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# MHC polymorphism in a songbird

## Fitness, mate choice, and sexual conflict

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Abstract <p>Sex differences in immune responses have been observed across a wide range of animal species, with the general tendency that males have weaker immune responses than females. These differences are at least partly caused by immune-regulating effects of sex hormones, and have been associated with an increased prevalence of autoimmune disorders in females and with a general tendency for males to be parasitized more often than females. Because of these differences, male and female phenotypes may be regarded as different immunological environments, however it has not previously been investigated whether sex differences in immune responses may lead to sexually antagonistic selection on immune system genes.</p> <p>The first chapter of this thesis presents a literature study of the effects of sex hormones on the strength of immune responses in vertebrates, based on which we propose the hypothesis that sexual selection may drive sexually antagonistic selection on genes associated with the immune system. In the following chapters, we investigated this hypothesis using major histocompatibility complex class I (MHC-I) genes in a species subject to strong sexual selection, a socially polygynous songbird, the great reed warbler <i>Acrocephalus arundinaceus</i>.</p> <p>MHC genes play an important role in vertebrate adaptive immunity where they enable recognition and elimination of pathogens. Due to an ongoing co-evolution between hosts and their pathogens, the MHC genes are among the most variable genes known in vertebrates. It has been hypothesized that hosts benefit from having high MHC diversity, because this confers protection against a wider range of pathogens. Interestingly, we found evidence for a sexual conflict and for sexually differential selection on MHC-I diversity in our great reed warbler study population.</p> <p>It has been predicted that genes associated with disease resistance should be advantageous in terms of sexual selection, and this prediction is central to our hypothesis that sexual selection may drive sexually antagonistic selection on genes associated with the immune system. We therefore investigated whether MHC-I genes were associated with sexual selection in great reed warblers. Our results indicated that MHC-I diversity (i) conveys a 'good genes' benefit to females that select older males and males with large song repertoires, and (ii) affects the ability of males to acquire attractive territories. These results confirmed that MHC-I genes are associated with sexual selection, and thereby corroborated our hypothesis that sexual selection may be driving the observed sexual conflict over MHC-I diversity via immune regulating effects of sex hormones.</p> <p>Finally, we investigated the source of MHC-I genotypic variation in great reed warblers by analyzing segregation of MHC-I haplotypes and performing phylogenetic reconstruction on MHC-I alleles. We identified five distinct clades in a phylogenetic tree that indicate the presence of several divergent MHC-I loci in the great reed warbler genome. Analyses of positive selection implied that each putative MHC-I locus may have evolved slightly different functions. Importantly, variation in MHC-I diversity between haplotypes was largely explained by variation within two specific clades, suggesting that the sexual conflict over MHC-I diversity may be caused by sexually antagonistic effects associated with alleles from these clades in particular.</p> <p>Our results suggest that sexually antagonistic selection is an important force in the evolution of vertebrate adaptive immunity, which may be important for a comprehensive, evolutionary understanding of autoimmune diseases and other costs associated with immune responses in vertebrates. The results presented in this thesis invite further studies that investigate the generality of sexually antagonistic selection over immune system genes in other species, as well as more detailed studies of the mechanisms underlying such sexual conflicts.</p>		
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# MHC polymorphism in a songbird

Fitness, mate choice, and sexual conflict

Jacob Roved



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- I. Roved, J., Westerdahl, H., & Hasselquist, D. 2017. Sex differences in immune responses: Hormonal effects, antagonistic selection, and evolutionary consequences. *Hormones and Behavior* 88: 95-105. <http://doi.org/10.1016/j.yhbeh.2016.11.017>
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- III. Roved, J., Hansson, B., Tarka, M., Hasselquist, D., & Westerdahl, H. MHC-I functional divergence is positively associated with life span and fitness in male great reed warblers: support for the divergent allele advantage hypothesis. Manuscript.
- IV. Roved, J., Hansson, B., Hasselquist, D., & Westerdahl, H. MHC class I genes and mate choice in great reed warblers. Manuscript.
- V. Roved, J., Hansson, B., Hasselquist, D., & Westerdahl, H. MHC haplotypes unravel patterns of selection and genetic structure in great reed warblers. Manuscript.

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## List of contributions

- I. The authors jointly conceived the study; J.R. conducted the literature search and review and wrote the paper, with substantial input from H.W. and D.H.
- II. J.R., B.H., H.W. and D.H. conceived and designed the study; H.W. designed and supervised the MHC genotyping protocols; all authors contributed to designing the data analysis protocols; J.R. carried out bioinformatics, analyzed the data, and wrote the paper, with substantial input from all authors.
- III. J.R., B.H., D.H., and H.W. jointly conceived and designed the study and data analysis protocols; J.R. carried out bioinformatics, analyzed the data, and wrote the paper, with substantial input from all authors.
- IV. J.R. conceived and designed the study and the data analysis protocols; J.R. carried out bioinformatics, analyzed the data, and wrote the paper, with substantial input from all authors.
- V. The authors jointly conceived the study; J.R. designed the data analysis protocols and constructed the haplotypes; J.R. carried out bioinformatics, analyzed the data, and wrote the paper, with substantial input from all authors.





# General introduction

## Mate choice and sexual selection

When females choose a partner among potential mates, they may base their choice on conditions that are important for successful breeding, e.g. the quality of the territory or breeding site, or the amount of parental care provided by the male (Bensch, 1996; Bensch & Hasselquist, 1992; Emlen & Oring, 1977; Kokko, Brooks, Jennions, & Morley, 2003; Tregenza & Wedell, 2000). However, they could also be attracted by signals of good genes that will benefit the quality or attractiveness of their offspring (Hamilton & Zuk, 1982; Trivers, 1972; Weatherhead & Robertson, 1979; Zahavi, 1975). Selecting a mate on the basis of external conditions may provide the females with direct benefits that are advantageous in terms of their own reproduction, whereas selecting mates on the basis of good genes may provide indirect benefits that are advantageous to their offspring. Thus, genetic benefits achieved when selecting a mate for good genes often increase the viability or the fitness of the offspring and may be achieved by choosing mates who are heterozygous, possess specific advantageous alleles, including alleles that make the offspring attractive as mates, or possess alleles that are compatible to the female's own genotype (Hasselquist, 1998; Penn & Potts, 1999; Tregenza & Wedell, 2000; Weatherhead & Robertson, 1979).

### *Hamilton & Zuk*

For females to be able to choose mates for good genes, there must be genetic variation among males in the focal genes, and females must be able to evaluate this variation. Furthermore, there must be factors that maintain the variation in the focal genes, otherwise the sexual selection imposed by the females would rapidly exhaust this genetic variation or drive populations towards genetic equilibria (Andersson, 1994; Kirkpatrick & Ryan, 1991; Kokko et al., 2003; Tregenza & Wedell, 2000). Hamilton and Zuk (1982) showed that the expression of male secondary sexual characters was associated with the incidence of chronic blood infections in seven surveys conducted on North American songbirds, and suggested that females use the expression of such characters to evaluate potential mates for good genes. They argued that the expression of secondary sexual characters is dependent on health and vigor, and thus only males with good genes for disease resistance may afford strong expression of such characters. Hence, by choosing males with more elaborate secondary sexual characters, females could achieve increased viability of their offspring through increased resistance to pathogens. Hamilton and Zuk (1982) argued that such genes for disease resistance are likely to be involved in constant,

cyclical coevolution between hosts and pathogens, whereby genetic variation for fitness could be maintained. They also argued that health and vigor may not only reveal itself in the expression of physical secondary sexual characters, but may also show in behavioral traits such as song or be conveyed by success in competition or fights (Hamilton & Zuk, 1982).

### *The immunocompetence handicap hypothesis*

The hypothesis by Hamilton and Zuk (1982) predicted that males with more striking display characters should have lower pathogen burdens, however, in some cases this prediction was not supported in empirical tests of the hypothesis (e.g. Hausfater et al., 1990; Ressel & Schall, 1989). A weakness of the hypothesis was that failed to include a cost that could ensure the honesty of secondary sexual characters as indicators of genetic quality (cf. Zahavi, 1977, 1975). The immunocompetence handicap hypothesis (ICHH) presented by Folstad and Karter (1992) extended Hamilton and Zuk's theory by suggesting that the androgen sex hormone testosterone is a key mediator of the expression of secondary sexual characters. The ICHH proposed that immunosuppressive effects of testosterone increase the susceptibility to and pathogenicity of infections in males (cf. e.g. Alexander & Stimson, 1988; Grossman, 1985), arguing that if secondary sexual character expression would be associated with an obligate immunosuppressive effect, it would introduce a cost that would ensure the honesty of the system (Folstad & Karter, 1992). As with the hypothesis by Hamilton and Zuk, the general prediction of the ICHH was still that secondary sexual characters advertise good genes for disease resistance to females. However, Folstad and Karter's theory hinged the character expression on testosterone levels and specified physiological positive or negative feedback mechanisms between the endocrine system, the immune system, secondary sexual characters, and pathogens. They suggested that these feedback mechanisms enable the system to be self-regulatory, so that the testosterone-dependent expression of sexual characters could be adjusted according to the costs of increased pathogenicity and the benefits of increased reproductive success (Folstad & Karter, 1992). The essence of the ICHH is that males with good genes for disease resistance can afford to maintain high levels of testosterone, by which they achieve strong expression of secondary sexual characters or a more dominant behavior. These males with good genes for disease resistance can also better afford the costs associated with immunosuppression caused by high testosterone levels. In contrast, males with lower quality genes would have to reduce their testosterone levels, because strong immunosuppression would be too costly. In this way, the immunosuppressive effect of testosterone ensures that only males with good quality genes for disease resistance can afford a strong expression of secondary sexual characters. Folstad and Karter argued that the ICHH model is evolutionarily stable because cheaters (i.e., males that maintain higher levels of testosterone than optimal, given the quality of their disease resistance genes) (i) will be more likely to pay a

cost in the long term than non-cheaters and (ii) pass on weaker genes for disease resistance to their offspring, and therefore will have a reduced inclusive fitness (i.e., measured over several generations) relative to that of non-cheaters with similar levels of character expression. They furthermore argued that the ICHH was applicable also to mating systems in which intrasexual selection affects female mate choice, because (i) health and vigor is conveyed honestly in competitive behavior or fights, (ii) high levels of testosterone are associated with aggressive and dominant behavior, and (iii) secondary sexual characters serve as badges of quality and strength that may serve as warnings to opponents of the same sex (Folstad & Karter, 1992).

## MHC genes and mate choice

A set of genes that are regarded as good candidates for mating preferences are those of the major histocompatibility complex (MHC) (Penn & Potts, 1999; Potts & Wakeland, 1993; Tregenza & Wedell, 2000; Zelano & Edwards, 2002). The MHC is a set of closely linked genes that serve a key function in the adaptive immune system, as MHC molecules enable evaluation of what is self and non-self. MHC molecules present peptide fragments on cell surfaces to passing T-cells, and if an MHC molecule presenting a foreign peptide (i.e., not derived from the host's own tissues) is bound by a matching T-cell receptor (TCR), the immune system mounts a response aimed at the peptide presented by the MHC molecule (Murphy & Weaver, 2016) (see Box I for a brief introduction to the vertebrate immune system and the role of MHC genes in triggering adaptive immune responses). The genes of the MHC are among the most polymorphic genes known in animals, and this polymorphism is believed to be maintained mainly by balancing selection from pathogens (Borghans, Beltman, & De Boer, 2004; Ejsmond & Radwan, 2015; Hamilton & Zuk, 1982; Hedrick, 2002; Potts & Wakeland, 1990). Four major hypotheses have been suggested to explain how such a pathogen-driven balancing selection on the MHC could work.

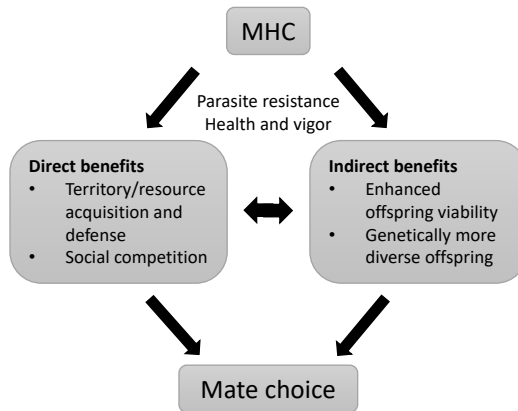
### *Rare allele advantage*

The rare allele advantage hypothesis suggests that the large polymorphism is maintained by negative frequency dependent selection from pathogens. An MHC allele that confers resistance to certain pathogens will be selected for in the host population. However, as it becomes more abundant, selection will favor pathogens that evolve to escape the recognition from that particular allele. However, these pathogens will then be recognized by other, less common alleles in the host population. This interaction results in co-adaptational cycles between hosts and pathogens (known as the Red Queen effect), in which rare MHC alleles convey a

fitness advantage over common ones, and the constant cycling of the system ensures that a large diversity of alleles is maintained (Apanius, Penn, Slev, Ruff, & Potts, 1997; Borghans et al., 2004; Ejsmond & Radwan, 2015).

### *Heterozygote advantage*

The heterozygote advantage (or overdominance) hypothesis suggests that it is advantageous for an individual to be heterozygous at the MHC loci and thereby have as many different MHC alleles as possible, because each MHC molecule is capable of binding only a limited repertoire of peptide antigens. Heterozygote individuals will therefore in most instances be resistant to a larger number of pathogens than homozygotes, because their MHC molecules together present a wider repertoire of antigens to the immune system (Doherty & Zinkernagel, 1975; Hughes & Nei, 1988, 1989, 1992). However, variation in the number of different MHC alleles per individual may also be caused by variation in the number of MHC gene copies, which is evident from the exceptionally high MHC diversity in many songbird species (Biedrzycka et al., 2017; Karlsson & Westerdahl, 2013; O'Connor, Strandh, Hasselquist, Nilsson, & Westerdahl, 2016; Sepil, Moghadam, Huchard, & Sheldon, 2012; Westerdahl, 2007). Although the term heterozygote advantage was originally used in its literal sense, the principle behind the heterozygote advantage hypothesis (i.e., that it is advantageous for individuals to have many different MHC alleles) is often applied universally, irrespective of the source of variation in the number of MHC alleles. The rare allele advantage hypothesis and the heterozygote advantage hypothesis are not mutually exclusive, as it may be beneficial both to have advantageous rare alleles and have a larger number of different alleles, and both models may contribute to the maintenance of population-wide polymorphism (Apanius et al., 1997; Ejsmond & Radwan, 2015; Potts & Wakeland, 1990). However, theoretical models suggest that negative frequency-dependent selection based on rare allele advantage potentially maintains a larger degree of polymorphism than selection based on heterozygote advantage, when pathogens have high mutation rates (Borghans et al., 2004; Ejsmond & Radwan, 2015). The heterozygote advantage hypothesis invites the question of why evolution has not favored an infinite increase in the number of MHC gene copies. This paradox has led researchers to hypothesize that having too many different MHC alleles may be disadvantageous, either because (i) it increases the risk of autoimmunity or (ii) it increases the negative selection during maturation of T-cells in the thymus, and thereby diminishes the total repertoire of T-cell receptors that bind antigen-presenting MHC molecules (Kubinak, Ruff, Hyzer, Slev, & Potts, 2012; Nowak, Tarczyhorno, & Austyn, 1992; Penn & Potts, 1999). So rather than favoring unlimited numbers of MHC gene copies, evolution is thought to have favored levels that optimize the trade-off between costs and benefits associated with the number of different MHC alleles per individual.



**Fig. 1** The MHC genotype may affect mate choice via condition dependent effects on both direct and indirect (genetic) benefits (figure adapted from Zelano and Edwards (2002)).

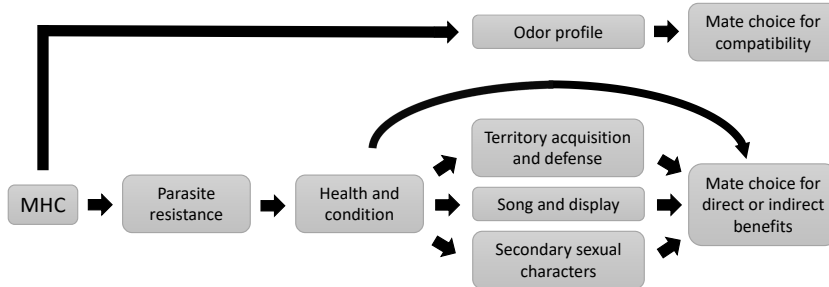
### *Divergent allele advantage*

Wakeland et al. (1990) presented the divergent allele advantage hypothesis, which predicts that MHC alleles with antigen binding repertoires that are dissimilar from other alleles convey a selective advantage over alleles with more similar antigen binding repertoires. The hypothesis arose from an empirical study, in which Wakeland and co-workers analyzed the structure and binding properties of MHC alleles from eight species/sub-species of the genus *Mus* and discovered that selection favored the maintenance of high functional divergence between MHC alleles (i.e., MHC alleles with dissimilar antigen-binding properties). The hypothesis has been confirmed by theoretical models and computations based on empirical data (Lenz, 2011; Pierini & Lenz, 2018; Richman, Herrera, & Nash, 2001), and has found support in empirical studies suggesting that more divergent MHC genotypes are favored by selection in natural populations (Eizaguirre, Lenz, Kalbe, & Milinski, 2012; Froeschke & Sommer, 2012; Kamath, Turner, Kusters, & Getz, 2014; Lenz, Eizaguirre, Kalbe, & Milinski, 2013; Lenz, Wells, Pfeiffer, & Sommer, 2009; Richman et al., 2001). Divergent allele advantage is predicted to amplify the effects of the two previously mentioned models of balancing selection, heterozygote advantage and rare allele advantage, in maintaining MHC polymorphism. Specifically it is expected to preserve functionally divergent alleles over alleles with a higher degree of resemblance (Lenz, 2011; Pierini & Lenz, 2018; Richman et al., 2001; Wakeland et al., 1990).

