university of groningen

University Medical Center Groningen

University of Groningen

Fitting genes

Schut, Elske

IMPORTANT NOTE: You are advised to consult the publisher's version (publisher's PDF) if you wish to cite from it. Please check the document version below.

Document Version
Publisher's PDF, also known as Version of record

Publication date:
2012

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):
Schut, E. (2012). Fitting genes: Sexual selection in the blue tit Groningen: s.n.

Copyright
Other than for strictly personal use, it is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), unless the work is under an open content license (like Creative Commons).

Take-down policy
If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

Downloaded from the University of Groningen/UMCG research database (Pure): http://www.rug.nl/research/portal. For technical reasons the number of authors shown on this cover page is limited to 10 maximum.

Download date: 10-02-2018

Fitting genes

Sexual selection in the blue tit: the role of the MHC and post-copulatory effects

This PhD project was carried out at the Behavioural Ecology and Self-Organization group and the Animal Ecology Group, both part of the Centre for Ecological and Evolutionary Studies of the University of Groningen (The Netherlands), according to the requirements of the Graduate School of Science (Faculty of Mathematics and Natural Sciences, University of Groningen).

Elske Schut received a bursary grant from the faculty of Mathematics and Natural Sciences following a top-masters programme. The research was supported by grants from GEBACO (FP6/2002–2006, no. 28696) and INCORE (FP6–2005-NEST-Path, no. 043318) to Jan Komdeur and from the Dr. J. L. Dobberke Stichting, the Schure- Beijerinck-Popping Fonds and Stichting Nicolaas Mulerius fonds to Elske Schut.

Lay-out and figures: Dick Visser
Cover design: Douwe Terluin/Elske Schut
Paintings on cover: Bep Wieringa (Oma/grandmother)
Cover pictures: Elske Schut
Other pictures: All by Elske Schut except bottom picture at cover of part II (by Juan Rivero) and pictures in chapter 2.
Printed by: Van Denderen BV, Groningen

ISBN: 978-90-367-5391-3
ISBN: 978-90-367-5392-0 (electronic version)

RIJKSUNIVERSITEIT GRONINGEN

Fitting genes

Sexual selection in the blue tit: the role of the MHC and post-copulatory effects

PROEFSCHRIFT

ter verkrijging van het doctoraat in de
Wiskunde en Natuurwetenschappen
aan de Rijksuniversiteit Groningen
op gezag van de
Rector Magnificus, dr. E. Sterken,
in het openbaar te verdedigen op
vrijdag 23 maart 2012
om 16.15 uur

door

Elske Schut

Promotor:	Prof. dr. Ir. J. Komdeur
Copromotor:	Dr. M. J. L. Magrath
Beoordelingscommissie:	Dr. T. Pizzari Prof. dr. E. Svensson Prof. dr. J. L. Olsen

Contents

CHAPTER 1	General introduction	6
BOX A	Immune recognition and the genetic structure of the MHC	19
PART I:	**Sperm availability**	
CHAPTER 2	Unhatched eggs: methods for discriminating between infertility and early embryo mortality	27
CHAPTER 3	No evidence for decreased egg fertility due to female sperm depletion over the laying period in the blue tit (*Cyanistes caeruleus*)	41
CHAPTER 4	Sperm numbers in the reproductive tract of female blue tits (*Cyanistes caeruleus*) are repeatable within the clutch and related to partner size	53
BOX B	Cloacal protuberance in the blue tit as an indication of sexual activity	63
PART II:	**The Major Histocompatibility Complex in the blue tit**	
CHAPTER 5	Characterization of MHC-I in the Blue Tit (*Cyanistes caeruleus*) reveals low levels of genetic diversity and trans-population evolution across European populations	71
CHAPTER 6	MHC-I screening in blue tits (*Cyanistes caeruleus*) using Reference Strand mediated Conformation Analysis (RSCA)	89
PART III:	**MHC based mate choice**	
CHAPTER 7	Evidence for assortative mate association according to MHC-I heterozygosity in the blue tit (*Cyanistes caeruleus*)	107
BOX C	Could passerines use olfaction in MHC-recognition?	114
BOX D	Heritability of MHC-I heterozygosity in the blue tit	119
CHAPTER 8	Synthesis	123
	References	137
	Nederlandse samenvatting	155
	Dankwoord-Acknowledgements	163
	Addresses of co-authors	167

CHAPTER 1

General introduction

E. Schut

Sexual selection

Males in many species (particularly bird species) are known to possess conspicuous ornaments, that may impose a fitness cost in terms of survival, e.g. because the ornaments limit their mobility or make them better visual to predators. A well known example of such an ornament is the peacock's conspicuous tail. Darwin (1871) explained the evolution of these ornaments by the theory of sexual selection. Sexual selection is a form of natural selection that works on the ability of individuals to gain access to mates and to obtain offspring. Sexual selection can take two forms: competition between individuals for access to individuals of the opposite sex and selection of mates by one of the sexes (although it may not always be possible to distinguish the two, e.g. because the preferred mate is the individual most successful in competition; Andersson 1994). This thesis focuses on sexual selection through mate selection.

In the majority of species, the number of copulations that optimizes reproductive success is lower for females than it is for males, because females invest more in their gametes but produce fewer of them. Hence, females are generally expected to be more selective in their mate choice (i.e. they are "the choosy sex"; Andersson 1994). However, the sex that is more choosy depends also on the relative investments of the sexes in offspring and on the mating system. Relative investment in offspring differs between species, and male choosiness or mutual choosiness, as well as female ornaments are known to occur (Clutton-Brock 2009). Females may benefit from being choosy in two ways: through direct benefits (e.g. an increase in the number of offspring surviving, through paternal care) or indirect, genetic benefits (i.e. enhanced offspring survival or reproductive success, assuming that male attractiveness is heritable). Several theories have been proposed to explain how indirect benefits arise (reviewed in Andersson 1994). Good genes models (also called indicator models or handicap models; Andersson 1994) are based on the idea that females obtain genetic benefits from mating with ornamented males. The good genes model only explains the evolution of male ornaments in case male ornaments form an honest indicator of male quality, e.g. if there is a cost ("handicap"; Zahavi 1975) of carrying the ornaments so that only males of high quality can carry them or if the genes determining ornament expression are associated with genetic benefits. Under the good genes model, a universal female preference for certain male characteristics is expected. If females select high quality males as their partners, their offspring will be of high quality (i.e. high survival probability and/or attractive), which increases the eventual number of (grand) offspring that originates from these females. In contrast, the genetic compatibility model suggests that the genetic quality of offspring depends on the specific interaction between paternal and maternal genotypes and therefore the preferred male genotype will differ between females (Trivers 1972). There are several reasons why the maternal and paternal genotypes may not be optimally compatible. In extreme cases, such as inbreeding, offspring may possess two copies of a recessively lethal allele, which may result in

embryo mortality in an early developmental stage. In less extreme cases, such as outbreeding depression, the combination of parental alleles may results in offspring that are not optimally adapted to the environment they live in and will have reduced survival probabilities. By creating a favourable combination of alleles, females may increase the survival and reproductive success of their offspring (Zeh and Zeh 1996; Tregenza and Wedell 2000). The good genes and genetic compatibility models are not necessarily mutually exclusive and mate choice according to both models could potentially exist within a single species (Mays and Hill 2004).

Post-copulatory sexual selection

Sexual selection may operate both before copulation (pre-copulatory sexual selection) and after copulation has taken place (post-copulatory sexual selection). In passerine birds monogamy may be the predominant mating system, but the occurrence of offspring sired by a male other than the social male (extra-pair offspring) is a well known phenomenon (a fact that became apparent after the development of molecular parentage testing), with between 10-20% of offspring being extra-pair in most passerines (Birkhead and Møller 1992; Westneat and Webster 1994; Griffith *et al.* 2002b). Hence, we know that females frequently copulate with more than one male within a single breeding cycle. The opportunity for females to have their eggs fertilized by multiple males is increased by the fact that female birds possess special structures, called sperm storage tubules (SST), in which they can store sperm for up to several weeks (Birkhead and Møller 1992). The fact that female birds possess SST increases the period during which copulation may lead to the fertilization of one or more ova. When a female has obtained sperm from more than one male, post-copulatory sexual selection can determine which male will father the majority of offspring.

Post-copulatory sexual selection can include both a female and a male component (called cryptic female choice, Eberhard 1996, and sperm competition, Birkhead and Møller 1992; Birkhead and Møller 1998, respectively), that are often hard to distinguish (Birkhead 1998b; Birkhead 2000). In general, there is some distance between the site of sperm insemination and the site of fertilization. In birds, sperm are inseminated into the cloaca and traverse the first part of the female reproductive tract before being stored in the SST. After release from the SST, sperm need to traverse the site of fertilization, where sperm penetrate the layers around the ovum and fertilization occurs (See Birkhead and Brillard 2007; figure 1.1 for details). Females can influence the process during which sperm traverse the female reproductive tract at several stages. It is well known that female birds may eject sperm immediately after copulation and store only a minute percentage of the sperm inseminated (Brillard 1993; Birkhead *et al.* 1993b; Bakst *et al.* 1994) and it is possible that females selectively store sperm from different males, e.g. in domestic fowl (*Gallus domesticus*) the likelihood that females eject sperm immediately after

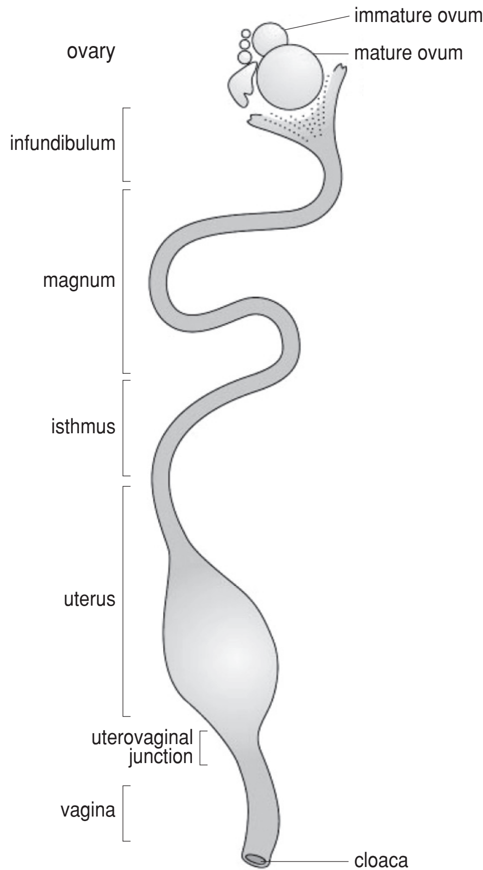


Figure 1.1 Overview of the female reproductive tract indicating the main anatomical divisions. The SST are located at the uterovaginal junction, the infundibulum is the site of fertilization. Figure from (Birkhead 1998a).

copulation is determined by male dominance (Pizzari and Birkhead 2000). Furthermore, females may selectively release the sperm of different males from the SST and interactions between sperm and ovum before fertilization may occur selectively (for details see Eberhard 1996). While females may exercise some (incomplete) control over fertilization, the process is thought to occur largely according to a fair-raffle model (Parker 1970; Parker 1998) when females have stored sperm from several males. According to this model, the proportion of sperm in the SST that belongs to a certain male directly relates to the likelihood that he will fertilize one or more eggs. Hence, males may influence their chances at fathering offspring e.g. by adjusting their ejaculate size (Birkhead and Møller 1998). In birds, the last male to copulate has a fertilization advantage (last male sperm precedence; Birkhead *et al.* 1988) and both males and females may bias paternity by adjusting the timing of copulation. Sperm mobility is another important determinant of paternity (Birkhead *et al.* 1999) and males may increase their fertilization success by producing highly mobile sperm.

In monogamously breeding birds, males commonly attempt to improve their chances of fertilizing the females' ova by guarding their social female to prevent her from mating with other males. Other mechanisms to enhance the likelihood of paternity include frequent copulation, and the removal of sperm from other males (Birkhead and Møller 1992; e.g. in the dunnock (*Prunella modularis*) where males peck at the females cloaca before copulation, which may induce the female to release a drop of sperm; Davies 1983).

During post-copulatory sexual selection, sperm interact with (cells present in) the female reproductive tract and there is scope for interaction at the cellular level (Zeh and Zeh 1997). An example is the interaction of sperm with the female immune system. In humans, an immune response against sperm within the female tract may reduce the fertility of couples (Naz and Menge 1994) and evidence exists for a similar immune response in birds (reviewed in Birkhead and Brillard 2007). Whether an immune response to sperm is initiated likely depends on molecules expressed on the sperm surface (e.g. Steele and Wishart 1992) and therefore on the male genotype. In addition, different males may vary in the efficiency of the interaction of their sperm with the layers around the ovum, which is required for the entering of sperm into the ovum before fertilization. Most likely such differences are caused by genetically determined differences in surface glycoproteins and semen composition (Barbato *et al.* 1998; Hammerstedt *et al.* 2001). There also is *in vivo* evidence that male fertilization success is dependent on genotype. For instance in field crickets (*Gryllodes supplicans*), where males who were siblings to the female had a reduced fertilization probability when in competition with a male not related to the female, compared to the situation where the same males were in competition with another sibling (Stockley 1999). In the arctic charr (*Salvelinus alpinus*; a species with external fertilization, which allows experimental manipulation of the number of sperm that males contribute) male fertilization success was associated with genotype of the Major Histocompatibility Complex (Skarstein *et al.* 2005). Hence, female selection of males with good or compatible genes may occur post-copulatory (Zeh and Zeh 1997).

To conclude, both male and female may exercise some control over fertilization (and the review of post-copulatory sexual selection mechanisms given here is by no means complete), but their control is incomplete and the combination of male and female post-copulatory processes determines which male will father the majority of offspring. Post-copulatory sexual selection is thought to have shaped both male and female reproductive characteristics over evolutionary time scales, e.g. male sperm morphology and semen composition and the length of the female reproductive tract.

The Major Histocompatibility Complex

Studies testing the good/compatible genes theories frequently explore the significance of the Major Histocompatibility Complex (MHC). In vertebrates, the MHC is

the gene complex that regulates immune recognition. The MHC is a highly diverse gene complex, with a high number of loci, high polymorphism at those loci and a high sequence diversity compared to other gene regions (Klein 1986; Nei and Hughes 1991). The MHC can be divided into several classes of genes, of which class I and II are involved in the specific immune system. MHC molecules bind foreign peptides and present them to T-cells, which then initiate an immune response (Goldsby *et al.* 2000; Janeway *et al.* 2008; see Box A for more details).

