data of the other nine most common functional MHC alleles exerted a minor effect (RVI values <0.7 and p-values >0.05).

DAY 12: In nestlings that were 12 days old (N=188), body mass was related to clutch number (p<0.001, RVI=1.0), but not to clutch size or the MHC allele 'seq25259'. Nestlings that were born in a later clutch had lower weight. We also found that tarsus size on day 12 was related to clutch number (p<0.001, RVI=1.0) but not to clutch size or the MHC allele 'seq25259'. Nestlings that were born in a later clutch had a smaller tarsus. The MHC and microsatellite heterozygosity, clutch size, sex and the presence/absence data of the ten most common functional MHC alleles exerted a minor effect (RVI values <0.7 and p-values >0.05).

Discussion

We found that hatching success and growth were associated with one specific MHC allele ('seq25259'). This allele seems to be maladaptive in the early developmental period. Hatching success was lower in individuals that possessed this functional MHC allele. The same allele was also associated with lower weight and a smaller tarsus in 6 days old chicks. However, its significance seemed to disappear in older nestlings, highlighting the importance of this allele especially during the incubation period and the first days of life. We could not find an influence of this allele on survival on day 6 and 12. Similarly, the influence of this allele on growth seems to decline in older nestlings.

In hole nesting house sparrows, mortality of eggs and nestlings can exceed 50% (Dyer et al. 1977). Pinowski et al. (1994) found similar results in a house sparrow population,

where egg mortality was about 30% and nestling mortality ranged between 24 and 30%. 67% of the unhatched eggs were infested with pathogenes such as *Escherichia coli* and *Staphylococcus epidermitis*. Also a great deal of nestlings that were infected with pathogenic microorganisms, had a lower growth rate or died, highlighting the negative effects of pathogenic microorganisms on hatching failure, survival and growth. Growth rates of young within a clutch can be related to brood size and hatching order (review: Lack 1966). Also parasitism is a common selective pressure than can influence growth rates and survival in nestlings (Price 1980, Møller 1994). These parasites could be viral, bacterial and fungal (Meijerhof 1992, Cook et al. 2003, Beissinger et al. 2005, Cook et al. 2005). With our data it is not possible to predict if hatching failure was caused by microbial infections, but since also growth was negatively correlated with the same functional MHC allele 5 days later, a microbial infection is likely. In further studies it would be interesting to link bacterial loads on egg shells and in nestlings to MHC alleles in parents and/or chicks.

MHC has become a major study subject of evolutionary and ecological immunology across a large variety of taxa (Sommer 2005, Piertney and Oliver 2006, Spurgin and Richardson 2010). The highly polymorphic genes at MHC loci are believed to be under some form of balancing selection maintaining the high variation, such as pathogen-driven selection (Penn et al. 2002). Pathogen-driven MHC diversity can be maintained in two ways. Individuals heterozygous at a MHC locus theoretically recognize twice as many foreign peptides than homozygotes (Hughes 1992, Milinski 2006). Also specific rare MHC alleles that provide a stronger immune response to a specific antigen can gradually increase in frequency in a population, maintaining high diversity (Takahata and Nei 1990, Hedrick 2002). It has been shown that in some species, especially common MHC alleles are associated with lower survival and higher parasite loads (Paterson et al. 1998, Froeschke and Sommer 2005, Schad et al. 2005). In our population, we also found one common functional MHC allele (16.05%) that had negative effects in early development.

At a first glance it is surprising that in our population individuals with higher MHC heterozygosity did not have higher survival or faster growth rates, but depending on the composition of the parasite community, either MHC heterozygosity or specific MHC alleles might be more beneficial. It has already been shown that specific MHC supertypes (clustered according to their antigen-binding motifs) were associated with different components of individual fitness and survival (Southwood and Sidney 1998, Sette and

Sidney 1999, Trachtenberg et al. 2003, Lund et al. 2004, Schwensow et al. 2007). In birds, individuals with specific MHC supertypes had higher suvival rates and lifetime reproductive success (LRS), but another supertype was associated with reduced LRS (Sepil et al. 2013).

In this study we used MHC class I alleles, which are responsible for adaptive cell-mediated immunity, but especially young nestlings mainly depend on the innate immune system. Therefore it is remarkable that one specific functional MHC allele was associated with reduced growth and lower hatching success, indicating that it is in linkage disequilibrium with other immune genes or genes involved in body condition. Consequently, why specific MHC alleles remain in a population may not only depend on single observed costs or benefits. It would be necessary to disentangle the influence of MHC genes itself or any other genes in linkage disequilibrium with it.

We predicted that hatching success and survival should be highest with low MHC similarity in parents (tested as the proportion of functional MHC alleles shared, genetic sequence distance and functional distance), but we could not find an effect of incompatibilities at the MHC. It has been shown that incompatibilities between mates could cause embryonic mortality (Hedrick and Black 1997, Komlos et al. 1977, Knapp et al. 1996, Hendry et al. 2000), but depending on the population (in- or outbreed), these effects may be hard to observe. To control for genome-wide genetic variation, we also included neutral heterozygosity at 10 polymorphic microsatellite loci in the analysis, but according to our predictions, we could not find an association between neutral heterozygosity and hatching success, survival or growth.

There was also influence of environmental variables, which we could not control for in our set-up, but statistically. Clutch number and clutch size seem both important to explain variation in growth and survival. Chicks from earlier clutches were more likely to survive day 1 and 12 and had more weight and a larger tarsus on day 6 and 12. Since we provided the same amount of food during the whole breeding season, one explanation might be that birds allocated more resources to egg production to their first clutch compared to later clutches (Hipfner 2001, Milonoff 1991). Chicks from clutches with fewer eggs had a higher probability to survive on day 6 and also had more weight on day 6. Clutch size has been shown to influence juvenile survival. On one hand, high-quality parents may be able to produce and raise more offspring with higher survival (Lescroël et al. 2010). On the other

hand, offspring from larger broods have more competition for parental care and food than offspring from smaller broods. Accordingly, studies about clutch size and juvenile survival had mixed results (Maness and Anderson 2013). Also sex was an important predictor of survival of 6 days old birds and females were more likely to survive. Sex-biased survival of several species of birds has already been shown especially during the period of parental care, but depending on the species or even population, survival rates of males or females can be different (Clutton-Brock 1991).

Conclusions

We found that hatching success and growth were associated with the MHC, more precisely with one specific MHC allele. Hatching success was lower in individuals that possessed the functional MHC allele 'seq25259'. The same functional MHC allele was also associated with lower weight and a smaller tarsus in 6 days old chicks. However, these correlations disappeared in older chicks, highlighting the importance of this allele especially during the incubation period and the first days of life. These results indicate that this MHC allele or another gene linked to it might be responsible for a higher susceptibility to a specific pathogen which is most severe during incubation and in the first days in the nest, but these negative effects disappear later on. Consequently, why specific MHC alleles remain in a population may not only depend on single observed costs or benefits.

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Figures and Tables

Figure 1: Influence of the functional MHC allele ‘seq25259’ on hatching success (binominal confidence interval)

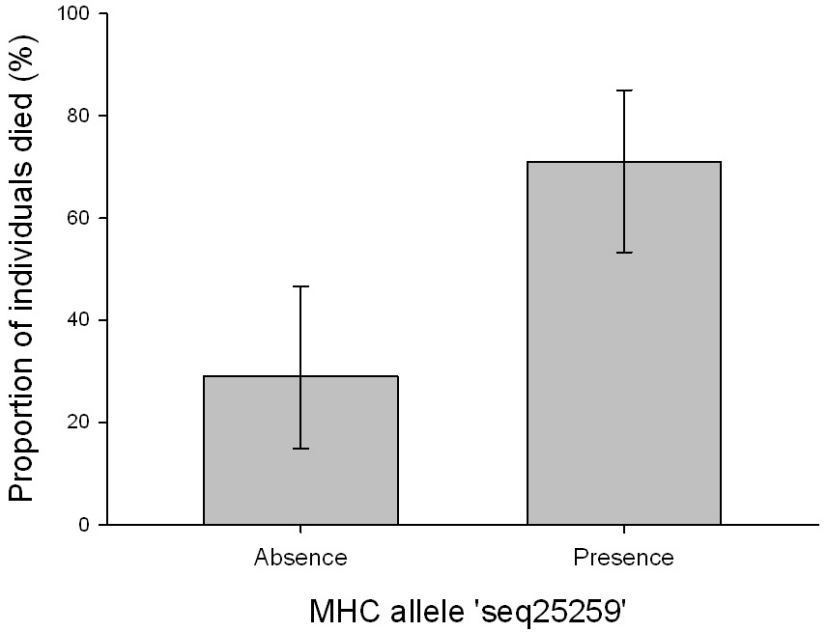


Figure 2: Influence of the functional MHC allele ‘seq25259’ on body mass in 6 days old chicks

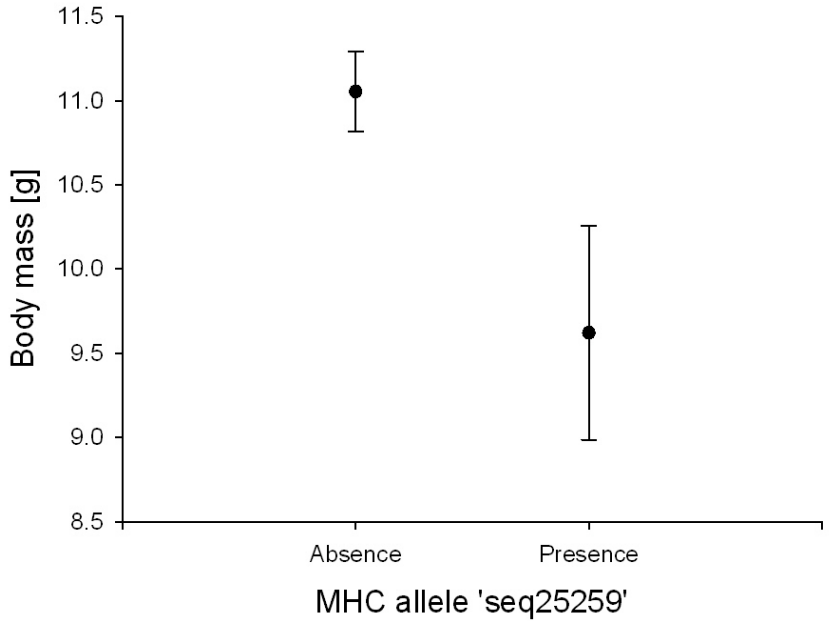
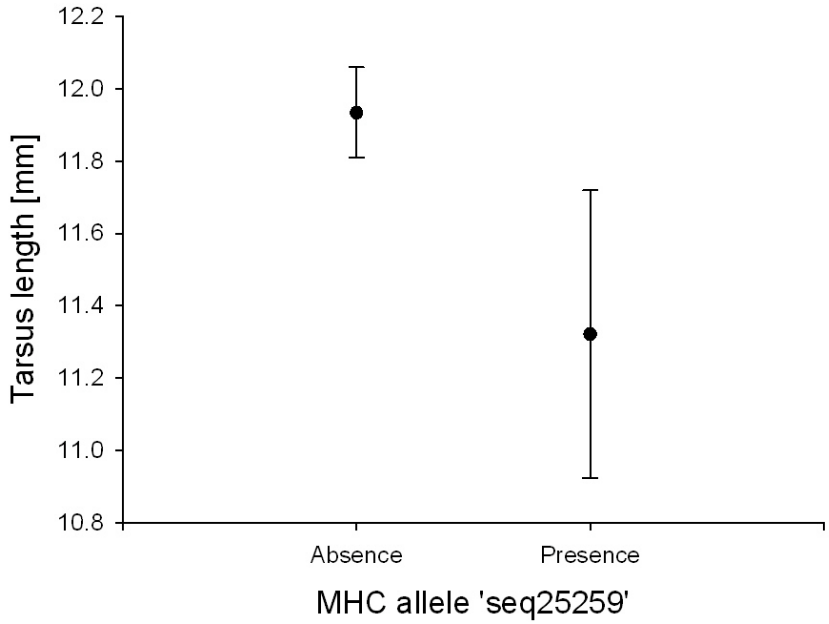


Figure 3: Influence of the functional MHC allele ‘seq25259’ on tarsus length in 6 days old chicks



Supplementary Information

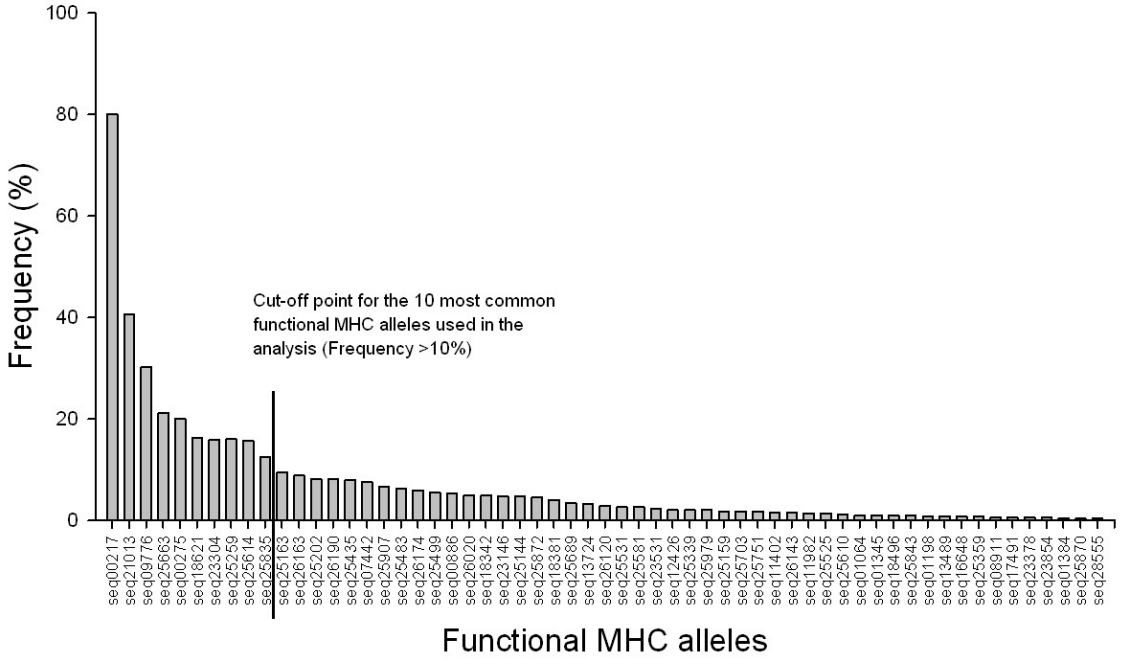
Supplementary Table 1: Genome-wide genetic variation

Primer set	PCR Mix	Size range	Number of alleles/locus	Primer set reference
P2D/P8	1	316 & 358	2	Dawson et al. (2012), see (Griffiths, Double et al. 1998)
Ase18 '	1	185-249	17	Richardson et al. (2000), see Griffith et al. (2007)
Pdoμ1	1	156-200	18	Neumann and Wetton (1996)
Pdoμ3	1	113-181	18	Neumann and Wetton (1996)
Pdoμ5	1	202-264	18	Griffith et al. (1999)
Pdoμ6*	1			Griffith et al. (1999)
Pdo9 '	1	362-424	15	Griffith et al. (2007)
Pdo10	1	102-152	12	Griffith et al. (2007)
Pdo16A	2	270-302	15	Dawson et al. (2012)
Pdo17	2	192-244	21	Dawson et al. (2012)
Pdo19	2	173-183	4	Dawson et al. (2012)
Pdo22	2	92-130	16	Dawson et al. (2012)
Pdo27	2	224-248	12	Dawson et al. (2012)
Pdo40A	2	298-328	15	Dawson et al. (2012)

* excluded due to amplification problems

' deviated from Hardy-Weinberg Equilibrium

Supplementary Figure 1: Frequency distribution of the 59 functional MHC class I alleles and cut-off point for the ten most common alleles used in the analysis (Frequency <10%)



Chapter III

Major Histocompatibility Complex: An appropriate tool to assess immunocompetence

Manuscript by

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Major Histocompatibility Complex: An appropriate tool to assess immunocompetence

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Abstract

A well-functioning immune defence is crucial for survival and reproduction, but to date it still remains challenging to assess the quality and function of the entire immune system as common immunological tests can only explain a fraction of it. This study is an attempt to find a more suitable and general predictor of immunocompetence, the ability of a host to produce an immune response following exposure to an antigen, with genes of the highly polymorphic major histocompatibility complex (MHC). MHC genes might be a proper tool because they are involved in T-cell mediated immune responses and crucial in pathogen recognition. Also, surveying wild bird populations may lead to methodological problems. Therefore we used an aviary population of house sparrows (*Passer domesticus*) to account for several environmental factors. MHC class I alleles were identified and to properly mirror the immunocompetence, several tests were conducted that correspond to the innate and the adaptive (cell-mediated and humoral) defence, one was done repeatedly in different life cycles. Additionally survival rate was monitored as an indicator of fitness. The results reveal that MHC diversity is reflected in several immunological responses as well as survival probability. Some of these immune responses, specifically those typically representing a measure for an innate response were correlated with the same MHC allele. A different set of MHC alleles correlated with repeated phytohaemagglutinin (PHA) injections, suggesting that the PHA test measures different areas of the immune system, innate and adaptive depending on the life stage. Our results indicate that using MHC might be a good candidate to further measure and quantify immunocompetence and to elucidate the complex interactions within the immune system.