### *MHC-based mating preferences*

In addition to pathogen mediated selection, mating preferences have been suggested to also be responsible for maintaining high levels of population-wide MHC polymorphism (Hedrick, 1992; Penn & Potts, 1999; Potts, Manning, & Wakeland, 1991; Reusch, Häberli, Aeschlimann, & Milinski, 2001). Individual variation in MHC genes may influence mate choice based on both direct and indirect benefits (Fig. 1) (Zelano & Edwards, 2002). This has been suggested to work through mechanisms that involve several independent properties of MHC genes: (i) the number of different MHC alleles per individual, (ii) the selective advantage of particular alleles or combinations of alleles, or (iii) genetic compatibility with potential mates (Penn & Potts, 1999; Piertney & Oliver, 2006). Under (i), heterozygote advantage predicts that males with more different MHC alleles should be more resistant to pathogens, which may improve their physical condition. Via effects on health and condition, the MHC genotype of males may influence territory acquisition and defense, and other male characters that may be favored by females seeking direct benefits (e.g. age) (Zelano & Edwards, 2002) (Fig. 2). However, the advantages of having many different MHC alleles may also ensure that such males are better able to afford costly ornaments, courtship displays, or intensive song, and may therefore also be favored by females that seek indirect benefits (Zelano & Edwards, 2002) (Fig. 2). Furthermore, because high functional divergence between the MHC alleles within an individual increases the peptide binding repertoire, we expect that high MHC functional divergence enhances the ability of males to provide both direct and indirect benefits to females in similar ways as having many different MHC alleles (Lenz, 2011; Pierini & Lenz, 2018; Wakeland et al., 1990). Under (ii), particular MHC alleles or combinations of alleles may be associated with improved resistance to common or particularly virulent pathogens, and rare allele advantage may favor new or rare MHC alleles in particular (Borghans et al., 2004; Penn & Potts, 1999; Westerdahl, Hansson, Bensch, & Hasselquist, 2004). Males that possess specific favorable alleles or combinations of alleles may therefore also be in better physical condition and thus have improved abilities to provide both direct and indirect benefits to females (Zelano & Edwards, 2002) (Fig. 2). By choosing mates that are genetically compatible in the MHC to themselves (iii), females may increase the genetic quality of the offspring. Studies in both humans, mice, fish, and birds have shown that olfactory cues offer a mechanism by which females may discriminate male MHC genotypes (Boehm & Zufall, 2006; Carroll, Penn, & Potts, 2002; Leclaire, Strandh, Mardon, Westerdahl, & Bonadonna, 2017; Milinski et al., 2005; Reusch et al., 2001; Wedekind, Seebeck, Bettens, & Paepke, 1995; Wedekind & Furi, 1997) (Fig. 2). In particular, females that prefer males with MHC genotypes that are dissimilar from their own may ensure that offspring inherit high numbers of different MHC alleles and/or high MHC functional divergence (Lenz, 2011; Penn & Potts, 1999; Pierini & Lenz, 2018; Wakeland et al., 1990). Furthermore, mate choice for MHC dissimilarity may also reduce the risk of

inbreeding and in small populations; it may even be a mechanism that favors mating with immigrants, which may contribute new or rare MHC alleles to the offspring (Penn & Potts, 1999; Potts & Wakeland, 1993). Finally, by choosing mates with dissimilar MHC genotypes, females may ensure that the MHC genotypes of the offspring are divergent from the genotype of either parent, which may increase the probability that the offspring are resistant to pathogens that have adapted to the genotypes of the parents (Penn & Potts, 1999).



**Fig. 2** The MHC genotype may affect mate choice both via effects on individual body odor and via differences in resistance to parasites and pathogens, which in turn affect individual health and condition (figure adapted from Zelano & Edwards (2002)).

## MHC genes and mating systems

To what extent MHC mating preferences are based on direct benefits or indirect (genetic) benefits, may be difficult to disentangle, because the ability of a male to provide either may be affected by his health and condition (Fig. 2). However, it is likely that good genes (either in terms of number of different MHC alleles, MHC functional divergence, or specific beneficial alleles) play a major role both in mating systems, where intrasexual selection is strong, and in mating systems with intersexual selection, where females base their choice on secondary sexual characters, because health and condition is important for both competition, song and display, and for developing secondary sexual characters. In contrast, mate choice for MHC compatibility may be more prominent in species where intrasexual selection is weak and secondary sexual characters are less important. In such species, body odors may provide females with cues about the MHC genotype of a potential mate and enable her to select a mate with a genotype compatible to her own (disassortative mating) (Boehm & Zufall, 2006; Carroll et al., 2002; Leclaire et al., 2017; Milinski et al., 2005; Reusch et al., 2001; Wedekind et al., 1995;



Wedekind & Furi, 1997). An exception, where MHC mate choice for genetic compatibility may work also in mating systems with strong intrasexual or intersexual selection, is in populations where inbreeding avoidance is favored, e.g. small and bottlenecked populations (Penn & Potts, 1999; Potts & Wakeland, 1993). Because of the extreme polymorphism of MHC genes, individuals that have similar MHC genes are likely to be related, and females may therefore avoid inbreeding by selecting mates with dissimilar MHC genotypes from their own (Penn & Potts, 1999).

## Box I

### *The vertebrate immune system*

The vertebrate immune system is highly conserved across species and consists of two major compartments, the innate and the adaptive immune systems.

*The innate immune system* is constitutively expressed and provides an immediate response to infection based on recognition of conserved features unique to pathogens. It is therefore renewed and active (i.e., inducing costs) even when the individual is not infected by any pathogens, as its crucial role is to be the front-line defense against novel infections (Murphy & Weaver, 2016; Schmid-Hempel, 2011). Innate immune cells express so-called pattern recognition receptors (PRR) that recognize molecular structures associated with pathogens or damaged tissue (Murphy & Weaver, 2016). The functions of the innate immune system are mediated by monocytes, macrophages, dendritic cells (DC), granulocytes (neutrophils, eosinophils, mast cells (MC), and basophils), natural killer (NK) cells, and endothelial cells (Murphy & Weaver, 2016). Major non-cellular components of the innate immune system are cytokines, platelets (thrombocytes), and the complement system (Murphy & Weaver, 2016).

*The adaptive immune system* provides a highly specific response to each type of infection (Murphy & Weaver, 2016). Because the adaptive immune system expands greatly in response to pathogens, the maintenance cost is relatively low in the resting state, while the major cost of the defense is paid only during infections (Schmid-Hempel, 2011). The need for activation and proliferation of the adaptive immune system means that it requires more time to be fully activated and mount a significant response to an infection. The response mechanisms of the adaptive immune system are divided into two major types that are either cell-mediated or based on antibodies (i.e., humoral immunity). Cell-mediated adaptive immune responses are mainly employed against intracellular pathogens and has two types of effector cells. CD8<sup>+</sup> cytotoxic

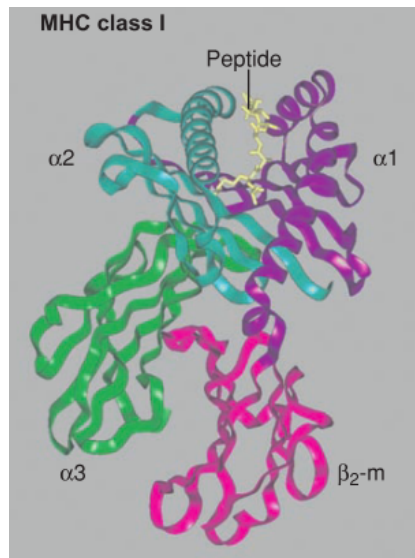
T-cells (CTLs) kill infected host cells, while CD4<sup>+</sup> helper T-cells stimulate macrophages to exterminate ingested microbes (Murphy & Weaver, 2016). In humoral immunity, the effector molecules are antibodies which are produced by B-cells upon stimulation from CD4<sup>+</sup> helper T-cells. The antibodies bind to extracellular pathogens and toxins and facilitate elimination of these (Murphy & Weaver, 2016).

CD4<sup>+</sup> helper T-cells are divided into four main groups: Th1, Th2, Th17 cells, and T-regulatory cells (Treg). The differentiation of naïve CD4<sup>+</sup> helper T-cells is induced by cytokine signals from innate immune cells, which are produced in response to the specific type of pathogen that is detected (Murphy & Weaver, 2016; Zhu & Paul, 2008). Th1, Th2, and Th17 cells stimulate different types of adaptive immune responses, type 1, type 2, and Th17-responses. The cytokine signals are amplified by positive feedback loops with the differentiating helper T-cells, and because cytokines associated with one type of helper T-cells (e.g. Th1) suppress the differentiation of other types (e.g. Th2 or Th17), adaptive immune responses are predominantly of one type (i.e., type 1, type 2, or Th17) (Murphy & Weaver, 2016; Zhu & Paul, 2008). Type 1 immune responses are characterized by strong cell-mediated responses, but also stimulate B-cells to produce a certain class of antibodies (Murphy & Weaver, 2016). In type 2 immune responses, IL-4 and IL-5 secreting Th2 cells stimulate B-cells to produce antibodies of different classes than those involved in type 1 responses (Murphy & Weaver, 2016). Th17-responses are especially involved in combatting infections at mucosal surfaces, and in this type of responses, Th17 cells stimulate other immune cells to produce cytokines (particularly IL-17 and IL-22) that induce strong inflammatory reactions (Murphy & Weaver, 2016). Treg cells are selected in the thymus and are involved in regulation of immune responses, e.g. in peripheral tolerance that serves to prevent immunopathology (Murphy & Weaver, 2016).

#### *The role of the MHC in triggering adaptive immune responses*

The genes of the major histocompatibility complex (MHC) code for glycoprotein receptor molecules that bind peptide fragments from proteins that have been degraded in the cell (Fig. 3). The MHC molecule presents the bound peptide on the cell surface to T-cells, and if the peptide-MHC complex is recognized by a T-cell receptor and a co-stimulatory signal is supplied, the T-cell is activated and an adaptive immune response is initiated (Murphy & Weaver, 2016). In contrast to the B-cell receptor, which recognize antigen in its naïve form, the T-cell receptor only recognizes peptide antigen presented by MHC molecules, and this makes the peptide binding repertoire of the

MHC molecules central to the function of the adaptive immune system (Frank, 2002; Murphy & Weaver, 2016).



**Fig. 3** MHC-I molecule with bound peptide. Figure adapted from Mehra & Kaur (2003).

There are two major classes of MHC molecules. MHC class I are expressed on all nucleated cells of the body and bind peptide antigens of cytoplasmic origin, i.e., from intracellular pathogens, while MHC class II only appear in macrophages, dendritic cells, and B-cells, and bind peptide antigens derived from extracellular pathogens (Willey, Sherwood, & Woolverton, 2008). MHC class I molecules present their antigen to CTLs and thereby trigger a cascade of responses, in which CTLs kill the infected cells (Murphy & Weaver, 2016). However, activated CTLs also release cytokines, particularly IFN- $\gamma$  which serves as stimuli to CD4<sup>+</sup> helper T-cells and activates macrophages that are recruited to the area of infection. Activated macrophages help eliminate the infection by ingesting and exterminating pathogens (Murphy & Weaver, 2016). MHC class II molecules, present their antigens to naïve CD4<sup>+</sup> helper T-cells, which differentiate into Th1 effector cells if stimulated by IFN- $\gamma$  and IL-12, Th2 effector cells if stimulated by IL-2 and IL-4, or Th17 effector cells if stimulated by TGF $\beta$ , IL-6, IL-21, and IL-23 (Zhu & Paul, 2008). Thus, the MHC class I is associated with type 1 adaptive immune responses, while MHC class II activation of T-helper cells may elicit either type 1, type 2, or Th17 responses, depending on the cytokine stimulus from other cells involved in the immune response.

# Aims of the thesis

- a. When formulating the ICHH, Folstad and Karter (1992) suggested that immunosuppressive effects of testosterone mediate the cost of secondary sexual characters and/or male competitive behavior. This would ensure that such characters or behavior could function as honest cues of genetic quality in loci related to disease resistance. However, Folstad and Karter did not consider the effects of the disease resistance genes when expressed in females, where no testosterone-mediated immunosuppression occurs. Sex hormones have been shown to affect immune responses and may underlie important sex differences in immunity observed in many vertebrate taxa (Foo et al., 2016; Hasselquist, 2007; Klein & Roberts, 2010; Roberts, Buchanan, & Evans, 2004; Schuurs & Verheul, 1990). In **Paper I**, I reviewed the literature and summarized our current knowledge about the effects of sex hormones on different aspects of vertebrate immune systems. Based on this, I proposed the new hypothesis that regulatory effects of sex hormones on immune responses may create a potential for sexually antagonistic selection on immune system genes that are shared between the sexes, and thus result in a genetic sexual conflict.
- b. The genes of the major histocompatibility complex are central to disease resistance in vertebrates and are highly polymorphic. Therefore, MHC-genes form good candidates for genes that underlie phenotypic variation between males that forms the basis of female mate choice (Hamilton & Zuk, 1982; von Schantz, Wittzell, Goransson, Grahn, & Persson, 1996). Given such an effect of the MHC genes, the hypothesis presented in **Paper I** predicts that MHC genes could be subject to sexually antagonistic selection. In **Paper II and III**, I therefore investigated our sexual conflict hypothesis using data from our long-term, detailed field study of the population great reed warblers *Acrocephalus arundinaceus* that breed in in Lake Kvismaren in southern Central Sweden. We focused our studies on MHC class I (MHC-I) genes, which have previously been shown to play an important role for disease resistance in this species (Westerdahl et al., 2005; Westerdahl, Asghar, Hasselquist, & Bensch, 2012).
- c. In **Paper IV**, I investigated whether female mate choice is associated with variation in MHC-I genes in great reed warblers, and specifically whether variation in the MHC-I genes affects female choice for direct and indirect benefits. We also investigated whether variation in the MHC-I genes is associated with male characters known to be correlated with male pairing

and breeding success (i.e., age, song repertoire size, and territory attractiveness) (Hasselquist, 1998; Hasselquist, Bensch, & von Schantz, 1996).

- d. MHC-genes have been shown to be in linkage disequilibrium in both humans and other species, and this may indicate that selection acts on multi-locus haplotypes rather than on single MHC genes (Begovich et al., 1992, 2001; Hollenbach et al., 2001). In the final paper of the thesis (**Paper V**), I characterized segregating MHC-I haplotypes from 119 great reed warbler families and used this data to investigate (i) whether MHC-I genes are under linkage disequilibrium in great reed warblers, (ii) whether variation in the number of different MHC-I alleles and MHC-I functional divergence on the diploid genotype level was reflected in variation on MHC-I haplotypes, and (iii) whether and how a multi-locus structure affected the MHC-I diversity on MHC-I haplotypes in terms of number of different MHC-I alleles and functional divergence.



**Fig. 4** Great reed warbler male singing long song in a typical position at the top of a reed (photo by Jacob Roved).

# The great reed warbler as a study system for investigating the effects of sexual selection on MHC genes

The great reed warbler is a polygynous songbird that breeds in Europe from late April to early August and migrates from late July to mid September to winter quarters in sub-Saharan Africa (Bensch, 1996; Koleček et al., 2016; Lemke et al., 2013). Males of this species defend large territories in reed beds of eutrophic lakes, and the most attractive territories are occupied by the individuals that arrive early from spring migration to the breeding area (Bensch, 1996; Bensch & Hasselquist, 1991; Cramp et al., 1992; Hasselquist, 1998; Koleček et al., 2016; Lemke et al., 2013). Once they have established a territory, the males produce a loud, complex, and variable song (called ‘long song’) in order to attract females (Fig. 4). This vocal ornament is physically highly demanding, as the males sing intensively almost without interruption throughout the daylight hours during most of the breeding season (in southern Central Sweden this is up to 21 hours per day) (Hasselquist & Bensch, 2008; Hasselquist, Bensch, & Ottosson, 1993). Females arrive on average 11 days later than the males to the breeding area and visit a number of territories when searching for a social mate (Bensch & Hasselquist, 1992; Tarka, Hansson, & Hasselquist, 2015). They appear to evaluate the songs of the males, the quality of the territories, and which status they would receive in the harem of a socially polygynous male before making their mate choice (Bensch, 1996; Bensch & Hasselquist, 1991, 1992; Hasselquist, 1998).

## *Hasselquist 1996 suggested mate choice for good genes*

In our study population of great reed warblers it has been shown that males with larger song repertoires had a larger proportion of their offspring survive until breeding age and that females preferred males with more variable song both as social and extra pair mates (Hasselquist, 1998; Hasselquist et al., 1996). The discovery that song repertoire size was associated with offspring survival implied that females earn indirect genetic benefits for their offspring by selecting males with more variable song (Hasselquist et al., 1996). Testosterone is involved in the development and expression of male song in birds (Ball, Castelino, Maney, Appeltants, & Balthazart, 2003; Wingfield & Farner, 1993), and thus mate attraction song in songbirds might be associated with costs related to immunosuppression, as hypothesized by Folstad and Karter (1992), which would ensure the honesty of this behavioral trait as a cue for genetic quality related to disease resistance (Ball et al., 2003; Marler, Peters, Ball, Dufty, & Wingfield, 1988).

*Other studies suggested that additive genetic variance for fitness exists*

Further studies in our great reed warbler study population found high levels of genetic variation in the MHC class I genes (MHC-I), and furthermore, fluctuating allele frequencies between cohorts suggested that MHC-I alleles are subject to frequency dependent selection (Westerdahl, Wittzell, von Schantz, & Bensch, 2004; Westerdahl et al., 2004; Westerdahl, Wittzell, & von Schantz, 1999). Evidence also suggested that individuals with a specific MHC-I allele and/or high number of different MHC-I alleles in the MHC-I were more resistant to a strain of malaria parasites (Westerdahl et al., 2005; Westerdahl et al., 2012). This indicated that MHC-I genes play an important role for disease resistance in this species, which further supports the hypothesis that MHC genes may be associated with 'good genes' effects and thus be relevant for mate choice.

*Potential also for mate choice for direct benefits*

However, the MHC genotype of great reed warbler males may also influence qualities evaluated by females to gain direct benefits through their mate choice (Fig. 2). Another study showed that the territory quality of males predicted both their social mating success (in terms of harem size) and their reproductive success (Hasselquist, 1998). Because acquiring a high quality territory is dependent on early arrival from spring migration (Bensch & Hasselquist, 1991; Hasselquist, 1998), the MHC genotype may influence the ability of males to acquire and control attractive territories (i.e., territories with better resources and lower nest predation) (Hansson, Bensch, & Hasselquist, 2000; Zelano & Edwards, 2002). Thus, the conditions for MHC-based mate choice in great reed warblers are present, and it appears that the effects of the MHC genotype potentially could be mediated both via direct and indirect benefits, as suggested by Zelano & Edwards (2002) (Fig. 2).

*Previous study found no mate choice for MHC-I diversity*

MHC-based mate choice has previously been investigated in great reed warblers (Westerdahl, 2004). In the previous study it was investigated whether females chose males with high number of different MHC-I alleles ('good genes as heterozygosity'), or males with numbers of different MHC-I alleles that were complementary to the females' own. However, the results showed no evidence for mate choice based on either number of different MHC-I alleles or MHC-I compatibility. The genotyping method employed in the study (denaturing gradient gel electrophoresis, DGGE) was not based on DNA sequencing of the MHC alleles, and thus allowed only investigations of the influence of allelic diversity in the MHC, while it was not possible to test effects related to the functional divergence between alleles, sensu Wakeland et al. (1990).

# General methodology

In the following paragraphs, I give a summary of the methods and the data analysis protocols used in my empirical studies of our great reed warbler data set. For further details, please see the methods sections of Paper II-V.

## *The study population and field methods*

We conducted our studies using data from a wild population of great reed warblers at Lake Kvismaren (59°10'N, 15°25'E) in southern Central Sweden that has been closely monitored in an exhaustive field study from 1984 until present (Bensch, Hasselquist, Nielsen, & Hansson, 1998; Tarka, Akesson, Hasselquist, & Hansson, 2014). Our data set consisted of 140 adult males and 126 adult females that had been observed, sampled, and ringed in the period 1984-2004, as well as 290 chicks that constituted the 1998 and 1999 cohorts from the same population, with addition of one nest from each of the years 1992 and 1996. In our study population, we kept detailed records on arrival dates, breeding behaviors, and identities of all individuals, based on observations from nearly daily visits to all territories throughout the breeding period from late April to late July (Bensch & Hasselquist, 1991; Hasselquist & Bensch, 1991). Furthermore, almost all nests were located and precise records were kept for all breeding attempts (Bensch et al., 1998). Molecular techniques were used to verify the paternity and maternity of all chicks (Arlt, Bensch, Hansson, Hasselquist, & Westerdahl, 2004; Hasselquist et al., 1996). Great reed warblers generally have low dispersal rates, and this allowed us to produce solid estimates of offspring recruitment, i.e., whether or not offspring return to breed in the study population when  $\geq 1$  year old (Hansson, Bensch, Hasselquist, & Nielsen, 2002). As estimates of Darwinian fitness, we used (i) *lifetime number of fledglings*, defined as an individual's lifetime sum of fledged offspring, and (ii) *lifetime number of recruits*, defined as an individual's lifetime sum of recruiting offspring. We also analyzed fitness in terms of (a) *life span*, defined as the age of an individual the last time it was observed in our study area, (b) *offspring fledging success* (i.e., lifetime number of fledglings in models including life span as covariate), and (c) *offspring recruitment success* (i.e., lifetime number of recruiting offspring in models including lifetime number of fledglings as covariate). Territory attractiveness ranks were estimated annually by averaging the relative occupation order for each territory in the two years flanking the given year.