The first bird species for which the MHC was characterized was the chicken, whose MHC is relatively simple compared to that of humans (with fewer MHC-I and MHC-II loci and fewer introns; Kaufman *et al.* 1995; Kaufman *et al.* 1999). Now that more bird species have been characterized, it has become clear that passerine species are far more diverse at their MHC than chickens and other galliform species (with the exception of the Japanese quail, *Cortunix japonica*; Shiina *et al.* 1995; Westerdahl *et al.* 2000; Westerdahl 2007).

Since the MHC plays an important role in immune recognition, it is likely to have important effects on the survival and reproductive success of individuals. Passerines (including the blue tit, *Cyanistes caeruleus*) commonly suffer from avian malaria infections, which may seriously affect individual survival (Merino *et al.* 2000; Cichon and Dubiec 2005; Tomás *et al.* 2007; Arriero *et al.* 2008; Knowles *et al.* 2010). Several studies of passerines provide evidence for a link between MHC-I genotype and malaria resistance (Westerdahl *et al.* 2005; Bonneaud *et al.* 2006b; Loiseau *et al.* 2008), suggesting that the MHC genes affect individual survival. However, these studies did not test whether MHC genotype was associated with survival. Brouwer *et al.* (2010) demonstrated that more MHC-I heterozygous individuals of the Seychelles warbler (*Acrocephalus sechellensis*) were more likely to survive the juvenile stage. In addition they found that individuals with one specific MHC-I allele had a longer life expectancy compared to individuals lacking the allele.

Since selection usually decreases genetic polymorphism (i.e. the number of alleles that remain in the population), the high MHC diversity is thought to be maintained by balancing selection, (i.e. selection that prolongs the lifetime of alleles in the population; Potts and Wakeland 1990; Hedrick 2002). Two mechanisms of balancing selection have been proposed: pathogen driven selection and mate choice based on the MHC. The pathogen driven selection hypothesis proposes that individuals with high MHC diversity or with rare alleles have a selective advantage due to a better disease resistance. The mate choice hypothesis also proposes that selection is driven by pathogens, but it further predicts that mate choice is the mechanism through which parents ensure that their offspring are optimally adapted to their pathogen environment. To achieve that their offspring are optimally adapted, females should select a mate on the basis of his MHC characteristics. A female preference for certain MHC characteristics may prevent alleles from going to extinction and therefore leads to balancing selection.

MHC-based mate choice

Hamilton and Zuk (1982) were the first to suggest that pathogen resistance, as reflected in male secondary sexual characters, could be selected through female mate choice. Following from this, Siva-Jothy and Skarstein (1998) proposed that the MHC genes may be the target of mate choice. Several mechanisms have been proposed by which the immune system may influence the production of male ornaments (Folstad and Karter 1992; Westneat and Birkhead 1998), including the immunocompetence handicap hypothesis. This hypothesis argues that males trade-off the development of ornaments against immunocompetence and that this trade off may be mediated by testosterone. Testosterone stimulates the production of ornaments, while it suppresses the immune system. Hence, individuals suffering from infections may have reduced testosterone production and thereby decreased ornament expression (Folstad and Karter 1992; see also box C).

Evidence for mate choice based on the MHC has been found in a range of species (see box C; but see e.g. Paterson and Pemberton 1997; Westerdahl 2004). Early studies on MHC based mate choice used laboratory strains of mice and rats. Hence, it is perhaps not surprising that evidence of inbreeding avoidance was found in many early studies, as genetic variation was relatively low and the risk of inbreeding was high. The findings from studies in non-model species have been mixed. There are several ideas about the male MHC features beneficial to the female (Penn and Potts 1999; Ekblom *et al.* 2004; Milinski 2006), two of which are based on the good genes hypothesis. According to this hypothesis, females should favour males with (1) high MHC heterozygosity, since this yields more diverse offspring or, (2) certain (rare) alleles that offer protection to infections prevalent in the population. Alternatively, genetic compatibility hypotheses state that females should choose a mate based on the compatibility of the males' MHC genotype with her own MHC genotype. Consequently, females should (1) avoid genotypic incompatibilities, (2) avoid inbreeding and outbreeding or (3) obtain the optimal number of alleles for their offspring, since there is evidence that the highest immunocompetence is achieved at intermediate levels of MHC heterozygosity and that individuals with too many MHC alleles may suffer auto-immune reactions (Nowak *et al.* 1992; Milinski 2006; Stjernman *et al.* 2008) .

There is supporting evidence for most of these hypotheses, e.g. in both the Savannah sparrow (*Passerculus sandwichensis*, MHC class II; Freeman-Gallant *et al.* 2003) and the Seychelles warbler (MHC class I; Richardson *et al.* 2005) extra-pair males (i.e. males that do not provide benefits other than genetic benefits) were more MHC diverse than the females' social partner. In the house mouse (*Mus musculus domesticus*) MHC recognition (involving both MHC classes) has been shown to serve as a means to recognize related individuals to avoid inbreeding (Yamazaki *et al.* 1988; Potts *et al.* 1991) and in the Savannah sparrow (*Passerculus sandwichensis*) and the house sparrow (*Passer domesticus*), females also avoided genetically similar partners (Freeman-Gallant *et al.* 2003; Bonneaud *et al.* 2006a).

Sticklebacks (*Gasterosteus aculeatus*) select a partner that will optimize the number of MHC class II alleles of their offspring (Aeschlimann *et al.* 2003). In the great snipe (*Gallinago media*), females were shown to prefer males with specific MHC class II alleles that offered resistance to common pathogens (Ekblom *et al.* 2004). However, it remains unclear how female birds may be able to recognize the MHC genotype of potential partners (see Box C).

While a relationship between mate choice and the MHC has been reported in a range of species, it is generally impossible to determine whether the MHC genes themselves are the target of mate choice. It is very well possible that genes closely linked to the MHC are the target of mate choice, resulting in MHC based mate association (Jordan and Bruford 1998). Decoupling effects of the MHC and loci closely linked to it is almost impossible (except in model species such as mice and rats, where strains can be produced that differ solely in the MHC genes; Yamazaki *et al.* 1978).

Sexual selection in the blue tit

This thesis addresses questions regarding (post-copulatory) sexual selection in the blue tit (*Cyanistes caeruleus*). Sexual selection is commonly studied in birds, due to the fact that males often exhibit conspicuous plumage colouration and complex song that are hard to explain under other forms of natural selection, as they are likely to attract predators and compromise survival. The blue tit is commonly studied in the context of sexual selection, due to the frequent occurrence of extra-pair offspring (e.g. Kempenaers *et al.* 1992; Charmantier *et al.* 2004; Brommer *et al.* 2007; Magrath *et al.* 2009; Vedder *et al.* 2011), which enhances the opportunity for mate choice, since ecological factors may constrain individuals in their choice of a social partner (Gowaty 1996; see below), but not (or to a lesser extent) in their choice of an extra-pair partner. Furthermore because of the occurrence of extra-pair offspring, it is certain that at least some females mate with more than one partner within a single reproductive cycle, which is a requirement for post-copulatory sexual selection (Birkhead and Møller 1992). Malaria parasites are known to negatively affect survival and reproductive success in the blue tit (Merino *et al.* 2000; Tripet *et al.* 2002; Stjernman *et al.* 2004; Tomás *et al.* 2007; Arriero *et al.* 2008; Knowles *et al.* 2010). Hence it seems plausible that an individuals' MHC genotype affects fitness and that blue tits choose a (genetic) partner on the basis of MHC genotype to improve offspring fitness.

While the majority of blue tits forms socially monogamous pairs, some males (15.4% in our population; Brommer *et al.* 2007; Vedder *et al.* 2011) are polygynous (i.e. they father offspring and provide parental care in several nests). Furthermore, extra-pair offspring occur commonly (in 44% of nests in our population; Brommer *et al.* 2007; Vedder *et al.* 2011). Vedder *et al.* (2011) demonstrated that the occurrence of polygyny and extra-pair paternity enhances the potential for sexual selec-

tion, as it amplifies reproductive skew (i.e. the difference in reproductive success between males). Blue tits are sexually dimorphic in size (with males being larger than females (Cramp and Perrins 1993), ultraviolet (UV) colouration of the blue crown (males have more UV shifted and more UV chromatic crowns; Hunt *et al.* 1998a) and vocalizations (males primarily sing during the so-called dawn chorus; Cramp and Perrins 1993).

Several studies (in wild and captive birds) in the blue tit provided evidence for a role of the crown UV in mate choice and for mutual mate choice (e.g. Andersson *et al.* 1998; although seasonal variation may confound the observed patterns in this study). In addition, evidence has been reported for a role of UV colouration in differential allocation (e.g. differences in offspring provisioning rate by the female in response to male crown UV and differences in the amount of androgens deposited in the egg yolk; e.g. Kingma *et al.* 2007), biasing of the offspring sex ratio by the female (e.g. Sheldon *et al.* 1999; Korsten *et al.* 2006) and male-male competition (e.g Vedder *et al.* 2010b). However, not all studies report such relationships with UV crown colour (e.g. Korsten *et al.* 2007; Vedder *et al.* 2008) and findings have varied between study populations and experimental set-ups (reviewed in Vedder 2011). Hence, the question whether UV crown colouration is sexually selected in blue tits is far from resolved.

Blue tit males sing during the beginning of the breeding season. Males sing throughout the day, but the highest singing intensity occurs during the so-called dawn chorus (Poesel *et al.* 2001). Males start singing early in the morning, positioned near the nest box where the female is roosting. The dawn chorus usually ends when the female appears from the nest box (Poesel *et al.* 2001). Like in other passerines, the song of the blue tit is thought to play a role in territory defense and sexual selection (Catchpole and Slater 2008). Previous studies on blue tit song revealed several correlations between male song characteristics (e.g. strophe length, repertoire size, drift), and reproductive success (e.g. male success at obtaining paternity; Kempenaers *et al.* 1997, clutch size and timing of egg laying; Poesel *et al.* 2001 and brood sex ratio; Dreiss *et al.* 2005). It has been proposed that song characteristics represent male quality and/or condition, e.g. because singing is energetically costly (e.g. Lambrechts and Dhondt 1988; Oberweger and Goller 2001). Bijnens (1988) suggested that strophe length was associated with male survival, since older males sang longer strophes, while there was no evidence that strophe length increased with age within individual.

Several studies found evidence for an effect of male tarsus length (e.g. “the part between the knee and ankle joint”; a measure of structural body size) and age (first year or older) in mate choice (but see Charmantier *et al.* 2004; Krokene *et al.* 2004 for studies that found no effect). Kempenaers *et al.* (1992) found that males with longer tarsi were less likely to be cuckolded. Kempenaers *et al.* (1997) and Foerster and Kempenaers (2004) found that older males were more likely to gain extra-pair paternity and extra-pair males had longer tarsi (although Foerster and Kempenaers 2004 found this effect only in extra-pair males that were neighbours). In a study in

our blue tit population in *De Vosbergen*, Vedder *et al.* (2011) also found that older males were more successful at gaining extra-pair copulations, more likely to be polygynous and were also cuckolded less frequently when they were polygynous. There was a tendency for males with longer tarsi to lose less within-pair paternity when they were polygynous.

The above mentioned studies on mate choice in the blue tit investigated male characteristics that potentially indicate male quality. However, these studies did not address the question whether these characteristics indeed reflect genetic differences in male quality. Evidence that blue tits use genetic characteristics in their mate choice was provided by García-Navas *et al.* (2009), who found a positive correlation between male and female heterozygosity, although their study found no evidence that relatedness between individuals was used in mate choice (i.e. to avoid inbreeding or outbreeding). Foerster *et al.* (2003) showed that extra-pair offspring were more heterozygous than within-pair offspring for microsatellite loci, suggesting that females choose extra-pair mates based on heterozygosity. Indeed, several previous studies have suggested an association between genotype (based on microsatellites) and individual quality in the blue tit. Foerster *et al.* (2003) reported that more heterozygous offspring were more likely to be recruits in the following breeding season (thought this study was based on only six microsatellite loci). A positive correlation between reproductive effort and microsatellite heterozygosity was described by García-Navas *et al.* (2009). Olano-Marin *et al.* (2011b) found that more heterozygous females had larger clutches, more heterozygous males fathered more offspring in the population and more heterozygous individuals produced a higher number of recruits. Surprisingly, Olano-Marin *et al.* (2011a) found negative effects of microsatellite heterozygosity on the probability of eggs hatching and on the local recruitment of females, but a positive effect on adult survival.

Thesis outline

The first general aim of this study was to gain insight into the process of post-copulatory sexual selection. To help address this aim, the number of sperm trapped on eggs was used to estimate the number of sperm present in the female reproductive tract at the time of fertilization (see below for details). These data were first used to determine whether females had adequate sperm in storage to fertilize their entire clutch. Second, the data were used to investigate whether any differences between females in the number of sperm in the female reproductive tract exist and whether they are associated with characteristics of the females' social partner. The other general aim of this study was to determine whether the MHC plays a role in mate choice in the blue tit. To do so, we first acquired background information on the blue tit MHC and developed a genotyping method. Then we applied this method to data from our study population.

The study was conducted on an established nest box population at *"De Vosbergen"* estate near Groningen (53°08'N, 06°35'E) in 2001. The study area consisted of a mixture of deciduous and coniferous forest, interspersed with open grassland. Throughout the 54 ha of the study area, 189 blue tit nest boxes had been placed at a height of approximately two meters. About half of these nest boxes were occupied by nesting blue tits each breeding season (April-June). By checking the content of nest boxes on a daily basis, the stage in the breeding cycle was assessed and soon after eggs hatched, blood samples were obtained from offspring and the parents were trapped inside the nest box before the young fledged.

Part I. Sperm availability

When a female has copulated with more than one male during a single breeding cycle, post-copulatory effects will determine which male fathers the majority of offspring (Birkhead and Møller 1998). An important determinant of paternity is the proportion of sperm from each male that the female has in storage (Parker 1970; Parker 1998). Part I of this thesis investigates the number of sperm that females have available for fertilization. If females have inadequate sperm to fertilize their entire clutch, this may result in infertility among the last eggs of their clutch. **Chapter 2** describes a method to distinguish between eggs that fail to hatch due to inadequate sperm or due to the embryo dying at an early stage. By dissecting the layers surrounding the egg yolk (inner- and outer perivitelline layer), the number of sperm present at the site of fertilization and the number of sperm that penetrated the inner perivitelline layer can be determined. Dissection of the germinal disc (the part of the yolk that includes the female nucleus, where fertilization takes place and where embryo development starts) allows one to determine the presence or absence of embryonic cells. Hence, one can determine whether embryonic development started and therefore whether the egg has been fertilized or not. **Chapter 3** uses some of the methodology described in chapter 2 to determine whether hatching failure due to a lack of sperm occurs in the population in De Vosbergen. Because copulation ceases or occurs much less frequently after the start of egg laying in most passerines (Birkhead *et al.* 1987) and because the blue tit lays an extraordinarily large clutch, we hypothesized that females may become sperm depleted towards the end of their laying sequence. Hence, we expected to find an increased likelihood of unfertilized eggs occurring towards the end of the laying sequence.