Introduction

For ecologists it is highly important to analyze host-parasite interactions of their study species, since they have an important impact on several life-history traits. To reveal the complex variations of the immune system among individuals, a concept called immunocompetence was introduced as an important determinant of fitness (Altizer et al. 2003, Bernatchez and Landry 2003). It grades the ability of a host to prevent or control infection by pathogens and parasites and its central prediction is that immune function is energetically costly. Especially when (physiological) resources are limited, it may constrain investment in other energetically costly life-history traits, such as secondary sexual traits, growth or reproduction (Lochmiller and Deerenberg 2000, Norris and Evans 2000, Owen et al. 2010).

It remains challenging to identify suitable methods to "measure" and classify immunocompetence in non-model species because single immunological tests can only explain a small part of the immune defence and our knowledge about interactions within the immune system is still limited. Measures of immunological responses do not directly assess the ability and capacity of the immune system to limit or resist an infection. Although a multitude of assays is available, most studies in birds focused on only a small proportion of immunological tests. Using only a single component of the immune system can be misleading because of its complexity, a trade-off between life-history traits unknown and varying energetic costs. It is always possible that no costs are observed because compensation within the immune system has taken place or an organism can compensate with an extra intake of food, which highlights the importance of using several tests reflecting different areas of the immune system simultaneously (Schmid-Hempel and Ebert 2003, Norris and Evans 2000, Keil et al. 2001).

The immune system can be divided into three main components: innate, cell-mediated and humoral immunity. Especially genes that are involved in the immune defence could provide important information and serve as tools for further investigations to quantify immunocompetence. In particular, genes of the major histocompatibility complex (MHC) could be suitable, because they are present in almost all vertebrates and they play a central role in the immune system and pathogen resistance (Klein 1986). More specifically, they are responsible for cell-mediated immune responses that are mediated by T-lymphocytes that either regulate the function of B-lymphocytes and phagocytes or

destroy infected host cells through interactions with antigens present on the surface of these cells (Roitt et al. 1998). There are three classes of MHC genes but in this study we only concentrated on MHC class I. These cell surface molecules typically present peptides derived from the degradation of intracellular pathogens (such as viruses or some protozoa) to antigen-specific CD8+ T lymphocytes that thereafter degrade the infected host cells (reviewed by: Sommer 2005).

There is evidence that immunocompetence is directly related to the MHC and that MHC heterozygosity is responsible for a slower development, more effective clearance and reduced pathogenicity of numerous bacterial and viral infections or parasites in a multitude of vertebrate species. Also specific MHC alleles have been shown to provide higher resistance/susceptibility or varying severity to numerous diseases (reviews by: Sommer 2005, Piertney and Oliver 2006). Individuals heterozygous at a MHC locus theoretically recognize twice as many foreign peptides than homozygotes. This "heterozygote advantage" gives a greater ability to respond to multiple simultaneous infections (Hughes 1992, Milinski 2006, Penn et al. 2002). There is evidence that the highest immunocompetence is achieved at intermediate levels of MHC heterozygosity (Penn and Potts 1999, Milinski 2006, Wegner et al. 2003). Extremely high diversity might lead to a reduced fitness by over depletion of the T-cell repertoire during self-tolerance induction and a reduced potential to mount an immune response to foreign antigens (Nowak et al. 1992).

The aim of this study was to demonstrate that the MHC is a suitable tool to measure and quantify immunocompetence and to gain more insight in interactions within the immune system. Specifically we were interested to find relationships of specific MHC alleles and MHC heterozygosity on several immunological tests and survival using a captive house sparrow population. To answer this question we used a variety of immunological tests that affect different areas of the immune system and monitored individual survival.

Methods

In 2010, wild house sparrows (*Passer domesticus*) were captured in Vienna, Austria. These birds were housed in large outdoor aviaries (10 birds/aviary; aviary size: 3.5m × 3.5m × 3m) that were equipped the same way with vegetation, perches, commercial food

for granivorous passerines (*ad libitum*) and water. In 2012, these birds were breeding and we used their offspring for several immunological tests. In 11 days old chicks we tested skin-swelling response to phytohaemagglutinin (PHA). Offspring were also housed in aviaries, but in same sex flocks. After one year (July 2013), additional immune tests were conducted with these 128 sparrows (52 females and 76 males) derived from 42 different families (average: 3 chicks/family) and born in different clutches (clutch 1-4). Our aviary population of wild house sparrows allowed us to control for several environmental factors that might otherwise interfere with our results, like food availability (food was offered *ad libitum*), predation and differences in microclimate as well as socio ecological factors including population density and age, to highlight the genetic influence on the immune system. Also, we could use a wider range of tests that requires recapture and would be a problem in the wild. Following immune tests were done with one year old birds: hematocrit (HCT), erythrocyte sedimentation rate (ESR), bacterial killing assay (BKA) and heterophil/lymphocyte (H/L) ratio. Due to stress and potential reciprocity, we had 2 different groups where we either tested first and second skin-swelling response to PHA in one year old birds (group 1) or haemagglutination titers after sheep red blood cell (SRBC) injections (group 2) (see Table 1 for experimental design). These tests and surveys correspond to the innate and the adaptive (cell-mediated and humoral) immune defence (see Table 2 for details). Since longer handling stress could affect different immunological measurements, blood was taken within 20 minutes after capture (Buehler et al. 2008). For each bird, we collected blood samples from the brachial vein (100 µl per bird) into sterile, heparinized capillary tubes. The immunological tests were performed prior to MHC screening and therefore blindly with respect to individuals' genotypes. We additionally measured tarsus length and body mass to calculate a condition factor (residuals of weight/tarsus). After one winter (March 2014) survival status was assessed. All animal experiments were in accordance to the Austrian Law (Geschäftszahl: BMWF-68.205/0081-ll/3b/2012).

Immune tests

ESR/HCT: To measure the immunological structure we analyzed ESR and HCT (in duplicate) with standard methods used for hematological parameters (Schalm 1975). We took fresh blood samples (about 20 µl) from the brachial vein and collected the blood into heparinized capillary tubes and immediately sealed them with wax at the bottom. The capillary tubes were positioned vertically for 4 h at 4°C. ESR was measured with a digital

calliper to the nearest 0.01 mm and calculated as the distance occupied by plasma divided by the total length of the blood column (red blood cells + plasma). Capillary tubes were centrifuged for 10 min at 12,000 g and HCT was measured as the relative amount of red blood cells divided by total blood volume (red blood cells + plasma) with a digital calliper the nearest 0.01 mm.

BKA: We measured the constitutive innate immunity by assessing the capacity of plasma to kill microorganisms in vitro for gram-negative bacteria (*Escherichia coli*), using spectrophotometry (according to Liebl and Martin 2009). Plasma was extracted by centrifuging fresh blood at 12,000 g for 10 min at 4°C and collected into new sterile 1.5 µl microcentrifuge tubes. Plasma samples were frozen at -80°C within 2 h and analyzed in triplicate within 5 days after collection. *E.coli* bacteria (American Type Culture Collection ATCC8739, Microbiologics Inc.), supplied as lyophilized pellets (6.6 x 10^7 colony forming units (CFU) per pellet) were reconstituted per manufacturer instructions in 10 ml sterile PBS (phosphate buffered saline, P4417 Sigma-Aldrich). Before each assay, microbial stocks were diluted to a working concentration of $1x10^5$ microbes/ml). Plasma was diluted 1:23 with sterile PBS (1.5 µl plasma with 34.5 µl PBS) and microbial working solution (12.5 µl) was added, vortexed and incubated (37°C) for 30 min. All procedures were performed in a laminar flow hood, providing a sterile working environment in the laboratory. After incubation, all samples were removed, vortexed and 250 µl of sterile TSB (tryptic soy broth, 22092 Sigma-Aldrich) was added to each sample. Broth was vortexed again and incubated (12 h at 37°C). The number of microbes in the initial inoculum served as a positive control (12.5 µl working culture + 36 µl PBS + 250 µl of TSB). Solutions of 250 µl sterile TSB and 48.5 µl sterile PBS were used as blanks. Microbial concentrations were determined by measuring the absorbance of each sample at 300 nm using a Nanodrop. The anti-microbial activity of plasma and blood was calculated as 1-(absorbance of sample⁄absorbance of control), or the proportion of microbes killed in samples relative to positive controls.

H/L ratio: Circulating cellular immunity was assessed using differential leucocyte counts of heterophils and lymphocytes (H/L ratio) by examining blood smears. Blood slides were prepared within 2 min after the blood was taken using the standard two slide wedge procedure. All samples were air-dried and fixed with absolute methanol and stained with Giemsa (Roth, T862.1). Differential leucocyte counts of 100 heterophils or lymphocytes were carried out using a light microscope (1000× magnification with oil immersion).

PHA: The PHA skin-swelling test was used to estimate cell-mediated immunity and to screen proinflammatory potential of individuals (Tella et al. 2008, Vinkler et al. 2010). Following assay was used (according to Bonneaud et al. 2005): The left wing web was injected with 0.02 mg of PHA-P (Sigma-Aldrich: L8754) in 0.04 ml of PBS, while the right wing web was injected with 0.04 ml of PBS as a control. Thickness of swelling was measured 24 h after immunization with a pressure-sensitive spessimeter to the nearest of 0.01 mm. Intensity of the response was assessed as the difference in swelling between the PHA injected and the control wing. This was done for 11 days old chicks and adults. Adults received two injections, the second PHA injection seven days after the first.

SRBC: In vivo humoral immune response was assessed by measuring primary antibody response to intraperitoneal injections of a suspension of sheep red blood cells (Sigma-Aldrich: R3378), with a standard haemagglutination method (Pap et al. 2008). We took a blood sample and injected birds with 100 µl of a 10% SRBC suspension. After 8 days we took another blood sample to assess the primary response to SRBC. After centrifugation, plasma was stored at -80°C for less than one month. We estimated the production of anti-SRBC antibodies using the haemagglutination method using duplicates. The plasma was heated to 56°C for 30 min to prevent lysis of red blood cells by the complement. After pipetting 15 µl sterile PBS in each well of a U-shaped 96-well microtitre plate, 15 µl plasma was pipetted into the first column. Plasma was then serially diluted in PBS (dilution: 1/2 to 1/2048 with one negative control (PBS) per row). We then added 15 µl of a 1% SRBC suspension to these dilutions. After agitation, the plates were covered by parafilm and incubated at 37°C for 60 min. Agglutination was visually determined and when sufficient antibody activity was present, antibodies agglutinated the SRBC. Titres were expressed as the log2-transformed reciprocal of the highest dilution of plasma showing positive haemagglutination.

Molecular Methods

Avian blood was stored in 95% ethanol and DNA was extracted using the DNeasy Blood & Tissue Kit (Qiagen).

MHC genotyping

MHC characterization was carried out using 454 amplicon sequencing. MHC class I exon 3 sequences that encode parts of the peptide binding region in MHC molecules were

amplified using individually tagged 454-adapted primers (according to Galan et al. 2010). The MHC-specific primer stretches used were forward primer (longfw2): GTCTCCACACTGTACAGYGGC; and reverse primer (rv3): TGCGCTCCAGCTCCYTCTGCC) (Karlsson and Westerdahl 2013). These primers amplify 222-225bp long (primers not included) classical MHC alleles in house sparrows (Karlsson and Westerdahl 2013). Using Qiagen Multiplex MasterMix each 15µl PCR reaction contained 7.5µl of Hot Start Master Mix, 0.6µl (5µmol) each of the forward and reverse primer, 1µl of template (25ng genomic DNA) and 5.3µl dd H_2O. The PCR conditions were: 95°C/15 min, 35x(95°C/30 s, 65°C/1 min, 72°C/1 min), 60°C/5 min. PCR products were run on a 2% agarose gel and products with positive amplifications were purified using the MinElute PCR Purification Kit (Qiagen). Samples were pooled in eights and DNA was quantified with a Nanodrop instrument and adjusted accordingly before a second pooling for the 454 quadrants. The number of samples in the 454 quadrants was aimed at a sequence coverage of 300 reads per individual (according to Borg et al. 2011). 491 individuals were run in two different sequencing reactions (run 1 (R1) and run 2 (R2)) and these runs were filtered separately (see below). R1 contained 272 samples (243 individuals + 29 technical replicates (11.9%)) and R2 contained 286 samples (248 individuals + 38 technical replicates (15.3%)). 454 sequencing was done at Lund University Sequencing Facility (Faculty of Science), Sweden.

MHC - bioinformatics and data processing

After 454 sequencing, the data was extracted and assigned to samples using the program jMHC (Stuglik et al. 2011). To get rid of artefacts generated during the initial PCR, the emulsion PCR and the 454 sequencing reaction the raw data from the 454 run was filtered. Filtering steps were in accordance with Galan et al. 2010, Zagalska-Neubauer et al. 2010 and Sepil et al. 2012.

First, sequences in low abundances (<3 reads) were deleted. Identical sequences within individuals were detected and merged using the web-applications "seqeqseq" (http://mbio-serv2.mbioekol.lu.se/apps/seqeqseq.html) and "mergeMatrix" (http://mbio-serv2.mbioekol.lu.se/apps/mergeMatrix.html).

Second, we only wanted to keep sequences with a suitable sequence depth. Since the maximum number of classical MHC alleles is eight in house sparrows we chose a threshold of 104 reads per sample, this gives a genotype score of 99.9% (m=8, Galan et

al. 2010). Here we used the web-application "popMatrix" (http://mbio-serv2.mbioekol.lu.se/apps/popMatrix.html) and filter 1. This web application has several filers, filters 1-4.

Third, true alleles are likely to occur more frequently than artifact alleles. Therefore we wanted to set a threshold that keeps true alleles but disregards artifact alleles. We used our replicates to set these thresholds (2% in R1 and R2) and all alleles that occurred in lower than 2% per sample were deleted using filter 3.

Forth, to verify MHC alleles they should occur in at least two independent PCRs. We worked with family data (189 adults produced about 300 chicks), these individuals were distributed across R1 and R2) and every allele is therefore likely to occur several times. Therefore we used the criteria that every allele should be found at least twice, here we used filter 4.

Fifth, the obtained sequences were examined in BioEdit (v7.2.0) and all sequences that did not meet the expected length of 222-225bp or displayed indels that were not multiples of three base pairs were deleted. The next step was to look for PCR recombinants (chimeras) and 1-bp substitutions. Sequences were examined starting with the lowest frequencies at the population level (Sepil et al. 2012). All sequences that occurred in a frequency below 2% at the population level were checked in all individuals for chimeras and 1-bp substitutions. 50% of the sequences between a frequency of ≥ 2% to 25% were checked but no chimeras or 1-bp substitutions were found. Therefore, we assume that sequences with a frequency of ≥ 2% are true alleles. Additionally, alleles were verified and controlled for with inheritance data of families (189 adults produced about 300 chicks).

The filtered MHC DNA sequences were translated into amino acid sequences; this left 78 different sequences in the dataset. Only potentially functional sequences with no stop codons or frame shifts were kept for the analyses. We were especially interested in functional MHC diversity, because amino acids that are found in the peptide-binding regions (PBR) are involved in interactions with antigens and therefore crucial for the immune system (Doytchinova and Flower 2005, Schwensow et al. 2007). They can be converted into five physicochemical descriptor variables: $z1$ (hydrophobicity), $z2$ (steric bulk), $z3$ (polarity), $z4$ and $z5$ (electronic effects) (Sandberg et al. 1998). The 16 amino acids from each sequence that corresponded to the PBR in chicken (Wallny et al. 2006)

were extracted; this left 59 different functional sequences in the dataset. MHC diversity was evaluated as: 1) the number of unique functional sequences/alleles per individual and 2) the presence/absence data of the 10 most common functional sequences/alleles with a frequency higher than 10% at the population level.

Genome-wide genetic variation

For the assignment of genome-wide individual heterozygosity, all individuals were typed at 13 highly polymorphic neutral microsatellite loci. The quality of loci (Hardy-Weinberg Equilibrium and Linkage Disequilibrium) was tested using the programs Cervus (v3.0.3) and Fstat (v2.9.3.2). Individuals were genotyped in two multiplex reactions according to the protocol of Dawson et al. (2012) (Supplementary Table 1). The loci used in this protocol are known to be distributed across at least seven different chromosomes and thus likely reflect the genome-wide level of neutral genetic variation (Dawson et al. 2012). Forward primers were fluorescently labeled using different dyes (YYE, FAM or AT550, Microsynth). The two multiplex PCRs were carried out using a Multiplex Kit (Qiagen) in a final PCR reaction volume of 6 µl containing 3µl of the Qiagen enzyme, 2µl of the primer mix and 1µl of the template (25 ng genomic DNA). PCR cycling scheme and primer concentrations of the 2 multiplex PCRs were carried out according to Dawson et al. (2012). PCR products were diluted (1:20) and analyzed in an ABI genetic analyzer (Applied Biosystem 3130xl) automated sequencer. Allele sizes were determined using GeneMapper (v3.0) with reference to an in-house DNA Size Standard (HMROX). We successfully amplified 12 loci (Supplementary Table 1). However, we excluded loci Pdoµ6 due to amplification problems.