Finally, the mate attraction songs (i.e., the long song) of  $> 95\%$  of all territorial males in the study area were recorded in the period 1987-1998, and the song repertoire size of each male was estimated from visual analysis of sonograms by D. Hasselquist. The song of the great reed warbler is highly variable, and composed of



a number of different syllables (i.e., different sounds), that are expressed in strophes of variable length (Catchpole, 1983). The song repertoire size was estimated as the cumulative number of different syllables occurring within 150 syllable switches in the song of a given male (Hasselquist et al., 1996). The cumulative number of different syllables increases with the number of syllable switches observed, however, there's a limit to the repertoire size of great reed warblers, and it has previously been shown that the cumulative number of different syllables levels off after 10-12 strophes (Catchpole, 1983, 1986). In comparison, 150 syllable switches corresponds to 18-22 strophes, and we therefore expect that the analyses conducted for our present studies provide solid estimates of the song repertoire sizes individual males (D Hasselquist et al., 1996).

### *MHC-I genotyping*

Blood samples of 20-80  $\mu$ l were collected each year from almost all individuals in our study area. To genotype the individuals in our data set, we extracted genomic DNA from blood samples and used previously designed primers (HNalla-HN46) to target a 262 bp region of MHC-I exon 3 with standard PCR amplification. The MHC-I exon 3 encodes the alpha two domain, which forms one half of the peptide binding groove on the MHC-I molecule (Fig. 3). We sequenced MHC-I exon 3 using high throughput sequencing (HTS). Samples from 88 adult males, 100 adult females, and 145 chicks were sequenced in a Roche 454 GS FLX (F. Hoffmann-La Roche AG, Basel, Switzerland), and samples from 53 adult males, 32 adult females, and 150 chicks (including 11 samples that were replicated from the 454-sequencing run) were sequenced in two independent runs using the Illumina MiSeq platform (Illumina Inc., San Diego, CA, USA). We added tag-sequences to our sequencing primers to be able to reassign sequences to our samples after multiplexing (Parameswaran et al., 2007).

PCR amplification and high-throughput DNA-sequencers such as the 454- or MiSeq-platforms produce erroneous sequences and therefore careful filtering and screening of the output data is critical for the success of MHC amplicon sequencing experiments. We used two different bioinformatics protocols to filter our data sets. The 454-sequencing data was demultiplexed using the software jMHC (Stuglik, Radwan, & Babik, 2011) and filtered according to the method in Galan et al. (2010). The data was then thoroughly screened by eye to remove non-functional alleles and any remaining low-quality and artificial sequences. The output from the Illumina data sets was filtered using the using the R package DADA2 (Callahan et al., 2016), after removing adapters, primers, and tag-sequences with the software Cutadapt (Martin, 2011). I developed the tools ReplMatch and GetReplStats for fast optimization of the filtering process by replicate comparison (published in the R package MHCtools (Roved, 2017)) and employed these tools to find the most appropriate settings in DADA2. Subsequently, we filtered the sequences output

from DADA2 by their per-amplicon frequencies. Again, the optimization tools were employed to find the most appropriate threshold for this filtering step. Finally, the data was thoroughly screened by eye to remove non-functional alleles and any remaining low-quality and artificial sequences.

We estimated a repeatability of 0.94 for the 454-sequencing experiment and a near-perfect repeatability of 0.998 for the Illumina sequencing experiment. By comparing 11 samples that were replicated between the Illumina and 454-sequencing experiments, we estimated a repeatability of 0.96, indicating that the output from the two experiments was comparable. In total, we found 390 unique great reed warbler MHC-I exon 3 sequences in the data set. To my knowledge, our study is the first to have used DADA2 for filtering amplicon sequencing data in an MHC study.

### *Estimating positive selection*

We used the recombination analysis tool RAT (Etherington, Dicks, & Roberts, 2005) to test for recombination break points within the MHC-I sequences. We then used the software PhyML (Guindon et al., 2010; Guindon & Gascuel, 2003) to run 12 different phylogenetic models, and inferred the best model for our data set by comparing Akaike Information Criterion values calculated from the log-likelihood ratios obtained for each model. The tree inferred by the best phylogenetic model was employed in the software codeml from the PAML package (Yang, 1997, 2007), specifying the nested site models M1, M2, M7, and M8. The nested models were compared using likelihood ratio tests to estimate probabilities of positive selection on the MHC-I sequences. Finally, we inferred codons under positive selection from model M8 (positively selected sites, PSS) by Bayes Empirical Bayes analysis (Yang, Wong, & Nielsen, 2005).

### *MHC-I diversity measures*

We used two different measures of MHC-I diversity, (i) the number of different MHC-I exon 3 alleles per individual and (ii) the functional divergence between the alleles observed in each individual.

When peptide antigens are bound by MHC molecules, specific amino acid codons in the peptide binding groove, so called anchor residues, form direct associations with the antigen (Bjorkman et al., 1987; Brown et al., 1993; Chappell et al., 2015; Follin et al., 2013). Theoretical models have shown that the peptide binding repertoire of two MHC molecules increases with the proportion of differences in the anchor residues, suggesting that individuals with more divergent MHC alleles may be able to recognize a broader repertoire of antigen peptides (Lenz, 2011; Pierini & Lenz, 2018). The positions of the anchor residues have been well studied for the human leukocyte antigen (HLA, the human equivalent of the MHC) and recently also for the chicken MHC, but detailed studies are yet lacking for the vast majority of non-model species (Bjorkman et al., 1987; Brown et al., 1993; Chappell et al.,

2015). We inferred the amino acid codons of our great reed warbler MHC-I sequences from the anchor residues of the peptide binding region (PBR) from HLA-A, following Westerdahl et al. (1999), and calculated the MHC-I functional divergence PBR as the mean proportion of amino acid differences in these codons between all MHC-I alleles in each individual. Furthermore, we also estimated the functional divergence between all alleles in each individual using the codons estimated to be under positive selection, by calculating the mean proportion of amino acid differences in these codons between all MHC-I alleles in each individual (MHC-I functional divergence PSS).

### *Haplotype inference*

Songbirds have evolved high levels of MHC diversity compared to other species, yet little is known about the structure of the genomic region of the MHC in songbirds (O'Connor et al., 2016; Westerdahl, 2007). Analyses of segregating haplotype patterns in the MHC-I of songbirds would address this issue by providing knowledge on how alleles are linked. However, only two such analyses have previously been attempted on songbirds (Karlsson & Westerdahl, 2013; Westerdahl et al., 2004). Haplotype analyses in species with highly diverse MHC pose numerous challenges due to the error rate associated with genotyping and due to the high prevalence of some MHC alleles, which makes their segregation patterns difficult to resolve. However, our large data set, including a detailed pedigree spanning multiple generations, enabled me to conduct this analysis with good accuracy in great reed warblers.

When genotyping the MHC-I of the great reed warblers in our data set, we used primers that target multiple class I loci (Westerdahl et al., 2004). We inferred segregating MHC-I haplotypes from 119 great reed warbler families by comparing the presence/absence of individual alleles in parents and offspring (Fig. 5 shows an example of MHC-I allele segregation in one great reed warbler family). I developed the analysis tool HpltFind to automate this process (published in the R package 'MHCtools' (Roved, 2017)), and after applying this tool on all families in our data set, we resolved uncertain allele assignments and haplotype incongruences by comparing segregation patterns across generations and matching putative identical haplotypes (the protocol is illustrated in Fig. 6). The analyses confirmed linkage disequilibrium for the MHC-I in great reed warblers, with two observations of recombination among 668 gametes.

Nest 28 of the 1999 cohort						
Alleles	Mother	Father	Offspring 1	Offspring 2	Offspring 3	
Acar-UA*144	X	X	X	-	X	
Acar-UA*4	X	X	X	X	X	
Acar-UA*155	X	X	X	X	X	
Acar-UA*9	X	X	X	X	X	
Acar-UA*12	X	-	X	X	-	
Acar-UA*122	X	-	X	X	-	
Acar-UA*125	X	-	X	X	-	
Acar-UA*133	X	-	X	X	-	
Acar-UA*201	X	-	X	X	-	
Acar-UA*276	X	-	X	X	-	
Acar-UA*296	X	-	X	X	-	
Acar-UA*133	X	-	X	X	-	
Acar-UA*201	X	-	X	X	-	
Acar-UA*276	X	-	X	X	-	
Acar-UA*296	X	-	X	X	-	
Acar-UA*340	X	-	X	X	-	
Acar-UA*348	X	-	X	X	-	
Acar-UA*79	X	-	X	X	-	
Acar-UA*153	X	-	-	-	X	
Acar-UA*157	X	-	-	-	X	
Acar-UA*223	X	-	-	-	X	
Acar-UA*239	X	-	-	-	X	
Acar-UA*31	X	-	X	-	-	
Acar-UA*285	-	X	X	-	-	
Acar-UA*23	-	X	X	-	-	
Acar-UA*119	-	X	-	X	X	
Acar-UA*271	-	X	-	X	X	
Acar-UA*94	-	X	-	X	X	

Putative segregating haplotypes	
Mother A	Mother B
Acar-UA*12	Acar-UA*144
Acar-UA*122	Acar-UA*153
Acar-UA*125	Acar-UA*157
Acar-UA*133	Acar-UA*223
Acar-UA*201	Acar-UA*239
Acar-UA*276	Acar-UA*31
Acar-UA*296	Acar-UA*4
Acar-UA*340	Acar-UA*55
Acar-UA*348	Acar-UA*9
Acar-UA*4	
Acar-UA*55	
Acar-UA*79	
Acar-UA*9	

Final haplotypes	
Mother A	Mother B
Acar-UA*12	Acar-UA*144
Acar-UA*122	Acar-UA*153
Acar-UA*125	Acar-UA*157
Acar-UA*133	Acar-UA*223
Acar-UA*201	Acar-UA*239
Acar-UA*276	Acar-UA*31
Acar-UA*296	Acar-UA*4
Acar-UA*340	Acar-UA*55
Acar-UA*348	Acar-UA*9
Acar-UA*55	
Acar-UA*79	
Acar-UA*9	

Putative segregating haplotypes	
Father A	Father B
Acar-UA*144	Acar-UA*119
Acar-UA*285	Acar-UA*271
Acar-UA*4	Acar-UA*4
Acar-UA*55	Acar-UA*55
Acar-UA*9	Acar-UA*9
Acaru-UA*23	Acar-UA*94

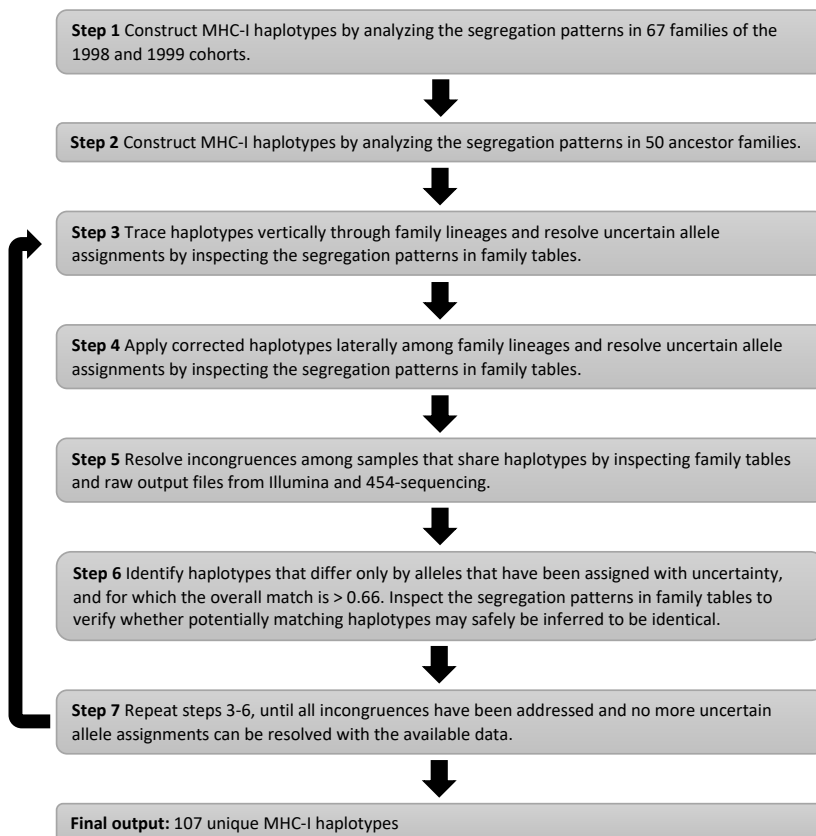
  

Final haplotypes	
Father A	Father B
Acar-UA*144	Acar-UA*119
Acar-UA*285	Acar-UA*271
Acar-UA*4	Acar-UA*4
Acar-UA*55	Acar-UA*55
Acar-UA*9	Acar-UA*9
Acaru-UA*23	Acar-UA*94

**Fig. 5** Family table from nest number 28 of the 1999 cohort showing MHC-I allele segregation patterns with inferred putative segregating MHC-I haplotypes (Mother A, Mother B, Father A, Father B) marked by different colors. Dark gray color indicates that a segregation pattern could not be determined for an allele, because it was present in both parents and in all offspring (uncertain allele). In the final haplotypes, uncertain alleles had been resolved by applying steps 3-6 in the haplotype inference protocol (from Paper V, Fig. 2).

### *Inference of putative clades among the MHC-I alleles in our data set*

We plotted the tree of our MHC-I sequences produced by the best phylogenetic model from the analyses in PhyML (see the paragraph ‘Estimating positive selection’) using the R package ggtree version 1.12.7 (Yu, Smith, Zhu, Guan, & Lam, 2017). We looked for clades in the tree that (a) were monophyletic, (b) showed reasonable SH-aLRT support values (Shimodaira & Hasegawa, 1999), and (c) did not contain extreme proportions of the total number of alleles (each allele is represented as a branch tip in the tree) or the total genetic variation (illustrated by the length of the branches). The decisions underlying the inference of the final clades are reported in the methods section of Paper V and lists of alleles assigned to each clade are presented in Appendix II of that paper.



**Fig. 6** Flow chart of our haplotype inference protocol (from Paper V, Fig. 1).

## Data analysis

All statistical analyses were conducted in R versions 3.1.2., 3.4.2., and 3.5.1 (R Core Team, 2014, 2017, 2018) including the packages *car* version 2.1.6 (Fox & Weisberg, 2011), *ggplot2* version 3.0.0 (Wickham, 2016), *Hmisc* version 4.1.1 (Harrell Jr., 2018), *lattice* version 0.20.35 (Sarkar, 2008), *lme4* version 1.1.14 (Bates, Mächler, Bolker, & Walker, 2014; Bates, Maechler, Bolker, & Walker, 2014), *lmodel2* version 1.7-3 (Legendre, 2018), *MASS* version 7.3.47 (Venables & Ripley, 2002), *MHCtools* (Roved, 2017), *openxlsx* versions 4.0.17 and 4.1.0 (Walker, 2017), and *pbrktest* (Halekoh & Højsgaard, 2014).

### *Fitness/life history analyses - Paper II and III*

We used linear regression models and generalized linear models to analyze sex-dependent effects of the number of different MHC-I alleles and MHC-I functional divergence on life span, lifetime number of fledglings, lifetime number of recruits, offspring fledging success, and offspring recruitment success. These models follow a model design suggested in a recent review paper on sex differences in disease genetics (Gilks, Abbott, & Morrow, 2014). Linear mixed effects models were employed to analyze the effects of MHC-I diversity on territory attractiveness rank for each sex. Generalized linear mixed models were used to analyze sex-dependent effects of the number of different MHC-I alleles and MHC-I functional divergence in the offspring on their recruitment success.

Both MHC-I functional divergence PBR and MHC-I functional divergence PSS showed a strong negative correlation with the number of different MHC-I alleles, and in order to avoid a possible confounding influence on the results, number of different MHC-I alleles was included as a covariate in all models in Paper III analyzing the effects of MHC-I functional divergence.

### *Mate choice analyses - Paper IV*

We used linear mixed effects models to test whether females select males with higher number of different MHC-I alleles or MHC-I functional divergence than the mean among all adult males. We also used linear mixed effects models to test whether females select males with number of different MHC-I alleles and MHC-I functional divergence that are complementary to their own. In order to test whether females selected males to maximize the joint pair number of different MHC-I alleles or joint pair MHC-I functional divergence, or to minimize the number of shared alleles, we generated 1,000 simulated data sets, in which females were paired to random males. We then used linear mixed effects models to test whether the values in the true mate pair differed from the observations in the randomly generated pairs. The same approach was used to test whether females selected males with more attractive territories, with larger song repertoire sizes, or males that were older. We

tested for correlations between male territory rank and age, number of different MHC-I alleles, and MHC-I functional divergence, respectively, using linear mixed effects models. Likewise, we also tested for correlations between male song repertoire size and age, number of different MHC-I alleles, and MHC-I functional divergence, respectively, and also for a correlation between male song repertoire size and MHC-I functional divergence, when age was included in the model. Finally, we used linear mixed effects models to analyze whether females select males with larger MHC-I functional divergence, when male age was accounted for in the model, and whether true mate pairs had larger joint pair functional divergence than randomly generated pairs, when random males were selected from the same age category as the true social male.

Because individual number of different MHC-I alleles and MHC-I functional divergence were correlated, the analyses of mate choice for larger or complementary MHC-I functional divergence were conducted using the residuals from linear regressions between MHC-I functional divergence (PBR or PSS, respectively) and number of different MHC-I alleles. When analyzing the effects of MHC-I functional divergence on territory attractiveness and song repertoire size, number of different MHC-I alleles was included as a covariate in the models.

#### *Haplotype analyses – Paper V*

We tested whether the number of different MHC-I alleles and functional divergence were correlated on MHC-I haplotypes using an ordinary least squares linear regression model. We then generated *in silico* simulations of the haplotype data set in order to investigate whether the correlation was an effect of non-random association of alleles on haplotypes. First, we generated 1,000 simulations in which alleles were randomly assigned to haplotypes, while maintaining the total number of alleles per haplotype. Subsequently, we generated 1,000 simulations in which the same number of alleles was assigned from each putative clade in the MHC-I exon 3 tree as was present in the haplotypes in the real data. From the simulated data sets, we estimated the probability of observing correlations by chance, that were more extreme than that observed in the real data set.

We compared the functional divergence between alleles within haplotypes to the functional divergence between alleles that were compared between haplotypes using a student's t-test. The number of alleles present on each haplotype from each clade was visualized with a heat map, and a Levene's test was used to test for equality of the variances of this number for each clade. Major axis linear regression models were used to test for (i) correlations between the number of alleles observed from each clade and the total number of alleles on each haplotype, and (ii) correlations between the number of alleles observed on each haplotype from different clades.

We compared the number of alleles observed from each clade on each real haplotype to the expectations from 1,000 simulated data sets in which alleles were randomly assigned to haplotypes, while maintaining the total number of alleles for each haplotype. The relationship between the mean number of alleles observed from each clade across all simulations and the number of alleles observed in the real data was visualized in scatterplots with a line indicating equality. Furthermore, we used the data set simulations to test whether the number of haplotypes that had each clade represented deviated from random expectations. We estimated probabilities for the observed numbers in the real data set by calculating the proportion of more extreme observations in the corresponding simulated data sets.