In **chapter 4** we explore whether characteristics of the females' social partner may influence variation in the number of sperm different females have in storage. One may expect that mechanisms of post-copulatory sexual selection (e.g. selective sperm storage of females; Eberhard 1996) lead to such differences.

Part II. The blue tit MHC

The MHC genes are frequently investigated as candidate genes to test models of sexual selection, such as the good/ compatible genes hypotheses. When this project

commenced, no published data were available on the blue tit MHC. In order to investigate questions regarding the role of the MHC in mate choice, a method was required to quickly and accurately determine the MHC genotype of individuals. In order to develop such a method, background knowledge about the structure of the MHC is required, but usually such knowledge is unavailable when working on a non-model species like the blue tit (Babik 2010). We started off by cloning and sequencing both MHC-I and MHC-II, but developed a screening method for MHC-I only. We chose to work with MHC-I, mainly for the practical reason that primers were available that allowed us to sequence the major part of the blue tit MHC-I and we managed to develop primers that selectively amplify the subset of alleles that are under selection. Molecules derived from MHC-I are expressed in all body cells and are involved in the recognition of inter-cellular pathogens (see Box A). Both inter- and intra-cellular pathogens are likely to occur in passerines, so investigation of MHC-I is as important as studying MHC-II. Developing a screening method for both MHC-I and MHC-II was beyond the scope of this project. **Chapter 5** describes the gene structure of MHC-I, which we obtained after cloning and sequencing. Sequences were obtained from individuals originating from three locations: Spain, the Netherlands and Sweden. **Chapter 6** describes how we adapted the MHC screening method Reference Strand mediated Conformation Analysis (RSCA) to quickly genotype MHC-I in blue tit individuals.

Part III. MHC-I based mate choice

In **chapter 7**, the RSCA method was used to screen MHC genotypes of 14 pairs to determine whether MHC based mate choice occurs in the blue tit. Because previous evidence from blue tits indicated that microsatellite heterozygosity was positively correlated between social partners (García-Navas *et al.* 2009), we tested whether the same was true for the MHC-I alleles. In addition, we tested whether the likelihood that a female produced extra-pair offspring was associated with the heterozygosity of her social partner and the genetic similarity between the female and her social partner.

A synthesis of the most important results in this thesis is provided in **chapter 8**. My findings are put into context and directions for future research are provided.

Immune recognition and the genetic structure of the MHC

E. Schut

The MHC is a gene complex that is part of the immune system in vertebrates. The MHC can be divided into three classes of genes, class I (MHC-I), class II (MHC-II) and class III (MHC-III). MHC-I and MHC-II derived molecules are involved in the specific immune system (i.e. the system that recognizes peptides derived from pathogens as foreign), while MHC-III derived molecules are involved in the non-specific immune system (i.e. creating general barriers to pathogens, such as in mucosal tissues that prevent pathogens from entering the body; Goldsby *et al.* 2000; Janeway *et al.* 2008). MHC-I molecules are present in the majority of cells, while MHC-II molecules are mainly expressed in cells of the immune system. MHC-I and II molecules function to bind foreign peptides and present them on the cell surface for recognition by T-cells (a class of white blood cells), which then activate an immune response (Figure A.1). MHC-I derived molecules bind and present peptides that originate from foreign proteins in the cytoplasm (mainly intracellular pathogens), while MHC-II derived molecules bind and present foreign peptides derived from the degradation of molecules in intracellular vesicles and extracellular space (mainly extracellular pathogens; but so-called cross-presentation does occur and infections exist that activate both MHC-I and II, e.g. malaria). This box focuses on MHC-I, since our work on the blue tit MHC focuses primarily on MHC-I.

MHC molecules consist of several proteins that are the gene products of exons within the MHC. Within the MHC-I genetic structure of passerines, there are five exons, interspersed by introns (Westerdahl 2003; Figure A.2). Multiple gene loci can exist within an exon. The three dimensional shape of the proteins derived from the exons depends on the amino acid sequence (which is determined by the nucleotide sequence). Hence, different alleles form different three dimensional shapes. In class I, exons 2 and 3 encode the peptide-binding region (PBR). The PBR is the part of the MHC molecule that binds foreign peptides in order to present them to T-cells. The amino acid sequences of exons 2 and 3 determine the shape of the protein binding cleft and determine which foreign peptides can be bound. Since exon 2 and 3 determine which foreign peptides can be bound, these are the regions of the MHC that are likely to be under selection, as it is important that they are able to bind peptides derived from infections prevalent in the population. Hence, exons 2 and 3 are the MHC regions that are most often studied (Janeway *et al.* 2008).

Each individual inherits one set of chromosomes from his father and one set from his mother, resulting in two copies of each gene. This means that individuals are either homozygous (i.e. they have the same allele twice) or they are heterozygous (i.e. they have two different alleles) at a certain gene locus. The expression of MHC alleles is co-dominant, which means that in heterozygous individuals, both MHC alleles are expressed, i.e. MHC molecules including proteins derived from each allele are present in cells. Each MHC molecule binds a limited number of peptides and therefore, more polymorphic individuals should be able to recognize more pathogens (Janeway *et al.* 2008).

The high number of gene loci at the MHC is thought to have arisen through the duplication of genes. Therefore, the MHC often consists of several gene loci that are

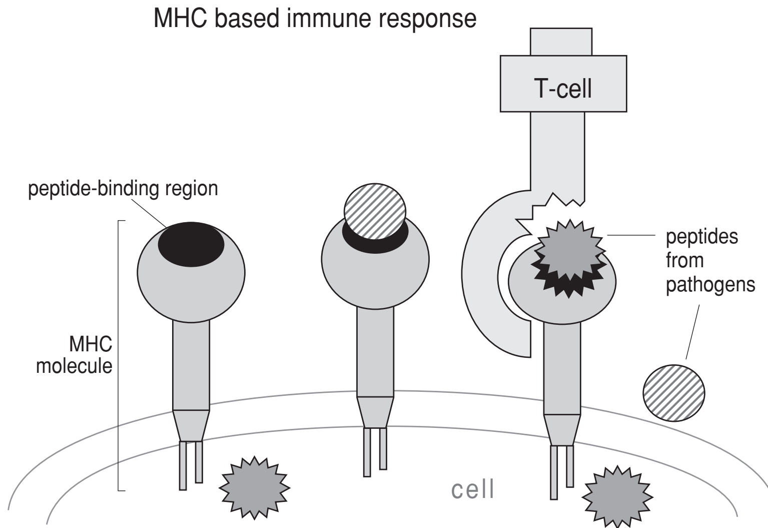


Figure A.1 MHC-I molecules bind peptides from the cytoplasm and present them to T-cells in the bloodstream. The T-cells then proliferate and an immune response is initiated to kill infected cells. Figure from Westerdahl (2003).

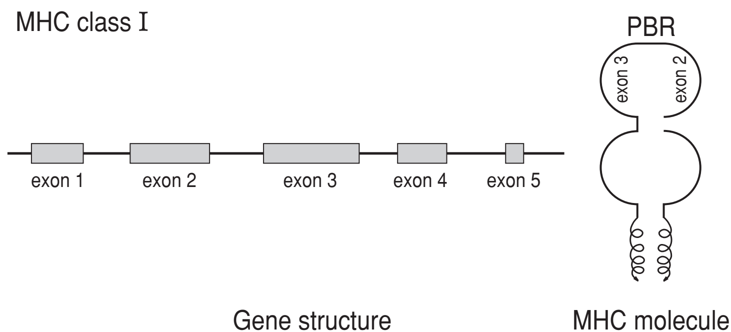


Figure A.2 Schematic representation of the DNA structure of MHC-I. Figure adapted from Westerdahl (2003).

highly similar in sequence and the same allele may be present at several loci (Beck *et al.* 1999; Hess and Edwards 2002; Fig. A.3.). By definition, genes belong to the MHC when they share a common history with genes involved in adaptive immunity and therefore cluster with known MHC alleles in a phylogenetic tree, even after they have lost their function (Hess and Edwards 2002). The fact that several highly similar gene loci exist within the MHC complicates the study of the MHC, even though distinguishing between loci is not necessary to assess functional diversity of the MHC, since functional loci will be very similar (Westerdahl 2007). Researchers

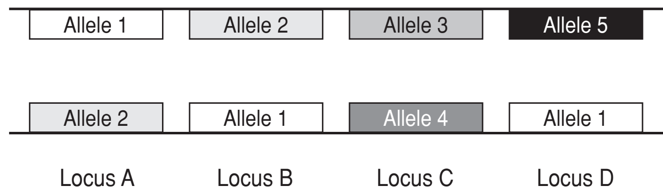


Figure A.3 Example of how MHC alleles may be distributed over several loci. The same MHC allele can be present at more than one locus. In this example, our heterozygosity estimate would be five different alleles.

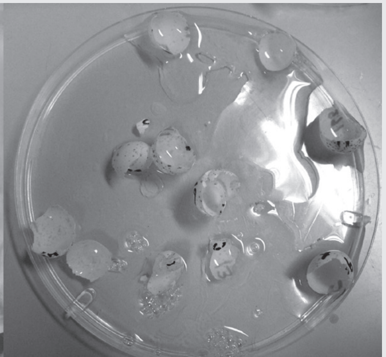
cannot determine how many gene loci they are amplifying at the same time when working on non-model species for which no genome map exists (Babik 2010). The closest approximation of the number of loci amplified may be the maximum number of alleles detected in an individual, divided by two (as individuals are diploid). MHC heterozygosity is often defined as the number of different alleles detected and can vary markedly between individuals (i.e. between 2 and 7 in the blue tit, this thesis, Chapter 5).

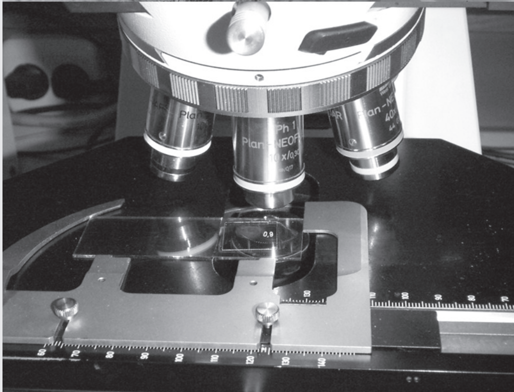
It is important to note the difference in the definition of heterozygosity between most other gene loci and studies of the MHC. The general definition of heterozygosity is the proportion of gene loci at which an individual is heterozygous (Freeland 2005). During the process of meiosis (the production of ova and sperm), a single copy of each locus ends up in each gamete (Weaver and Hedrick 1995). Since the two copies of each locus are then separated, heterozygosity according to the general definition is not directly heritable (but the heritability of heterozygosity depends on allele frequencies in the population, see Box D). Since we cannot determine from which locus an MHC allele originates, we cannot use this definition for MHC heterozygosity. Hence, the number of different MHC alleles found is used as

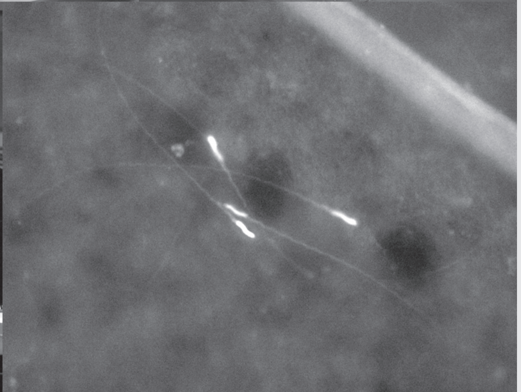
Table A.1 Explanation of terms used in this box (definitions based on Weaver and Hedrick 1995).

Term	Definition
Gene	Genetic unit encoding a single trait.
Locus	The position of a gene on a chromosome, at each locus two alleles are present (one inherited from the mother and one inherited from the father).
Alleles	Different forms of the same gene.
Transcription	The process by which an RNA copy of a gene is made, the RNA copy is later used to produce a protein (in the process called translation).
Intron	A region that interrupts the transcribed part of a gene.
Exon	A region of the DNA that is transcribed; a coding region.
Haplotype	A set of linked genes or DNA sequences that tend to be inherited together.

an estimate of heterozygosity (e.g. Richardson *et al.* 2005; Promerová *et al.* 2011, chapter 7, this thesis), because this is determined by the number of MHC alleles at which the individual is heterozygous. However, since heterozygosity in the MHC is calculated over several loci that are inherited together (the alleles on one of the strands together are called a haplotype), MHC-heterozygosity is (to a certain extent) heritable, since more heterozygous individuals are more likely to have heterozygous haplotypes. Hence, more MHC-heterozygous individuals are likely to have more MHC-heterozygous offspring, although there is also a possibility that one of the haplotypes contains many alleles, while the other contains few. In that case, some of the offspring are likely to be less diverse than their parents, while average heterozygosity in a brood is associated with parental heterozygosity (Partridge 1983).







Part I

Sperm availability

Chapter 2

Unhatched eggs: methods for discriminating between infertility and early embryo mortality

T. R. Birkhead, J. Hall, E. Schut and N. Hemmings

Abstract

Unhatched eggs are a common phenomenon in birds and are often referred to as being 'infertile', which (confusingly) can mean at least two things: (1) that the ovum has not been fertilized or (2) that the embryo has died during development. These two broad categories of hatching failure can be difficult to distinguish, particularly in the early stages of embryo development. We describe methods to distinguish between infertility (due to insufficient sperm) and early embryo mortality in passerine eggs using the Zebra Finch *Taeniopygia guttata* as a model. We also describe how we successfully adapted these methods for use on eggs from a wild species, the Tree Sparrow *Passer montanus*, collected after the incubation period, and show that sperm can be visualized on the perivitelline layer of unhatched eggs even several weeks after laying.

Ibis (2008), 150: 508–517

Introduction

Hatching success is an important component of avian reproduction and it is not unusual for one or more unhatched eggs to be found in nests after chicks have fledged (e.g. Koenig 1982). Confusingly, ornithologists often refer to unhatched eggs as 'infertile' when they show no sign of development (e.g. blood vessels or embryonic tissue) on visual inspection, and in general researchers rarely attempt to distinguish between eggs in which fertilization has occurred and those in which it has not. Further, since the contents of unhatched eggs incubated to full term are often 'addled' (i.e. yolk and albumen have amalgamated, and tissues have begun to decompose), field ornithologists generally assume such material cannot be usefully examined. Consistent with this view, Small et al. (2000) found that it was impossible to visualize sperm on the perivitelline layer (PVL) of Domestic Turkey *Meleagris gallopavo* eggs incubated for just 7 days, probably because the sperm cells had degraded (but see Wishart 1997).