Statistical analysis

Treatment effect on weight (SPSS v.20): Since we had 2 different treatment groups (PHA or SRBC injections), we tested if there was an effect on body mass. Weight was measured on day 0 and 7/8 (group 1/2) to see if the different treatments influenced weight. We conducted a GLM with sex and treatment group as fixed factors and weight difference as dependent variable. Sex and the interaction of sex and treatment group were not significant. Non significant results were removed subsequently. There was a significant treatment effect on the weight difference between the 2 groups ($p=0.023$) during the treatment time. While individual weight in group 2 (SRBC injections) was stable (N= 62, MW +0.06g, stabw 0.90), individuals of group 1 (PHA injections) gained

more weight after the first PHA injection (N=62, MW +0.45g, stabw 0.99). To test if the weight difference between treatment groups remained for a longer time (8 months), we also measured weight after one winter (March 2014) and conducted a GLM with weight difference before the experiments and after one winter as dependent variable and sex and treatment group as fixed factors. We found that there was only a significant sex effect on the weight difference after one winter (p=0.001). Not the treatment group or the interaction of sex and treatment group were significant, indicating that the different immunological treatments did not affect physical health over a longer time. Male weight was stable (N= 57, MW +0.09g, stabw 1.27) while females lost more weight over the winter (N=46, MW -0.77g, stabw 1.29). The sex differences in weight changes might be a result of different investment strategies or parasite/infection pressures between sexes. Gaining weight is not an uncommon reaction to immunological challenges since individuals might try to compensate for the higher physiological needs with higher food intake. Disease (e.g. anemia or parasites) could lead to a higher food intake and might mask potential negative fitness effects (Norris and Evans 2000). PHA treatment has already been shown to increase food intake in birds (Martin et al. 2003, Barbosa and Moreno 2004).

Influence of MHC on the immune system and survival:

We used linear mixed models within the R package v.3.0.3 x64 (R Core Team 2013) to analyze the influence of MHC on several immunological parameters and survival. In order to evaluate and compare different models, we used the Akaike Information Criterion corrected for small sample sizes (AIC_c, Burnham and Anderson 2002). In all cases there was not one clearly best model, so we used methods of model averaging and multimodel inference (Burnham and Anderson 2002). These methods allow inference over all models considered, but this was weighted according to model support by the data. Additionally, these methods do not only estimate standard errors unconditionally for a single model, they also provide the probability for single variables being in the unknown "true" model (the so-called relative variable importance - RVI). Unlike variable selection based on p-values these techniques have a sound mathematical basis and are increasingly recommended (Symonds and Moussalli 2011). We conducted these calculations in R using the package MuMIn (Bartón 2013). Additionally, we inspected the residuals for normality visually using histograms and QQ-plots. H/L ratio and ESR were log-transformed to achieve normality. Since we included the 10 most common functional

MHC sequences in our analysis (frequency on the population level >10%), we had to do a pre-model selection of variables. In the first model we evaluated the relevance of the 10 sequences, while all other factors (e.g. number of functional MHC sequences, microsatellite heterozygosity, clutch number and condition) were fixed. We then included only functional MHC sequences in the final model that had RVI values larger than 0.5. Since early development could have an important influence on immune parameters, the day they were born (day numbers in 2011) as well as the clutch number (we used birds that originated from clutch 1-4 of specific pairs) were recorded. Since breeding was highly synchronized, these two parameters were correlated ($p<0.001$) and only clutch number was used. In the results section, only variables that have RVI values >0.6 and p-values <0.05 are discussed.

Influence of MHC on the immune system: We used linear mixed models to analyze which of the variables (number of functional MHC sequences, presence/absence data of the ten most common functional sequences, microsatellite heterozygosity, clutch number, condition and family origin as a random effect) correlated with the following immune parameters that were tested in different models: ESR, HCT, BKA, H/L ratio, PHA (chicks and first and second response after one year) and SRBC as dependent variable. In cases where the standard deviation of the random effect "family" was very small, we used a model with fixed effects only.

Influence of MHC on survival: We used a logistic mixed model to analyze which of the variables (number of functional MHC sequences, presence/absence data of the ten most common functional sequences, microsatellite heterozygosity, clutch number, H/L ratio and family and treatment group (group 1 or 2) as random effects) correlated with survival (0) or death (1) over one winter. Since the standard deviation of the random effect "treatment group" was very small, we removed it from the model. We included the H/L ratio in our analysis because it has been show that birds with high H/L ratios might have reduced survival rates (Kilgas et al. 2006, Ochs and Dawson 2008).

Covariance of different immune measures: We conducted a principle component analysis (PCA) with individuals of group 1 (N=64) to gain insight into the complex relationships within different immune measures with SPSS (v.20). It is a method to derive linear combinations of original variables in a dataset to summarize variation. Covariance can be identified among more than two variables and this method is increasingly used to

summarize data taken from multiple measures of immune function (Matson et al. 2006, Buehler et al. 2011). We used varimax rotation to maximize contrasts of variable loadings between factors. We were especially interested in relationships of the immune system for individuals of group 1, since we wanted to see the relationship of the repeated PHA measurements.

Results

MHC characterization

We had a total of 75894 sequence reads of 19200 unique sequences in 454 run 1 (R1) that had complete tags and primers. In run 2 (R2) we had a total of 125752 sequence reads of 34670 unique sequences. After filtering the data, a total of 85 different MHC alleles were found that could be translated into 59 unique functional MHC alleles, based on their chemical properties (Sandberg et al. 1998). There was an average of 4.2 functional MHC alleles/individual (ranging from 1-8). Males had on average 4.2 alleles (sd: 1.4), females had 4.3 (sd: 1.4). Adults possessed on average 4.3 alleles (sd: 1.5), chicks had 4.2 (sd: 1.3). In adults and chicks, there were 10 functional MHC alleles that occurred in frequencies larger than 10% at the population level, these were included in the analysis.

Influence of MHC on the immune system

Innate immunity – ESR and **HCT (**N=128): One functional MHC sequence 'seq09776' negatively correlated with the ESR (p=0.012, RVI=0.90; Fig. 1). Individuals possessing this sequence had a smaller ESR (see Tab. 3 for details). The same functional MHC sequence positively correlated with the HCT (p=0.039, RVI=0.76; Fig. 2). Individuals possessing this sequence had a higher HCT (see Tab. 4 for details). Since ESR and HCT are closely linked it is not surprising that there was a correlation between them (linear regression $p<0.001$) and the same MHC sequence correlated with both.

Innate immunity – BKA (N=122): We found a negative correlation of the same functional MHC sequence ('seq09776') that also correlated with ESR and HCT (p=0.011, RVI=0.88) and a positive correlation of 'seq25663' (p=0.011, RVI=0.87) on BKA (Fig. 3). Individuals with the functional sequence 'seq09776' had lower, while individual with 'seq25663' had higher rates of *E. coli* bacteria killed by plasma (see Tab. 5 for details).

Innate and adaptive (cell-mediated) immunity – H/L ratio (N=128): We found a positive correlation between the functional MHC sequence 'seq00217' and the H/L ratio (p=0.020, RVI=0.82; Fig. 4). Individuals possessing this sequence had a higher H/L ratio (see Tab. 6 for details).

Innate and adaptive (cell-mediated) immunity –PHA: In chicks (N=125), again the functional MHC sequence 'seq09776' (p=0.021, RVI=0.82; Fig. 5) that also correlated with ESR, HCT and BKA had a negative correlation with PHA (see Tab. 7 for details). In adults (N=62) we could not find a correlation with the first response to PHA and any other variables (see Tab. 8 for details). The second response to PHA was negatively correlated with MHC heterozygosity (p<0.001, RVI=1.00; Fig. 6) and the functional MHC sequence 'seq23304' (p=0.018, RVI=0.86; Fig. 7). Individuals possessing more different functional MHC sequences and/or the sequence 'seq23304' produced a smaller secondary response to the mitogen (see Tab. 9 for details). PHA response in chicks was not correlated with the first (linear regression: p=0.496) or second (linear regression: p=0.956) PHA response after one year. First and second response to PHA in adult birds were correlated and almost significant (linear regression: p=0.051). Second swelling response to PHA was higher than first response (paired t-test: p=0.001), first response in adults was higher than the response in chicks ((paired t-test: p<0.001) (Fig. 8).

Adaptive (humoral) immunity - SRBC (N=62): We found a positive correlation of the functional MHC sequence 'seq18621' (p=0.008, RVI=0.93; Fig. 9) on agglutination of SRBC (see Tab. 10 for details). Individuals with this sequence had higher antibody activity to agglutinate the SRBC.

Survival (N=121): We found a positive correlation of the functional MHC sequence 'seq25835' (p=0.048, RVI=0.67; Fig. 10) and the H/L ratio (p=0.032, RVI=0.83; Fig. 11) on survival. Individuals with the functional MHC sequence 'seq25835' and high H/L ratios had lower survival rates (see Tab. 11 for details).

Covariance of different immune measures: Our principal component analysis identified three PCs with eigenvalues > 1 that cumulatively accounted for 63% of the total variation (see Tab. 12). The patterns of loadings on these three PCs revealed that ESR, HCT and H/L correlated with PC1 (26.2% of total variation), PHA in chicks and BKA correlated with PC2 (18% of total variation) and PHA first and second response in adults correlated with PC3 (18% of total variation). PC1 might reflect measurements of blood

parameters, while PC2 with PHA in chicks and BKA could reflect innate immunity and PC3 with the two PHA measurements in adults might reflect adaptive cell-mediated immunity.

Discussion

In this study we could demonstrate that MHC is a suitable tool to measure and quantify immunocompetence and to gain more insight in interactions within the immune system. We found that different functional MHC sequences and MHC heterozygosity correlated with the innate and adaptive immunity, including: HCT/ESR, BKA, H/L ratio, PHA and SRBC.

Since it has been shown that antibody production against several antigens (e.g. *Salmonella enteritis*, *Brucella abortus*, bovine serum albumin, SRBC) and also cellmediated immunity (e.g. PHA) is linked to the MHC in different bird species (Bonneaud 2005, Zhou and Lamont 2003) we investigated the interaction of MHC class I on different immune responses. Although birds under natural conditions will not be challenged with novel antigens that are commonly used in immunological tests, some are related with actual diseases. Chicken lines that were selected to produce high antibody titers to SRBC showed also higher antibody levels against the Newcastle disease virus and were more resistant to some naturally occurring pathogens (e.g. *Mycoplasma gallisepticum*, *Eimeria necatrix*) and feather mites (Gross et al. 1980). Long-term selection for antibody response to SRBC also leads to a change in allelic frequencies of MHC genes in chicken (Dunnington et al. 1984).

Especially one functional MHC sequence correlated with different immune parameters (HCT, ESR, BKA, and PHA in chicks). Individuals possessing the sequence 'seq09776' had a lower ability to kill *E.coli* bacteria in vitro (BKA), a lower ESR, a lower swelling response to PHA in chicks, but a higher HCT. The negative relationship between ESR and HCT is logic, since they are physiologically dependent. That ESR and HCT explain a similar section of the variation is also indicated by a principle component analyses (PCA), where both factors are correlated to PC1. Similarly, BKA and PHA in chicks were correlated to PC2. Thus PC2 may be interpreted as an innate response factor, which is supported by the fact that *E. coli* is primarily attacked by cellular components of the

blood and it relies mainly on soluble blood components rather than phagocytosis (Millet et al. 2007). This significant MHC sequence occurred in a high frequency (32.81%) and was the third most common functional sequence in our population. The association of one specific functional MHC sequence on to the intensity of different immune responses is at a first glance surprising, but it might be in linkage disequilibrium with other immune genes, e.g. MHC class II or genes that are involved in body condition. Because of its high complexity, we cannot interpret smaller or bigger immune reactions as negative or positive effects or an advantage or disadvantage, since our knowledge about the underlying physiological mechanisms and relationships within the immune system are still limited.

We also found evidence that extremely high MHC heterozygosity might lead to reduced fitness. Since the heterozygote advantage theory predicts that individuals heterozygous at a MHC locus theoretically recognize twice as many foreign peptides than homozygotes (Hughes 1992, Milinski 2006), it is surprising that in our population individuals with higher MHC heterozygosity had a weaker response against a second PHA injection. These results indicate that the expression of numerous sequences might be costly. Extremely high diversity might lead to a reduced fitness by over depletion of the T-cell repertoire during self-tolerance induction and a reduced potential to mount an immune response to foreign antigens (Nowak et al. 1992).

Individual year to year survival directly correlated with stress indicated by the H/L ratio, but at the same time also with one functional MHC sequence (‘seq25835’). Birds with a high H/L ratio had a lower chance to survive the winter. This pattern has been also found in other bird species, where breeding individuals with higher H/L ratios did not survive the winter (Kilgas et al. 2006, Ochs and Dawson 2008). Our results are even more interesting since the severe conditions in the natural environment should be less prominent in our captive population. We could further show that the H/L ratio itself was also correlated with a specific functional MHC sequence (‘seq00217’). Thus individuals appointed with this MHC sequence had to suffer from higher H/L ratios that correlated with lower survival. But also individuals appointed with the functional MHC sequence ‘seq25835’ had lower survival rates. Specific functional MHC sequences that correlated with survival rates have also been found in voles (Kloch et al. 2013). Since we only concentrated on MHC class I, which is responsible for cell-mediated immunity, it is remarkable that also other areas of the immune system (innate and humoral) were linked

to MHC class I, highlighting the linkage of different genes. Unfortunately, with our study design it is not possible to disentangle the influence of MHC genes itself or any other genes in linkage disequilibrium with it.

Impact of the immune tests in detail

The ESR is increased during any cause of inflammation and individuals with the functional MHC sequence 'seq09776' had a lower ESR. The same functional MHC sequence 'seq09776' also correlated with HCT. Individuals possessing this functional sequence had a higher HCT. The hematocrit value reflects blood oxygen-carrying capacity, it can be low in acute or chronic diseases (e.g. anemia), nutritional deficiencies, in parasite infested individuals (in blood or gastrointestinal) and bacterial infections (review: Fair et al. 2007). Individuals that possessed the functional sequence 'seq09776' had higher HCT values. These two blood measurements that correlated with the same functional MHC sequence ('seq09776') were also correlated (shown by a linear regression and a PCA).

The BKA assay is a useful tool to broadly characterize an individual's capacity to prevent infections once they have reached the bloodstream. *E.coli* bacteria commonly infect house sparrows and their plasma contains proteins (globulins) and lysozyme (enzyme) that can fight these infections. *E. coli* is primarily attacked by cellular components of the blood and it relies mainly on soluble blood components rather than phagocytosis. The observed variation in cell-mediated microbial killing likely reflects individual variation in cellular defences against a variety of microbes rather than the ability to recognize specific pathogens (Millet et al. 2007). In our population, the same functional MHC sequence ('seq09776'), that also correlated with ESR and HCT and the sequence 'seq25663' correlated with the BKA whereby individuals with 'seq09776' had lower, and individuals with 'seq25663' had higher rates of *E. coli* bacteria killed.

The H/L ratio has widely been used as an indicator of stress in birds. In response to stressors, the relative number of heterophils in peripheral blood increases while the relative number of lymphocytes decreases (Gross and Siegel 1983). These effects are mediated by glucocorticoids (Siegel 1985). It can be increased during times of low food availability, in parasite-infested, migrating or breeding birds (review by Davis et al. 2008). High H/L ratios might also lead to a depression of some immune indices (Müller et al. 2011, Krams et al. 2012) and can cause higher mortality rates (Kilgas et al. 2006, Ochs

and Dawson 2008). In our population, individuals with the functional sequence 'seq00217', the most common sequence, had higher H/L ratios.

The PHA skin-swelling test is widely used to estimate cell-mediated immunity and it is a robust tool for a quick screening of an individuals' proinflammatory potential (i.e. the ability to mount an inflammatory response) (Tella et al. 2008, Vinkler et al. 2010). The swelling response is highly complex, involving both cells of the adaptive and innate immunity and inflammation is as much a consequence of the recruitment of granulocytes as lymphocytes over varying time periods (Martin et al. 2006). In our population we found that the functional MHC sequence 'seq09776' that correlated with ESR, HCT and BKA, also correlated with the PHA swelling response in chicks. In adults, only the second injection of PHA was correlated with the MHC. Although MHC heterozygosity had no effect on the primary response to PHA in adults, individuals possessing more different functional MHC sequences produced a smaller secondary response to the mitogen. PHA response of chicks was smaller and not correlated with the PHA response in adults, also different MHC sequences correlated with the results. Also Haussmann et al. (2005) found that a PHA response in immature birds was lower compared to the PHA response of one year old adults in different bird species. In adults, first and second response to PHA was correlated and they explained one component of the PCA. The swelling response of the second PHA injection was significantly higher. Tella et al. (2008) also found that a second PHA injection showed stronger swelling responses compared to the first PHA injection in a variety of parrot species, indicating that repeated PHA injections may lead to an acquired immunity that could be explained by an increase of circulating lymphocytes. First and second response to PHA in adults and PHA response in chicks might not measure the same area of the immune system. In chicks, swelling response might depend more on the innate response, in adults, adaptive immunity could be more important. In a meta-analysis, Møller and Saino (2004) showed that survival of different bird species was positively correlated with PHA responses.