Finally, we conducted tests for positive selection on subsets of the MHC-I sequences corresponding to clades A-E inferred from the phylogenetic tree (see the paragraph 'Inference of putative clades among the MHC-I alleles in our data set'). The tests were conducted with *codeml* from the PAML software package (Yang, 1997, 2007) as described above (see the paragraph 'Estimating positive selection'). Trees inferred from the phylogenetic model selected for the total data set were used as inputs to *codeml*. For the subsets corresponding to clades B-E, we inferred codons under positive selection from model M8 by Bayes Empirical Bayes analysis (Yang et al., 2005).



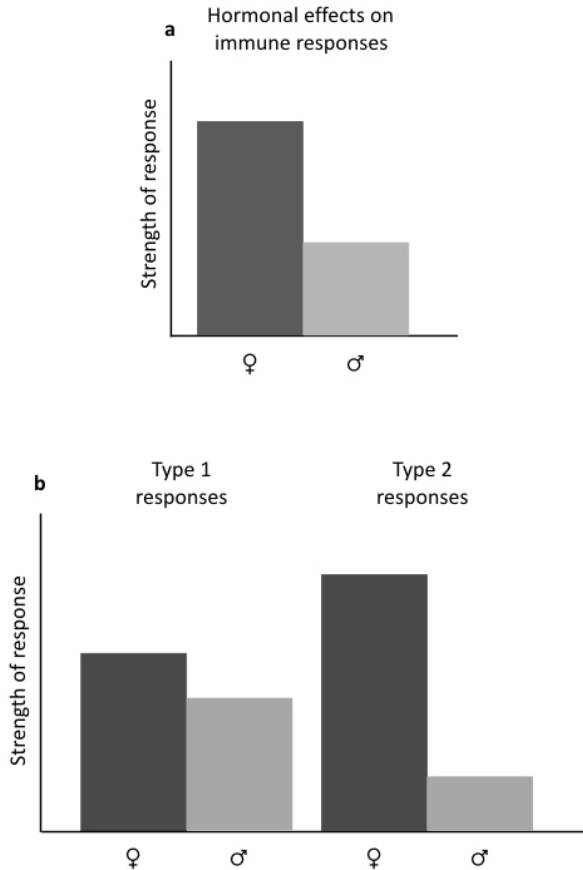


# Results and discussion

## The effects of sex hormones on immune systems

Steroid sex hormones have pronounced and multifaceted regulatory effects on the vertebrate immune system (Foo et al., 2016; Klein & Roberts, 2010; Roberts et al., 2004; Schuurs & Verheul, 1990). In Paper I, we reviewed the literature on such effects, and found support for the generally immuno-suppressive effects of the male sex hormone testosterone, that are central to the concept of the ICHH by Folstad and Karter (1992) (Foo et al., 2016). The female sex hormone estrogen has been found to have some generally immuno-enhancing effects, although this tendency needs to be confirmed in a wider selection of species (Foo et al., 2016). When reviewing the effects of sex hormones on specific immune functions, we found that estrogen and progesterone (the other major sex hormone in females) favor functions associated with type 2 adaptive immune responses and disfavor functions associated with type 1 responses (see Fig. 1, Table S2, S3 in Paper I (Appendix A)). In contrast, testosterone disfavors type 2 responses while it shows an inconsistent pattern for type 1 responses (see Fig. 1 and Table S1 in Paper I (Appendix A)) (see Box I for an introduction to the vertebrate immune system). Altogether, the reviewed literature suggested that immune responses differ systematically between the sexes. Males tend to have generally suppressed immune responses compared to females, and the differences should be particularly pronounced for immune functions associated with type 2 adaptive immune responses (Fig. 7).

In this sense, males and females to some extent constitute different immunological environments, and differences in immune responses may cause the balance between costs and benefits of genes associated with immunity to play out differently between the sexes. Testosterone-suppressed immune responses are likely to enhance natural selection on specific immune functions in males to mitigate an increased cost of pathogen infections. In females, however, enhanced immune responses may increase the costs associated with the immune responses per se (e.g. in terms of energy, nutrients, or risk of immunopathology and autoimmunity). This may also enhance natural selection on specific immune functions, but in contrast to the males, the main selection in females may be to mitigate the costs associated with the immune responses. As a consequence, there may be different optima in males and females for natural selection on genes associated with immunity. If such genes are shared between the sexes (e.g. if located on autosomal chromosomes), there is potential for sexually antagonistic selection, which occurs when there are different optima for a heritable trait in males and females, and the trait is prevented from

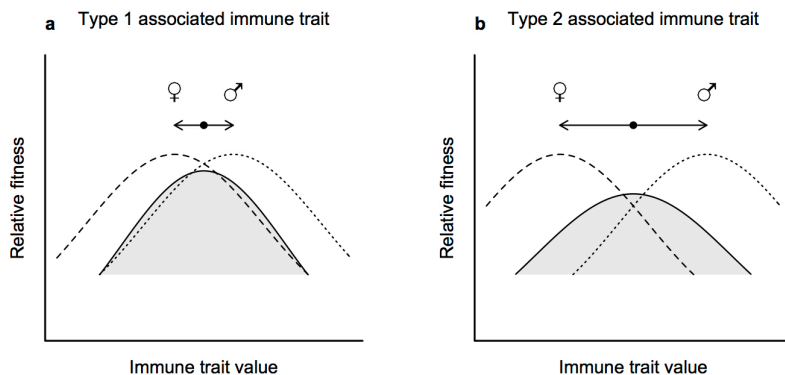


**Fig. 7** Sex differences in the strength of immune responses. **a** Immune responses are generally stronger in females (dark grey) than in males (light grey), which may be a consequence of the general immunosuppressive effects of testosterone. **b** However, the sex differences are not predicted to be of similar magnitude in type 1 and type 2 associated adaptive immune responses; we predict that the difference in the strength of immune responses between females and males is larger for type 2 associated immune responses (right graph, type 2) than for type 1 associated immune responses (left graph, type 1) (from Paper I, Fig. 2).

reaching its optimal expression in either sex (Bonduriansky & Chenoweth, 2009; Cox & Calsbeek, 2009; Gilks et al., 2014; Lande, 1980).

Our review of the literature suggested that differences in the strength of immune responses between males and females depend on whether the immune function under investigation is involved in type 1 or type 2 adaptive immune responses (Fig. 7 b). As a consequence, we predicted that the difference between male and female optima for immune functions associated with type 2 adaptive immune responses is larger than for functions associated with type 1 adaptive immune responses (Fig. 8). Accordingly, the potential for sexually antagonistic selection may vary for genes

associated with type 1 or type 2 adaptive immune responses. Interestingly, the predicted differences in the optima between males and females correspond well with empirical observations of sex differences in autoimmune diseases in humans. Autoimmune diseases are generally overrepresented in females, which is thought to be partly due to regulatory effects of sex hormones on immunity (Ngo, Steyn, & McCombe, 2014; Whitacre, 2001). Furthermore, androgens in general promote autoimmune diseases with a type 1 cytokine profile, while estrogen promotes autoimmune diseases with a type 2 cytokine profile (Almeida Gonzalez et al., 2010; Ngo et al., 2014).

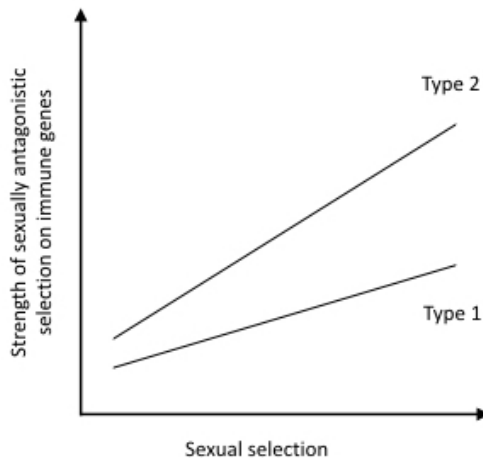


**Fig. 8** Hypothetical sex specific optima of immune traits associated with type 1 and type 2 adaptive immune responses (curves with broken/dotted lines), and the realized trait distribution when the trait is encoded by genes shared by both sexes (e.g., autosomal genes) (solid line, grey-shaded distribution). The hypothetical optima of type 1 and type 2 associated immune traits are predicted to differ between the sexes because of the differences in the effects of sex hormones on the immune system. The difference between the optima of females (broken line) and males (dotted line) are predicted to be smaller for (a) type 1 associated, than for (b) type 2 associated immune traits. This should create stronger sexually antagonistic selection in type 2 associated immune traits compared to type 1 associated immune traits (illustrated by the length of the horizontal arrows). The realized trait distributions (of immune genes shared by the sexes) are predicted to differ between type 1 and type 2 immune responses, as increased strength of sexually antagonistic selection should create a wider trait distribution with decreased mean fitness (from Paper I, Fig. 3).

MHC genes have been given particular attention among studies of genes associated with adaptive immune responses and disease resistance. High MHC genetic diversity has been hypothesized to be advantageous because increased diversity may increase disease resistance, but it has also been associated with disadvantages such as increased risk of autoimmunity and negative selection of T-cells (Baum, Davies, & Peakman, 1996; Benoist & Mathis, 1998; Doherty & Zinkernagel, 1975; Hughes & Nei, 1992; Nowak et al., 1992; Woelfing, Traulsen, Milinski, & Boehm, 2009). Accordingly, several studies have found evidence for optimal MHC diversity (Kalbe et al., 2009; Kloch, Babik, Bajer, Sinski, & Radwan, 2010; Madsen & Ujvari, 2006; Woelfing et al., 2009). However, the potential for sex differences in immune

responses to affect the nature of selection on MHC diversity has so far not been considered. The genomic region of the MHC has been found on autosomal chromosomes in chicken, mice, and humans, and thus, MHC diversity constitutes a trait that is shared between the sexes (Kaufman et al., 1999; Kumanovics, 2007; The MHC sequencing consortium, 1999). As a consequence of this, we proposed that sex differences in the strength of immune responses could create a potential for sexually antagonistic selection over MHC diversity (Fig. 8).

Furthermore, we proposed that sexually antagonistic selection may be more pronounced for MHC class II than for MHC class I genes. This prediction is based on the observation that the sex difference in type 2 adaptive immune responses is larger than in type 1 responses (Fig. 7 b), and furthermore that MHC class II genes are more associated with type 2 responses (which are largely effected via antibodies) than with type 1 responses (in which cell-mediated immunity plays a larger role), while MHC class I genes are exclusively associated with type 1 responses (see Box I).



**Fig. 9** The strength of sexually antagonistic selection on immune genes in relation to the strength of sexual selection. We predict a larger increase in the strength of sexually antagonistic selection for type 2 than for type 1 associated immune traits (from Paper I, Fig. 4).

### *Behavioral and ecological implications*

Previous studies have predicted that the amount of circulating testosterone in males should increase with the strength of sexual selection (Hasselquist, 2007; Rolff, 2002; Zuk, 1990; Zuk & McKean, 1996). In species, where intra-sexual selection is strong (i.e., where males fight or compete with each other for access to females) or

where females favor males with strong expression of secondary sexual characters, there is selection on males to produce high levels of testosterone to become dominant, large, or produce striking ornaments to increase their mating success. In contrast, testosterone levels are predicted to be lower in species with no or only weak sexual selection (e.g. socially monogamous species). Because of the immune-suppressive effects of testosterone, this means that strong sexual selection may be associated with increased differences between the sexes in the strength of immune responses (Fig. 7) (Hasselquist, 2007; Zuk, 1990). This implies that mating behavior may influence selection on immune genes, and we predicted that genes associated with immunity are under stronger sexually antagonistic selection in species where sexual selection is strong (e.g. in socially polygynous species or species with lek mating systems). In species with weak sexual selection, the hormonal effects on immune responses should be less prominent, and we predicted sexually antagonistic selection to be weaker. Because of the stronger potential for sexually antagonistic selection on genes associated with type 2 adaptive immune responses (Fig. 8), we furthermore predicted a stronger correlation between the strength of sexual selection and sexually antagonistic selection on genes associated with type 2 adaptive immune responses compared to type 1. We have illustrated these relationships in Fig. 9.

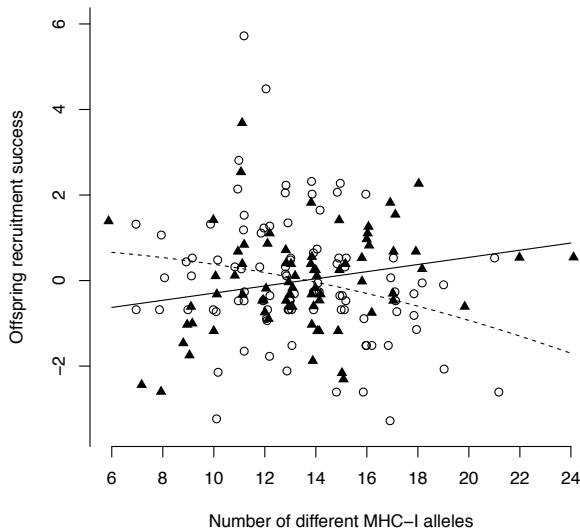
Latitudinal patterns of infections risk may also influence the predicted strength of sexually antagonistic selection on immune genes, because species that live or overwinter in tropical areas are exposed to more pathogens than species in temperate or arctic areas (Hasselquist, 2007; Piersma, 1997; Ricklefs, 1992; Westerdahl et al., 2014). Furthermore, the slow pace-of-life in tropical species select for increased investment in immunity (Ricklefs, 1992; Ricklefs & Wikelski, 2002). We argued that these mechanisms may amplify sexually antagonistic selection on immune system genes, particularly genes associated with type 2 adaptive immune functions. This prediction gained support from previous observations that humoral immunity is more closely correlated with latitudinal and pace-of-life patterns than cell-mediated immunity (Hasselquist, 2007; Owen-Ashley, Hasselquist, Råberg, & Wingfield, 2008).

In the final section of the review paper, we argued that increased sexual selection in host species may increase the fitness of their pathogens. When increased testosterone levels cause immune responses to be suppressed in males, pathogens would normally be expected to respond by lowering their virulence (Frank, 2002). However, females maintain efficient immune responses and this selects for pathogens to retain at least some of their virulence. Therefore, males may be more susceptible to pathogen infection in species with strong sexual selection, and this may be particularly true for infections with pathogens targeted by type 2 adaptive immune responses (e.g. many food-borne pathogens such as helminths).

## Sexual conflict over MHC-I diversity in great reed warblers

In Paper II, we investigated the effects of the number of different MHC-I alleles per individual on three measures of Darwinian fitness in our study population of great reed warblers in Lake Kvismaren in southern Central Sweden. The data set for this study consisted of 188 adults that bred during the years 1984-2004 and 145 chicks from the cohort of 1998. The number of different MHC-I alleles per individual varied between 6 and 24 different alleles per individual with a mean of 13.4. Among adult birds, we found no effect of number of different MHC-I alleles on life span or on offspring fledging success (lifetime number of fledged offspring in models with life span as covariate), but there was a significant effect on offspring recruitment success (lifetime number of offspring recruited to the breeding population in models with lifetime number of fledged offspring as covariate) in both males and females, with a highly significant interaction between sex and number of different MHC-I alleles (Fig. 10). The analysis showed that males benefit from having a higher number of different MHC-I alleles, while females benefitted from having a lower number of different MHC-I alleles. Despite this difference in selection between the sexes, there was no difference in the mean number of different MHC-I alleles per individual between the sexes (Fig. S1, Paper II), and our results therefore provided evidence of an unresolved genetic sexual conflict over the number of different MHC-I alleles per individual in great reed warblers (cf. (Bonduriansky & Chenoweth, 2009; Cox & Calsbeek, 2009)).

In our review of the effects of sex hormones on immune systems, we described the potential for sexually antagonistic selection on immune system genes caused by differences in hormonal effects on the strength and nature of immune responses (Paper I). In accordance with the mechanisms described there, high testosterone levels in males of this polygynous species may increase their optimal level of number of different MHC-I alleles considerably above the optimum for the females. Because the number of different MHC-I alleles per individual is a shared, autosomal trait, this genetic sexual conflict cannot be solved on the genomic level and female great reed warblers may end up with numbers of different MHC-I alleles above their optimum and thus have increased costs associated with type I immune responses, for example costs related to immunopathology. This may affect the phenotypic condition in the females, and negatively affect their ability to provide parental care for the offspring. Phenotypic costs associated with immune responses in females have previously been found to negatively affect the reproductive success in wild pied flycatchers *Ficedula hypoleuca* and nestling feeding rates in wild blue tits *Cyanistes caeruleus* (Ilmonen, Taarna, & Hasselquist, 2000; Raberg, Nilsson, Ilmonen, Stjernman, & Hasselquist, 2000).

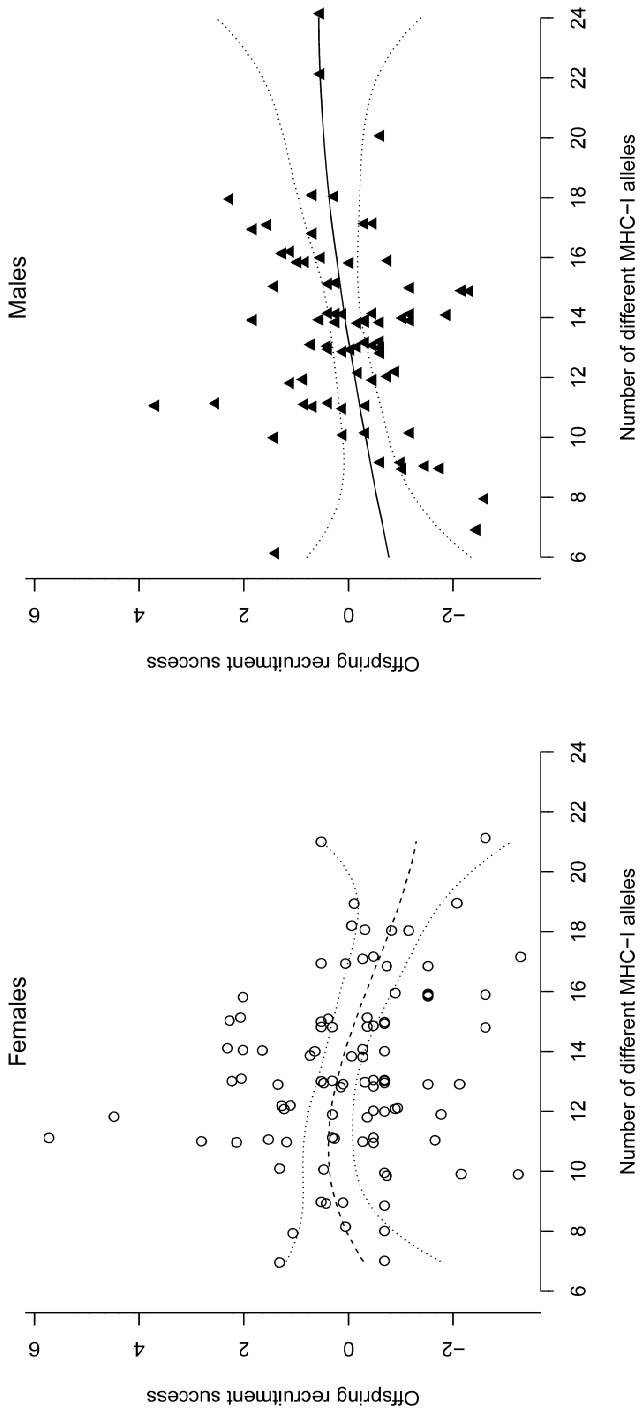


**Fig. 10** The relationship between the number of different MHC-I alleles per individual and offspring recruitment success in great reed warblers. Females are shown with open circles and males are shown with black triangles. The lines show the predictions from regression models of offspring recruitment success on number of different MHC-I alleles in females (broken line) and males (solid line). Offspring recruitment success is illustrated using the residual lifetime number of recruiting offspring from a regression with lifetime number of fledglings. Jitter was added to the number of different MHC-I alleles to distinguish individual data points (from Paper II, Fig. 1).