Hatching failure results from two main causes; ova may fail to be fertilized, or fertilized eggs may fail to hatch. Fertilization is defined by the events that occur between penetration of the ovum by sperm and fusion of the male and female pronuclei (also referred to as syngamy). Embryonic mortality can occur at any stage of development and for a variety of reasons (Table 2.1). In eggs incubated for more than about 48 h, the presence of embryonic tissue and early blood vessel formation is usually sufficient to confirm that fertilization has taken place (Sellier *et al.* 2005). It is much more difficult to distinguish infertility (see Table 2.1, categories a–g) from early embryo mortality (EEM) that has occurred before any visible signs of embryo development are apparent (see Table 2.1, categories h and i). Studies of domesticated birds indicate that most embryo mortality is likely to occur either very early (including the period between fertilization and oviposition) or late in the incubation period, immediately prior to hatching (Lodge *et al.* 1971, Christensen 2001, Brun *et al.* 2004, Sellier *et al.* 2005).

Distinguishing between infertility and early embryo mortality is important for ornithologists interested in reproductive success (Bensch *et al.* 1994, Kempenaers *et al.* 1999, Morrow *et al.* 2002, Spottiswoode & Møller 2004), and has important implications for conservation (Gee 1983, Pletschet & Kelly 1990, Jamieson & Ryan 2000, Briskie & Mackintosh 2004). Infertility may be due to insufficient sperm (including, for example, sperm ejection by the female; Pizzari & Birkhead 2000), some defect of the sperm (which may in turn be a function of male health, condition, age, or contamination with toxic substances; Brillard 1990, Bakst & Wishart 1994, Bakst & Cecil 1997, Ackerman *et al.* 1999), or incompatibilities between the sperm and ovum (Zeh & Zeh 1997), resulting in biochemical rejection of sperm at the PVL. Technically, only a single sperm is necessary for fertilization in most organisms, but in birds, relatively large numbers are typically associated with the ovum at the time of fertilization, possibly because the female pronucleus within the germinal disc (GD) is a relatively small 'target' on the large, yolky ovum (see

Birkhead *et al.* 1994, Steele *et al.* 1994). In the Domestic Fowl *Gallus domesticus*, Wishart (1987) showed that following a single insemination of 100×10^6 sperm, the first ovum (surface area: 2828 mm^2) may have as many as 99 000 sperm associated with its PVL; successive ova had fewer sperm on their PVL, and by the time the number of sperm had fallen to about 200, an ovum had only a 50% chance of being fertilized. In the Zebra Finch *Taeniopygia guttata* – whose ovum is much smaller (surface area: 226 mm^2) than that of the fowl – the ovum had a 50% chance of being fertilized when 20 sperm were present on the PVL (Birkhead & Fletcher 1998).

Hatching failure due to embryo mortality may result from a variety factors, including exposure of eggs to variable temperature, humidity and microbial infection prior to the onset of full incubation (Meijerhof 1992, Cook *et al.* 2003, Beissinger *et al.* 2005, Cook *et al.* 2005), poor female condition or genetic incompatibility between the male and female, such as that resulting from inbreeding or outbreeding, including interspecific crosses (e.g. Lodge *et al.* 1971, Bensch *et al.* 1994, Etches 1996, Kempenaers *et al.* 1996, Kempenaers *et al.* 1996, Brillard *et al.* 1998, Christensen 2001, Sellier *et al.* 2005, Birkhead & Brillard 2007).

To date, few studies of wild birds have attempted to establish the cause of hatching failure (but see Birkhead *et al.* 1995, Svensson *et al.* 2007), whereas several studies of domestic birds have examined this issue in detail (e.g. Lodge *et al.* 1971, Kirby & Froman 1990, 1991, Bakst *et al.* 1997, 1998, Christensen 2001, Sellier *et al.* 2005). In the past, poultry biologists have claimed to be able to distinguish between infertile eggs and EEM either by candling eggs (see Sellier *et al.* 2005) or by visual inspection of the germinal disc in newly laid eggs, using criteria first

Table 2.1 Hatching failure categories and descriptions

Status	Hatching failure categories
Infertility	a) failure of the male to transfer sperm to the female
	b) failure of the sperm to be stored in the female's sperm storage tubules
	c) failure of stored sperm to be transported to the infundibulum (the site of fertilization)
	d) failure of sperm in the infundibulum to interact with the perivitelline layer (PVL) of the ovum
	e) failure of sperm to undergo the acrosome reaction and penetrate the PVL
	f) failure of the sperm pronucleus to locate the female pronucleus
	g) failure of the male and female pronuclei to fuse (syngamy)
Embryo mortality	h) embryo mortality occurring between fertilization and egg-laying
	i) embryo mortality occurring between egg-laying and the point where signs of embryonic development become apparent to the naked eye
	j) embryo mortality between egg-laying and hatching
	k) mortality during hatching

described by Kosin (1944). However, more recent studies have questioned the accuracy of conclusions drawn by these methods, which may overlook cases of EEM (Bakst & Akuffo 2002, Sellier *et al.* 2006).

If eggs that are infertile or have experienced EEM are incubated to full-term, their contents often begin to decompose (as is the case with many unhatched eggs found in the wild), rendering methods such as those used by poultry biologists inapplicable, because they depend upon examining fresh eggs. Species in which hatching failure is high are sometimes endangered, so it may not be appropriate to sacrifice potentially viable eggs to get fresh material on which the available techniques may be applied. For this reason, it has been suggested that the techniques developed by poultry researchers have limited value in conservation research (Small *et al.* 2000). The aim of the present study is two-fold. First, we present criteria for distinguishing unequivocally between the different causes of hatching failure (infertility and early embryo mortality) in passerine eggs. We describe methods for establishing these causes of hatching failure using the Zebra Finch as a model passerine. Secondly, we test the applicability of these techniques on fully incubated, unhatched eggs from a wild species, the Tree Sparrow *Passer montanus*, to establish the amount of information that may be obtained from unhatched eggs remaining in nests after chicks have hatched.

Methods and results

Part 1: Criteria and methods for discriminating between the possible causes of hatching failure

The initial part of this study was conducted on captive, domesticated Zebra Finches. The birds were part of a long-established population maintained at the University of Sheffield since 1985, in which there is no evidence that the level of inbreeding is any different from that of a wild population (see Birkhead *et al.* 2006). The eggs of paired and unpaired females were used to obtain fertilized and unfertilized eggs, respectively. Unpaired females had been isolated from males for at least 6 months. Since the maximum duration of fertility in the Zebra Finch is around 13 days (Birkhead *et al.* 1989), these females could not have laid fertile eggs.

Eggs were taken within 1–2 h of oviposition and placed in an artificial incubator at 37.5°C and examined after variable periods of time (0–120 h incubation) to establish the optimum duration of incubation before the eggs are examined using the methods described by Kosin (1944), Bakst and Akuffo (2002) and Sellier *et al.* (2006) (see Procedures, below). Our studies showed that incubation for 50–55 h provided an optimum compromise between: (1) sufficient development to be sure an egg was fertile and (2) sufficient disintegration of the PVL in fertile eggs to prevent the assessment of holes in the PVL (see below; also Jamieson & Ryan 2000). However, in eggs that were infertile or had undergone EEM at or before 50–55 h, the PVL maintained its integrity and could be examined after at least 120 h incubation and

probably longer (see Methods, Part 2). This indicates that disintegration of the PVL may be linked to development rather than temperature.

To establish unequivocally the cause of hatching failure, three aspects of an egg must be examined: (1) the gross appearance of GD, (2) the presence or absence of sperm and holes on the PVL over the GD, and (3) the presence or absence of cell nuclei in the GD tissue.

The criteria (summarized in Table 2.2) for distinguishing between eggs of different status were:

Fertile and developing normally. The GD at 50–55 h of incubation typically comprises an embryo at the primitive streak stage (stage IV–V according to Hamburger & Hamilton's (1951) scheme; see Fig. 2.1A). At 50–55 h of incubation, blood vessels are not apparent (these first appear at 72 h and can be seen on candling). Holes and sperm are present on the PVL over the GD and large numbers of cell nuclei are present in the GD (Fig. 2.1D–F).

Fertile but dead – early embryo mortality. The appearance of the GD after 50–55 h of incubation is quite variable, ranging from that of an unincubated egg (Fig. 2.1C) to that of an egg incubated for 50–55 h (see above). Sperm and holes are present in the PVL over the GD. Cell nuclei are present in the GD, their number depending on the stage of development at which embryo death occurred.

Table 2.2 Criteria for distinguishing between eggs of different fertility status. GD, germinal disc.

Status	GD appearance after 50-55 h incubation	Sperm/holes in PVL	Nuclei in GD
(a) Fertile and developing normally	Embryo at stage IV-V (Hamburger & Hamilton 1951) - primitive streak formed See Fig. 2.1A	Present; including holes in PVL over GD	Abundant nuclei present Nuclei regular and round in appearance; sometimes elongated
(b) Fertile but dead	Embryo at any stage up to IV-V (i.e. disparity between incubation period and developmental stage)	Present; including holes in PVL over GD	Nuclei present but relatively fewer than in a normally developing egg
(c) Infertile	Dense white appearance No rings visible Vacuoles present; sometimes appears granular See Fig. 2.1B	None or very few (i.e. insufficient) sperm/ holes in PVL	Nuclei absent (but see (d))
(d) Partheno-genesis	Resembles an infertile GD See Fig. 1B	No sperm/holes in PVL	Small number of irregularly shaped nuclei present (Schut *et al.* 2008)

Infertile. The appearance of the GD at 50–55 h of incubation is distinctive, comprising a dense asymmetric whitish spot with no visible rings (Fig. 2.1B; cf. the unincubated, fertile egg in Fig. 2.1C). The GD of an infertile ovum often contains vacuoles, giving it a granulated appearance (Fig. 2.1B). However, the main criteria

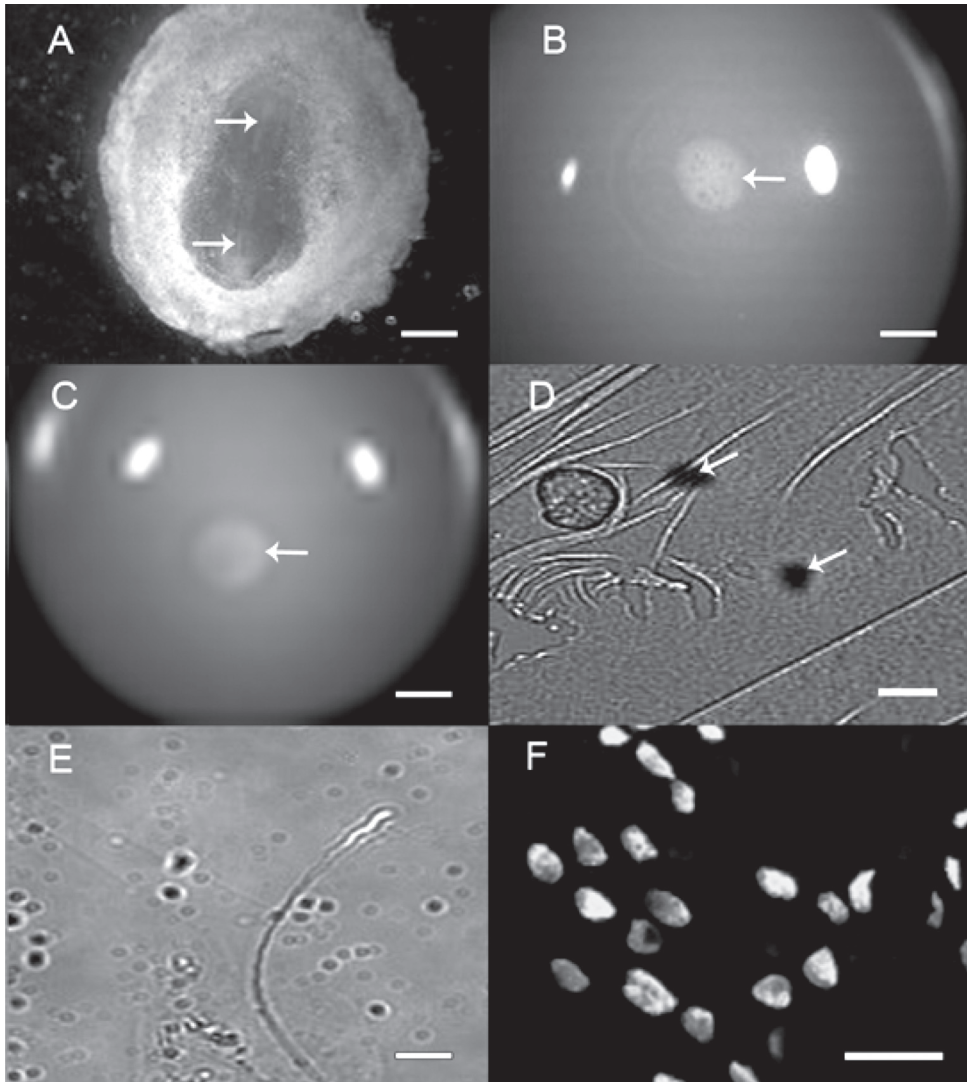


Figure 2.1 Zebra Finch. (A) Fertile germinal disc (GD) after 55 h of incubation showing primitive streak (arrows; scale bar = 1 mm). (B) Infertile GD after 55 h of incubation (scale bar = 1 mm). (C) Fertile, unincubated GD (scale bar = 1 mm). (D) Holes in the inner perivitelline layer (PVL) made by sperm entering the ovum (scale bar = 10 μm). (E) Sperm on outer PVL stained with Hoechst 33342 (scale bar = 10 μm). (F) nuclei from developing GD stained with Hoechst 33342 (scale bar = 10 μm). Note: Additional bright spots on yolk in (A–C) are reflections from the light-source.

distinguishing an infertile ovum is the complete absence of sperm and holes in the PVL over the GD, and of nuclei from the GD. Occasionally parthenogenetic development occurs in infertile Zebra Finch eggs (Schut *et al.* 2008; see Table 2.2 (d)), and, when it does, the appearance of the GD at 50–55 h of incubation is similar to that of an infertile ovum. Parthenogenetic eggs have no holes in the PVL over the GD, but relatively small numbers of irregular nuclei are present in the GD (Kosin 1945, Schut *et al.* 2008).

Procedures

Here we describe methods for obtaining the required information on the GD and the PVL, together with a brief account of the relevant biology.

Germinal disc (GD)

Cut the egg open with fine scissors and discard the albumen. Place the yolk in a small Petri dish with the GD uppermost and examine using a low power (10– 20×) binocular dissecting microscope illuminated with gooseneck lighting (e.g. Olympus KL 1500 halogen cold light source) fitted with blue filters to increase the contrast between the GD and surrounding yolk. Compare the GD with the images in Figure 2.1.