In vivo humoral immune response was assessed by measuring primary antibody response to intraperitoneal injections of SRBC. It gives information about the antibody-mediated immunity (IgM and IgG). Like skin tests, measurement of antibody titers following immunization with an antigen integrates a large number of immunological functions and events. If sufficient antibody activity is present, the antibodies will agglutinate the SRBC, which causes a visible spreading of the SRBC on the bottom of the wells. The functional

MHC sequence ‘seq18621’ positively correlated with SRBC and individuals with this sequence had higher antibody activity to agglutinate the SRBC. This sequence did not correlate with any other tests of the innate and cell-mediated response and seems to influence a different area of the immune system. Also other studies found a correlation of specific MHC sequences on SRBC (Bonneaud 2005, Dunnington et al. 1984).

Conclusions

In this study we could demonstrate that MHC is a suitable tool to further measure and quantify immunocompetence and to gain more insight in interactions within the immune system. With our aviary population, we were able to show different interactions within the immune system in relation to MHC diversity, while other factors could be controlled for, highlighting the genetic influence. We especially focused on functional MHC diversity which is based on properties of amino acids in the PBR. We found that different functional MHC sequences and MHC heterozygosity influenced survival and different areas of the innate and adaptive immunity, reflected by: HCT/ESR, BKA, H/L ratio, PHA and SRBC. Some of these immune measures, especially of the innate immunity, were correlated with the same functional MHC sequence (‘seq09776’). Also, one immune measure (PHA) correlated with different functional MHC sequences in several life stages, indicating that this test measured different immune areas (innate – adaptive). This could additionally been shown by a PCA. We also found evidence that extremely high MHC heterozygosity might implicate a disadvantage for the immune system.

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Figures and Tables

Figure 1: Influence of the functional MHC sequence ‘seq09776’ on ESR (log-transformed)

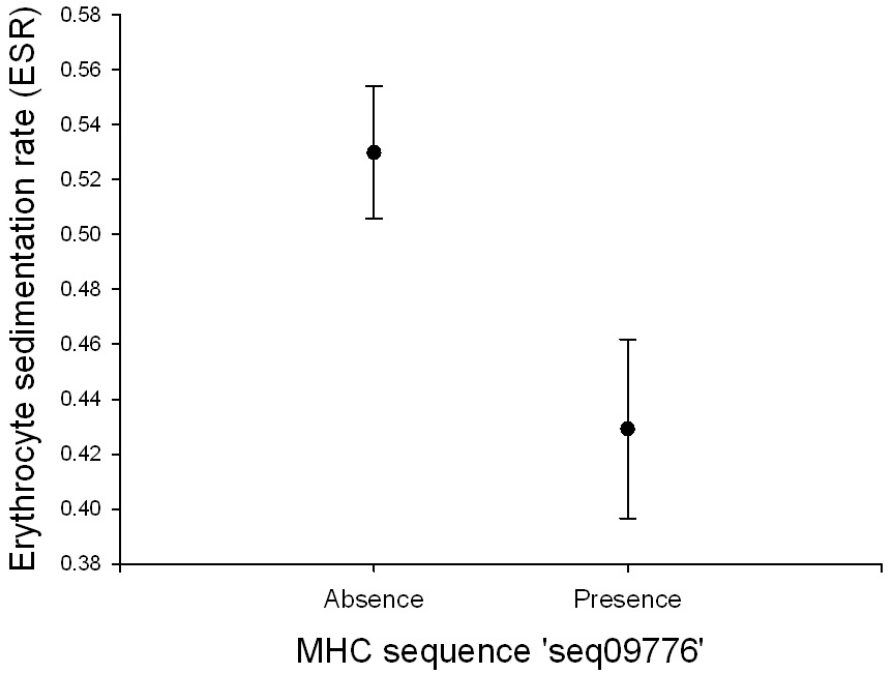

Figure 2: Influence of the functional MHC sequence ‘seq09776’ on HCT

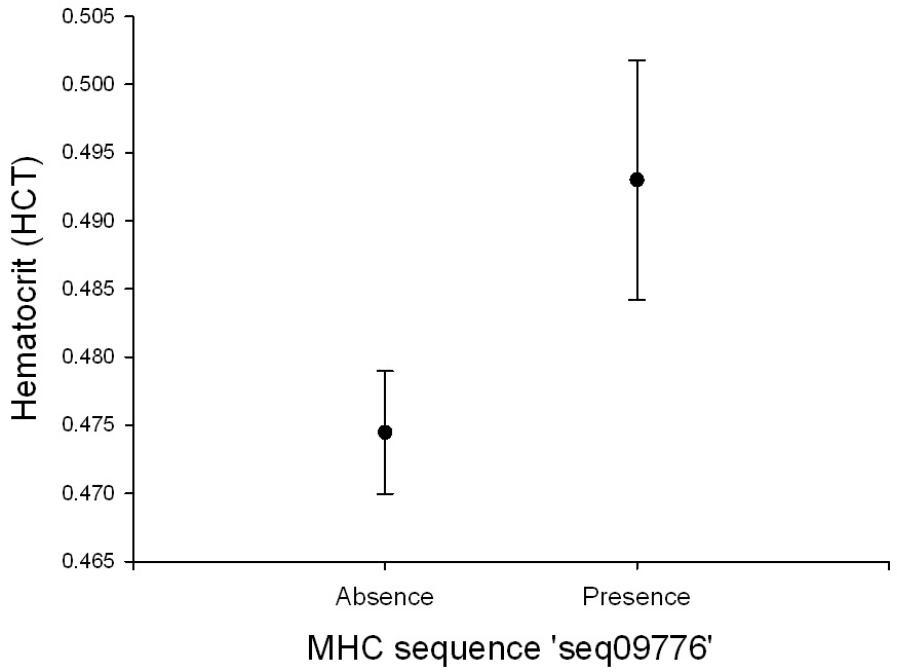

Figure 3: Influence of the functional MHC sequences ‘seq09776’ and ‘seq25663’ on BKA

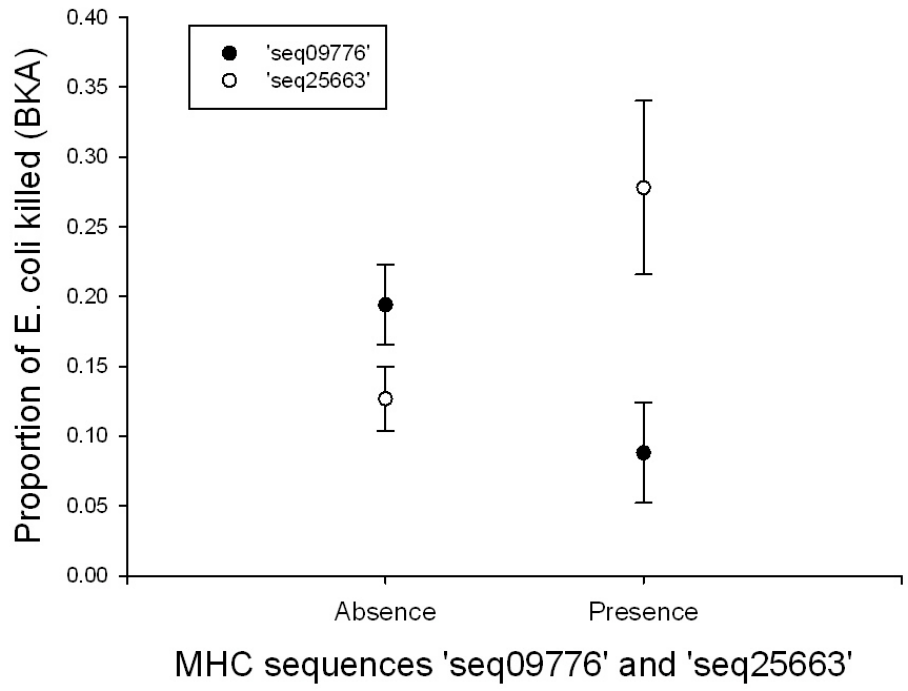

Figure 4: Influence of the functional MHC sequence ‘seq00217’ on H/L ratio (log-transformed)

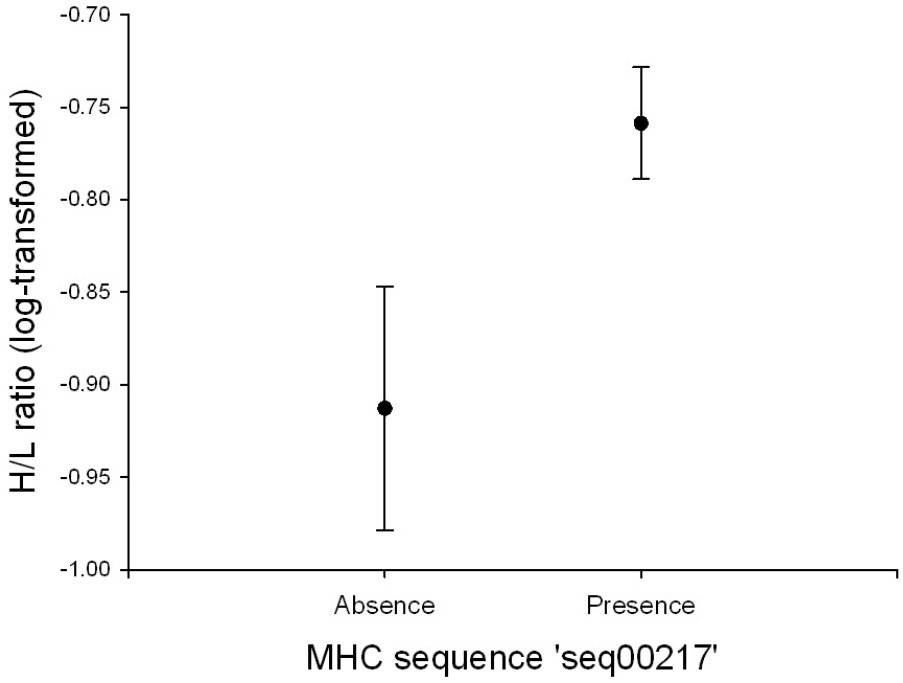

Figure 5: Influence of the functional MHC sequence ‘seq09776’ on PHA response in chicks

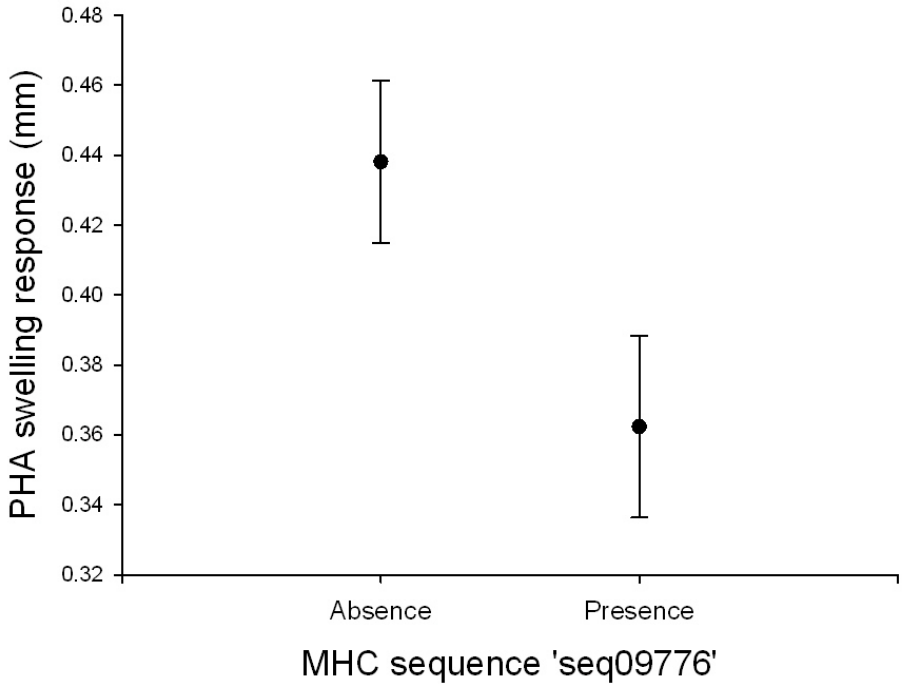

Figure 6: Influence of the functional MHC heterozygosity on the second PHA response in adults

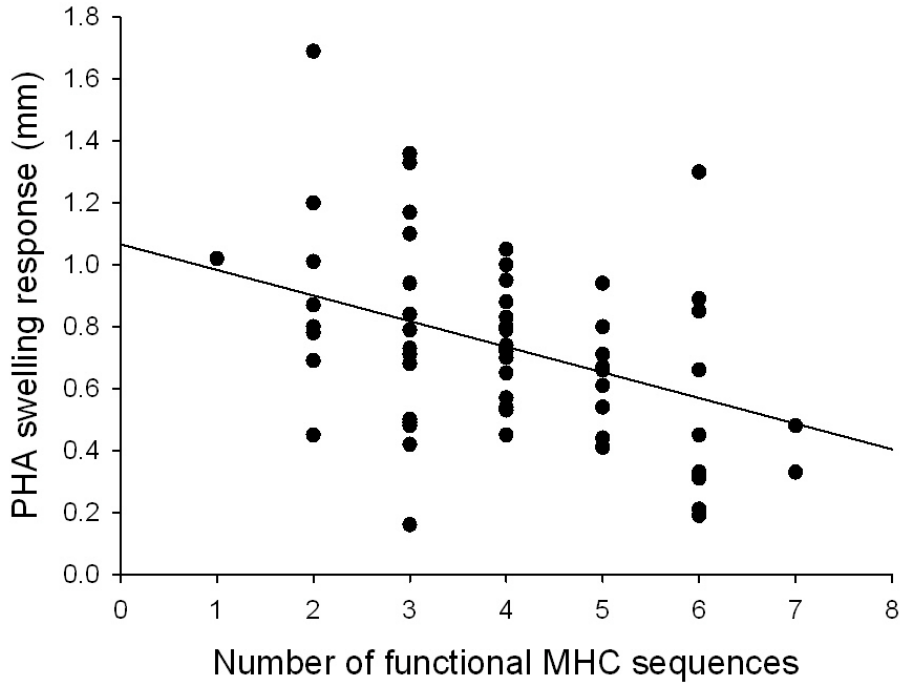

Figure 7: Influence of the functional MHC sequence ‘seq23304’ on the second PHA response in adults

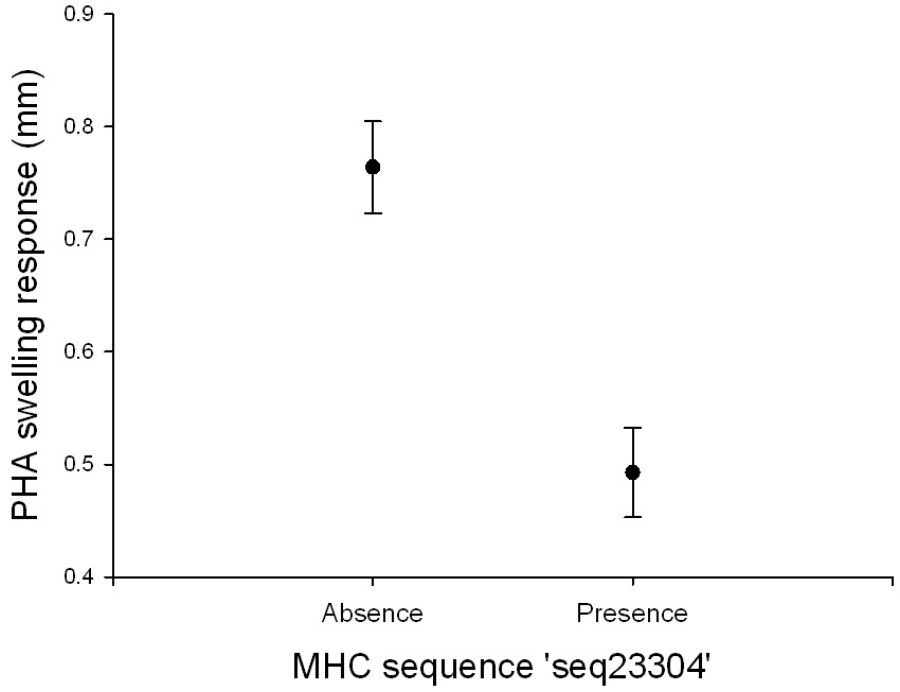

Figure 8: PHA response in chicks and adults (first and second response)