Our statistical models showed no evidence of optimal number of different MHC-I alleles in either males or females (Fig. 10), but this may be due to a lack of samples towards the edges of the distribution of the number of different MHC-I alleles preventing us from detecting such effects with adequate statistical support. A cubic spline visualization of the correlation between number of different MHC-I alleles and offspring recruitment success indicated that optima for the number of different MHC-I alleles for females and males may be located on either side of the population mean, as expected for an unresolved sexual conflict (Fig. 11) (Bonduriansky & Chenoweth, 2009; Cox & Calsbeek, 2009). The sexual conflict over number of different MHC-I alleles could potentially be solved by differential gene expression in males and females, however our results indicate that such a mechanism, if present in great reed warblers, has not evolved to an extent that resolves the conflict.

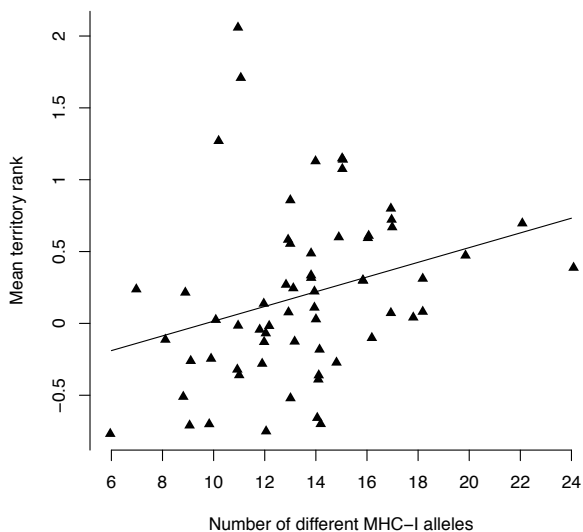
We also tested if the number of different MHC-I alleles in the offspring affected their probability of survival until recruitment, but found no such effect (and no interaction between sex and number of different MHC-I alleles). This indicates that the effect of the number of different MHC-I alleles in the parents on offspring survival is caused by phenotypic effects in the parents and is not significantly





**Fig. 11** The relationship between the number of different MHC-I alleles per individual and offspring recruitment success in females (a) and males (b) showing predictions ( $\pm 2$  s.e.) from cubic spline models. Offspring recruitment success is here illustrated using the residual lifetime number of recruiting offspring from a regression with lifetime number of fledglings. Jitter was added to the number of different MHC-I alleles to distinguish individual data points (from Paper II, Fig. 2).

affected by genes passed on to the offspring. We found no evidence of mate choice for genetic compatibility related to number of different MHC-I alleles (disassortative mating), but there was a highly significant positive correlation between territory attractiveness and number of different MHC-I alleles among males (Fig. 12). However, we found no correlation between number of different MHC-I alleles and territory attractiveness rank in females. One factor that could explain the lack of such a correlation is that, while males compete amongst each other for the best territories, females evaluate the quality of the territory of potential mates while also taking into account the rank that she will get in the harem of any polygynous males, as well as their song repertoire size (Bensch, 1996; Bensch & Hasselquist, 1992; Hasselquist, 1998). This absence of a straightforward competition among females suggests that territory quality is probably less dependent on physical condition in females than it is in males.



**Fig. 12** The relationship between the mean territory rank and the number of different MHC-I alleles per individual in males with predicted values from a linear regression model. The relationship is illustrated using the lifetime mean of age-standardized territory attractiveness rank values. Jitter was added to the number of different MHC-I alleles to distinguish individual data points (from Paper II, Fig. 3).

The results that we present in Paper II are in accordance with previous hypotheses that sexually antagonistic selection may be an important force generating and maintaining genetic variation both in general (Brommer, Kirkpatrick, Qvarnstrom, & Gustafsson, 2007; Rice & Chippindale, 2001) and in traits related to disease resistance (Gilks et al., 2014). Our empirical results also enforced our view that sex

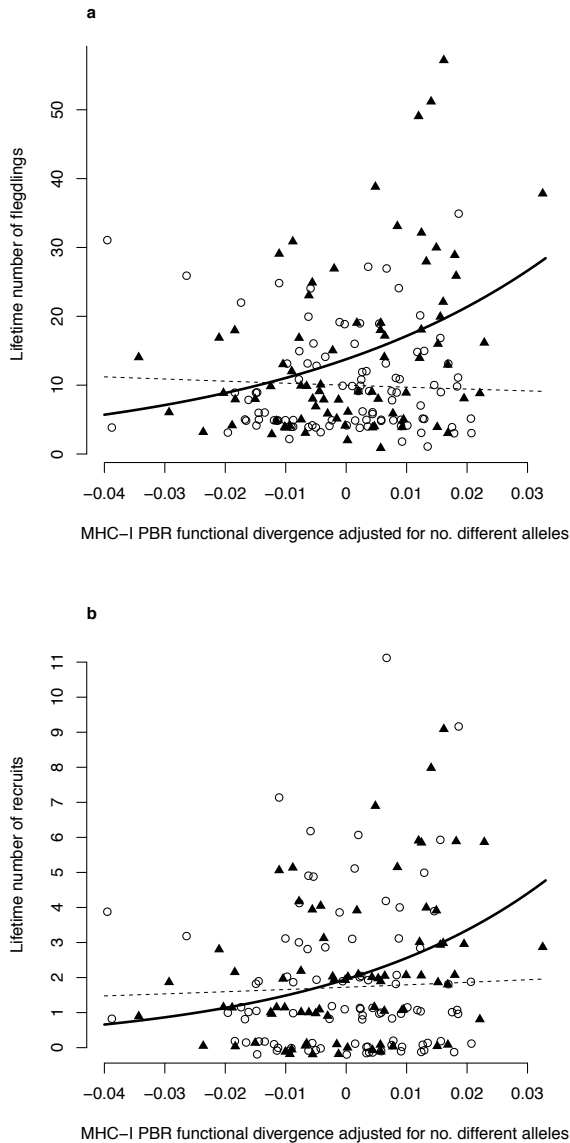
differences may be central to investigations of the selective forces that maintain and constrain MHC diversity (Paper I).

## The effects of MHC-I functional divergence

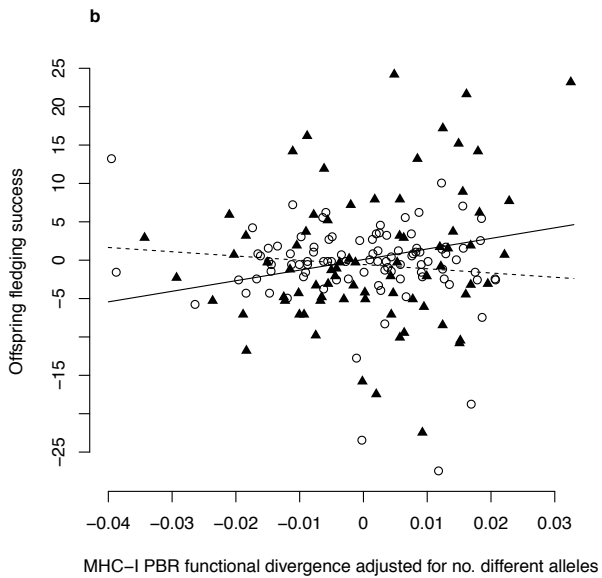
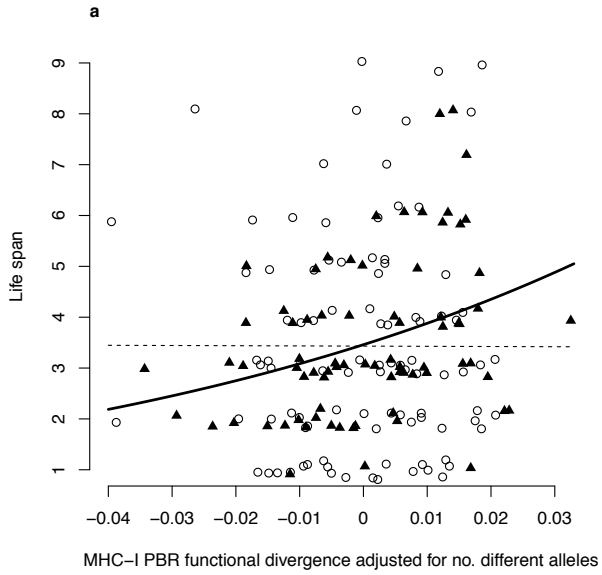
In 1990, Wakeland and co-workers presented the divergent allele advantage hypothesis, in which they predicted that having MHC alleles with highly divergent antigen binding properties should be advantageous in terms of disease resistance (Wakeland et al., 1990). They argued that increased functional divergence would be advantageous because an individual with more divergent MHC alleles is able to cover a wider spectrum of the antigenic space, thereby minimizing the amounts of gaps where pathogens may escape recognition. This prediction has recently found support in theoretical models (Lenz, 2011; Pierini & Lenz, 2018; Wakeland et al., 1990).

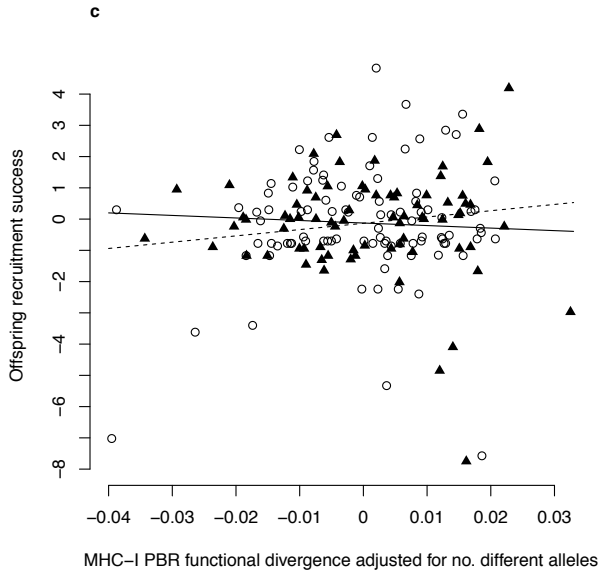
In Paper III, we investigated the effects of MHC-I functional divergence on Darwinian fitness using the same data set on great reed warblers as in the study on the number of different MHC-I alleles (Paper II). We found a strong effect of MHC-I functional divergence on Darwinian fitness in adult males, but not in females. Males with a higher MHC-I functional divergence lived longer, had more fledged offspring, and more offspring recruited to the breeding population, while no such effects were found in females (Fig. 13, Fig. 14). MHC-I functional divergence in adult birds did not significantly affect offspring fledging success (lifetime number of fledged offspring in models with life span as covariate) or offspring recruitment success (lifetime number of offspring recruited to the breeding population in models with lifetime number of fledged offspring as covariate) (Fig. 14). The lack of any such effects in the males indicates that the fitness advantage of having a high MHC-I functional divergence in male great reed warblers is an effect of increased life span, which increases the number of breeding attempts.

The fact that we found no fitness effects of MHC-I functional divergence in female great reed warblers indicates differential selection between the sexes on this property of the MHC-I. Our results did not indicate that increased MHC-I functional divergence was disadvantageous to females, however, we did find a significant difference between the sexes in the effect on lifetime number of fledged offspring (Fig. 13 a). The results that we presented in Paper II show that the mean number of different MHC-I alleles per individual in our study population is below the optimum for males. In light of this, our present results suggest that higher MHC-I functional divergence may be favored as a compensation for suboptimal numbers of different MHC-I alleles in males, as it enables recognition of a broader range of pathogens. In contrast, the mean number of different MHC-I alleles per individual in our study



**Fig. 13 a** The relationship between the lifetime number of fledglings and MHC-I functional divergence in the PBR (O = females, ▲ = males). The lines show the predictions from a generalized linear model of lifetime number of fledglings on MHC-I functional divergence with sex as a fixed factor and including the interaction MHC-I functional divergence × sex. **b** The relationship between the lifetime number of recruits and MHC-I functional divergence in the PBR (O = females, ▲ = males). The lines show the predictions from a generalized linear model of lifetime number of recruits on MHC-I functional divergence with sex as a fixed factor and including the interaction MHC-I functional divergence × sex. In each figure, the solid line shows the predicted values for the males and the broken line the predicted values for the females. Thick lines indicate significant correlations. MHC-I functional divergence was adjusted for number of different MHC-I alleles per individual. Jitter was added to lifetime number of fledglings and lifetime number of recruits, respectively, to distinguish individual data points (from Paper III, Fig. 1).





**Fig. 14 a (opposite page)** The relationship between life span and MHC-I functional divergence in the PBR (○ = females, ▲ = males). The lines show the predictions from a generalized linear model of life span on MHC-I functional divergence with sex as a fixed factor and including the interaction MHC-I functional divergence × sex. **b (opposite page)** The relationship between the offspring fledging success (the lifetime number of fledged offspring adjusted for life span) and MHC-I functional divergence in the PBR (○ = females, ▲ = males). The lines show the predictions from a linear model of the offspring fledging success on MHC-I functional divergence with sex as a fixed factor and including the interaction MHC-I functional divergence × sex. **c** The relationship between the offspring recruitment success (the lifetime number of recruited offspring adjusted for the lifetime number of fledged offspring) and MHC-I functional divergence in the PBR (○ = females, ▲ = males). The lines show the predictions from a linear model of the offspring recruitment success on MHC-I functional divergence with sex as a fixed factor and including the interaction MHC-I functional divergence × sex. In each figure, the solid line shows the predicted values for the males and the broken line the predicted values for the females. Thick line indicates a significant correlation. MHC-I functional divergence was adjusted for number of different MHC-I alleles per individual. Jitter was added to the values of life span to distinguish individual data points (from Paper III, Fig. 2).

population appeared to be closer to the optimum for females, i.e., closer to their optimal solution to the trade-off between disease resistance benefits and immunity costs (e.g. immunopathology), and this may explain why we observed no directional selection on MHC-I functional divergence in females.

We also investigated whether MHC-I functional divergence in the offspring affected their probability of survival until recruitment, but found no such effect and also no effect of sex in this analysis. This indicates that the fitness advantage of increased MHC-I functional divergence that was found for adult males only is realized with sexual maturity. This suggests that such advantages of a superior phenotypic condition may be particularly important in association with the stresses of breeding behavior.

Previous empirical studies that investigated the divergent allele advantage hypothesis have found associations between MHC functional divergence and pathogen resistance or effects of MHC functional divergence in relation to mate choice (Bollmer, Dunn, Freeman-Gallant, & Whittingham, 2012; Froeschke & Sommer, 2012; Kamath et al., 2014; Lenz et al., 2009; Sepil et al., 2015). Furthermore, two studies have investigated MHC functional divergence in relation to fitness using between-year measures of survival and reproductive success, but these studies obtained contrasting results (Knafler, Clark, Boersma, & Bouzat, 2012; Lenz, Mueller, Trillmich, & Wolf, 2013). Our present results provided evidence for strong selection for high MHC-I functional divergence in male great reed warblers, and these results are novel in the sense that they provide empirical support for the divergent allele advantage hypothesis in a wild vertebrate population, where breeding individuals were studied in detail over their entire lives. Furthermore, the increased fitness of males with high MHC-I functional divergence implies that selection maintains a high degree of polymorphism with highly divergent MHC-I alleles in the population, in accordance with the prediction by Wakeland et al. (1990).

In the present study, we quantified MHC-I functional divergence in peptide-binding codons inferred from the human HLA-A (MHC-I functional divergence PBR). To verify the validity of this method, we tested for signatures of positive selection on the great reed warbler sequences in our data set using codeml from the software package PAML (Yang, 1997, 2007) and found considerable overlap between the inferred peptide-binding codons and codons estimated to be under positive selection (positively selected sites, PSS). We repeated all models using MHC-I functional divergence quantified in the PSS, and verified that the results from these models did not differ qualitatively from the results obtained using MHC-I functional divergence PBR.

## MHC and mate choice

In Paper IV, we investigated the association between variation in MHC-I genes and mate choice. The aims of this study were to determine whether females selected males based on specific properties of their MHC-I genes and whether variation in MHC-I genes affected male characters that were previously shown to be preferred by females.

Our results showed that males selected by females as social partners (i.e., social males) had larger MHC-I functional divergence than the average among all adult males (Fig. 15, Table 1). We also found that females selected males with whom they had a larger joint pair MHC-I functional divergence than with randomly selected

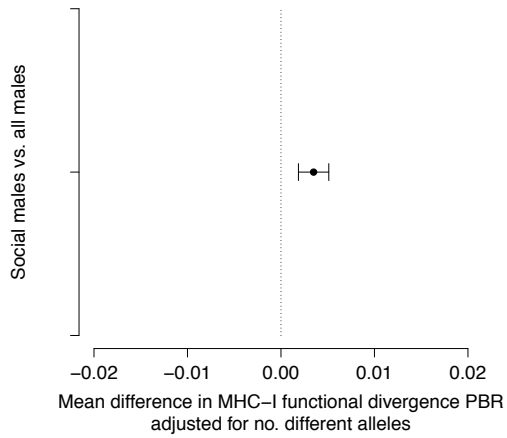
males (Fig. 16, Table 1). In contrast, we found no evidence that females select males based on any aspect of MHC-I allelic diversity (i.e., number of different MHC-I alleles in the social male compared to the mean among all adult males, joint pair number of different MHC-I alleles, or proportion of shared alleles) and no evidence that females select males with numbers of different MHC-I alleles or levels of MHC-I functional divergence that are complementary to their own (Table 1).

Male age, song repertoire size, and territory attractiveness have previously been found to be associated with annual pairing and breeding success in our study population (Hasselquist, 1998; Hasselquist et al., 1996), and our present results confirmed these findings by showing that social males were older and had larger song repertoires and more attractive territories than randomly selected adult males (Fig. 17, Table 2). We also showed that both song repertoire size and territory attractiveness ranks were positively correlated with male age (Table 3).

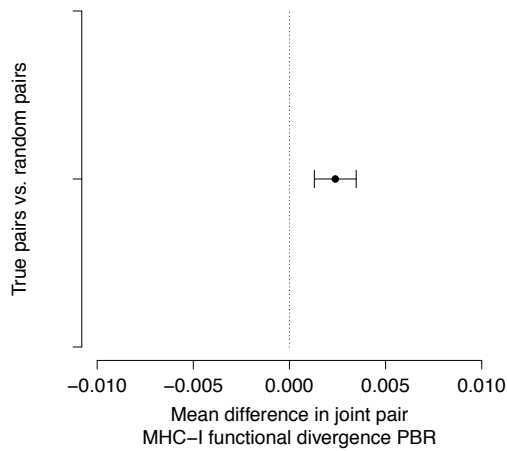
In Paper II, we showed that male territory attractiveness rank was positively associated with the number of different MHC-I alleles (Fig. 12). The results of our analyses in Paper IV confirmed this finding using a larger data set (Table 3). Furthermore, we found that song repertoire size was positively associated with MHC-I functional divergence, and this association tended to be significant even when age was included as a covariate in the model (Fig. 18, Table 3).