Fertilization occurs in the infundibulum (Olsen & Neher 1948). In chicken, turkey, and most passerines, including the Zebra Finch, the egg is laid (oviposited) 20–26 h after fertilization (Romanoff 1960, Eyal-Giladi & Kochav 1976, Gupta & Bakst 1993, T.R. Birkhead pers. obs.). Between fertilization and egg-laying the ovum is transported down the oviduct while the albumen and shell form around it (Romanoff 1960). During this same period the fertilized GD undergoes rapid cell division resulting in the formation of a blastoderm prior to egg-laying (Eyal-Giladi & Kochav 1976, Watt *et al.* 1993). In fertile eggs of the chicken, turkey, Zebra Finch, and probably most other avian species, the *area opaca* and the *area pellucida* are visible upon oviposition (Watt *et al.* 1993). The *area opaca* is a dense white ring in the periphery of the GD, whereas the *area pellucida* is the paler centre of the fertilized GD (Fig. 2.2).

Nuclei in the GD

To check for the presence of cell nuclei, and hence the presence of a developing embryo, examine the GD as follows (from Gupta & Bakst 1993). Place a doughnut-shaped piece of filter paper, whose inner hole is about 1 mm larger in diameter than the germinal disc and whose outer ring is about 2 mm wide, on the yolk over the GD. Then, using a small pair of scissors, cut through the PVL around the outer edge of the filter paper ring. Using a pair of forceps, lift the filter paper with the PVL attached away from the yolk and place, with the GD uppermost, into a Petri dish containing phosphate-buffered saline (PBS). Using a hair-loop (i.e. a piece of human hair taped to a cocktail stick to form an oval loop about 2 mm across and 5 mm long), gently remove the excess yolk from the GD, which appears whitish when

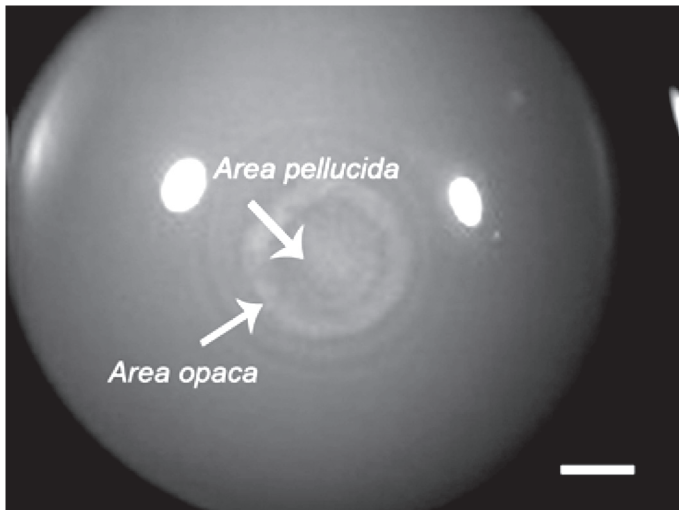


Figure 2.2 Fertile Zebra Finch egg after 24 h of incubation showing the opaque outer ring, the area opaca, and the pale inner area, the area pellucida (scale bar = 1 mm).

clean. Separate the GD from the PVL and place the GD on a microscope slide with 10 μL of Hoechst 33324 fluorescent dye (0.05 mg/ml). Disperse the GD material in the dye by gentle flushing in and out of a pipette, then add a coverslip and leave the slide to incubate in the dark for approximately 5 min. Examine the slide using a fluorescence microscope with a BP 340–380 excitation filter and a LP 425 suppression filter. Any nuclei present will be stained bright blue (Fig. 2.1F). Numbers of nuclei can be counted either systematically by scanning the slide (when numbers are low), or by counting fields of known area and extrapolating (when numbers are higher), to provide a crude index of the stage of embryo development (Kochav *et al.* 1980).

In normally developing eggs, cell division begins 6–8 h after fertilization (Eyal-Giladi & Kochav 1976, Watt *et al.* 1993) and oviposition occurs 16–18 h later. The fertile GD therefore contains numerous cell nuclei at oviposition and the number of nuclei continues to increase as development proceeds (Kochav *et al.* 1980, Liptói *et al.* 2004). In the Zebra Finch, the mean number of nuclei (± SD) per egg was highly variable and increased, as expected, with the duration of incubation (0 h eggs, 15565 ± 7755, n = 9 eggs; 24 h eggs, 26547 ± 7048, n = 9; 48 h eggs, 102812 ± 9444, n = 7). The shape and size of nuclei should be recorded to distinguish between normal and parthenogenetic development (Schut *et al.* 2008).

Holes and sperm on the PVL overlying the GD

Remove the PVL overlying the GD from the doughnut-shaped piece of filter paper and separate the inner and outer layers of the PVL by gentle agitation in PBS,

teasing the layers apart with fine forceps. Examine the inner PVL using a light microscope with dark field optics to check for holes (left after sperm have entered the ovum; Bramwell *et al.* 1995; Fig. 2.1D). Place the outer PVL on a microscope slide with a drop of Hoechst dye and view in the same way as the GD material (see above) to visualize any sperm nuclei (Fig. 2.1E).

When sperm reach the ovum, the sperm plasma-lemma binds to a sperm receptor on the outer surface of the inner PVL overlying the GD. The sperm then undergoes an acrosome reaction (Franklin 1970, Stepinska & Bakst 2006) and hydrolyses a path through the inner PVL leaving a hole. The sperm head enters the GD region of the ovum and is engaged by the microvilli formed by the oolemma. After a number of sperm have penetrated the ovum, the outer PVL is laid down, preventing the entrance of further sperm. Sperm that have not entered the ovum are trapped between the inner and outer PVLs (Wishart 1987).

Part 2: Applicability to unhatched eggs found in the field

The second part of this study was conducted on the Tree Sparrow, a small sexually monomorphic passerine in which high levels of hatching failure have been reported (Svensson *et al.* 2007). Our study population was one of those described by Svensson *et al.* (2007), located on organic farmland just outside Uppsala, Sweden. The present study was carried out in mid- to late June 2007 so all eggs were likely to have been from second clutches.

Unhatched eggs were removed from nests containing chicks aged greater than 2 days including nests from which chicks had fledged. Within Tree Sparrow broods hatching is synchronous, with most eggs hatching on the same day (Summers-Smith 1995), so all removed eggs were likely to remain unhatched. We obtained 40 unhatched eggs from 20 different pairs. By estimating the age of the live chicks in the nest (from overall body size, M. Svensson pers. comm.), and adding this to the incubation period (*c.* 12 days; Summers-Smith 1995), an estimate of the 'age' (i.e. period since being laid) of unhatched eggs was obtained. By the time of examination, the eggs were 15–25+ days old ($n = 40$) (after being laid) and had experienced either incubation or ambient temperatures, or both.

The unhatched Tree Sparrow eggs were kept in cool storage for 2–15 days to limit further deterioration. Originally, we intended to examine eggs using the techniques described above (Part 1). However, due to their age and possibly the effect of transportation to the lab, the egg contents had begun to degrade and the yolk and albumen had amalgamated (Fig. 2.3A), so these methods were not applicable. We therefore adapted our methods, as follows.

In all cases the yolk had disintegrated and become mixed with the albumen (Fig. 2.3A). This mixture was emptied into a Petri dish and a small amount of PBS added to dilute it. The remains of the degraded GD and PVL appeared as a small dense white bundle amongst the yolk and albumen mixture, visible with the naked eye (Fig. 2.3A). This bundle was removed using fine forceps and placed in PBS. The GD matter was then separated from the PVL under a dissecting microscope using fine

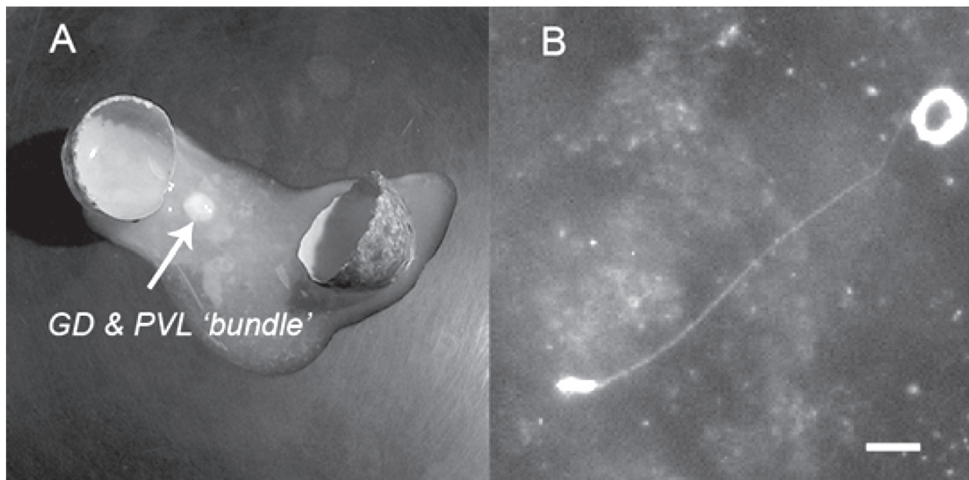


Figure 2.3 Tree Sparrow. (A) The contents of a fully incubated, addled tree sparrow egg on opening, showing the GD and PVL 'bundle'. (B) Sperm on PVL of fully incubated, addled egg (scale bar = 5 μm).

forceps and a hair-loop. In most cases the GD was strongly adhered to the PVL and difficult to remove, meaning it was not always detached in its entirety. The removed GD matter was placed on a microscope slide with a drop of the fluorescent dye Hoechst 33342, and examined for the presence of any nuclei as described in Part 1.

The remaining PVL was then cleaned by washing gently in PBS and placed on a microscope slide. Probably as a result of degradation, the PVL left in the egg appeared to be only a small portion of the original entire PVL. As it was strongly attached to the GD, it is likely that this was the PVL overlying the GD. The PVL was examined in the same way as described in Part 1 to visualize and count any sperm present.

On opening, the egg contents were disintegrated in all but one egg, which contained an almost fully formed chick. No other eggs showed any obvious sign of development, although six eggs contained some dark red/brown matter that was strongly adhered to the GD, possibly including degraded blood vessels or embryonic tissue. Material from the first four eggs was not successfully salvaged and examined, due to initial difficulties in adapting the techniques. Sufficient GD and PVL material for examination was therefore obtained from 36 (90%) of the 40 eggs. In all of these eggs, nuclei and sperm were found in the GD material and PVL respectively. Sperm appeared similar to those found in the fresh eggs, with the head and midpiece still intact and tail attached in most cases (Fig. 2.3B), and the number of sperm was generally high (total number on the area of PVL above GD ranged from 21–202; mean = 88.87 ± 47.04 SD, n = 36). However, holes could not be visualized, presumably because of degradation of the inner PVL.

Discussion

The methods we describe in Part 1 of this study can be used to distinguish between passerine eggs that are either infertile or have undergone EEM prior to, or during, the first 50–55 h of incubation. After 50– 55 h of incubation the appearance of the GD alone can be used to identify eggs that have been fertilized and are developing normally. When a disparity exists between the duration of incubation and the appearance of the GD, EEM can be established from the presence of holes and sperm in the PVL, and the presence of nuclei in the GD. The fact that, in Tree Sparrows at least, sperm can be detected on the outer PVL, and nuclei detected in the GD, of fully incubated eggs (up to 25 days old followed by a further 15 days in cool storage) is an important finding, since it has previously been assumed that unhatched eggs incubated to full-term, whose contents have become 'addled', cannot provide useful information about fertilization (e.g. Small *et al.* 2000). The techniques described in Part 1 of this study, to examine fertility status of passerine eggs, were based on the yolk remaining intact and therefore not applicable to the Tree Sparrow eggs we examined in Part 2. However, the techniques in Part 1 were successfully adapted and used to yield similar results as they would on fresher eggs. Despite a certain degree of degradation of both GD and PVL matter, both nuclei and sperm could still be clearly seen under the microscope. The only apparent limitation to examining such addled eggs was that holes in the inner PVL could not be seen, presumably due to degradation of the inner PVL. Although it was not possible to examine the inner PVL for holes, it was still possible to infer whether fertilization had or had not occurred from the numbers of sperm present on the PVL. Birkhead and Fletcher (1998) found that the Zebra Finch ovum had a 50% chance of being fertilized when 20 sperm were present on the entire PVL; this number may be used as a 'threshold' value, i.e. with more than 20 sperm on the PVL and nuclei present in the GD, it is likely that fertilization has occurred. In contrast, with fewer than 20 sperm on the PVL, we cannot exclude the possibility that any nuclei found in the GD may be of parthenogenetic origin. This threshold number of sperm needs to be adjusted in different species depending on ovum size; birds with relatively larger or smaller ova (the yolk in a laid egg) will require relatively more or fewer sperm to achieve fertilization (Birkhead *et al.* 1994).

The efficacy of the techniques described in Part 1 decreases for fertile eggs as developmental stage advances; by the time blood vessels are beginning to form (around 72 h), the PVL has started to disintegrate, precluding its examination (although, by com-parison, the developing embryo appears to retain much of its integrity). However, because disintegration of the PVL appears to be halted if development stops (that is, if EEM occurs), despite incubation continuing, a longer window of opportunity exists for assessing the status of undeveloped eggs. Our data indicate that, for eggs that are not developing, it is still possible to establish the presence or absence of holes and sperm on the PVL after 4–5 days (96– 120 h) of incubation and hence distinguish unequivocally between infertile and EEM eggs.

Further, after a much longer period of incubation and/or ambient temperature (>25 days), sperm on the PVL and nuclei in the GD are still visible; only the inner PVL has completely degraded by this time, so holes cannot be seen. These techniques would now benefit from further experimental validation; for example, by comparing fully incubated eggs (1) in which EEM has been experimentally induced with (2) those known to be infertile due to lack of sperm.

Our findings provide a protocol for researchers aiming to establish the causes of hatching failure in wild birds. If the species under investigation is of conservation concern, the best course of action may be to allow eggs to be incubated to full-term, removing unhatched eggs for examination after the other chicks in the brood have hatched, or a few days beyond the normal incubation period if no eggs hatch. Here we show that sufficient information can be obtained from such eggs to infer, with a reason-able degree of confidence, whether or not the eggs had been fertilized, from the presence of sufficient sperm on the PVL and nuclei in the GD. This method avoids the risk of sacrificing potentially viable eggs, but does not provide unequivocal evidence of sperm–ovum interaction (i.e. the presence of holes in the inner PVL).