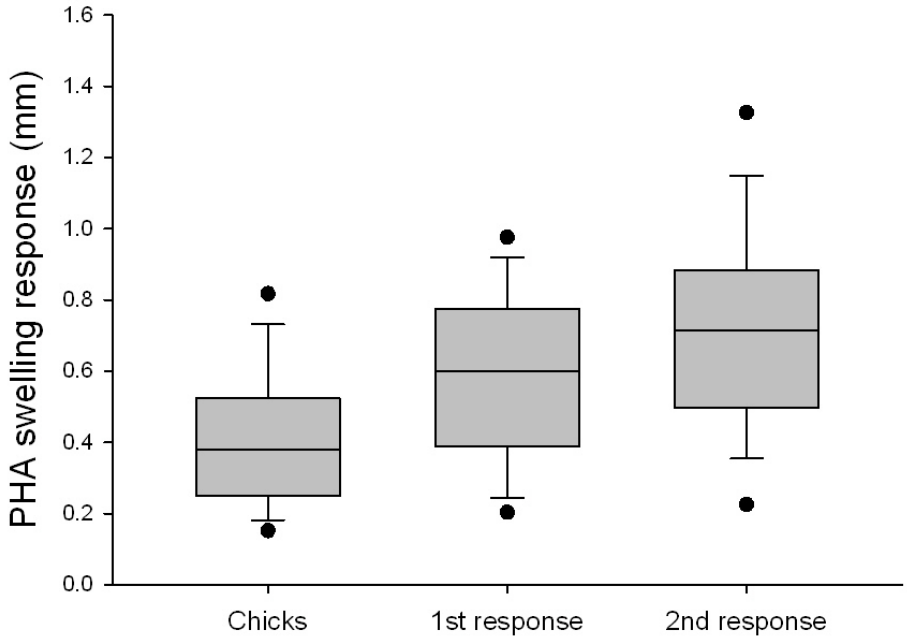

Figure 9: Influence of the functional MHC sequence ‘seq18621’ on SRBC

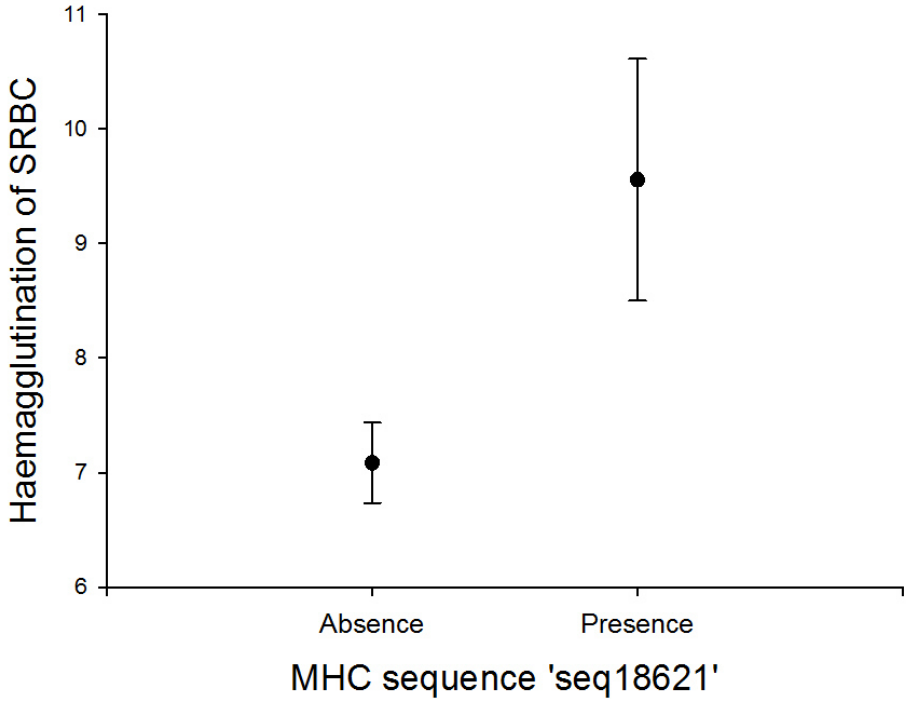

Figure 10: Influence of the functional MHC sequence ‘seq25835’ on survival

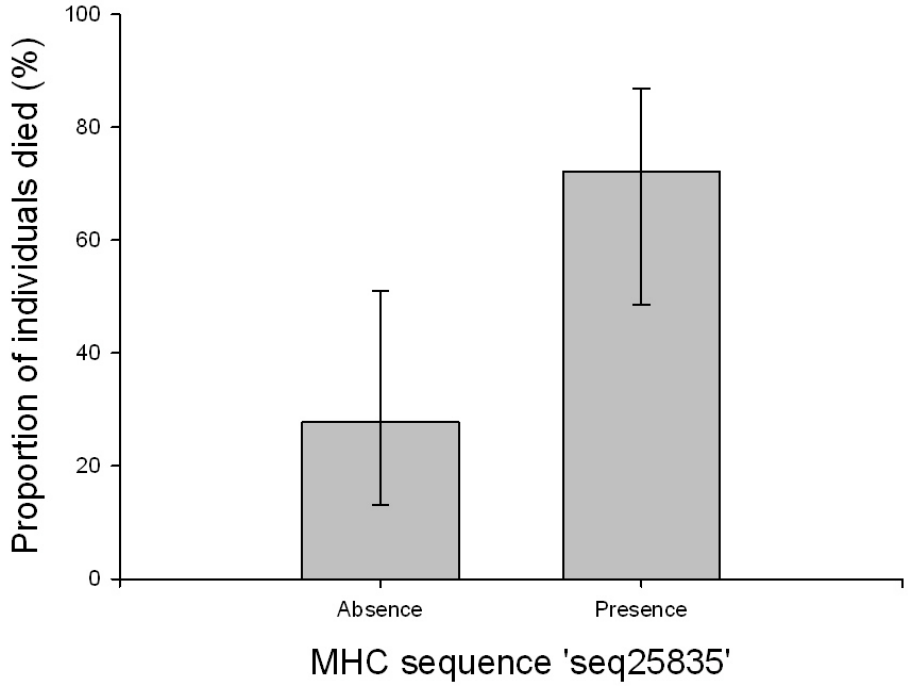

Figure 11: Influence of the H/L ratio on survival

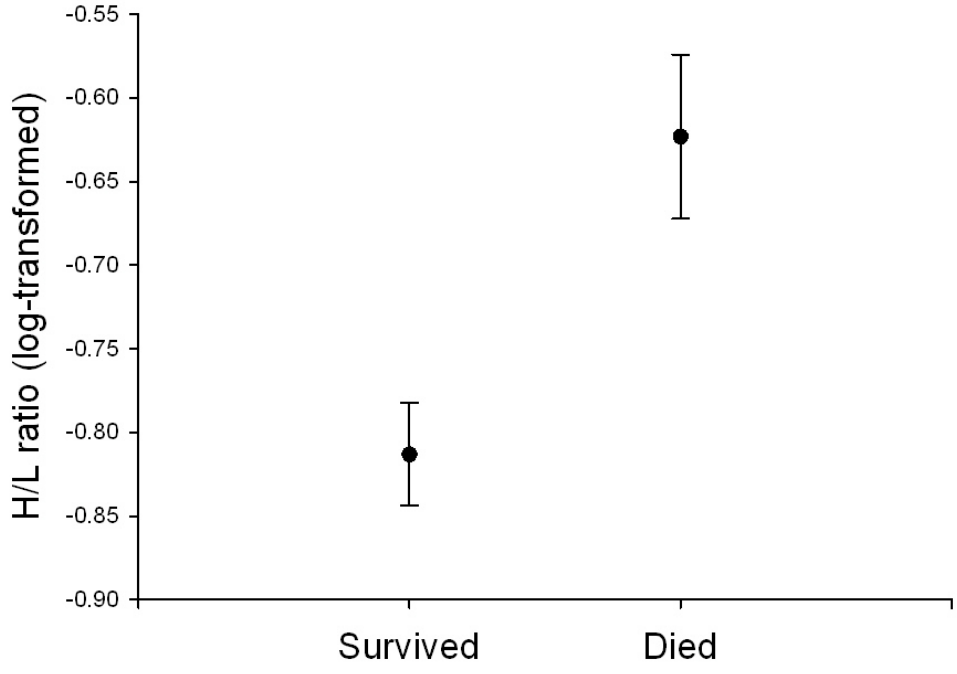

Table 1: Schematic presentation of the experimental design

Schedule	Group 1+2 (N=128) (M=76, F=52)	Group 1 (N=64) (M=31, F=33)	Group 2 (N=64) (M=45, F=19)
2012 (Spring) - Day 0	PHA injection (chicks) + Body measurements		
- Day 1	PHA response		
2013 (July) - Day 0	Blood (100µl) + Body measurements + HCT+ESR+H/L+BKA	1st PHA injection	SRBC injection
- Day 1		1st PHA response	
- Day 7		2nd PHA injection + Body mass	
- Day 8		2nd PHA response	Blood (100µl) + Body mass + SRBC (Agglutination)
2014 (March)	Survival status + Body mass		

Table 2: Immune tests used correspond to the innate and adaptive (cell-mediated and humoral) immune response in relation to MHC class I which is influencing cell-mediated adaptive immunity

Immune tests	Innate immunity	Adaptive immunity	
		cell-mediated	humoral
HCT	X		
ESR	X		
H/L	X	X	
BKA	X		
PHA (chick)	X	X	
PHA (1st response)	X	X	
PHA (2nd response)	X	X	
SRBC			X
MHC class I		X	

Table 3: Factors influencing ESR (linear model)

Model-averaged coefficients	Estimate	Adjusted SE	p value	RVI
(Intercept)	0.71006	0.32554	0.0292 *	
Seq09776	-0.10816	0.04304	0.0120 *	0.90
Seq21013	-0.09683	0.06129	0.1142	0.60
MHC heterozygosity	0.03360	0.02179	0.1231	0.58
Condition	0.02273	0.01445	0.1158	0.55
Microsatellite heterozygosity	-0.45273	0.33616	0.1781	0.47
Sex	-0.05018	0.04014	0.2112	0.43
Clutch	-0.02560	0.02312	0.2683	0.39

Table 4: Factors influencing HCT (linear model)

Model-averaged coefficients	Estimate	Adjusted SE	p value	RVI
(Intercept)	0.432299	0.067873	<2e-16 ***	
Seq09776	0.019559	0.009468	0.0388 *	0.76
Condition	-0.005749	0.003024	0.0573 .	0.69
Seq23304	-0.021207	0.011238	0.0592 .	0.68
Clutch	0.008507	0.004935	0.0847 .	0.61
Seq25614	-0.018122	0.012098	0.1341	0.52
Microsatellite heterozygosity	0.099452	0.071690	0.1654	0.48
MHC heterozygosity	-0.002676	0.003184	0.4006	0.33
Sex	-0.001839	0.008679	0.8322	0.25

Table 5: Factors influencing BKA (linear mixed model)

Model-averaged coefficients	Estimate	Std. Error	p value	RVI
(Intercept)	0.151083	0.229034	0.5095	
Seq09776	-0.127715	0.050279	0.0111 *	0.88
Seq25663	0.142886	0.055995	0.0107 *	0.87
Sex	0.056375	0.043910	0.1992	0.42
MHC heterozygosity	0.022142	0.019435	0.2546	0.40
Clutch	-0.015986	0.025881	0.5368	0.28
Microsatellite heterozygosity	-0.177387	0.406425	0.6625	0.26
Condition	-0.004224	0.016051	0.7924	0.25

Table 6: Factors influencing H/L ratio (linear mixed model)

Model-averaged coefficients	Estimate	Std. Error	p value	RVI
(Intercept)	-1.192771	0.376807	0.00155 **	
Seq00217	0.162643	0.069913	0.02000 *	0.82
Seq00275	0.131868	0.068118	0.05288 .	0.68
MHC heterozygosity	0.032166	0.019922	0.10640	0.51
Sex	0.081832	0.053788	0.12817	0.51
Microsatellite heterozygosity	0.476336	0.488317	0.32933	0.35
Clutch	-0.004279	0.033594	0.89864	0.25
Condition	0.003246	0.019762	0.86954	0.25

Table 7: Factors influencing PHA (11 day old chicks) (linear mixed model)

Model-averaged coefficients	Estimate	Std. Error	p value	RVI
(Intercept)	0.414186	0.181454	0.0225 *	
Seq09776	-0.095537	0.041412	0.0211 *	0.82
Seq25614	-0.085068	0.048177	0.0774 .	0.61
Clutch	-0.025792	0.020598	0.2105	0.42
Condition (11 days)	-0.005870	0.007771	0.4500	0.30
Microsatellite heterozygosity	0.177364	0.315973	0.5746	0.28
MHC heterozygosity	0.004327	0.014213	0.7608	0.27
Sex	0.005909	0.033212	0.8588	0.25

Table 8: Factors influencing PHA (1st adult response) (linear model)

Model-averaged coefficients	Estimate	Adjusted SE	p value	RVI
(Intercept)	1.0422902	0.5438147	0.0553 .	
Microsatellite heterozygosity	-0.8667956	0.4998592	0.0829 .	0.61
Condition	-0.0216268	0.0210471	0.3042	0.36
MHC heterozygosity	0.0100895	0.0224706	0.6534	0.26
Sex	0.0276417	0.0635688	0.6637	0.26
Clutch	-0.0003175	0.0391710	0.9935	0.24

Table 9: Factors influencing PHA2 (2nd adult response) (linear model)

Model-averaged coefficients	Estimate	Adjusted SE	p value	RVI
(Intercept)	1.69933	0.65737	0.009737 **	
MHC heterozygosity	-0.08943	0.02490	0.000328 ***	1.00
Seq23304	-0.26386	0.11202	0.018497 *	0.86
Clutch	-0.07405	0.04314	0.086082 .	0.60
Microsatellite heterozygosity	-0.99927	0.58543	0.087840 .	0.60
Sex	0.06276	0.07116	0.377805	0.31
Condition	-0.01359	0.02542	0.592879	0.26

Table 10: Factors influencing SRBC (linear model)

Model-averaged coefficients	Estimate	Adjusted SE	p value	RVI
(Intercept)	7.22247	3.87159	0.06211 .	
Seq18621	2.81106	1.05698	0.00783 **	0.93
MHC heterozygosity	-0.43126	0.27017	0.11043	0.56
Clutch	-0.43712	0.39563	0.26922	0.37
Microsatellite heterozygosity	4.20192	6.19283	0.49745	0.28
Sex	0.10316	0.78406	0.89532	0.24
Condition	-0.04194	0.27046	0.87676	0.24

Table 11: Factors influencing survival (logistic mixed model)

Model-averaged coefficients	Estimate	Std. Error	p value	RVI
(Intercept)	-0.12456	3.27256	0.9696	
H/L ratio	2.46867	1.14965	0.0318 *	0.83
Seq25835	1.52465	0.77062	0.0479 *	0.67
Condition	0.30523	0.21725	0.1600	0.49
Clutch	0.44801	0.32553	0.1688	0.46
Sex	-0.35386	0.66193	0.5929	0.29
Microsatellite heterozygosity	-2.62228	5.33464	0.6230	0.27
MHC heterozygosity	-0.09571	0.23462	0.6833	0.27

Table 12: Principal-component loadings after varimax rotation for seven different measures of innate and adaptive immunity (group 1, N=62)

	PC1	PC2	PC3
PHA (chicks)	-0,022	**0,807**	0,006
HCT	**-0,777**	0,104	-0,207
WBClog	**0,674**	-0,216	-0,269
BKA	0,038	**-0,756**	0,051
PHA (adults) 1st response	0,136	-0,164	**0,726**
PHA (adults) 2nd response	-0,074	0,103	**0,791**
ESR	**0,835**	0,193	0,071
Variance per component (%)	26,238	18,741	18,055
Cummulative variance (%)	26,238	44,978	63,034

Supplementary Information

Supplementary Table 1: Genome-wide genetic variation

Primer set	PCR mix	Size range	Number of alleles/locus	Primer set reference
Ase 18	1	185-249	17	Richardson et al. (2000), see Griffith et al. (2007)
Pdoμ1	1	156-200	18	Neumann and Wetton (1996)
Pdoμ3	1	113-181	18	Neumann and Wetton (1996)
Pdoμ5	1	202-264	21	Griffith et al. (1999)
Pdoμ6*	1			Griffith et al. (1999)
Pdo9	1	362-424	18	Griffith et al. (2007)
Pdo10	1	102-152	14	Griffith et al. (2007)
Pdo16A	2	270-302	15	Dawson et al. (2012)
Pdo17	2	192-244	22	Dawson et al. (2012)
Pdo19	2	173-183	4	Dawson et al. (2012)
Pdo22	2	92-130	17	Dawson et al. (2012)
Pdo27	2	224-248	12	Dawson et al. (2012)
Pdo40A	2	298-328	15	Dawson et al. (2012)

* had to be excluded due to amplification problems

Chapter IV

Ornament size reflects male malaria resistence: good news for choosy females looking for a mate

Manuscript by

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Ornament size reflects male malaria resistence: good news for choosy females looking for a mate

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Abstract

Only high quality males will be able to produce a large ornament and overcome the negative effects associated with it (e.g. stress and reduced immune function). But a large ornament could also provide valuable information about the adaption of a males' immune system in a given environment ("badge of immune system"). Since infection with parasites might be costly, males with specific resistance alleles that help them to evade infection could allocate more resources in the development of a large badge. These resistance alleles against parasites might be found at the major histocompatibility complex (MHC), a cell surface molecule crucial for pathogen resistance. In this study we investigate if MHC class I resistance alleles against a common blood parasite, the avian malaria strain SGS1 also influence badge size in a captive house sparrow population. We additionally investigated neutral heterozygosity (13 microsatellite loci) to control for other genetic influences. We found that one specific MHC allele was responsible for resistance against SGS1 in our population. Due to this fitness advantage, this allele was also responsible for the development of a larger badge in one year old males. This indicates a signal function of the badge about the functionality and adaption of the immune system in a specific environment.