Hasselquist (1998) investigated factors that increase male mating success and fitness in our study population, and found that the attractiveness of a male's territory predicted both the fledging success of his offspring and production of offspring recruits. This suggests that female great reed warblers obtain direct benefits that are advantageous in terms of the phenotypic quality of the offspring when selecting males with attractive territories (Hasselquist, 1998). In contrast, male song repertoire size predicted post-fledging survival of offspring, which suggests that male song may be advertising indirect, genetic benefits to females (Hasselquist, 1998; Hasselquist et al., 1996). Male age was associated with number of female mates and annual number of fledglings (Hasselquist, 1998). Older males were found to occupy on average more attractive territories and have larger song repertoires compared to younger males, and may be preferred by females because of benefits associated with these properties (Hasselquist, 1998). However, females may also show a direct preference for older males to get genetically superior offspring or because older males provide better parental care (Hasselquist, 1998; Järvi, Roskaft, & Slagsvold, 1982; Searcy, 1982; Weatherhead & Boag, 1995; Weatherhead, 1984; Weatherhead & Robertson, 1981; Wetton, Burke, Parkin, & Cairns, 1995). The emerging trend from these early investigations is that female great reed warblers select males to gain both direct and indirect benefits (Hasselquist, 1998; Hasselquist et al., 1996).





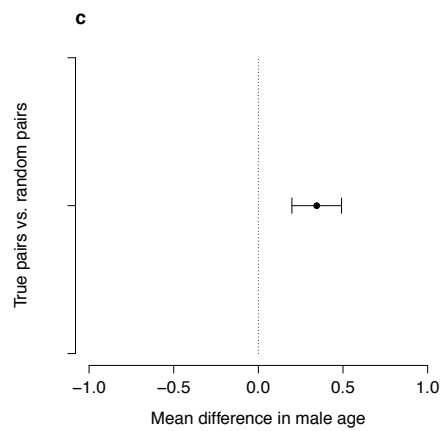
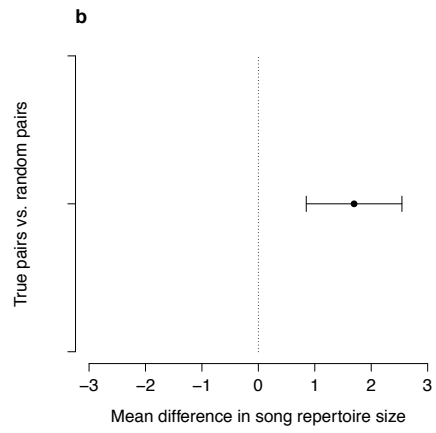
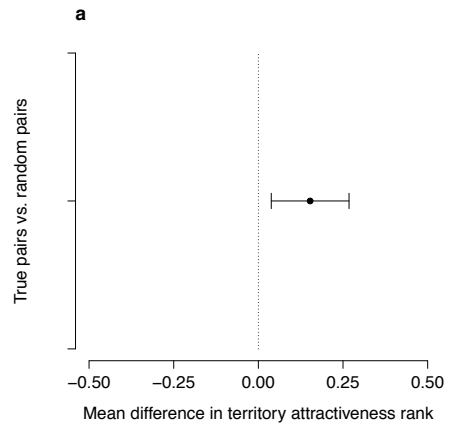
**Fig. 15** The mean differences ( $\pm 2$  S.E.) in MHC-I functional divergence PBR between social males and all adult males in the population (from Paper IV, Fig. 1 b).



**Fig. 16** The mean differences ( $\pm 2$  S.E.) in joint pair MHC-I functional divergence PBR between social mate pairs and random pairs generated in 1,000 population simulations (from Paper IV, Fig. 2 b).

**Table 1** Mate choice in relation to number of different MHC-I alleles and MHC-I functional divergence. Summary of the statistical models. P-values < 0.1 are in italic with \* indicating  $p < 0.05$ , \*\* indicating  $p < 0.01$ , and \*\*\* indicating  $p < 0.001$ . S.E. = standard error. lm = linear regression model, lmm = linear mixed effects model, d.f. = residual degrees of freedom. For mixed effects models,  $N_{obs}$  = number of observations,  $N_f$  = number of females. PBR = functional divergence among codons of the peptide binding region, PSS = functional divergence among codons estimated to be under positive selection. Details of all models including complete summaries from correlation models are presented in Paper IV (from Paper IV, Table 1).

Male property	Alternative hypothesis	Model	Estimate	S.E.	t-value	P-value	d.f. (N)
Number of different MHC-I alleles	Females select social males with larger number of different MHC-I alleles than the mean among all males	lm	-0.16	0.18	-0.87	0.39	d.f. = 283
	Females select social males with number of different MHC-I alleles complementary to their own	lm	0.059	0.055	1.08	0.28	d.f. = 282
	Females aim to maximize joint pair number of different MHC-I alleles	lm	-0.089	0.19	-0.48	0.63	d.f. = 283
	Females aim to minimize the proportion of shared alleles	lm	0.0013	0.005	0.25	0.80	d.f. = 283
MHC-I functional divergence	Females select social males with larger MHC-I functional divergence than the mean among all males	PBR	0.0035	0.00081	4.3	< 0.0001***	$N_{obs} = 284$ $N_f = 126$
		PSS	0.0015	0.00077	1.93	0.054	$N_{obs} = 284$ $N_f = 126$
	Females select social males with levels of MHC-I functional divergence complementary to their own	PBR	0.09	0.061	1.48	0.14	$N_{obs} = 284$ $N_f = 126$
		PSS	-0.031	0.065	-0.47	0.64	$N_{obs} = 284$ $N_f = 126$
	Females aim to maximize joint pair functional divergence	PBR	0.0024	0.00054	4.38	< 0.0001***	d.f. = 283
		PSS	0.001	0.00052	2.02	0.044*	$N_{obs} = 284$ $N_f = 126$



**Fig. 17 (opposite page)** **a** Mean difference ( $\pm 2$  S.E.) in territory attractiveness rank of the social male between social mate pairs and random pairs generated in 1,000 population simulations. **b** Mean difference ( $\pm 2$  S.E.) in song repertoire size of the social male between social mate pairs and random pairs generated in 1,000 population simulations. **c** Mean difference ( $\pm 2$  S.E.) in age of the social male between social mate pairs and random pairs generated in 1,000 population simulations (from Paper IV, Fig. 3).

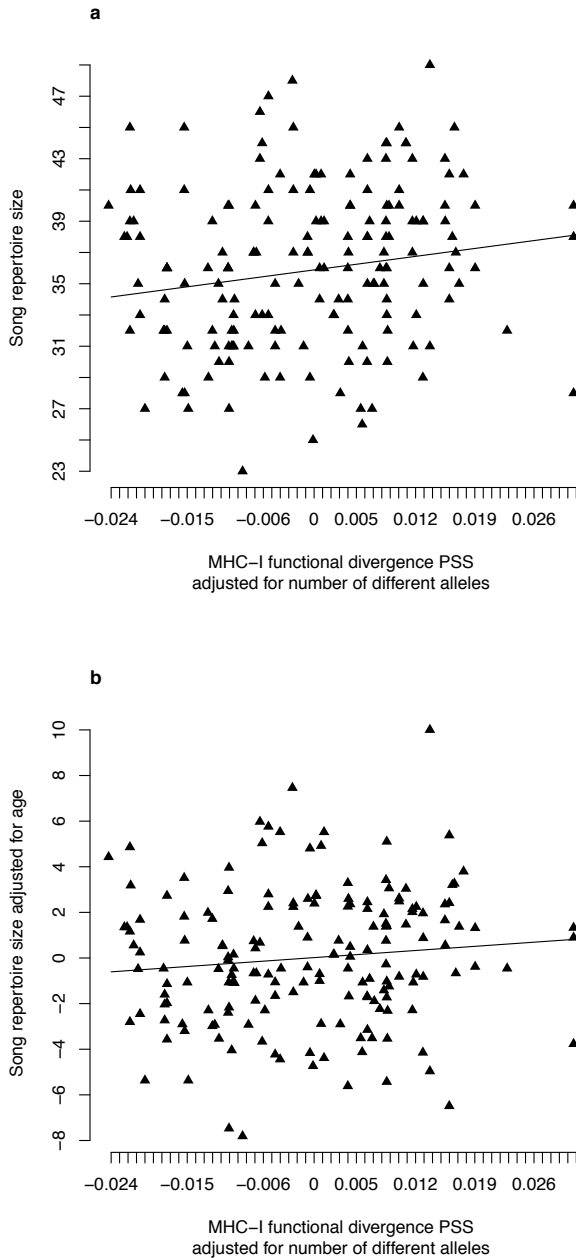
**Table 2** Mate choice in relation to male territory attractiveness, song repertoire size, and age. Summary of the statistical models. \*\* indicates  $p < 0.01$  and \*\*\* indicates  $p < 0.001$ . S.E. = standard error. lm = linear regression model. d.f. = residual degrees of freedom. Details of all models are presented in Paper IV (from Paper IV, Table 2).

Alternative hypothesis	Model	Estimate	S.E.	t-value	P-value	d.f.
Females select social males that have more attractive territories	lm	0.15	0.057	2.66	0.0084**	d.f. = 196
Females select social males with larger song repertoires	lm	1.70	0.42	4.00	< 0.0001***	d.f. = 147
Females preferentially select older males as social mates	lm	0.34	0.073	4.70	< 0.0001***	d.f. = 283

Our present results confirmed the findings in a previous study of MHC-based mate choice in great reed warblers, where no evidence was found for mate choice based on either number of different MHC-I alleles in males or compatibility in the number of different MHC-I alleles between male and female social pair mates (Westerdahl, 2004). However, our present results showed that variation in MHC-I genes is associated with mate choice through effects on both territory acquisition, male song repertoire size, and life span (Fig. 12; Fig. 14 a; Fig. 17 a, b, c; Fig. 18). Furthermore, we found evidence for female mate choice for larger joint-pair MHC-I functional divergence (Fig. 16), which tended towards significance even when male age was accounted for in our mate pair simulations. This could potentially hint at a possibility that olfactory cues may be involved in female mating preferences (Boehm & Zufall, 2006; Carroll et al., 2002; Leclaire et al., 2017; Milinski et al., 2005; Reusch et al., 2001; Wedekind et al., 1995; Wedekind & Furi, 1997). Overall, our results indicated that MHC-I genes affect mate choice in great reed warblers both by conveying indirect (genetic) benefits and by affecting the ability of males to provide direct benefits. To my knowledge, our study is the first to show such a universal effect of MHC genes in relation to mate choice, a scenario that was predicted by Zelano and Edwards in 2002 (Fig. 1). A reason that this has not previously been investigated may be the scarcity of study systems that offer sufficiently detailed records on mating events and male properties associated with female choice.

**Table 3** The effects of age, number of different MHC-I alleles, and MHC-I functional divergence on male territory attractiveness and song repertoire size. Summary of the statistical models. P-values < 0.1 are in italic with \* indicating  $p < 0.05$ , \*\* indicating  $p < 0.01$ , and \*\*\* indicating  $p < 0.001$ . S.E. = standard error. Imm = linear mixed effects model, Im (quad) = quadratic regression model, Imm (quad) = quadratic mixed effects model. For quadratic models, we report the estimated coefficients of the quadratic terms and the linear terms, denoted a and b, respectively. d.f. = residual degrees of freedom. For mixed effects models,  $N_{obs}$  = number of observations,  $N_m$  = number of males. PBR = functional divergence among codons of the peptide binding region, PSS = functional divergence among codons estimated to be under positive selection. Details of all models including complete summaries are presented in Paper IV (from Paper IV, Table 3).

Male property	Independent variable	Model	Estimate	S.E.	t-value	P-value	d.f. (N)	
Territory attractiveness	Age	Im (quad)	a	-0.059	0.015	-3.91	<i>0.00012***</i>	d.f. = 293
			b	0.59	0.11	5.59	<i>&lt; 0.0001***</i>	
	Number of different MHC-I alleles	Imm	0.039	0.016	2.45	<i>0.014*</i>	$N_{obs} = 244$ $N_m = 118$	
	Number of different MHC-I alleles w. age as covariate	Imm	0.037	0.014	2.54	<i>0.011*</i>	$N_{obs} = 244$ $N_m = 118$	
	MHC-I functional divergence	PBR	Imm	2.97	4.15	0.71	0.47	$N_{obs} = 244$ $N_m = 118$
		PSS	Imm	-2.35	4.57	-0.51	0.61	$N_{obs} = 244$ $N_m = 118$
Song repertoire size	Age	Imm (quad)	a	-0.44	0.14	-3.08	<i>0.0026**</i>	$N_{obs} = 186$
			b	4.66	0.88	5.31	<i>&lt; 0.0001***</i>	$N_m = 118$
	Number of different MHC-I alleles	Imm	0.099	0.13	0.76	0.45	$N_{obs} = 172$ $N_m = 106$	
	MHC-I functional divergence	PBR	Imm	63.1	33.1	1.91	<i>0.056</i>	$N_{obs} = 172$ $N_m = 106$
		PSS	Imm	74.8	35.5	2.11	<i>0.035*</i>	$N_{obs} = 172$ $N_m = 106$
	MHC-I funct. div. w. age as covariate	PBR	Imm	34.8	30.5	1.14	0.25	$N_{obs} = 172$ $N_m = 106$
		PSS	Imm	60	32.2	1.86	<i>0.063</i>	$N_{obs} = 172$ $N_m = 106$



**Fig. 18 a** The relationship between song repertoire size and MHC-I functional divergence PSS in adult males with predictions from a linear mixed effects model. **b** The relationship between song repertoire size adjusted for age and MHC-I functional divergence PSS in adult males with predictions from a linear mixed effects model. In each figure, MHC-I functional divergence PSS was adjusted for number of different MHC-I alleles per individual (from Paper IV, Fig. 5).

In Paper I, we presented the hypothesis that regulatory effects of sex hormones on immune systems could drive sexually antagonistic selection on immune system genes. This hypothesis was derived from the ICHH, by predicting patterns of selection on immune system genes in either sex, when sexual selection and the effects of sex hormones of immune responses were taken into account (Paper I). A central prediction in the ICHH is that males with dominant behaviors or strong expression of secondary sexual characters preferred by females advertise high quality genes for disease resistance (Folstad & Karter, 1992; Hamilton & Zuk, 1982). Previous results have indicated that MHC-I genes are important for disease resistance in our great reed warbler study population (Westerdahl et al., 2005, 2004; Westerdahl et al., 2012), and in combination with this, the connections between MHC-I genes and mate choice (Paper IV) implies that MHC-I genes fulfill the role of disease resistance genes predicted in the ICHH. Consequently, we propose that female preference for testosterone-mediated traits and behaviors (i.e., song, territory acquisition) generates a selective pressure on MHC-I genes to compensate for testosterone-mediated immune-suppression in males. This mechanism may be enhanced by the strong sexual selection in the socially polygynous great reed warbler and drive the number of different MHC-I alleles per individual to levels that are unfavorable for females, thereby causing the sexual conflict over number of different MHC-I alleles that we observed in Paper II. It also offers an explanation to the differential selection on MHC-I functional divergence between the sexes that we observed in Paper III.

## Investigations of MHC-I haplotypes

The MHC constitutes a multigene family in which multiple gene copies are usually organized in tandem in a tight genomic region (Trowsdale 1995, Kelley et al 2005). Studies in humans have revealed non-random association of MHC alleles on haplotypes, which suggests that natural selection favors combined effects of particular MHC alleles across multiple loci (Begovich et al., 1992, 2001; Buhler, Nunes, & Sanchez-Mazas, 2016; Hollenbach et al., 2001; Testi et al., 2015). Exceptionally high MHC diversity has been found in songbirds, with some species showing considerable variation in the number of different MHC alleles between individuals (Biedrzycka et al., 2017; Karlsson & Westerdahl, 2013; O'Connor et al., 2016; Sepil et al., 2012; Westerdahl, 2007). This was also found in our great reed warbler study population, where we observed between 6 and 25 MHC-I alleles per individual (Paper II-IV). Haplotypes are the ultimate source of variation in the number of different MHC alleles on the (diploid) genotype level, and this variation could arise either by variation in the number of MHC gene copies per haplotype or by haplotypes sharing larger or smaller numbers of identical alleles. If substantial

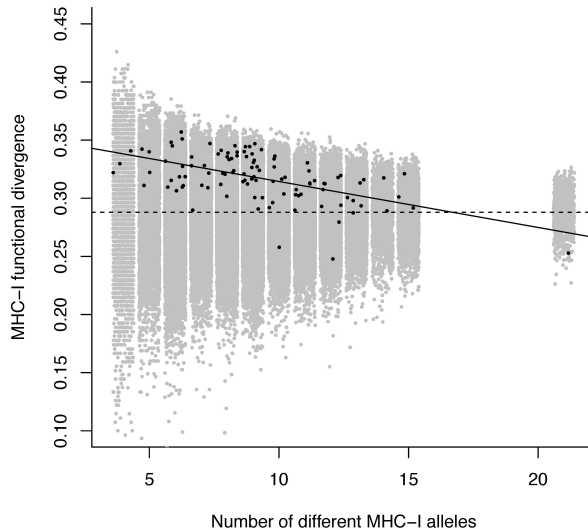
MHC gene copy number variation exists between haplotypes, an interesting question arises about which mechanism of natural selection that maintains low-diversity haplotypes in populations. According to the heterozygote advantage hypothesis, low-diversity haplotypes should be disadvantageous because they offer a smaller antigen binding repertoire compared to more diverse haplotypes (Doherty & Zinkernagel, 1975; Hughes & Nei, 1992). However, divergent allele advantage suggests that low diversity could be compensated for by increased functional divergence between the alleles on such haplotypes (Lenz, 2011; Pierini & Lenz, 2018; Wakeland et al., 1990). Alternatively, low-diversity haplotypes could be maintained by natural selection if they contain favorable combinations of alleles from different MHC loci, as suggested by studies in humans (Begovich et al., 1992, 2001; Testi et al., 2015). Finally, sexually antagonistic selection could also provide a mechanism that maintains variation in MHC-I haplotype diversity, if low diversity is favored in one sex and high diversity is favored in the other, as we observed in Paper II.

In Paper V, we examined the relative importance of these mechanisms by investigating the interaction between MHC-I haplotype structure, number of different MHC-I alleles, and MHC-I functional divergence in our data set. We characterized 107 unique MHC-I haplotypes by analyzing segregation patterns of MHC-I alleles in 119 great reed warbler families, and found considerable variation in the number of MHC-I gene copies with a minimum of four and a maximum of 21 different alleles per haplotype. The fact that we observed only two recombinant haplotypes among 334 offspring confirmed linkage disequilibrium for the MHC-I genes in great reed warblers.

We found a strong negative correlation between the number of different MHC-I alleles and MHC-I functional divergence on haplotypes (Fig. 19), similar to that observed in individuals on the (diploid) genotype level (Paper III). *In silico* data simulations, in which alleles were randomly assigned to haplotypes, showed that this correlation is highly unlikely to occur by chance, suggesting that increased functional divergence may be favored by natural selection on MHC-I haplotypes with low numbers of alleles. However, if low-diversity haplotypes are maintained because of favorable combinations of alleles from different MHC loci, divergence between such loci could cause the mean functional divergence on low-diversity haplotypes to be high by default, and this could offer an alternative explanation to the observed correlation.