If researchers wish to examine eggs for holes as well as sperm and GD nuclei, they may wait 4– 5 days after incubation begins and then candle eggs for development. The rate at which embryos develop differs both between species (Sellier *et al.* 2006, T.R. Birkhead pers. obs.) and (obviously) with the intensity of incubation. However, examining eggs 4–5 days after laying should in most cases be sufficient to establish whether any development (i.e. through the presence of blood vessels) has occurred. This method provides a means by which researchers can minimize the risk of sacrificing a fertile, developing egg without waiting until the end of the incubation period. In other words, researchers should candle eggs 4–5 days after laying to determine (as far as possible) whether embryo development has occurred (Wilson 1994). If the egg shows no signs of development, the egg can be opened for more detailed examination. For eggs with unpigmented shells, candling is relatively easy, and it is straightforward to establish whether a developing embryo or associated blood vessels are present, indicating that fertilization has occurred. However, if eggshells are heavily pigmented, candling is more difficult, and the researcher faces a greater risk of sacrificing a viable egg.

Acknowledgements

The authors thank M. Bakst, J.P. Brillard, R. Montgomerie, and two anonymous referees for valuable advice and comments on the manuscript, and M. Svensson for assistance with fieldwork. T.R.B. was funded by a grant from the Leverhulme Trust and J. Hall by a Nuffield Student Bursary.

CHAPTER 3

No evidence for declining fertilization success due to sperm depletion over the laying period in female blue tits (*Cyanistes caeruleus*)

Elske Schut, Oscar Vedder, Jan Komdeur and Michael J. L. Magrath

Abstract

Hatching success can be an important determinant of reproductive success in birds and key reasons for hatching failure include embryonic death and the failure of egg fertilization. A mismatch between male and female reproductive behaviour could be responsible for the occurrence of infertile eggs. Since in most passerines copulation activity declines or ceases after laying of the first egg, we hypothesised that the likelihood of fertilization failure may increase over the laying sequence. Using a passerine species with an extraordinarily large clutch, the blue tit (*Cyanistes cearuleus*), we investigated whether (i) eggs laid later in the laying sequence are more often infertile, (ii) sperm number trapped inside eggs decreases over the laying sequence and (iii) sperm number inside eggs relates to egg fertility. We found no pattern of fertilization likelihood over the laying sequence, nor evidence that larger clutches contained more infertile eggs. Similarly, there was no indication that the proportion of fertile eggs in a clutch correlated with the number of sperm within eggs, or that females laying larger clutches had stored more sperm. Moreover, within clutches, sperm number did not vary between the 2nd and 10th egg. The only factor associated with proportion of infertile eggs within clutches was mean egg volume, with a higher proportion of infertile eggs occurring in clutches of larger eggs. Our findings suggest that copulation continues throughout most of the laying period in the blue tit.

Introduction

Eggs that fail to hatch are a well known phenomenon in birds and may form an important limitation to reproductive success. A review of previous studies in passerine birds, reported an average of 8.8% unhatched eggs (Koenig 1982). There are two causes for hatching failure: (early) embryo mortality (e.g. due to genetic incompatibilities or improper incubation) and the eggs remaining unfertilized (Birkhead *et al.* 2008). Although potentially only one sperm is needed for fertilization, evidence from zebra finches (*Taeniopygia guttata*) suggests that eggs with less than 20 sperm have a 50% reduced hatchability (Birkhead and Fletcher 1998). Hence, egg laying females should attempt to have sufficient sperm available to fertilize each egg in their clutch.

It is a well-known phenomenon that female birds can store sperm for up to several weeks in specialized sperm storage tubules (SST) located at the utero-vaginal junction in the female reproductive tract (Bakst 1987). The capacity to store sperm increases the length of the period during which copulations may lead to the fertilization of one or more eggs in the females' clutch. Hence, females need not copulate immediately prior to each ovulation to fertilize consecutive eggs in their clutch but can instead store sperm following copulation and release it as required from the SST over the course of egg laying (Birkhead and Møller 1998). The absence of a need for females to copulate prior to the ovulation of each consecutive egg is reflected in the copulation patterns observed in the wild. In the majority of passerine species, copulation starts some days before the start of egg laying and ceases or declines considerably in frequency after the onset of egg laying (Birkhead *et al.* 1987).

Despite the capacity of females to store sperm, the number of sperm in the SST declines generally rapidly after the start of egg laying (Birkhead *et al.* 1993b; Briskie 1994; Birkhead *et al.* 1997). This decline is most likely due to a combination of the release of sperm for the fertilization of the remaining eggs, sperm leaking from the SST (passive sperm loss; Wishart 1987) and the degradation of sperm in the female tract (Birkhead *et al.* 1993b; Briskie 1994; Pellat 1997). Based on this decline of sperm in storage over the laying sequence, we hypothesize that female sperm depletion (Birkhead and Møller 1992) may on occasions limit the number of fertile eggs that a female can lay.

If females are subject to depletion, we expect that eggs produced later in the laying sequence will be at greater risk of fertilization failure. Additionally, a mismatch in the timing of male and female reproductive behaviour could result in infertility of the earliest eggs in the clutch if sperm production in the male partner is delayed (Cabezas-Díaz and Virgós 2007). Potential evidence for these processes was found in the pied flycatcher (*Ficedula hypoleuca*) where hatching failure due to infertility occurred more frequently in both the first and last eggs in a clutch (Potti and Merino 1996). Both in the house sparrow (*Passer domesticus*; Cordero *et al.* 1999) and the red-legged partridge (*Alectoris rufa*; Cabezas-Díaz and Virgós 2007), infertile eggs were most likely to occur in the first half of the clutch. It is unclear

whether these studies represent cases of inadequate sperm, since they did not differentiate between true infertility and embryonic mortality at a very early stage (See Birkhead *et al.* 2008).

Whether eggs have been fertilized or not can be determined using the method described by Wishart (1987); Birkhead *et al.* (2008). By dissecting the perivitelline layers around the yolk and staining them using a fluorescent dye, the number of holes created by sperm penetrating the ovum and the number of sperm trapped between the inner and outer perivitelline layers can be determined. The number of sperm trapped between the layers and the number of holes in the inner layer together provide an index of the number of spermatozoa present at the site of fertilization (Birkhead and Fletcher 1994). The number of holes in the inner layer correlates positively with the number of sperm trapped between the layers in birds (Birkhead and Fletcher 1994; Birkhead *et al.* 1994a) including the blue tit (*Cyanistes caeruleus*; Lifjeld *et al.* 2000).

This study investigates egg fertility in the blue tit, which has the largest average clutch known among passerines. Since copulation ceases early during the laying sequence in many passerine species, we hypothesize that eggs produced later in the laying order by female blue tits may be more likely to be infertile because of sperm depletion. Blue tits have a high copulation rate (7–28 copulations per day; Kempenaers *et al.* 1992), but it is unclear whether copulation continues throughout the egg laying phase. A previous study found that the number of spermatozoa on blue tit eggs declines over the laying sequence (Lifjeld *et al.* 2000), suggesting that copulation activity may cease or decrease before clutch completion. The aims of this study are to (i) determine whether the likelihood of egg infertility varies over the laying sequence, (ii) determine whether sperm number within eggs decreases with laying order and, (iii) determine whether the number of sperm within eggs predicts fertility.

Methods

Sample collection

The blue tit is a hole-nesting passerine that will breed in nest boxes when provided. We studied blue tits in a nest box population at "*De Vosbergen*" estate, near Groningen, the Netherlands (53°08′N, 06°35′E) in the breeding seasons of 2006, 2007 and 2009. All occupied nest boxes were checked daily from the beginning of April, and newly laid eggs were numbered to record the laying sequence. Blue tits in our population laid on average 12.2 eggs per clutch in the years 2006, 2007 and 2009 (range 5–19). The width and length of eggs were measured on the day of laying (to the nearest 0.1 mm). Egg volume (V) was calculated using the equation $V = 0.51 \times L \times W^2$ (Hoyt 1979). As part of other studies (Magrath *et al.* 2009), the majority of clutches was collected 11–12 days after the start of incubation and replaced with plastic model eggs to prevent the female from deserting the nest.

Eggs were hatched in separate compartments within incubators so that the hatching order could be determined (following the protocol in Magrath *et al.* 2009). Chicks were returned to their nest of origin within a few hours after hatching. Eggs remaining unhatched well past the expected hatching date were broken open to identify signs of embryo development. Eggs were scored as infertile when no signs of embryonic development were visible to the naked eye. Nests that failed completely, e.g. due to abandonment or predation, were excluded from our analyses. A total of 279 clutches was used for the analyses of the relationship between apparent egg fertility and laying order and clutch size.

Processing of eggs

To determine sperm counts, eggs were collected on the day they were laid and stored at 4°C until microscopic examination. Most eggs were processed within nine days of laying (range 0–24 days, well within the maximum period described by Birkhead *et al.* 2008). In 2007, the first half of several clutches was collected for a different experiment and we determined sperm count of seven first eggs, 20 second eggs, five third eggs, 30 fourth eggs, three fifth eggs and one sixth egg from a total of 35 clutches. In 2009, we specifically collected eggs 2 ($n = 30$) and 10 ($n = 32$) to determine sperm counts. The complete database contained eggs from 70 clutches.

We used the protocol described in detail in Chapter 4 to determine sperm counts. In short, we separated yolk and albumin and the egg yolk was floated in PBS (Invitrogen, California, USA). The yolk was cut into two halves and rinsed in PBS to clean yolk off the perivitelline layers. Subsequently, the first half of the perivitelline layers was spread out on a microscopic slide and a drop added of Hoechst 33258 dye (Invitrogen, California, USA). The layers were covered with a cover slip and left to incubate in the dark for approx. five minutes. The slide was then examined under a fluorescence microscope (Optech B5 PT, Orbi solutions, Aalten, the Netherlands) with a BP 340-380 excitation filter and LP 425 suppression filter. Sperm were counted by scanning through the sample using a 40× objective. The second half of the perivitelline layers was left in PBS and processed immediately after the first. The total number of sperm cells found in both halves was used for analyses and referred to as “sperm count”. Sperm counts were always performed by the same person (ES).

We used the method described by Wishart (1987); Birkhead *et al.* (2008) to count sperm on the perivitelline membranes of blue tit eggs. We did not, however, count holes in the inner perivitelline layer or search for microscopic signs of embryo development, to confirm that unhatched eggs were infertile. These procedures are laborious and time consuming and we elected to record whether or not there were signs of embryonic development that were visible to the naked eye. We should be able to detect an effect of sperm depletion on hatchability without separating between infertility (i.e. a lack of sperm) and inviability (i.e. failed embryo development) if the effect is strong enough. Throughout the manuscript, we use the term “infertile” for eggs that failed to hatch and showed no visible signs of embryo development.

Statistical analyses
To test whether egg infertility was related to laying order in the clutch, we used logistic regression to assess the relationship between egg fertility a) and absolute position in the laying sequence (both linear and quadratic), year of study, clutch size, egg volume and the relationship between egg fertility and b) relative position in the laying sequence (both linear and quadratic), year of study, clutch size and egg volume separately. Relative laying sequence provides a better measure of relative position within clutch because clutched differed considerably in size. To obtain relative egg number, we transformed egg number in the clutch to a scale of zero to one (i.e. egg number/total number of eggs laid). Since there is previous evidence that egg mass and egg hatchability are positively correlated (Potti and Merino 1996), we corrected for egg volume in all our analyses. Egg volume is strongly correlated with egg mass in this population (Magrath unpublished data). Since 28 females produced more than one clutch in our dataset over several years (23 females with two clutches, five females with three clutches), female identity was included as an additional random factor in the hierarchical models (see below).

To test whether females that laid larger clutches stored more sperm, we used a mixed model examining the effects of year, clutch size and egg volume on sperm count. The diameter of the yolk was previously shown to accurately predict the number of sperm on the PVL of an egg (across species). More sperm may be trapped by larger ova due to their larger surface, or since more sperm are released from storage for the fertilization of larger ova, as sperm get diluted on the larger surface and more sperm are required for fertilization (Birkhead *et al.* 1994a). Hence, we included egg volume in this analysis. It has been shown that egg mass and ovum mass are correlated in a passerine (Christians and Williams 2001) and since egg mass and egg volume are correlated (see above) we assume that egg volume provides an indication of ovum mass. We performed this same analysis using 10th eggs only, to test for the possibility that females producing larger clutches (also) copulated more during the second half of the laying period. We only dissected 10th eggs in 2009 so the variable study year was not included in this analysis. This dataset contained 10th eggs from four clutches of 11 eggs, seven of 12 eggs, seven of 13 eggs, five of 14 eggs, one of 15 eggs, two of 16 eggs and five clutches of 17 eggs. Sperm counts were log10-transformed to normalize the distribution of data.

To test whether sperm count was associated with egg fertility we used logistic regression to examine the effects of study year, clutch size, average egg volume and average sperm count on the likelihood that a clutch contained infertile eggs. Since we could not assess the fertility of eggs for which we counted sperm (because eggs were destroyed in the process), we could perform this analysis only between clutches rather than assessing within clutch differences at the level of the individual egg.

The above analyses were performed in MLwiN 2.02 (Rasbash *et al.* 2005) using mixed models with female, clutch and egg identity as levels for the within clutch analyses, clutch and egg identity as a level in sperm count analyses and clutch as a

level in the between clutch analysis. All logistic regressions used a binomial distribution with a logit link function. We used backwards elimination to derive final models, starting with full models that included all factors and eliminating non-significant terms. Significance was assessed using, the Wald statistic (approximating the χ^2 distribution) and two-tailed probability testing in all cases. Test statistics were presented as when re-entered variables into the final model.

We tested for a consistent change in sperm numbers between the 2nd and 10th eggs within clutches using a paired t-test in Statistica version 9 (StatSoft inc, Tulsa, USA).

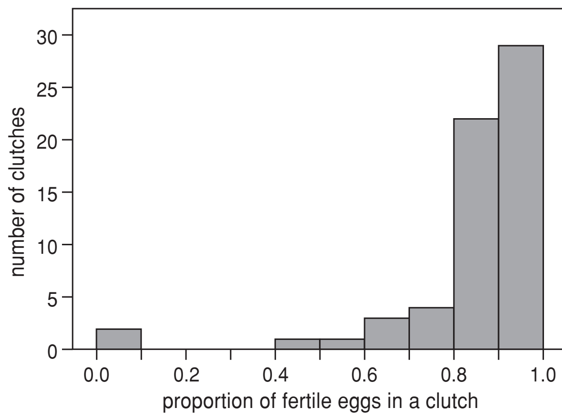


Figure 3.1 Frequency histogram for all clutches of the proportions of fertile eggs per clutch (Fertility was known for n = 271 clutches, n = 209 clutches in which all eggs were fertile were left out, 62 clutches with infertile eggs were included).

Table 3.1 The number of eggs showing no visible signs of embryo development over the three study years. The number of infertile eggs and the number of clutches containing infertile eggs are given as proportions of the total number of eggs and clutches, respectively.