Introduction

Showy ornaments are usually thought to be an outcome of sexual selection processes (Hamilton and Zuk 1982). They provide a "badge of status" to impress conspecific rivals or potential mating partners (Järvi and Bakken 1984, Rohwer 1975, Rohwer 1985, Møller 1987, Owens and Hartley 1991, Johnstone and Norris 1993, Botero et al. 2010, Dawkins and Krebs 1978, Liker and Barta 2001, Gonzalez et al. 2002, Nakagawa et al. 2007, Buchanan et al. 2010, Dolnik and Hoi 2010). In particular female choice is thought to be responsible for the evolution of exaggerated ornaments, frequently observed in males. An excessively (notoriously) stressed example in this context is the male peacock tail (Petrie et al. 1991). Bigger or brighter ornaments could by itself be attractive (Weatherhead and Robertson 1979) leading to more attractive sons (Saino et al. 1997, Petrie et al. 1991). However a key issue in mate choice is whether ornaments are informative about the quality of the bearer. One significant ornament feature in this context would be the health status e.g. reflected by immune system quality (Dufva and Allander 1995, Figuerola et al. 1999). A superior immune system for example may lead to viable offspring (Norris 1993, Møller 1994) with increased disease resistance (Hamilton and Zuk 1982, Andersson 1986, Møller 1990, Clayton 1991). However direct evidence for a "badge of immunity" is scarce.

Although a preference for brightly colored males has been reported in many species, the underlying purpose of this preference is not fully understood. Pale males, with respect to carotinoid-based ornaments for example, have been shown to suffer from parasitism and seem to be of low health status (review: Møller et al. 2000). In this context, Hamilton and Zuk (1982) proposed that plumage coloration has evolved as an honest signal of parasite and disease resistance and result in a negative relationship between parasite levels and ornament expression since only the healthiest individuals can afford the costs of developing a sexually selected trait. Alternatively, however, only the healthiest individuals may be able to invest into extravagant ornaments at the same time suffering high parasite loads. Ornaments can therefore likewise reflect individual quality exhibiting a badge of immuno handicap (Dufva and Allander 1995, Figuerola et al. 1999). Not surprisingly contradictory results have been found testing this relationship, which is further complicated by the fact that it is difficult to derive conclusions in particular when the negative effects on the host are not known (Clayton 1991). An ornament may hence either constitute a badge of handicap or a badge of health status, e.g. reflect parasite

intensity. Thus the relationship between the quality of the immune system and ornament expression and parasite resistance would be most relevant to understand a possible signalling function. A common genetic basis for ornament expression and immunocompetence has been rarely found. However, an ornament may provide specific information about individual resistance towards a specific disease (parasite).

The aim of this study was to test if ornament size, in our case the size of a melanin based black plumage badge of male house sparrows provides valuable information about the functionality of their immune system – "badge of immune system", in particular whether males, depending on ornament size, may have specific MHC alleles that prevent them from infection with common parasites. To do this we used house sparrows, captured from the wild and housed in aviaries. Their black badge (or bib) is a melanin-based ornament important in sexual selection (Buchanan et al. 2010, Dolnik and Hoi 2010, Griggio et al. 2010) and a secondary sexual signal that indicates individual quality. We identified a common threat, the avian malaria parasites *Plasmodium relictum* (mtDNA strain SGS1), and tested if specific major histocompatibility complex (MHC) alleles and overall MHV heterozygosity may provide resistance. The MHC is a large genomic region which contains the most diverse genes known in vertebrates. The gene composition and the genomic arrangement can vary widely between species and individuals. There are multiple MHC loci, most with many alleles. These genes play a central role in the immune system, disease resistance and autoimmunity (Klein 1986).

Avian malaria was chosen because it is a widely distributed Protozoan disease, caused by species of the genus *Plasmodium*. Infection of these parasites can decrease reproductive success (Marzal et al. 2005, Merino et al. 2000, Richner and Heeb 1995) and can cause anemia, morbidity or death (Atkinson and Van Riper 1991). Also chronic malaria infections with low parasite intensity (parasitemia) have shown to be costly in terms of reduced fitness and reproductive success (Asghar et al. 2011, Knowles et al. 2010). Finally and most important, specific major histocompatibility complex (MHC) alleles have been linked to increased resistance or susceptibility to common malaria strains in birds (Bonneaud et al. 2006, Loiseau et al. 2008). MHC alleles can influence presence/absence, but also intensity of infection (parasitemia) (Sepil et al. 2013, Westerdahl et al. 2012).

Methods

General methods

In March 2012, we assessed malaria infection status of 189 wild house sparrows (90 males and 99 females) that were captured in Vienna, Austria and housed in large outdoor aviaries (10-15 birds/aviary; aviary size: 3.5m × 3.5m × 3m). All aviaries were equipped the same way with vegetation, perches, commercial food for granivorous passerines (ad libitum) and water. These 189 birds were allowed to breed and from these chicks, we used 76 male house sparrows (originating from 37 pairs) to investigate badge size after their first winter (measured in June 2013) in relation to MHC resistance alleles and stress level (H/L ratio). We used one year old males because the development of an melanin-based ornament is dependent on testosterone (Bokony et al. 2008) and the effects of testosterone on plumage coloration may be age-dependent (Peters et al. 2006, Roberts et al. 2012, Alonso-Alvarez et al. 2009). Badge size of male sparrows usually increases during each molt (Summers-Smith 1988, Nakagawa et al. 2007, Buchanan et al. 2001). Roberts et al. (2012) demonstrated, that during the post-juvenile molt, the initial development of the first adult badge size was not significantly influenced by testosterone levels. Other factors, like environmental influences or genes might be important as well (Jensen et al. 2008). This was a clear advantage of our aviary population where we knew the age of the males and could account for several environmental factors. We additionally measured tarsus length and weight to get a body mass index as a condition factor (weight/tarsus residuals). The obvious benefit of an aviary population is that several factors potentially influencing the results (age, season, territory, diet, food availability, breeding status) could be controlled for. All measurements were performed prior to MHC screening and therefore blindly with respect to individuals' genotypes. All animal experiments were in accordance to the Austrian Law (Geschäftszahl: BMWF-68.205/0081-ll/3b/2012).

Blood sampling and H/L ratio

To test the stress level of birds, we assessed the H/L ratio, which reflects circulating cellular immunity by examining blood smears. The H/L ratio has widely been used as an indicator of stress in birds. In response to stressors, the relative number of heterophils in the peripheral blood increases while the relative number of lymphocytes decreases (Gross

and Siegel 1983). For each bird, we collected blood samples from the brachial vein (100 µl per bird in total) into sterile, heparinized capillary tubes within 20 minutes after capture, since longer handling stress could affect H/L ratio (Buehler et al. 2008). Blood slides were prepared within 2 min after the blood was taken using the standard two slide wedge procedure. All samples were air-dried and fixed with absolute methanol and stained with Giemsa (Roth, T862.1). Differential leukocyte counts of 100 heterophils or lymphocytes were carried out using a light microscope (1000× magnification with oil immersion).

Molecular Methods

Avian blood (collected from the brachial vein) was stored in 95% ethanol and DNA was extracted using the DNeasy Blood & Tissue Kit (Qiagen).

Malaria infection status

Primers and PCR conditions used are described by Bensch et al. (2000), Hellgren et al. (2004) and Waldström et al. (2004). All PCR reactions were carried out in 25µl reactions. Negative (deionized water) and positive (DNA samples from birds with known infection) control samples were used. The protocol consists of two PCR reactions, an initial 25 cycles PCR using the primers HaemNF1 and HaemNR3 and a final 35 cycles PCR using internally nested primers HaemF and HaemR2 (for *Haemoproteus* and *Plasmodium*) or HaemFL and HaemR2L (for *Leucozytozoon*). The first PCR included 2µl of the template (25 ng) of total genomic DNA, the second PCR included 2µl from the initial PCR as a template. Both included 1.5µl $MgCl_2$ (25mM), 2.5µl 10 X PCR buffer, 2.5µl dNTP's, 1µl of each primer (10µM), 0.1µl *Taq* DNA polymerase (25u/µl) and 14.4µl ddH_2O. The cycling profile consisted of an initial denaturation for 2 min at 94 °C, 30 sec at 94 °C, 30 sec at 50 °C, 45 sec at 72 °C for 25 (first PCR) or 35 (second PCR) cycles, followed by final extension at 72 °C for 10 min. Parasites were detected by a positive amplification on a 2% agarose gel (and visualized by a gel-red stain) and subsequently sequenced in an ABI genetic analyzer (Applied Biosystem 3130xl). Fragments were sequenced from the 5' end with the primer HaemF. The reaction included: 5.0 µl ddH_2O, 1.5 µl 5xbuffer, 0.5 µl primer (10 µM), 2 µl template (40-100 ng) and 1 µl BigDye Terminator Ready Reaction Mix. Following temperature profile was used: an initial denaturation for 1 min at 96°C, 10 sec at 96°C, 5 sec at 50°C and 4 min at 60°C for 25 cycles. Obtained

sequences of the cytochrome b (479 bp) were aligned using BioEdit (version 7.1.3.0.) and Mega 5 (version 5.05) and compared with the database MalAvi (Bensch et al. 2009).

MHC genotyping

MHC characterization was carried out using 454 amplicon sequencing. MHC class I exon 3 sequences that encode parts of the peptide binding region in MHC molecules were amplified using individually tagged 454-adapted primers (according to Galan et al. 2010). The MHC-specific primer stretches used were forward primer (longfw2): GTCTCCACACTGTACAGYGGC; and reverse primer (rv3): TGCGCTCCAGCTCCYTCTGCC) (Karlsson and Westerdahl 2013). These primers amplify 222-225bp long (primers not included) classical MHC alleles in house sparrows (Karlsson and Westerdahl 2013). Using Qiagen Multiplex MasterMix each 15µl PCR reaction contained 7.5µl of Hot Start Master Mix, 0.6µl (5µmol) each of the forward and reverse primer, 1µl of template (25ng genomic DNA) and 5.3µl dd H_2O. The PCR conditions were: 95°C/15 min, 35x(95°C/30 s, 65°C/1 min, 72°C/1 min), 60°C/5 min. PCR products were run on a 2% agarose gel and products with positive amplifications were purified using the MinElute PCR Purification Kit (Qiagen). Samples were pooled in eights and DNA was quantified with a Nanodrop instrument and adjusted accordingly before a second pooling for the 454 quadrants. The number of samples in the 454 quadrants was aimed at a sequence coverage of 300 reads per individual (according to Borg et al. 2011). 491 individuals were run in two different sequencing reactions (run 1 (R1) and run 2 (R2)) and these runs were filtered separately (see below). R1 contained 272 samples (243 individuals + 29 technical replicates (11.9%)) and R2 contained 286 samples (248 individuals + 38 technical replicates (15.3%)). 454 sequencing was done at Lund University Sequencing Facility (Faculty of Science), Sweden.

MHC - bioinformatics and data processing

After 454 sequencing, the data was extracted and assigned to samples using the program jMHC (Stuglik et al. 2011). To get rid of artefacts generated during the initial PCR, the emulsion PCR and the 454 sequencing reaction the raw data from the 454 run was filtered. Filtering steps were in accordance with Galan et al. 2010, Zagalska-Neubauer et al. 2010 and Sepil et al. 2012.

First, sequences in low abundances (<3 reads) were deleted. Identical sequences within individuals were detected and merged using the web-applications "seqeqseq" (http://mbio-serv2.mbioekol.lu.se/apps/seqeqseq.html) and "mergeMatrix" (http://mbio-serv2.mbioekol.lu.se/apps/mergeMatrix.html).

Second, we only wanted to keep sequences with a suitable sequence depth. Since the maximum number of classical MHC alleles is eight in house sparrows we chose a threshold of 104 reads per sample, this gives a genotype score of 99.9% (m=8, Galan et al. 2010). Here we used the web-application "popMatrix" (http://mbio-serv2.mbioekol.lu.se/apps/popMatrix.html) and filter 1. This web application has several filers, filters 1-4.

Third, true alleles are likely to occur more frequently than artifact alleles. Therefore we wanted to set a threshold that keeps true alleles but disregards artifact alleles. We used our replicates to set these thresholds (2% in R1 and R2) and all alleles that occurred in lower than 2% per sample were deleted using filter 3.

Forth, to verify MHC alleles they should occur in at least two independent PCRs. We worked with family data (189 adults produced about 300 chicks), these individuals were distributed across R1 and R2) and every allele is therefore likely to occur several times. Therefore we used the criteria that every allele should be found at least twice, here we used filter 4.

Fifth, the obtained sequences were examined in BioEdit (v7.2.0) and all sequences that did not meet the expected length of 222-225bp or displayed indels that were not multiples of three base pairs were deleted. The next step was to look for PCR recombinants (chimeras) and 1-bp substitutions. Sequences were examined starting with the lowest frequencies at the population level Sepil et al. 2013. All sequences that occurred in a frequency below 2% at the population level were checked in all individuals for chimeras and 1-bp substitutions. 50% of the sequences between a frequency of ≥ 2% to 25% were checked but no chimeras or 1-bp substitutions were found. Therefore, we assume that sequences with a frequency of ≥ 2% are true alleles. Additionally, alleles were verified and controlled for with inheritance data of families (189 adults produced about 300 chicks).

The filtered MHC DNA sequences were translated into amino acid sequences; this left 78 different sequences in the dataset. Only potentially functional sequences with no stop codons or frame shifts were kept for the analyses. We were especially interested in functional MHC diversity, because amino acids that are found in the peptide-binding regions (PBR) are involved in interactions with antigens and therefore crucial for the immune system (Doytchinova and Flower 2005, Schwensow et al. 2007). They can be converted into five physicochemical descriptor variables: *z*1 (hydrophobicity), *z*2 (steric bulk), *z*3 (polarity), *z*4 and *z*5 (electronic effects) (Sandberg et al. 1998). The 16 amino acids from each sequence that corresponded to the PBR in chicken (Wallny et al. 2006) were extracted; this left 58 different functional sequences in the dataset. MHC diversity was evaluated as: 1) the number of unique functional sequences/alleles per individual and 2) the presence/absence data of the 10 most common functional sequences/alleles with a frequency higher than 10% at the population level.

Genome-wide genetic variation

For the assignment of genome-wide individual heterozygosity, all one year old males (76) were typed at 13 highly polymorphic neutral microsatellite loci. The quality of loci (Hardy-Weinberg Equilibrium and Linkage Disequilibrium) was tested using the programs Cervus (v3.0.3) and Fstat (v2.9.3.2). Individuals were genotyped in two multiplex reactions according to the protocol of Dawson et al. (2012) (results shown in Supplementary Table 1). The loci used in this protocol are known to be distributed across at least seven different chromosomes and thus likely reflect the genome-wide level of neutral genetic variation (Dawson et al. 2012). Forward primers were fluorescently labeled using different dyes (YYE, FAM or AT550, Microsynth). The two multiplex PCRs were carried out using a Multiplex Kit (Qiagen) in a final PCR reaction volume of 6 µl containing 3µl of the Qiagen enzyme, 2µl of the primer mix and 1µl of the template (25 ng genomic DNA). PCR cycling scheme and primer concentrations of the 2 multiplex PCRs were carried out according to Dawson et al. (2012). PCR products were diluted (1:20) and analyzed in an ABI genetic analyzer (Applied Biosystem 3130xl) automated sequencer. Allele sizes were determined using GeneMapper (v3.0) with reference to an in-house DNA Size Standard (HMROX). We successfully amplified 12 loci (Supplementary Table 1). However, we excluded loci Pdoµ6 due to amplification problems.