We investigated this scenario further by analyzing the phylogenetic relationship between the MHC-I alleles in our data set using the software PhyML (Guindon et al., 2010; Guindon & Gascuel, 2003). We identified five putative clusters of alleles that constituted four monophyletic clades and one paraphyletic clade with high support values (Fig. 20). We labeled these clades A-E, and a group of 7 alleles that

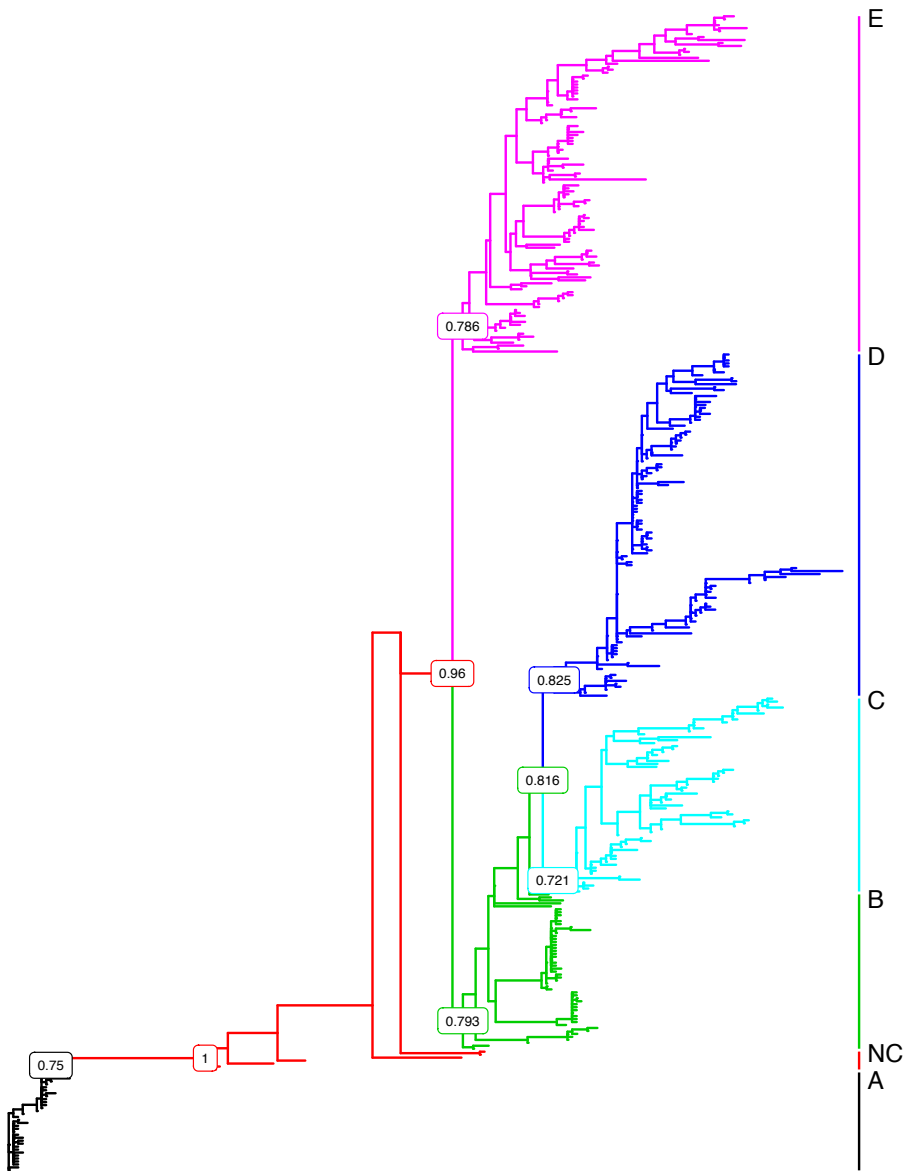




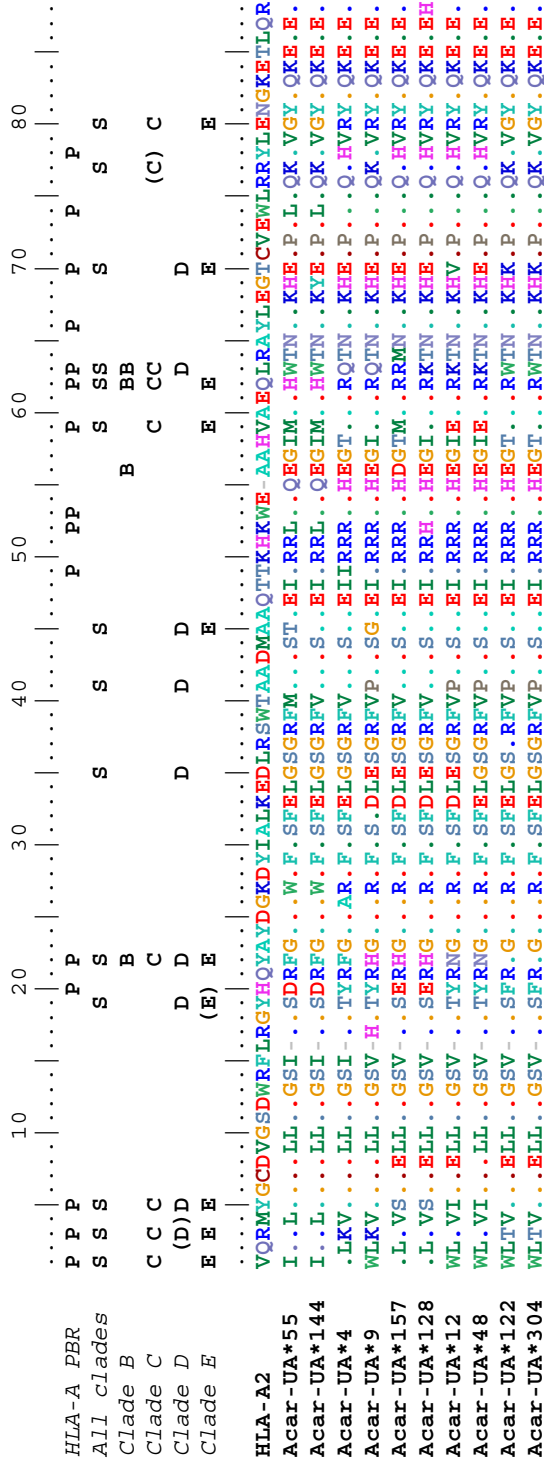
**Fig. 19** The correlation between the MHC-I functional divergence and number of different MHC-I alleles on haplotypes from the real data set (black dots) and from 1,000 simulated data sets in which alleles were randomly assigned to haplotypes (gray dots). The solid line shows predictions from a linear model based on the real data set and the broken line shows mean predictions from linear models based on the simulated data sets. Jitter was added to the total number of alleles per haplotype to distinguish individual data points (from Paper V, Fig. 3).

fell outside of the defined clades was labeled N.C. (non-clustering). We then tested for positive selection among the alleles belonging to each clade separately using *codeml* from the software package PAML (Yang, 1997, 2007). We found evidence for positive selection on alleles from clades B-E, but not on alleles from clade A. Furthermore, different codons were predicted to be under positive selection in clades B-E, indicating that MHC-I molecules from different clades may be specialized to bind antigens with different chemical and/or structural properties (Fig. 21). Based on these observations, we hypothesized that the identified clades represent distinct MHC-I loci in the great reed warbler genome.

For each haplotype in the data set we calculated the number of alleles represented from each clade, and found that this number was more or less stable for clades A and B, while it varied considerably for clades C, and in particular D and E (Fig. 22, Table 4). Alleles from clades A and B were present on almost all haplotypes, and every haplotype contained alleles from minimum three of the clades A-E (Fig. 22). Interestingly, haplotype diversity was largely explained by variation in the number of alleles from clades D and E (Fig. 23, Table 5).

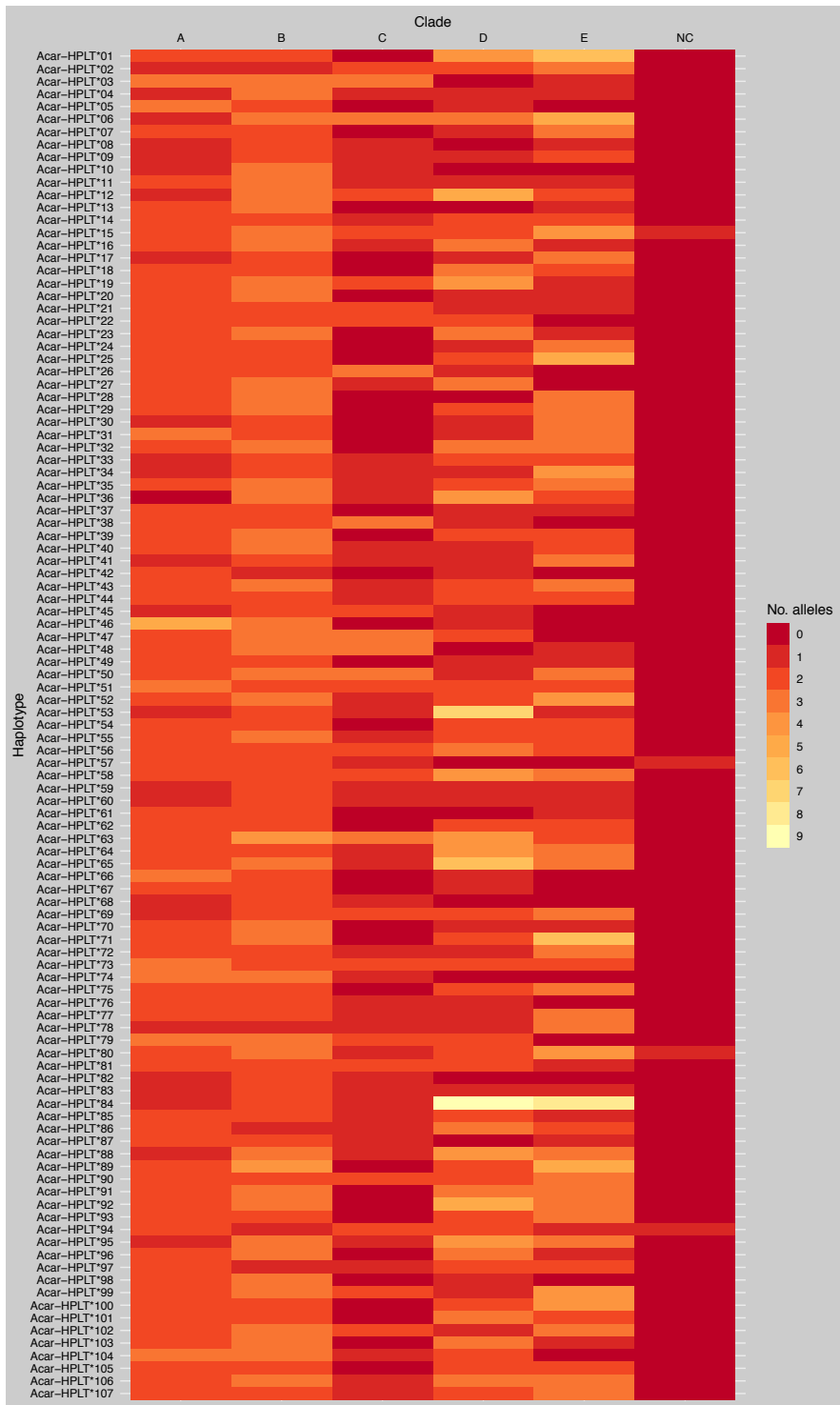


**Fig. 20** Unrooted GTR tree of the 390 MHC-I exon 3 sequences in our data set with SH-aLRT support values shown for selected nodes. Clades are designated by the following colors: black = A, green = B, cyan = C, blue = D, pink = E, and red = NC (non-clustering) (from Paper V, Fig. 5).



**Fig. 21** Extract from a human HLA-A2 protein sequence (IMGT/HLA accession no. HLA00005) (Robinson, Halliwell, McWilliam, Lopez, Parham, et al., 2013; Robinson, McWilliam, Lopez, & Marsh, 2013) aligned with the two most abundant sequences in our data set from each clade in the MHC-I exon 3 tree. Codons of the peptide binding region inferred from HLA-A are marked with 'P' (Bjorkman et al., 1987), and codons estimated to be under positive selection in a test on all 390 great reed warbler sequences (across all clades) are marked with 'S'. Codons estimated to be under positive selection in individual tests using sequences only from clades B, C, D, and E, respectively, are marked with corresponding letters. No codons were estimated to be under positive selection in a test using sequences only from clade A. Letters in parenthesis indicate p-values between 0.05 and 0.1 for that site. The positions of sites estimated to be under positive selection have been adjusted to account for the gap inserted in pos. 16 of the great reed warbler sequences (from Paper V, Fig. 6).

**Fig. 22 (opposite page)** Heatmap showing the number of alleles observed in clades A, B, C, D, E, and NC (non-clustering) for each haplotype (from Paper V, Fig. 7).



Our results indicated that MHC-I alleles on haplotypes originate from different loci in great reed warblers, and that gene duplication within two loci (clades D and E) causes most of the variation observed in the number of different MHC-I alleles on haplotypes. It is likely that these observations could explain the negative correlation between the number of different MHC-I alleles and MHC-I functional divergence on haplotypes. On haplotypes with only few alleles, the high mean functional divergence may be caused by the divergence between alleles from different loci, while on high-diversity haplotypes, gene duplication within the loci represented by clades D and E may decrease the mean functional divergence by increasing the proportion of more similar alleles. We tested this prediction using *in silico* data simulations, in which we assigned the same number of alleles from each putative clade to simulated haplotypes as was observed on the corresponding real haplotypes. The correlation between the number of different MHC-I alleles and the mean functional divergence between the alleles on haplotypes produced by these simulations was similar to the one observed in the real data set (Fig. 24).

**Table 4** Variances for the number of alleles per haplotype from each clade (from Paper V, Table 4).

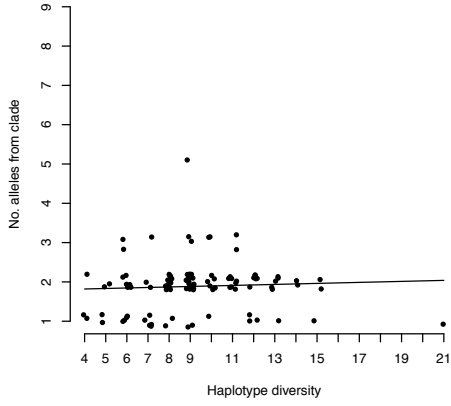
Variances for the number of alleles per haplotype						
	Clade A	Clade B	Clade C	Clade D	Clade E	Clade NC
Variance	0.417	0.392	0.829	2.221	2.396	0.036

**Table 5** Results from major axis linear regression models between the number of alleles observed on each haplotype from each clade and the total number of different alleles on each haplotype. P-values and 95% confidence intervals for the slope (C.I. lower and C.I. upper) were generated from 1,000 model permutations. Clade N.C. showed no variation in the number of alleles from each clade, and therefore no correlation with the total number of alleles per haplotype. Details of all models including complete summaries are presented in Paper V (from Paper V, Table 5).

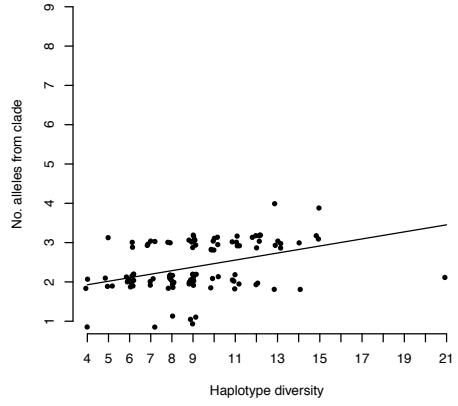
No. of alleles observed from each clade and total no. alleles on each haplotype							
Clade	Intercept	Slope	C.I. lower	C.I. upper	p-value	R <sup>2</sup>	N
A	1.77	0.013	-0.032	0.058	0.31	0.0031	106
B	1.57	0.090	0.048	0.13	< 0.001	0.15	107
C	0.87	0.065	0.0061	0.12	< 0.025	0.067	69
D	-2.00	0.44	0.36	0.52	< 0.001	0.55	95
E	-1.33	0.39	0.30	0.48	< 0.001	0.46	87
NC	1	0	-	-	-	-	4

**Fig. 23 (opposite page)** Correlations between the observed number of alleles from each clade and the total number of alleles per haplotype with lines showing predictions from major axis linear regression models. Jitter was added to the observed number of alleles from each clade and the total number of alleles per haplotype to distinguish individual data points (from Paper V, Fig. 9).

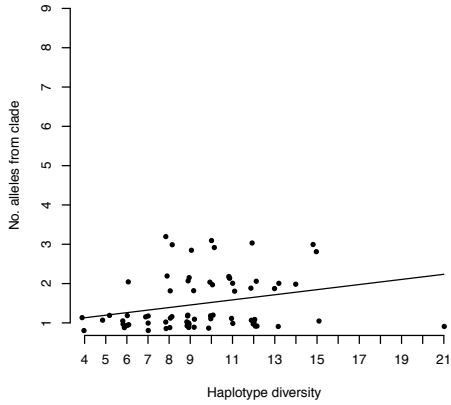
**Clade A**



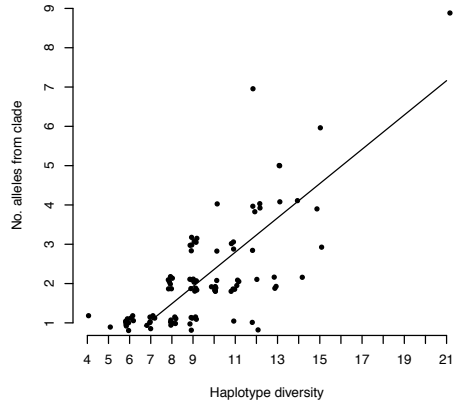
**Clade B**



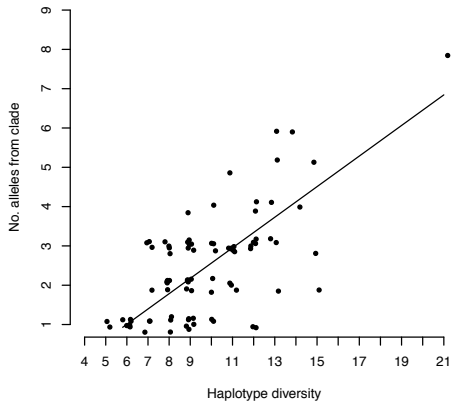
**Clade C**



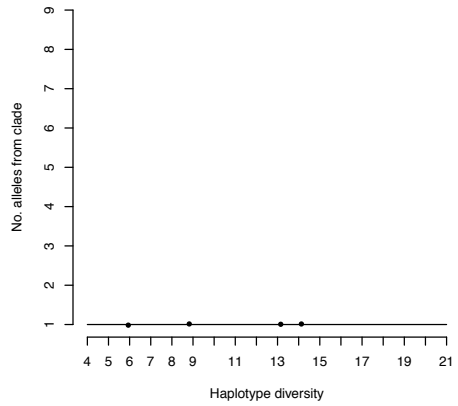
**Clade D**



**Clade E**



**Clade NC**

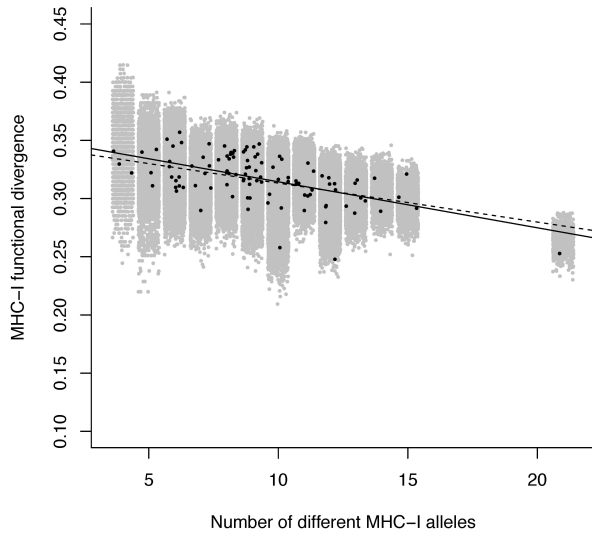


In Paper II, we presented evidence for a sexual conflict over MHC-I diversity. Having many different MHC-I alleles was shown to be advantageous to males, while females benefitted from having fewer alleles (Fig. 10). Our results also showed that males with higher numbers of different MHC-I alleles had on average more attractive territories, which is likely to increase their mating success and thereby have an overall positive effect on individual fitness (Fig. 12) (Paper II and IV). Thus, high-diversity haplotypes may be maintained in great reed warblers because they are advantageous to males, while, on the other hand, low-diversity haplotypes may be maintained because they are more advantageous to females.