Year:	2006	2007	2009
Number of infertile eggs	40/1051 (3.8%)	40/897 (4.5%)	25/999 (2.5%)
Number of nests containing infertile eggs	23/100 (23%)	23/90 (26.5%)	15/89 (17%)
Average number of infertile eggs per nest	0.40 (0-8 range)	0.44 (0-10 range)	0.28 (0-7 range)

Results

Egg infertility

In the three study years, the proportion of infertile eggs in the population ranged between 2.5 – 4.5%. Between 17.0 – 26.5% of all clutches contained infertile eggs and the average proportion of infertile eggs within a clutch ranged between 0.28 – 0.44 in the three years (table 3.1, figure 3.1).

Within clutch patterns of egg fertility

We hypothesized the last eggs in the clutch would be most likely to be infertile and to fail to hatch if females are sperm limited. However, we found no linear or quadratic relationship between the likelihood of egg infertility and laying sequence for either absolute or relative laying order (table 3.2). Nor was there a significant effect of year, clutch size or egg volume on the likelihood of egg infertility (table 3.2).

Sperm counts

We found no evidence for a consistent change in sperm count between the 2nd and 10th eggs of the same clutch (n = 27 clutches, average 2nd eggs: 290.4 ± 60.4 SE, average 10th eggs: 292.0 ± 38.4 SE; Paired t-test, t = –0.98, df = 26, p = 0.34). Similarly, there was no evidence that females laying a larger clutch stored more

Table 3.2 Logistic regression of year, clutch size, egg volume and a) position in the laying sequence (both linear and quadratic effect) and b) relative position in the laying sequence (both linear and quadratic effect) on the likelihood of being fertile.

a. Dependent variable: whether an egg is fertile or not				
Predictor:	Estimate (SE)	χ^2	df	p
Position in the laying sequence	0.011 (0.027)	0.18	1	0.67
Quadratic effect of position in the laying sequence	–0.004 (0.006)	0.47	1	0.49
Year		1.67	2	0.43
Clutch size	0.113 (0.078)	2.07	1	0.15
Egg volume	0.002 (0.002)	2.36	1	0.12
b. Dependent variable: whether an egg is fertile or not				
Relative position in the laying sequence	0.073 (0.035)	0.04	1	0.84
Quadratic effect of relative position in the laying sequence	–0.922 (1.337)	0.48	1	0.49
Year		1.67	2	0.43
Clutch size	0.113 (0.078)	2.07	1	0.15
Egg volume	0.002 (0.002)	2.36	1	0.12

sperm, as eggs from larger clutches did not differ in sperm count when using all eggs in our analysis or only 10th laid eggs. Average sperm count per clutch did differ between years (table 3.3), reflecting a difference in variation in sperm count between 2007 (196.8 ± 16.0 SE) and 2009 (282.1 ± 31.3 SE).

Clutch level correlates of infertility

Because copulation ceases early during the laying sequence in many passerine species, we hypothesized that, sperm depletion may result in a greater risk of egg infertility, especially in a species with a large clutch size. Our sperm count dataset contained 65 clutches for which fertility was known; five of these clutches contained infertile eggs. We found no evidence that the proportion of fertile eggs in a clutch was related to sperm count, clutch size or year (table 3.4). The only factor that was significantly associated with the proportion of infertile eggs was average egg volume (table 3.4); clutches with larger eggs contained a greater proportion of infertile eggs (figure 3.2).

Table 3.3 Mixed model of the effects of year, clutch size and egg size on the number of sperm counted on the perivitelline layers.

Dependent variable: sperm count (average for the eggs sampled from each clutch)				
Predictor:	Estimate (SE)	χ^2	df	*p*
Year	0.160 (0.072)	4.93	1	**0.03**
Clutch size	0.005 (0.019)	0.07	1	0.80
Average egg volume	0.000 (0.000)	0.05	1	0.82
Dependent variable: sperm count (value for the 10th egg)				
Clutch size	–0.014 (0.028)	0.244	1	0.62
Average egg volume	0.001 (0.001)	0.80	1	0.37

Table 3.4 Logistic regression of year, average sperm count, clutch size and average egg volume on the proportion of infertile eggs in a clutch.

Dependent variable: the proportion of infertile eggs in the clutch				
Predictor:	Estimate (SE)	χ^2	df	*p*
Year	1.219 (0.895)	1.856	1	0.17
Average sperm count	0.601 (0.889)	0.456	1	0.50
Clutch size	–0.111 (0.183)	0.368	1	0.54
Average egg volume	–0.011 (0.004)	9.347	1	**0.002**

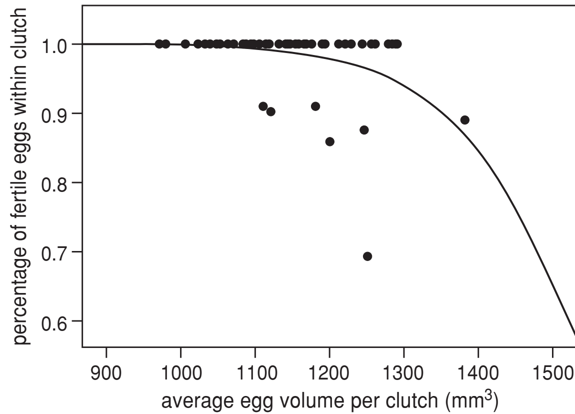


Figure 3.2 Scatter plot of the relationship between average egg volume in the clutch and the percentage of eggs that hatched ($n = 70$ clutches of the sperm database).

Discussion

In birds, copulation generally ceases or declines markedly in frequency after the onset of egg laying (Birkhead *et al.* 1987). Consequently, we hypothesized that females may become sperm limited late in the laying sequence resulting in a greater incidence of egg infertility, especially in birds that produce large clutches. We investigated this hypothesis in blue tits, which on average lay the largest clutches among passerines. However, we found no evidence for any pattern of egg infertility over the laying sequence for either absolute laying order or the relative laying order. This absence of any relationship between egg fertility and laying order suggests that sperm did not become limiting over the laying period in this population of blue tits.

Despite a previous study finding that egg sperm count declined over the laying sequence in blue tits (Lifjeld *et al.* 2000), we did not find a consistent change in sperm count between 2nd and 10th eggs within a clutch. The fact that sperm count did not decrease over the laying sequence in our population further suggests that copulations continued throughout the laying sequence. An alternative possibility is that females store enough sperm to fertilize their entire clutch by the start of the laying period and have adequate sperm to release a constant amount throughout their laying period. However, because studies in other species demonstrated that sperm are lost passively from the sperm storage tubules and also degrade within the female tract (Wishart 1987; Birkhead *et al.* 1993b; Briskie 1994; Pellat 1997), this explanation seems less likely.

We also found no relationship between the percentage of fertile eggs in a clutch

and sperm count on eggs sampled from that clutch, suggesting that females in our study population generally had sufficient sperm to fertilize their eggs even when sperm count was relatively low. In the zebra finch the likelihood of eggs being fertile declined when the egg sperm count fell below 30 and was 50% lower when only 20 sperm were present (Birkhead and Fletcher 1998). We found an average of 238 ± 18 (SE) sperm per egg (Schut *et al.* unpublished) and only three eggs with 30 sperm or less. Furthermore, we found no evidence that females laying a larger clutch stored more sperm, again suggesting that females in our population were not sperm limited.

Birkhead and Møller (1992) hypothesised that sperm depletion may be an important limitation to the number of successful copulations that male birds can perform. Since it is known that within-pair copulation rates in blue tits are high (7 – 28 copulations per day; Kempenaers *et al.* 1992) depletion of sperm in pair males could be a significant issue, especially if copulation continues throughout the laying period. Female blue tits may counter this potential problem by engaging in extra-pair copulations (i.e. from males whose pair female has finished laying) to limit the risk of infertility especially in late laid eggs when their pair male may be most vulnerable to sperm depletion (as suggested by: Wetton and Parkin 1991; Birkhead and Møller 1992; Birkhead *et al.* 1994b). However, in this scenario, we would expect to find more extra-pair offspring in the late laid eggs within clutches, yet the opposite was revealed to be the case in this population (Magrath *et al.* 2009).

The lack of sperm depletion in the study population is also supported by the relatively low incidence of egg infertility. Overall, we found only 3.5% of eggs laid were infertile compared to an average of 8.8% reported for other passerine species (Koenig 1982), but comparable to that reported in another blue tit population (4%; Kempenaers *et al.* 1996).

Kempenaers *et al.* (1996) showed that the level of genetic similarity between blue tit parents is an important determinant for the proportion of unhatched eggs in their clutch. Evidence for an inbreeding component to hatchability has also been presented for passerines (van Noordwijk and Scharloo 1981; Kruuk *et al.* 2002; Cordero *et al.* 2004; Hansson 2004; Spottiswoode and Møller 2004; Knape *et al.* 2008). Several of these studies found only a small number of pairs with high inbreeding coefficients and reduced hatchability (Kempenaers *et al.* 1996; Hansson 2004; Knape *et al.* 2008). This would be consistent with our result of finding relatively few pairs with a high number of infertile eggs. Hence, inbreeding may explain those few cases in our population where a high proportion of eggs were infertile.

In conclusion, the absence of any relationship between egg infertility and laying sequence in conjunction with the lack of evidence for a decline in sperm count between early and late eggs within clutches suggest that pair copulations continue during the entire laying period in blue tits. Copulation through the laying period is unusual among passerines (Birkhead *et al.* 1987), but is in line with observations from another blue tit population (B. Kempenaers pers. comm.; Kempenaers *et al.*

1995). Perhaps the large clutch size produced by female blue tits has selected for the continued participation in copulation after the onset of egg laying to minimise the risk of sperm depletion.

Acknowledgements

The animal experiments committee of the University of Groningen granted permission for all experimental procedures involving the handling of birds and eggs. We would like to thank Bart Eggen and Loes Drenth from the Department of Developmental Genetics at the University of Groningen for allowing us to use the department's fluorescence microscope. The authors would further like to thank the Kraus Groeneveld Stichting for permission to work in de Vosbergen and Evelien Jongepier, Daphne Niehoff, Anna Harts and Peter Wolfs for assistance with fieldwork. This research was financially supported by a Marie Curie International Incoming Fellowship to M.J.L.M. (CMIF1-CT-2005-008914) and grant number 028696 to J.K., both of which are components of the European Community's Sixth Framework Programme.

Chapter 4

Sperm numbers in the reproductive tract of female blue tits (*Cyanistes caeruleus*) are repeatable within the clutch and related to partner size

Elske Schut, Michael J. L. Magrath, Oscar Vedder, Marco van der Velde and Jan Komdeur

Abstract

While most passerine species are socially monogamous, genetic polyandry is widespread. The occurrence of clutches with mixed paternity provides evidence that females commonly have sperm from multiple males in their reproductive tract. Therefore, it is likely that paternity of the offspring is determined by post-copulatory sexual selection mechanisms. While post-copulatory sexual selection mechanisms have commonly been studied, our understanding of them remains incomplete. We used the number of sperm trapped between the perivitelline layers around the egg yolk ("sperm count") of freshly laid blue tit eggs to investigate whether characteristics of the females' social partner are associated with the number of sperm females have in storage. Our results indicate that sperm counts are repeatable between eggs of the same clutch and that sperm counts are higher in females paired to a larger social male. Because our method does not allow us to distinguish between the sperm of different males, several explanations for the latter result are possible. The fact that we found variation in sperm counts associated with male characteristics leads us to suggest that variation in sperm counts in the female reproductive tract represents (at least in part) copulation behaviour and/or post-copulatory tactics rather than only differences in female sperm storage capacity.

Introduction

While monogamy is the most common social mating system in passerine birds, genetic polyandry is widespread (Birkhead *et al.* 1987; Westneat *et al.* 1990; Griffith *et al.* 2002a). The function of females copulating outside the pair bond (i.e. engaging in extra-pair copulations; EPCs) is contentious, but it was proposed that females may obtain direct benefits (e.g. fertility insurance) or advantageous genes (i.e. good genes or compatible genes) for their offspring (reviewed in Jennions and Petrie 2000; Griffith *et al.* 2002a). The fact that female birds have specialized sperm storage organs (sperm storage tubules, SST), where they can store sperm for up to several weeks, extends the period during which copulations may lead to the fertilization of eggs within the clutch and hence increases their opportunity to copulate with multiple males within a single breeding cycle (Birkhead and Møller 1992; Birkhead and Møller 1998).

When a female copulates with more than one male, fertilization is thought to occur largely according to a "fair-raffle model" (Parker 1970; Parker 1998), i.e. a males' chance of fertilizing one or more eggs is proportional to the relative quantity of his sperm that the female retains in storage. Another important determinant of fertilization success is the timing of insemination relative to oviposition. Due to passive sperm loss from the SST, the sperm of males inseminating long before oviposition will (partly) be lost when the ovum is available for fertilization (Wishart 1987; Birkhead *et al.* 1993b) and last male sperm precedence (i.e. the majority of offspring being fathered by the last male to copulate) is a well-known phenomenon in birds (Birkhead *et al.* 1988). The mobility of his sperm is another important determinant of a males' fertilization success (Birkhead *et al.* 1999). Hence, when in competition with others, males can increase their fertilization success by adjusting the frequency and timing of copulation, their ejaculate size and the quality of their ejaculate (reviewed in Birkhead and Møller 1998). Females may also exercise some control over fertilization by influencing the timing of copulation, selectively retaining the sperm of different males and ejecting that of others and by selective release from the SST, amongst other possible mechanisms (Eberhard 1996). Despite the fact that such mechanisms of post-copulatory sexual selection have received much recent attention, our understanding of them remains incomplete.

Studying the selection processes occurring after the successful insemination of sperm is challenging, even when invasive methods are used (e.g. dissecting of the female tract). One method that is somewhat less invasive than most involves counts of the number of sperm trapped between the perivitelline layers (PVL) around the egg yolk (see Wishart 1987; Birkhead *et al.* 2008). Dissection of the PVL allows one to determine the number of holes created by sperm penetrating the ovum and to count the number of sperm trapped between the inner and outer PVLs. Together, the number of holes and sperm provide an index of the number of spermatozoa present at the site of fertilization. Since previous studies demonstrated that the

number of holes in the inner PVL correlates positively with the number of sperm between the inner and outer PVL (Birkhead and Fletcher 1994; Birkhead *et al.* 1994a; Lifjeld *et al.* 2000), we used only sperm numbers as an indication of the number of sperm present at the site of fertilization. The number of sperm present at the site of fertilization is correlated to the number of sperm in the female SST. Studies using artificial insemination provide evidence that sperm numbers on the PVL are also positively correlated to the number of sperm inseminated (Brillard and Bakst 1990; Birkhead *et al.* 1993b). Since in wild-living passerines, copulation may be frequent and sperm numbers inseminated are likely to be much higher than those used in artificial insemination, it is unclear whether sperm numbers on the PVL represent sperm numbers inseminated in wild-living passerines. An important advantage of the use of this method is that it does not require the sacrifice of adult females. Throughout the manuscript we will refer to the number of sperm between the inner and outer PVL as "sperm count".