Statistical analysis

We used linear mixed models within the R package v.3.0.3 x64 (R Core Team 2013) to analyze the effects on malaria infection status of 189 individuals from the parent generation and on badge size and H/L ratio of their offspring, 76 male house sparrows. In order to evaluate and compare different models, we used the Akaike Information Criterion corrected for small sample sizes (AIC_c, Burnham and Anderson 2002). In all cases there was not one clearly best model, so we used methods of model averaging and multimodel inference (Burnham and Anderson 2002). These methods allow inference over all models considered, but this was weighted according to model support by the data. Additionally, these methods do not only estimate standard errors unconditionally for a single model, they also provide the probability for single variables being in the unknown "true" model (the so-called relative variable importance - RVI). Unlike variable selection based on p-values these techniques have a sound mathematical basis and are increasingly recommended (Symonds and Moussalli 2011). We conducted these calculations in R using the package MuMIn (Bartón 2013). Additionally, we inspected the residuals for normality visually using histograms and QQ-plots. H/L ratio was log-transformed to achieve normality. Since we included the 10 most common functional MHC alleles in our analysis (frequency on the population level >10%), we had to do a pre-model selection of variables. In the first model we evaluated the relevance of the 10 alleles, while all other factors (e.g. number of functional MHC alleles, microsatellite heterozygosity, body condition and H/L ratio) were fixed. We then included only functional MHC alleles in the final model that had RVI values larger than 0.5.

Influence on stress level (H/L ratio)

We used a linear mixed model to analyze which of the variables (number of functional MHC alleles, presence/absence data of the 10 most common functional alleles, microsatellite heterozygosity, body condition and family origin as a random factor) influenced the H/L ratio.

Resistance to the malaria strain SGS1

We used a logistic mixed model to analyze which of the variables (number of functional MHC alleles, sex, presence/absence data of the 10 most common functional alleles and

microsatellite heterozygosity) influenced the presence (1) or absence (0) of the malaria strain SGS1.

Influence on badge size

We used a linear mixed model to analyze the effect of different variables (number of functional MHC alleles, presence/absence data of the 10 most common functional alleles, microsatellite heterozygosity, body condition and family origin as a random factor) on badge size.

Results

Malaria infection status

In the parent generation (189 individuals), we found that 101 birds (53.44 %) were infected with 4 different *Plasmodium* strains, the other 88 birds (46.56 %) were uninfected. Most of the infected individuals were infected with the strain SGS1 (68 birds – 67.33 %), followed by GRW11 (22 birds – 21.78 %), GRW6 (8 birds – 7.92 %), COLL1 (1 bird – 0.99 %) and one multiple infection between GRW11 and GRW6 (1 bird – 0.99 %). Since SGS1 was the most abundant malaria strain and because of that a potentially severe threat, we concentrated only on this one to find resistance genes occurring in this population.

MHC characterization

We had a total of 75894 sequence reads of 19200 unique sequences in 454 run 1 (R1) that had complete tags and primers. In run 2 (R2) we had a total of 125752 sequence reads of 34670 unique sequences. After filtering the data, a total of 85 different MHC alleles were found that could be translated into 59 unique functional MHC alleles, based on their chemical properties (Sandberg et al. 1998). There was an average of 4.2 functional MHC alleles/individual (ranging from 1-8). Males had on average 4.2 alleles (sd: 1.4), females had 4.3 (sd: 1.4). Adults possessed on average 4.3 alleles (sd: 1.5), chicks had 4.2 (sd: 1.3). In adults and chicks, there were 10 functional MHC alleles that occurred in frequencies larger than 10% at the population level, these were included in the analysis.

Influence on stress level (H/L ratio)

We found that badge size clearly affected stress level ($p<0.0001$, RVI=1.0, Fig. 1). The influence of all other variables was uncertain or unlikely (see Tab. 1 for details).

Resistance to the malaria strain SGS1

We found that the functional MHC allele 'seq00275' was the most important predictor of malaria infection status ($p=0.0389$, RVI=0.79). Individuals possessing this allele had a higher resistance against the malaria strain SGS1 (see Tab. 2 for details).

Influence on badge size

The presence of the functional MHC allele 'seq00275' had a strong positive effect on badge size ($p=0.0081$, RVI=0.91, Fig. 2). Individuals possessing this allele had a larger badge. The presence of the functional MHC alleles 'seq25259' ($p=0.045$, RVI=0.67) and 'seq18621' ($p=0.062$, RVI=0.63) and also microsatellite heterozygosity ($p=0.069$, RVI=0.061) might also have a positive effect (see Tab. 3 for details).

Discussion

Male ornament size reflects malaria resistance. This is the first evidence so far, that a male ornament predicts a specific disease resistance. Male house sparrows with bigger badges are more likely malaria free and posses an MHC allele responsible to conquer malaria. More specifically we identified one common blood parasite (SGS1) in our population that seems to be threatening and severe enough to cause a selective pressure, resulting in the occurrence of advantageous MHC resistance alleles. The functional MHC allele 'seq00275' has been detected as the most important predictor of malaria infection status. Individuals possessing this allele had a higher resistance against SGS1. MHC alleles associated with increased resistance or susceptibility to avian malaria have also been found in other populations of house sparrows with population specific effects that can be antagonistic across populations (Bonneaud et al. 2006, Loiseau et al. 2008, Sepil et al. 2013).

Furthermore we showed that ornament size is an honest signal of individual quality in our population. Ornament size is related to the H/L ratio which has been identified as an

indicator of stress in birds. In response to this stressor, the relative number of heterophils in peripheral blood increases while the relative number of lymphocytes decreases (Gross and Siegel 1983). These effects are mediated by glucocorticoids (Siegel 1985). High H/L ratios might also lead to a depression of some immune indices (Müller et al. 2011, Krams et al. 2012) and cause higher mortality rates (Kilgas et al. 2006, Ochs and Dawson 2008). It can be increased during times of low food availability, in parasite-infested birds, in migrating or breeding birds, that have large or experimentally increased broods (review by Davis et al. 2008).

Badge size turned out to be the strongest predictor of stress level in our population, indicating that individuals with a larger badge were also more stressed. This might be due to the fact that males with large badges have higher testosterone levels. Testosterone related costs can include more aggressive interactions with an increased risk of injury (Maynard Smith and Harper 1988, Jawor and Breitwisch 2003, Tibbetts and Dale 2004), a suppression of the immune system (Folstad and Karter 1992) or a depression of resistance to oxidative stress (Alonso-Alvarez et al. 2007). In our study we used only one year old males, since the genetic influences in comparison to testosterone might be more important for the development of the initial badge size (Jensen et al. 2008).

Ornament size and coloration can signal quality and parasite resistance (Hamilton and Zuk 1982, Andersson 1986, Møller 1990, Clayton 1991, Møller et al. 2000), but a lot of studies fail to show direct relationships between parasites and ornament size, because of the difficulty to identify suitable parasite species (Clayton 1991). We could demonstrate that the same functional allele ('seq00275'), which provided higher resistance against the blood parasite SGS1, was also a good predictor of ornament size and hence individuals possessing this allele had a larger badge. However a moderate positive effect was also found for the functional MHC alleles 'seq25259' and 'seq18621' and also for neutral heterozygosity (measured at 12 microsatellite loci). Also, these alleles could be involved in immune functions. Very likely other parasites or disease agents might be important threats in this population and these functional MHC alleles might provide resistance against.

Depending on the parasite community, either MHC heterozygosity or specific MHC alleles might be more beneficial. In fact there is indication across different populations that females tent to follow different tactics during mate choice. In house sparrows,

depending on the environment, males with larger or smaller ornaments might be preferred (Qvarnström 2001, Radovan and Hoi 2002, Endler 1988, Griggio and Hoi 2010). The highly polymorphic genes at MHC loci are believed to be under some form of balancing selection maintaining the high variation, such as pathogen-driven selection (Penn et al. 2002). Pathogen-driven MHC diversity can be maintained in two ways. Individuals heterozygous at a MHC locus theoretically recognize twice as many foreign peptides than homozygotes (Hughes 1992, Milinski 2006). Also specific rare MHC alleles that provide a stronger immune response to a specific antigen can gradually increase in frequency in a population, maintaining high diversity (Hedrick 2002). We used functional MHC alleles, because especially amino acids that are found in the peptide-binding regions (PBR) of MHC sequences are involved in interactions with antigens and are therefore crucial for pathogen resistance (Doytchinova and Flower 2005, Schwensow et al. 2007).

If a parasite would have severe fitness consequences for individuals, there would be a strong selective pressure to inherit a resistance allele to ones offspring. The badge size might reflect the ability to fight the most important and dangerous parasites present in a given population (Dufva and Allander 1995, Figuerola et al. 1999) and could signal valuable information about the capability of the immune system in a given population thus provide a "badge of immune system". For instance malaria infestation varies dramatically between different populations. Malaria is very frequent in our Austrian population and seems to be in general more frequent in middle Europe whereas malaria seems to be rare in Mediterranean populations. Schaschl et al. (unpublished) found that in 2007, the prevalence of a malaria infection in different house sparrow populations in Croatia was 17%, in Spain it was 21.8% and in Austria 78.8%. Such local variations may have consequences for the importance of e.g. malaria defence (resistance) and hence on the signalling function of an ornament. In a Mediterranean population, because of its low malaria prevalence, the signalling content may not include information on malaria resistance. Thus if female choice is based on ornament size, females should be adapted to the local situation otherwise she might misinterpret the signalling function of an ornament. In other words a Spanish female house sparrow might wrongly interpret the underlying characteristica expressed by an ornament of a dislocated male (e.g. a male from Middle Europe).

Conclusions

Ornament size can be good predictor of adaption in a specific environment and individuals with better disease resistance and health status might be able to build larger ornaments since only the healthiest individuals can afford the costs of developing this sexually selected trait. We showed that the ornament size is an honest signal, reflected by elevated H/L ratios and only high quality males might be able to develop a large badge. Quality can be measured as MHC heterozygosity (provides resistance to multiple parasites) or specific MHC alleles (provide resistance against specific parasites with severe fitness consequences). The badge size in males is influenced by the sum of specific alleles, especially if they are relevant for this population and provide e.g. higher resistance against specific parasites. It can signal proper immune function and a larger badge can indicate resistance against the most common and/or severe parasites in a specific population ("badge of immune system"). Since resistance to certain parasites is population dependent, the badge is only a good predictor of adaption in a specific environment.

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Figures and Tables

Figure 1: Influence of badge size on the H/L ratio (log-transformed)

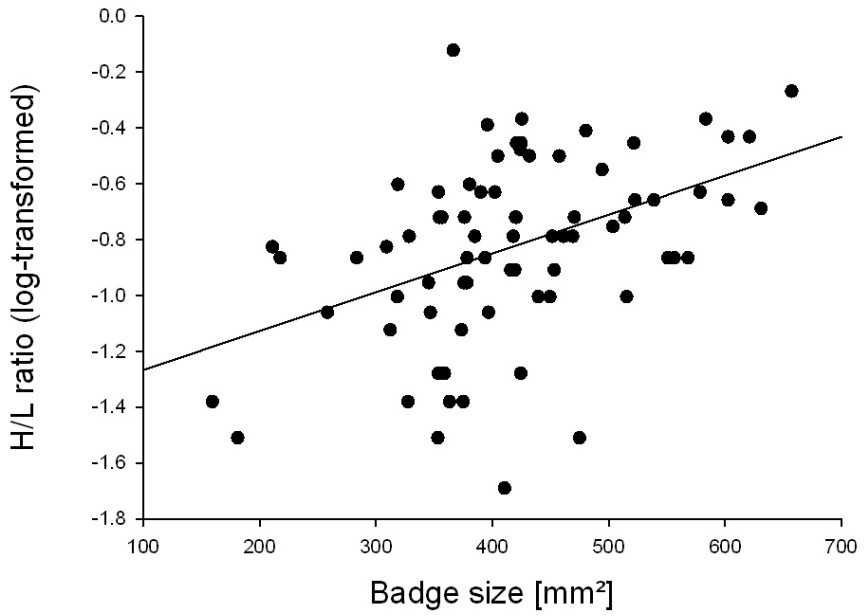

Figure 2: Mean badge size (with standard error bars) dependent on the absence/precence of the functional MHC allele 'seq00275'

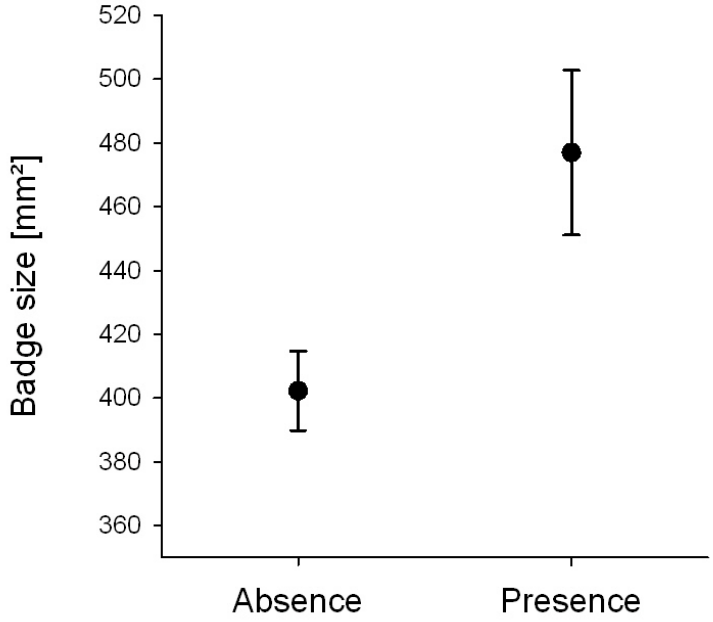

Table 1: Factors influencing H/L ratio (linear mixed model) with family origin as a random effect in one year old males

Model-averaged coefficients	Estimate	Adjusted SE	p value	RVI
(Intercept)	-1.6407	0.4126	6.99e-05***	
Badge size	0.0014	0.0004	0.0001***	1.0
MHC heterozygosity	0.0566	0.0366	0.1217	0.56
seq18621	-0.1708	0.1008	0.0901.	0.55
seq21013	-0.1344	0.1010	0.1833	0.47
Body condition	-0.0240	0.0229	0.2964	0.34
Microsatellite heterozygosity	0.5418	0.5812	0.3512	0.32

Table 2: Factors influencing the presence/absence of SGS1 (logistic mixed model) in the parent generation (two years or older)

Model-averaged coefficients	Estimate	Adjusted SE	p value	RVI
(Intercept)	0.0038	0.9474	0.9968	
seq00275	-0.9547	0.4622	0.0389*	0.79
MHC heterozygosity	-0.1800	0.1233	0.1442	0.53
seq25259	0.6400	0.4944	0.1955	0.46
Microsatellite heterozygosity	0.8839	1.5699	0.5734	0.29
Sex	0.0337	0.3325	0.9192	0.26

Table 3: Factors influencing badge size (linear mixed model) with family origin as a random effect in one year old males

Model-averaged coefficients	Estimate	Adjusted SE	p value	RVI
(Intercept)	181.511	223.813	0.4174	
seq00275	75.607	28.569	0.0081**	0.91
seq25259	72.901	36.415	0.0453*	0.67
seq18621	57.606	30.854	0.0619.	0.63
Microsatellite heterozygosity	367.970	202.556	0.0693.	0.61
seq25663	44.165	26.380	0.0941.	0.56
MHC heterozygosity	-1.413	9.534	0.8822	0.27
Body condition	5.036	7.372	0.4945	0.24

Supplementary Information

Supplementary Table 1: Genome-wide genetic variation

Primer set	PCR mix	Size range	Number of alleles/locus	Primer set reference
Ase 18	1	185-249	17	Richardson et al. (2000), see Griffith et al. (2007)
Pdoμ1	1	156-200	18	Neumann and Wetton (1996)
Pdoμ3	1	113-181	18	Neumann and Wetton (1996)
Pdoμ5	1	202-264	21	Griffith et al. (1999a)
Pdoμ6*	1			Griffith et al. (1999a)
Pdo9	1	362-424	18	Griffith et al. (2007)
Pdo10	1	102-152	14	Griffith et al. (2007)
Pdo16A	2	270-302	15	Dawson et al. (2012)
Pdo17	2	192-244	22	Dawson et al. (2012)
Pdo19	2	173-183	4	Dawson et al. (2012)
Pdo22	2	92-130	17	Dawson et al. (2012)
Pdo27	2	224-248	12	Dawson et al. (2012)
Pdo40A	2	298-328	15	Dawson et al. (2012)

* excluded due to amplification problems

General Discussion

The main focus of my project was to investigate the relationship between the genetic make-up at the major histocompatibility complex (MHC) and different life-history traits in house sparrows (*Passer domesticus*). We found that mating with a "MHC compatible" and/or MHC heterozygote partner can influence fertilization (Chapter I), hatching success (Chapter II) and growth and immunocompetence in offspring (Chapter II and III). Additionally we found a sexually selected ornament in males that signals MHC dependent parasite resistance (Chapter IV).

In Chapter I we could demonstrate that fertility in pairs was influenced by the MHC genotype. According to our results, a strong immune system in females constitutes a drawback in terms of fertilization success. This is, furthermore to our knowledge the first evidence for a sex specific effect of the immune system related to the MHC region on fertility. If heterozygote females are able to resist a wider range of pathogens (Hughes 1992, Milinski 2006), they could also initiate immune responses against a wider range of molecules expressed on the sperm surface of mating partners. These negative effects can be reduced by selecting sperm from more similar males. We found that more similar pairs at the peptide binding region (PBR) of the MHC seemed to benefit in terms of an increase in fertilization success, but there might be an optimum level of similarity. Past studies usually concentrated only on similarity determined as the percentage of shared MHC alleles (Bonneaud et al. 2006, Bichet et al. 2014, Løvlie et al. 2013), which might not necessarily be a suitable measure. Thus the incorporation of genetic and functional MHC distances in pairs is another novelty of this study that should be included in future studies about MHC-dependent mate choice and/or fertilization.