The fact that variation in the number of alleles from clades D and E explained most of the variation in haplotype diversity implies that the functions of alleles from these clades are particularly associated with the sexual conflict. In Paper III, we proposed that increased MHC-I functional divergence may be favored as a compensation for suboptimal numbers of different MHC-I alleles in males, because it enables recognition of a broader range of pathogens. The results of the tests for positive selection within each clade suggested that alleles from different clades may have evolved specialized peptide binding properties (Fig. 21). Therefore, compensation for suboptimal numbers of different MHC-I alleles in males should be mediated by increased divergence between alleles within clades D and E. Future investigations of the specific functions of these alleles may shed light on the proximate mechanisms that underlie the sexual conflict over MHC-I diversity. In contrast to clades D and E, clade A is the least diverse of the five clades in the phylogeny of the MHC-I alleles in our data set, and is highly divergent from the other clades (Fig. 20). The fact that the alleles in clade A show no signs of positive selection implies that they may be non-classical MHC-I alleles (Drews, Strandh, Råberg, & Westerdaahl, 2017), and further investigations should be conducted to examine the specific function of these alleles.

In Paper III and IV we presented evidence that MHC-I functional divergence was positively associated with life span and Darwinian fitness in great reed warbler males (Fig. 13, Fig. 14 a). Furthermore, in Paper IV we showed that MHC-I functional divergence was also positively associated with song repertoire size (Fig. 18). In the models behind those results, we included the number of different MHC-I alleles per individual as a covariate because of the strong negative correlation between that measure and MHC-I functional divergence. In the present analyses, we found that the correlation between the number of different MHC-I alleles and MHC-I functional divergence on MHC-I haplotypes was reproduced in data simulations that incorporated the inferred genetic structure of putative loci represented by clades A-E (Fig. 24). This indicates that including the number of different MHC-I alleles as covariate in the models in Paper III and IV had the effect of removing the variation in MHC-I functional divergence that was explained by divergence between the clades. This suggests that the advantages of increased MHC-I

functional divergence in male great reed warblers, that we presented in Paper III and IV (i.e., increased life span, Darwinian fitness, and song repertoire size), are in fact caused by increased divergence of the alleles within clades.



**Fig. 24** Correlation between the MHC-I functional divergence and number of different MHC-I alleles on haplotypes from the real data set (black dots) and from 1,000 simulated data sets in which alleles were randomly assigned to haplotypes taking allele affiliation with clades into account (gray dots). The solid line shows predictions from a linear model based on the real data set and the broken line shows mean predictions from linear models based on the simulated data sets. Jitter was added to the number of different alleles per haplotype to distinguish individual data points (from Paper V, Fig. 11).





# Conclusion and perspectives

Hamilton and Zuk (1982) suggested that females may gain genetic benefits related to disease resistance when choosing males with high expression of secondary sexual characters, and this idea was later refined by Folstad and Karter (1992) in the ‘immunocompetence handicap hypothesis’ (ICHH). Folstad and Karter proposed that the male sex hormone testosterone could provide a mechanistic link between immunocompetence and the expression of secondary sexual characters by imposing a handicap of suppressed immune responses in males. Sexual selection has strong influences on individual fitness in many species, yet it has not previously been investigated whether an association between sexual selection and hormonally mediated suppression of immune responses in males may affect selection on immune system genes. I therefore conducted a detailed review of the literature on regulatory effects of sex hormones on immune responses, and proposed the novel hypothesis that sex differences in the strength of immune responses may create a basis for sexually antagonistic selection on immune system genes (Paper I).

Genes of the major histocompatibility complex (MHC) code for receptors involved in self/non-self recognition by the adaptive immune system, and these genes are central to disease resistance in many vertebrate species. I took advantage of our detailed long-term study of a wild population of the socially polygynous great reed warbler to conduct an empirical investigation of the potential for sexual selection to drive sexually antagonistic selection on MHC genes (Paper II-IV). These investigations revealed an unresolved sexual conflict over the number of different MHC-I alleles per individual (Paper II), and positive associations between MHC-I functional divergence and life span as well as between MHC-I functional divergence and Darwinian fitness in males, but not in females (Paper III). In Paper IV, I showed that variation in MHC-I genes affected male characters associated with female mate choice for both direct and indirect (genetic) benefits, and that these characters were all associated with number of different MHC-I alleles or MHC-I functional divergence. Furthermore, my results suggested that females selected males with compatible genotypes, that would maximize MHC-I functional divergence in their offspring.

In the final paper of the thesis (Paper V), I successfully identified segregating MHC-I haplotypes to investigate the sources of variation in the number different MHC-I alleles and functional divergence, and how these properties are related. These investigations confirmed linkage disequilibrium for the MHC-I genes in great reed warblers. Furthermore, a phylogenetic analysis revealed that the MHC-I alleles in our data set cluster in five clades that showed different signatures of positive

selection, suggesting that these clades represent distinct MHC-I loci in the great reed warbler. I found substantial variation in the number different MHC-I alleles on each haplotype in the population, and it seems likely that this variation is maintained by the sexual conflict over the number different MHC-I alleles, because haplotypes with few alleles should be advantageous in females while haplotypes with more alleles should be advantageous in males. The tight linkage of the MHC-I on an autosomal chromosome generates a shared distribution of MHC properties between the sexes, which prevents resolution of the sexual conflict. Interestingly, variation in the number different MHC-I alleles on haplotypes was largely explained by variation in the number of alleles from two specific clades, indicating that future studies of the effects of alleles from these two clades may provide a more detailed understanding of the proximate mechanisms behind the sexual conflict over MHC-I diversity.

Overall, the results of my empirical studies indicated (i) that sexual selection for testosterone-mediated male traits and behaviors may enhance sex differences in immune responses and thereby generate a selective pressure on MHC-I genes to compensate for immune-suppression in male great reed warblers, (ii) that this selective pressure drives the number of different MHC-I alleles per individual to levels that are unfavorable to females, and thereby causes a sexual conflict, (iii) that increased functional divergence between MHC-I alleles is favored in males, possibly as a compensation for suboptimal numbers of different MHC-I alleles, and (iv) that the sexual conflict maintains variation in the number of alleles on MHC-I haplotypes in the population.

For the great reed warbler specifically, I would encourage further studies that investigate the possible differentiation of biological functions of the putative MHC-I loci presented in Paper V, including the ecological and evolutionary relevance of these. Furthermore, my empirical studies focused on the variation in exon 3 of the MHC-I genes. This exon encodes the alpha two domain, which forms one side of the peptide binding groove of the MHC-I molecule (Fig. 3). This is the most variable domain in several mammals, but it is not known if that is also the case in songbirds. A more complete picture of the functional variation in the MHC-I genes would be achieved by also genotyping the MHC-I exon 2, which encodes the alpha one domain, that forms the opposite side of the peptide binding groove. Finally, I also encourage studies that investigate the variation in MHC class II (MHC-II) genes. MHC-II functions are associated more with type 2 adaptive immune responses, and therefore MHC-II genes may be subject to sexually antagonistic selection that is even more pronounced than that observed in the MHC-I (Paper I).

Based on the predictions in Paper I, I expect that sexually antagonistic selection on immune system genes may be common among vertebrates, because both the overall architecture of the immune system and the effects of sex hormones on immune

responses are phylogenetically conserved. Sexually antagonistic selection may have played a significant role in the evolution of the vertebrate immune system, and I therefore propose that future studies investigate this topic across a broader range of species. Insights gained from such studies may help us understand how different properties of immune system genes influence costs and benefits of immune responses, which may be helpful e.g. in developing an evolutionary understanding of why immunopathological reactions occur, despite potentially adverse effects on individual fitness.



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# Swedish summary

I mitt forskningsprojekt har jag studerat generna i 'the major histocompatibility complex' (MHC). Generna i detta komplex kodar för molekyler som uttrycks på cellytan och dessa molekyler är avgörande för immunförsvarets förmåga att upptäcka och bekämpa patogener (sjukdomsalstrande mikroorganismer, som virus, bakterier och parasiter). Det övergripande syftet i min forskning har varit att undersöka betydelsen av den genetiska variationen inom MHC klass I (MHC-I) generna. MHC-I molekylerna gör det möjligt för immunförsvaret att upptäcka patogener som finns inuti värdens celler, t.ex. virus och malaria-parasiter.

MHC-I generna uttrycks i alla kroppens celler, och MHC-I molekylerna presenterar fragment av proteiner som har brutits ner i cellen. Dessa fragment kan härstamma från cellens egna komponenter eller från patogener. Om proteinfragmentet inte härstammar från cellen utan från ett patogen så kallas det antigen. När MHC-molekylerna presenterar antigen för T-celler påbörjas en immunförvarsreaktion. T-celler kan bara skilja på vad som är ett kroppseget proteinfragment och ett antigen när proteinfragmenten presenteras av MHC-molekyler. Varje MHC-molekyl kan presentera ett begränsat antal proteinfragment och därför är variationen i MHC-generna inom varje individ betydelsefull för hur väl skyddad denna individ är mot patogener.

MHC-generna är bland de mest variabla gener som finns hos ryggradsdjur, och det är olika varianter av patogener som upprätthåller denna variation. Patogenerna anpassar sig och undkommer de vanligaste MHC-genvarianter i en värdpopulation medan ovanliga MHC-genvarianter är fördelaktiga för bäraren. Detta förlopp mellan vanliga och ovanliga MHC-genvarianter är cykliskt och förändras hela tiden, olika MHC-genvarianter är fördelaktiga vid olika tidpunkter. Individer har en fördel av att ha en bred variation i sina MHC-gener för att kunna känna igen en så stor bredd av patogener som möjligt, medan patogenerna har en fördel sin snabba anpassningsförmåga som uppnås via en kort generationstid. Individer kan få en bred variation i sina MHC-gener antingen genom att ha många MHC-genkopior eller genom att ha kombinationer av MHC-genvarianter som ser olika ut (denna olikheten mellan MHC-genvarianter kallas MHC-divergens). Tidigare studier från bland annat människor har visat att individer med hög MHC-divergens kan känna igen fler patogener än individer med låg MHC-divergens.

Jag har utfört mina studier på en vild fågelart, trastsångare *Acrocephalus arundinaceus*, där det finns data insamlat från en population i våtmarksområdet Kvismaren, som har studerats i detalj sedan 1983. Detta projektets långsiktighet och detaljkunskap gör det unikt inom ekologisk grundforskning. Trastsångare är en



polygyn art, där framgångsrika hanar kan ha upp till fem honor i sitt harem. Honorna väljer sin partner baserat på hanarnas sång och ålder, och på hur attraktiva territorier hanarna har. Med detta val skapar honorna en fördel för hanar som är i bra fysisk kondition, och vi har funnit att variationen i MHC-I generna spelar en betydelsefull roll eftersom (i) antalet genvarianter (så kallade alleler) inom en individ har betydelse för hur bra territorier hanarna får och (ii) MHC-divergensen inom en individ har betydelse för hanens livslängd och hur varierat han sjunger. Våra resultat visar även att MHC-divergensen är högre hos hanar som valdes av honorna som sociala partners än genomsnittet bland alla hanar.

Våra resultat matchar bra mot tidigare framlagda vetenskapliga teorier av Hamilton och Zuk (1982) och Folstad och Karter (1992), som föreslog att gener som är betydelsefulla för sjukdomsresistens gynnas vid partnerval. Hamilton och Zuk (1982) hittade ett negativt samband mellan uttrycket av hanliga ornament (t.ex. mättnaden av fjädrarnas färger) och förekomsten av blodparasiter hos nordamerikanska sångfåglar, och utvecklade teorin att hanliga ornament ger honorna möjlighet att välja de hanar som är mest resistent mot sjukdomar. Folstad och Karter (1992) föreslog att det hanliga könshormonet testosteron spelade en viktig roll för fysiologin bakom Hamilton och Zuks teori, genom att testosteron både reglerar uttrycket av hanliga ornament och dämpar uttrycket av immunförsvaret. Dessa två effekter av testosteron gör att endast de hanar som har de bästa generna för sjukdomsresistens har råd med att ha både höga testosteronhalter och högt uttryckta hanliga ornament. Därmed visar högt uttryck av hanliga ornament att hanen är av god kvalitet.

Även om kopplingen mellan MHC-gener och partnerval har påvisats i många arter (det mest kända exemplet är kanske T-shirt experimentet som blev utfört på människor av Wedekind (1995, 1997) där försökspersoner föredrog partners med MHC-gener som var olika de egna MHC-generna, detta val var möjligt att göra endast genom att lukta på partners' T-shirts), så har det inte tidigare funnits en teori som tar hänsyn till vilken effekt de gener som ger hög sjukdomsresistens hos hanar har när de hamnar hos honor, där immunförsvaret inte är dämpat av testosteron. Tillsammans med mina handledare Helena Westerdahl och Dennis Hasselquist gjorde jag en litteraturstudie, där jag undersökte reglerande effekter av könshormoner på immunförsvaret hos ryggradsdjur (inklusive människor). Vi hittade att hanar generellt sett har ett lägre uttryck av immunförsvaret. Med andra ord är hanarnas immunförsvaret i regel inte lika effektivt som honornas. Men om hanar selekteras för att ha gener som ger hög sjukdomsresistens för att kompensera för deras dämpade uttryck av immunförsvaret, t.ex. hög MHC-variation, då finns det en risk att dessa gener ger ett alldeles för effektivt immunförsvarsuttryck hos honorna, vilket kan innebära en ökad risk för autoimmuna sjukdomar. Baserad på denna teori utvecklade vi hypotesen att stark sexuell selektion (d.v.s. starkt

diskriminerande partnerval) för ökad sjukdomsresistens hos hanar kan ge upphov till en genetisk konflikt mellan könen.

Vi undersökte denna hypotes på trastsångarna, och hittade att hanar som har många olika MHC-I alleler får avkommor som överlever i högre grad. Det omvända gällde för honor, då deras avkommor överlevde bättre om honan hade färre MHC-I alleler. Vi hittade även en könsskillnad i effekten av MHC-divergens, då hanar med hög divergens levde längre och därmed fick fler avkommor, medan ingen sådan fördel fanns hos honorna. Denna typ av könskonflikt i immunförsvaret har oss veterligen aldrig tidigare konstaterats hos djur, men eftersom de flesta ryggradsdjur, även människor, har samma uppbyggnad av sitt adaptiva immunförsvaret så bör liknande könsskillnader i styrkan av immunförsvarsresponsen finnas hos många olika arter.

I avhandlingens sista kapitel undersökte vi hur variationen mellan individer i antalet MHC alleler och MHC-divergens kan ha uppkommit hos trastsångarna. Detta gjorde vi genom att analysera nedärvningsmönster i MHC-I generna och därigenom identifiera så kallade haplotyper. Haplotyper är grupper av alleler som sitter tätt ihopkopplade på kromosomerna som nedärvs från respektive förälder. Genom att sammanställa resultaten från haplotyp-analysen med en släktskapsanalys av alla MHC-I allelerna som vi fann i trastsångarpopulationen, så kunde vi dela in MHC-I allelerna i fem olika grupper (betecknade A-E). Dessa fem grupper representerar sannolikt olika MHC-I loci (platser på kromosomerna), i trastsångarnas genom (alla kromosomerna, arvsmassan). Vi utförde även en analys som visade att den naturliga selektionen hade påverkat MHC-allelerna från de fem olika grupperna på olika sätt, vilket tyder på att MHC-molekylerna troligtvis har specialiserats på att binda olika typer av antigen. Vår analys visade att variationen i antalet alleler per MHC-I haplotyp förklarades av genduplicering inom två (D och E) av de fem förmodade MHC-I locina. Dessa två loci (D och E) innehöll fler alleler än de övriga tre (A-C). Våra resultat indikerar vidare att det är alleler från grupperna D och E som driver könskonflikten hos trastsångarna, konflikten där hanarna har en fördel av att ha många MHC-alleler medan honorna har en fördel av att ha få MHC-alleler. Detta möjliggör att framtida studier kan undersöka vilka sjukdomar som alleler från grupperna D och E skyddar emot, och vilka kostnader som de medför t.ex. i form av risk för autoimmunitet.

Sammanfattningsvis har våra studier påvisat att variationen i MHC-I generna är oerhört viktig för trastsångarna. Vi hittade en könskonflikt i relation till antalet MHC-I alleler på individnivå, en konflikt som sannolikt är kopplad till den effekt som könshormonerna testosteron och östrogen har på styrkan av immunförsvarsresponserna. Vi har dessutom lyckats karaktärisera ett stort antal MHC-haplotyper hos sångfåglar och vår metodutveckling i samband med denna analys har öppnat dörren för helt nya kunskaper och vinklingar av sångfåglares MHC-gener. Sådana kunskaper är värdefulla eftersom sångfåglar utgör unika

studiesystem, där det är relativt enkelt att studera individernas beteende och individernas framgång (de kan hållas i holkar, hittas och observeras relativt enkelt i naturen, många rör sig relativt lokalt, etc.). Ekologiska studier av vilda arter är dessutom betydelsefulla om man vill studera evolutionen av immunförsvaret, eftersom vilda djur lever med sina naturliga parasiter och sjukdomar.

# Many thanks!

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*Mac x 2 = science^2*

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## List of papers

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- I. Roved, J., Westerdahl, H., & Hasselquist, D. 2017. Sex differences in immune responses: Hormonal effects, antagonistic selection, and evolutionary consequences. *Hormones and Behavior* 88: 95-105. <http://doi.org/10.1016/j.yhbeh.2016.11.017>
- II. Roved, J., Hansson, B., Tarka, M., Hasselquist, D., & Westerdahl, H. 2018. Evidence for sexual conflict over MHC diversity in a wild songbird. *Proceedings of the Royal Society B* 285: 20180841. <http://doi.org/10.1098/rspb.2018.0841>
- III. Roved, J., Hansson, B., Tarka, M., Hasselquist, D., & Westerdahl, H. MHC-I functional divergence is positively associated with life span and fitness in male great reed warblers: support for the divergent allele advantage hypothesis. Manuscript.
- IV. Roved, J., Hansson, B., Hasselquist, D., & Westerdahl, H. MHC class I genes and mate choice in great reed warblers. Manuscript.
- V. Roved, J., Hansson, B., Hasselquist, D., & Westerdahl, H. MHC haplotypes unravel patterns of selection and genetic structure in great reed warblers. Manuscript.