Our results suggest that females might use different mating strategies depending on their own genetic make-up at the MHC. In particular females with a strong immune system should reduce possible fertilization costs by incorporating a second aspect in their mate choice process, namely to mate with a genetically more similar male. Females with fewer alleles in contrast may more likely benefit from a male with a strong immune system to gain heterozygote offspring. Such a female quality dependent mate choice strategy in relation to male MHC diversity has been in fact experimentally shown by Griggio et al. (2011). In this study female house sparrows with few MHC alleles showed a clear preference for males with a high number of MHC alleles, whereas females with many

MHC alleles did not. Griggio et al. (2011) explained the lack of a preference with their reduced benefits to gain immunocompetent offspring. Our results now provide a more satisfactory explanation why immunocompetent females did not show a clear preference for male MHC heterozygosity, namely they might suffer higher costs of reduced fertilization.

Male MHC composition did not seem to be important except for one specific MHC allele ('seq21013'). When solely the male in a breeding pair possessed it, fertilization success was higher. Also Skarstein et al. (2005) found that a specific MHC allele was associated with increased fertilization success in fish.

Our results will have important implications to understand MHC-based mate choice. All three hypotheses to explain MHC-dependent mate choice, may not be mutually exclusive and even individuals within a species might integrate different aspects in their mating decision, namely high allelic diversity of MHC alleles, genetic compatibility between partners as well as the occurrence of specific alleles (review in Piertney and Oliver 2006, Kamiya et al. 2014).

In Chapter II we could show that hatching success and growth in house sparrow chicks were associated with the MHC, more precisely with one specific MHC allele. Hatching success was lower in individuals that possessed the MHC allele ('seq25259'). The same allele was associated with lower weight and a smaller tarsus in 6 days old chicks. However, its significance on survival and growth seemed to disappear in older nestlings, highlighting the importance of this allele especially during the incubation period and the first days of life. These results indicate that this MHC allele or another gene linked to it might be responsible for a higher susceptibility to a specific pathogen which seems to be maladaptive in the early developmental period.

In hole nesting house sparrows, mortality of eggs and nestlings can exceed 50% (Dyer et al. 1977). Parasitism is a common selective pressure that can influence growth rates and survival in nestlings (Price 1980, Møller 1994). Pinowski et al. (1994) found that egg mortality, survival and growth rates in house sparrow chicks were related to pathogens such as *Escherichia coli* and *Staphylococcus epidermitis*. With our data it is not possible to predict if hatching failure was caused by microbial infections, but since also growth was negatively correlated with the same functional MHC allele 5 days later, a microbial

infection is likely. In further studies it would be interesting to link bacterial loads on egg shells and in nestlings to MHC alleles in parents and/or chicks.

The highly polymorphic genes at MHC loci are believed to be under some form of balancing selection maintaining the high variation, such as pathogen-driven selection (Penn et al. 2002) that can be maintained in two ways. Individuals heterozygous at a MHC locus theoretically recognize twice as many foreign peptides than homozygotes (Hughes 1992, Milinski 2006). Also specific rare MHC alleles that provide a stronger immune response to a specific antigen can gradually increase in frequency in a population, maintaining high diversity (Takahata and Nei 1990, Hedrick 2002). It has been shown that in some species, especially common MHC alleles are associated with lower survival and higher parasite loads (Paterson et al. 1998, Froeschke and Sommer 2005, Schad et al. 2005).

In our population, we also found one common MHC allele (16.05%) that had negative effects in early development, but it maintains in the population, indicating benefits outweighing this disadvantages. We used MHC class I alleles, which are responsible for adaptive cell-mediated immunity (Klein 1986), but especially young nestlings mainly depend on the innate immune system. Therefore it is remarkable that one specific functional MHC allele was associated with reduced growth and lower hatching success, indicating that it is in linkage disequilibrium with other immune genes or genes involved in body condition.

In Chapter III we could demonstrate that the MHC is a suitable tool to further measure and quantify immunocompetence in non-model species. Specific MHC alleles and MHC heterozygosity influenced survival and several immunological tests that represent the innate and adaptive immunity. Especially one MHC allele (‘seq09776’) seems to be in particular important, because it correlated with four different tests, specifically representing a measure for an innate response. Individuals with this allele had a lower ability to kill *E.coli* bacteria in vitro, a lower erythrocyte sedimentation rate, a lower swelling response to a phytohaemagglutinin (PHA) injection in chicks, but a higher hematocrit. Because of its complexity, we cannot interpret smaller or bigger immune reactions as negative or positive effects or an advantage or disadvantage, since our knowledge about the underlying physiological mechanisms and relationships within the immune system are still limited. This significant MHC allele occurred in a high frequency

(32.81%) and was the third most common allele in our population. The association of one specific MHC class I allele on to the intensity of different immune responses is at a first glance surprising, but it might be in linkage disequilibrium with other immune genes, e.g. MHC class II or genes that are involved in body condition.

Also other MHC alleles had an influence on our immune measures, like the heterophil/lymphocyte ratio and the haemagglutination test to sheep red blood cells. Also survival over one winter was influenced by one MHC allele. Since we only concentrated on MHC class I, which is responsible for cell-mediated immunity, it is remarkable that also other areas of the immune system (innate and humoral) were linked to MHC class I, highlighting the linkage of different genes.

One immune measure (PHA injection) correlated with two different MHC alleles in several life stages, indicating that this test measured different immune responses. In chicks, swelling response to PHA might depend more on the innate response, in adults, adaptive immunity could be more important.

We found evidence that extremely high MHC heterozygosity might implicate a disadvantage for the immune system and that the expression of numerous MHC alleles might be costly. While intermediate heterozygosity can be advantageous (Hughes 1992, Milinski 2006), extremely high diversity may lead to a reduced fitness by over depletion of the T-cell repertoire during self-tolerance induction (Nowak et al. 1992). In adults, the second injection of PHA was correlated with MHC heterozygosity and individuals possessing more different alleles produced a smaller immune response.

In Chapter IV we found that a sexually selected ornament (black throat badge) in males signals MHC dependent parasite resistance and immunocompetence. This is the first evidence so far, that a male ornament predicts malaria resistance in birds. Ornament size and coloration can signal quality and parasite resistance (Hamilton and Zuk 1982, Andersson 1986, Møller 1990), but a lot of studies fail to show direct relationships between parasites and ornament size, because of the difficulty to identify suitable parasite species (Clayton 1991).

We identified a common blood parasite (avian malaria strain SGS1) in our population that seems to be severe enough to cause a selective pressure, resulting in the occurrence of advantageous MHC resistance alleles. MHC alleles associated with increased resistance

or susceptibility to avian malaria have also been found in other populations of house sparrows (Bonneaud et al. 2006, Loiseau et al. 2008). The MHC allele 'seq00275' was the most important predictor of malaria infection status. Individuals possessing this allele had a higher resistance against SGS1. Moderate positive effects on ornament size were also found for two other MHC alleles. This could be explained by the fact that also other parasites might be important threats and that these MHC alleles provide resistance against them or could be involved in other immune functions. Depending on the parasite community, either MHC heterozygosity or specific MHC alleles might be more beneficial.

Furthermore we showed that ornament size is an honest signal of individual quality in our population. Ornament size was related to the H/L ratio which has been identified as an indicator of stress in birds (Gross and Siegel 1983). Badge size turned out to be the strongest predictor of stress level in our population, indicating that individuals with a larger badge were also more stressed. Only high quality males should be able to overcome the negative effects of developing a large badge (e.g. higher stress levels) (Hamilton and Zuk 1982).

The badge size can signal valuable information about the capability of the immune system and might reflect the ability to fight the most important and dangerous parasites present in a given population (Dufva and Allander 1995, Figuerola et al. 1999) and thus provide a "badge of immune system". Since resistance to certain parasites is population dependent, the badge is only a good predictor of adaption in a specific environment.

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Summary

In many species females are highly selective when choosing a mate, because in general, they invest more into reproduction compared to males. By choosing the right partner, females may gain direct benefits like paternal care, but also indirect benefits in the form of e.g. 'good genes' that could provide superior immunocompetence to their offspring. The major histocompatibility complex (MHC), a highly polymorphic gene region, is important for an appropriate immune response against pathogens. The main focus of my project was to investigate the relationship between the genetic make-up at the MHC and different life-history traits to test if mating with a 'MHC compatible' and/or MHC heterozygote partner provides superior immunocompetence in the offspring. To control for environmental factors, our study was carried out on an aviary population of house sparrows (*Passer domesticus*). MHC class I alleles were DNA sequenced using next generation sequencing (454 amplicon sequencing). In order to achieve the major study aims we i) tested if MHC composition of breeding pairs influenced their fertilization success, ii) monitored survival rates in their offspring, iii) conducted several immunological tests corresponding to the innate and adaptive defence and iv) investigated whether the expression of a sexually selected male ornament (black throat badge) may constitute an indicator of health status and MHC based resistance against parasites. We found that fertilization success was influenced by female MHC heterozygosity, MHC compatibility in pairs and one specific MHC allele in males. Functional MHC diversity correlated with survival rate at different life stages and several immunological tests. Furthermore, one specific MHC allele was associated with higher resistance against avian malaria and males with this allele also had larger badges, indicating that badge size can constitute a predictor of male parasite resistance and immunocompetence.

Zusammenfassung

Weibchen vieler Arten sind bei der Partnerwahl sehr wählerisch, da sie im Allgemeinen und im Vergleich zu Männchen mehr in die Fortpflanzung investieren. Durch passende Partnerwahl können Weibchen direkte Vorteile wie väterliche Fürsorge bekommen, aber auch indirekte Vorteile, wie z.B. „gute Gene“ die ihren Nachkommen eine überlegene Immunkompetenz bieten könnten. Der Haupthistokompatibilitätskomplex (MHC), eine hoch polymorphe Genregion, ist wichtig für eine angemessene Immunabwehr gegen Pathogene. Der Hauptfokus meines Projekts war es, die Beziehungen zwischen genetischer Zusammensetzung am MHC und verschieden Merkmalen in der Lebensgeschichte zu untersuchen um zu testen, ob die Verpaarung mit einem „MHC kompatiblen“ und/oder MHC heterozygoten Partners eine überlegene Immunkompetenz bei den Nachkommen bieten kann. Um den Einfluss von Umweltfaktoren zu reduzieren wurde unsere Studie an einer Volierenpopulation von Haussperlingen (*Passer domesticus*) durchgeführt. Mit Hilfe von „next generation sequencing“ (454 amplicon sequencing) wurden MHC class I Allele DNA-sequenziert. Um unsere Studienziele zu erreichen haben wir i) getestet ob die MHC Zusammensetzung in Brutpaaren deren Fruchtbarkeitserfolg beeinflusst, ii) die Überlebensraten ihrer Nachkommen überwacht, iii) verschiedene immunologische Tests durchgeführt, die zur angeborenen und adaptiven Abwehr gehören und iv) geprüft ob die Ausbildung eines sexuell selektierten Ornaments in Männchen (Brustlatz) ein Indikator für den Gesundheitszustandes und eine MHC basierende Resistenz gegen Parasiten darstellen könnte. Wir haben herausgefunden, dass der Fruchtbarkeitserfolg von der MHC Heterozygosität des Weibchens, der MHC Kompatibilität in Paaren und einem speziellen Allel in Männchen abhängig ist. Funktionelle MHC Diversität korrelierte mit der Überlebensrate in mehreren Lebensphasen und verschiedenen Immuntests. Außerdem haben wir herausgefunden, dass ein spezielles MHC Allel mit einer höheren Resistenz gegen Malaria assoziiert war und Männchen mit diesem Allel außerdem größere Brustlätze besaßen, was ein Hinweis darauf ist, dass der Brustlatz ein Anzeichen für Parasitenresistenz und Immunkompetenz bei Männchen darstellen kann.

Contributions

	Chapter I	Chapter II	Chapter III	Chapter IV
Study conception and design	BL, HH	BL, HH	BL, HH	BL, HH
Genetic analysis	BL, HW	BL, HW	BL, HW	BL, HW, SB
Run the experiment	BL	BL	BL	BL
Data analysis	BL, FK, MS	BL, MS	BL, MS	BL, FK, MS
Writing the manuscript	BL, HH, HW	BL, HH	BL, HH	BL, HH
Revising the manuscript	BL, HCW, HH, HW, MS, YM	BL, HCW, HH, HW, MS, YM	BL, HCW, HH, HW, MS, YM	BL, HH, HW, MS, SB

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HCW	Hans-Christoph Winkler
HH	Herbert Hoi
HW	Helena Westerdahl
MS	Maria Strandh
SB	Staffan Bensch
YM	Yoshan Moodley

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Curriculum Vitae

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Education

Since 2010	PhD candidate in Biology at the University of Vienna; PhD thesis at the Konrad Lorenz Institute of Ethology, Vienna (Title: "Costs and benefits of genetic mate choice")
2010	Graduation with the degree *Diplom-Ingenieur* (equiv. to MSc)
2006 - 2010	Master studies in Agricultural Biology at the University of Natural Resources and Life Sciences, Vienna; Diploma thesis at the Institute of Zoology (Title: „Short-term effects of recent land use changes in Eastern Austria on bird assemblages in a human-dominated landscape")
2008	Graduation with the degree *Magister* (equiv. to MSc)
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Publication list

Lukasch B, Westerdahl H, Strandh M, Winkler H, Moodley Y and Hoi H (submitted): Major histocompatibility complex genes partly explain hatching success and early nestling growth in house sparrows

Linsberger-Martin G, Lukasch B and Berghofer E (2012): Effects of high hydrostatic pressure on the RS content. Starch/Stärke 64:157-165

Lukasch B, Frank T and Schulze, CH (2011): Short-term effects of recent land use changes in Eastern Austria on bird assemblages in a human-dominated landscape. Biodiversity and Conservation 20:1339–1352

Conference contributions

08/2014	Oral presentation at the 26th International Ornithological Congress, Tokyo: Genetic background helps understanding immunocompetence and survival in house sparrows
08/2013	Poster presentation at the 14th Congress of the European Society of Evolutionary Biology, Lisbon: The influence of MHC on fertility success in house sparrows (*Passer domesticus*)
10/2012	Oral presentation at the Mini-symposium on bird malaria parasites, Lund: Influence of malaria infection on mate choice in house sparrows
08/2012	Oral presentation at the 14th International Behavioral Ecology Congress, Lund: Influence of malaria infection on mate choice in house sparrows
08/2011	Oral presentation at the 8th Conference of the European Ornithologists' Union, Riga: Short-term effects of recent land use changes in Eastern Austria on bird assemblages in a human-dominated landscape

Awards and Honors

08/2014	Travel grant (University of Vienna): 650 €
08/2014	Travel grant (IOC conference Tokyo): 100.000 ¥
10/2012 – 04/2013	Marietta Blau (OEAD): 8.400 €
08/2012	Travel grant (ISBE conference Lund): 800 $
01/2012	PhD grant (Kulturabteilung der Stadt Wien): 750 €
11-12/2011	KWA (University of Vienna): 1.300 €

Work experience

04/2014	Florida International University (USA): Bird banding and mist netting at Fairchild Tropical Botanic Garden
10/2012 – 05/2013	Lund University (Sweden): Visiting scientist; MHC characterization (next generation sequencing), paternity analysis and malaria screening
02/2012	University of Sheffield (UK): Visiting scientist; Identification of sperm on the perivitelline layer of bird eggs using fluorescent microscopy
11-12/2011	Lund University (Sweden): Visiting scientist; MHC characterization and malaria screening
09/2010	BirdLife International, Hellenic Ornithological Society (Greece): Bird banding and mist netting in Antikythira

06/2010	University of Vienna (Austria): Bird banding and mist netting in Illmitz, Austria
04-05/2010	BirdLife Austria: Bird surveys in north-eastern Austria
12/2009 - 05/2010	Konrad Lorenz Institute for Ethology, Austrian Academy of Sciences (Austria): Research assistant in the field of sexual selection in *Passer domesticus* and *Panurus biarmicus*
09-10/2009	Bird Studies Canada (Canada): Monitoring of monarch butterflies and bird banding and mist netting at the Long Point Bird Observatory
07-08/2009	Auring (Austria): Bird banding and mist netting at Ringelsdorf, Austria
05-06/2009	University of Natural Resources and Life Sciences (Austria): Research assistant in a project about the soil bank of *Ambrosia artemisiifolia*
03-06/2009	University of Vienna (Austria): Bird surveys and GIS data analysis in north-eastern Austria
08-09/2008	Juodkrante Bird Ringing Station (Lithuania): Bird banding and mist netting
01-07/2007	University of Natural Resources and Life Sciences (Austria): Food processing and chemical and physical analyses of starch
07/2005	Medical University of Vienna (Austria): Water analysis, analysis of baby food and renewal of the reference stocks