In this study we used this method to examine whether characteristics of the females social partner (among other factors) may influence the number of sperm available for fertilization in blue tits (*Cyanistes caeruleus*). The blue tit is an interesting study species in the context of post-copulatory mate choice, because extra-pair offspring (EPO) and hence post-copulatory sexual selection, occur commonly (Kempenaers *et al.* 1997; Leech *et al.* 2001; Delhey *et al.* 2003; Brommer *et al.* 2007; Magrath *et al.* 2009). Several previous studies used this method in free-living blue tits (Kempenaers *et al.* 1994; Birkhead *et al.* 1994a; Lifjeld *et al.* 2000; Johnsen *et al.* 2011), but we are the first to relate sperm counts to characteristics of the social partner. We hope that the identification of the male characteristics related to the number of sperm a female has available for fertilization, we will gain insight into female mating and post-copulatory tactics. Note, however, that this method does not enable us to distinguish the sperm of different males, so conclusions regarding post-copulatory sexual selection can only be tentative and must be carefully drawn.

We aim to determine whether the variation in sperm counts is smaller between eggs within the same clutch than for eggs produced by different females, i.e. whether consistent differences in sperm count between females exist. We also test whether females that produced EPO had higher sperm counts. We expected this to be the case because (i) males are likely to produce larger ejaculates during EPCs, since they have to compete with the sperm from at least the social male and (ii) the social male may increase his ejaculate size and copulation rate in response to his female engaging in EPCs (to ensure his fertilization success). Furthermore, we assessed whether sperm counts correlate with characteristics of the social male that may indicate his attractiveness. In this case the relation could be predicted to go in either direction. Females may store more sperm of an attractive partner, because (i) they want to ensure that this male has a high probability of paternity and (ii) attractiveness may indicate good health, and healthy males may produce larger ejaculates or copulate more frequently. However, a negative relationship between sperm count and the attractiveness of the social partner could result if (i)

the female is less likely to engage in EPCs when paired to an attractive partner, (ii) the pair male does not copulate with the female as frequently, because he has greater confidence of paternity, and (iii) the pair male has a small ejaculate size because he frequently engages in EPCs because of his attractiveness.

We use male age, body size (i.e. tarsus length) and UV chroma as indicators of male attractiveness. A previous study in our population showed that males with longer tarsi sired a larger proportion of the offspring in their social nest and that older males were more likely to sire EPO and to be polygynous while older males did not lose more paternity in their social nest (Vedder *et al.* 2011). There are indications that UV chroma is used as a signal in mate choice in blue tits (e.g. Andersson *et al.* 1998; Hunt *et al.* 1998a; Hunt *et al.* 1998b; Korsten *et al.* 2006; Delhey *et al.* 2007, but see Örnborg *et al.* 2002; Delhey *et al.* 2006) and in male-male competition (Alonso-Alvarez *et al.* 2004; Vedder *et al.* 2010b, but see Korsten *et al.* 2007; Vedder *et al.* 2008).

Methods

Field procedures

We used birds breeding in a wild net box population at De Vosbergen estate, near Groningen, the Netherlands (53º08′N, 06º35′E) in 2007 and 2009. Blood samples were collected from all adult birds and their offspring. All occupied nest boxes were checked daily from the beginning of April, and newly laid eggs were marked to record the laying sequence. A small blood sample (ca. 10 μL) was taken from the chicks within three days from hatching and was used to determine paternity (see below). Adults were trapped in their nest box during the chick feeding stage (10–12 days after hatching) and a blood sample was taken from the brachial vein. Blood samples were stored in 100% ethanol and at room temperature.

At the time of catching, tarsus length was recorded to the nearest 0.1 mm using sliding callipers and body mass was recorded to the nearest 0.1 g using a spring balance (Pesola). The age of the birds was determined following Svensson (1992), birds were classified as one year or older. Adult males were taken to the field station for UV measurements of the blue crown. A USB-2000 spectrophotometer with illumination from a DH-2000 deuterium-halogen light source (Avantes, Eerbeek, The Netherlands) was used to measure the spectral reflectance. The measurement probe was held at a right angle against the centre of the crown for illumination and recording. Five replicate measurements were taken for each individual. The probe was lifted from the crown and replaced in between each measurement. The running mean was calculated over 10 nm for each measurement to smooth the curve. The average of the UV chroma was calculated by dividing the sum of the reflectance between 320 and 400 nm by the sum of the reflectance between 320 and 700 nm. We chose to use UV chroma, rather than other UV measurements (i.e. hue, brightness) since there is evidence that this characteristic is used as a signal in

mate choice and male-male competition (see Introduction). UV measurements were taken by two different observers and statistically corrected for observer bias.

Processing of eggs

In 2007 eggs were collected from the first half of the clutch for a different experiment. We used seven first eggs, 20 second eggs, five third eggs, 30 fourth eggs, three fifth eggs and one sixth egg of in total 35 nests. In 2009, we collected eggs 2 (n = 30) and 10 (n = 32). All eggs were collected on the day of laying and stored in the fridge (4°C) until microscopic examination. All eggs were processed on average within 9 days after laying (range 0–24 days, well within the maximum period after which sperm can be counted, Birkhead *et al.* 2008). In 2009, eggs were first weighed to the nearest mg. Then the shell was broken in two halves and yolk and albumin were separated. As much albumin as possible was removed using a 200 μL pipette and the egg yolk was put in a petri-dish with PBS. The egg yolk was cut in two halves (taking care not to cut through the part overlaying the germinal disc, since it is known that this is the part containing most of the sperm (Birkhead *et al.* 1994a) and yolk was cleaned off the PVL Once all yolk was cleaned off the PVL, the first half was spread out on a microscopic slide (inner and outer PVL together), using watch-maker forceps and a drop of Hoechst 33258 dye (5 mg/ml, ± 5 μL per sample; Invitrogen, California, USA) was added on top, it was covered with a cover slip and put in a dark drawer for approx. five minutes to incubate. After five minutes, the slide was put under a fluorescence microscope (Optech B5 PT, Orbi solutions, Aalten, the Netherlands, with a BP 340–380 excitation filter and LP 425 suppression filter). Sperm were counted by scanning through the entire slide at 400× magnification (40× objective). The second half of the PVL was left in PBS and processed immediately after the first. The total number of sperm cells found in both halves was used for further analyses (See Wishart 1987; Birkhead *et al.* 2008) for details on the method).

Molecular work

A chelex extraction (Walsh *et al.* 1991) was used to extract DNA from the blood samples. A paternity analysis was performed on the basis of six microsatellite markers (Pca 3, Pca 7, Pca 8, Pca 9 (Dawson *et al.* 2000), Pocc 6 (Bensch *et al.* 1997) and Pdo 5 (Griffith *et al.* 1999). For details on polymerase chain reactions (PCR) and determination of microsatellite allele-sizes, see Magrath *et al.* (2009) for the year 2007 and Vedder *et al.* (2010a) for the year 2009.

The mean exclusion probability over all markers was calculated in Cervus 3.0 (Kalinowski *et al.* 2007). The mean exclusion probability for the female was 0.99934, while for the male it was 0.99997 (provided that the genotype of the female was known). Using microsatellite markers, all individuals could be assigned to their social mother. Offspring were scored as extra pair when the genotypes of the chick and its social father did not match at more than one microsatellite locus. For all nestlings from nests were no male was caught during the feeding stage,

Cervus 3.0 (Kalinowski *et al.* 2007) was used to attempt to assign paternity to one of the sampled males in the population. A male was assigned as the father when the male's genotype matched the offspring's genotypes at all 6 loci for those alleles that could not originate from the mother.

Statistical analyses

A one way ANOVA with nest identity as the explaining variable was performed in SPSS 16.0 to determine within and between clutch effects on sperm count. F-values from the ANOVA were then used to calculated repeatability of sperm count within nest following Lessells and Boag (1987). Analyses of variables associated with sperm count were performed using a mixed model in MLwiN 2.02 (Rasbash *et al.* 2005) with clutch identity as a random effect to account for the use of multiple eggs per clutch. We used backwards elimination, starting with a model including all factors we hypothesized to affect sperm count (year, male tarsus length, male age, male UV chroma and whether or not the clutch contained mixed paternity) and eliminating the least significant effect in each round. To test the significance of the factors in the model, the Wald statistic (approximating the χ^2 distribution) was used. A two-tailed test was used in all cases. Sperm counts were $\log_{10}$-transformed, to let the error distribution more closely resemble a normal distribution.

A total of 128 eggs of 70 clutches was used. Although we attempted to catch all males at the nest during offspring feeding, this was not always achieved. In some cases the identity of the male was recorded by colour ring observations, without the male being caught. In some other cases where a male was caught, not all measures (tarsus, age, mass, UV chroma) were recorded. Hence, the number of observations for the different male characteristics may differ. Paternity was known for 53 clutches (98 eggs). Tarsus length was known of 45 males (83 eggs), body mass was known of 45 males (81 eggs), UV chroma was measured of 43 males (78 eggs) and male age was recorded of 53 nests (97 eggs).

Results

Consistency of sperm count within clutches

We found an average number of 238 ± 18 (SE) sperm per egg (figure 4.1). Sperm count was strongly repeatable between eggs from the same clutch (for clutches with the 2nd and 10th egg only: R = 0.61 ± 0.12 SE, n = 27 clutches; for all clutches in the dataset: R = 0.59 ± 0.09 SE, n = 70 clutches; figure 4.2). Consistent with this finding, we found a significant effect of clutch identity on sperm count (F = 4.27, $p < 0.001$, n = 70 clutches). We were unable to establish whether sperm count was consistent between clutches of the same female or male in different years, because we had multiple clutches for very few individuals (two females and one male).

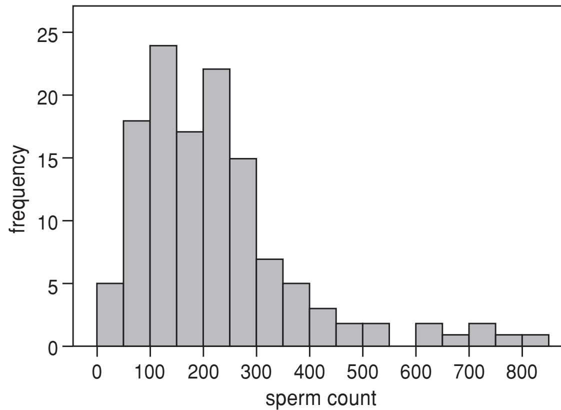


Figure 4.1 Histogram of all values for sperm count found (n = 128 eggs of 70 nests).

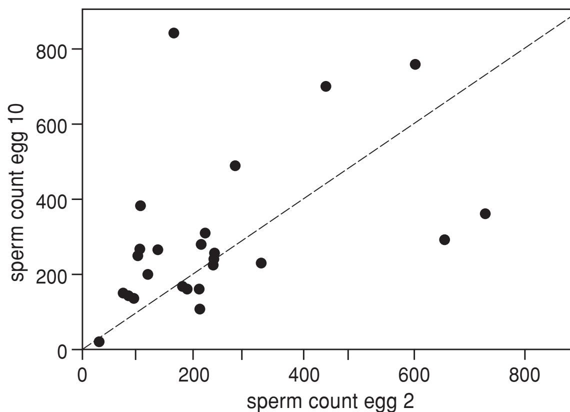


Figure 4.2 Sperm count of the 10th egg plotted against sperm count of the 2nd egg within a clutch (n = 27 clutches, F = 4.089, p = 0.0003, R = 0.61 ± 0.12 SE).

Sperm counts in relation to social male characteristics and extra-pair paternity

Sperm count was positively correlated to tarsus length of the females' social partner (p = 0.004, table 4.1, figure 4.3) and varied between study years (p = 0.028; table 4.1). Other characteristics of the social male (age, body mass and UV chroma) did not contribute significantly to explaining variation in sperm counts (table 4.1). The presence of EPO in the clutch was also unrelated to sperm counts of eggs sampled from that clutch (table 4.1).

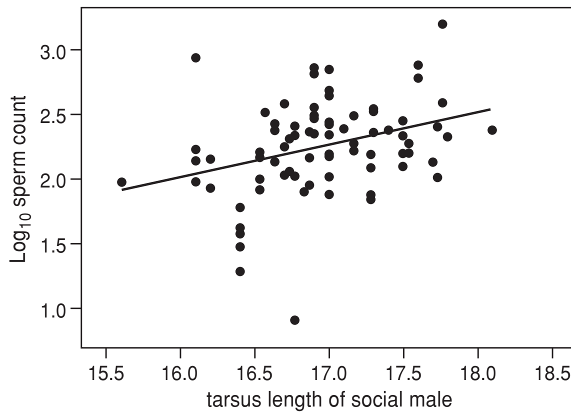


Figure 4.3 Log_{10} of sperm count plotted against the tarsus length of the social male.

Table 4.1 Summary of mixed model investigating factors associated with sperm count. The final model was obtained by backwards elimination and values of non-significant predictors are given as when the predictor is entered back into the final model.

Response variable: sperm count Predictor variable	Estimate (SE)	χ^2	df	*p*
Year	0.273 (0.094)	8.44	1	0.004
Male tarsus length	0.194 (0.089)	4.82	1	0.028
Male age	–0.072 (0.103)	0.49	1	0.484
Male body mass	–0.001 (0.087)	0.00	1	1.000
Male UV chroma	–2.214 (2.742)	0.65	1	0.420
Mixed paternity?	0.144 (0.092)	2.41	1	0.121

Discussion

We found a significant difference in sperm count between clutches and, hence, we found that sperm count was repeatable between consecutive eggs of the same clutch. Furthermore, sperm count was positively associated with male structural body size, but not with other male characteristics or the presence or absence of extra-pair paternity in the remainder of the clutch.

Several factors potentially determine the number of sperm present at the site of fertilization (i.e. in the infundibulum) when fertilization occurs and are thereby responsible for between clutch differences in sperm count on the PVL. Sperm counts may for instance vary due to differences in the number of sperm that were inseminated (i.e. differences in the number of copulations and ejaculate size), due to

differences in the proportion of the sperm that the female stores (i.e. in relation to male attractiveness) and due to the maximum sperm storage capacity of the SST. It was previously shown in the bearded tit that there is a limitation to the number of sperm that a female can store (Sax *et al.* 1998). Since the blue tit is a species with a relatively high copulation rate (7–28 copulations per day during the breeding cycle; Kempenaers *et al.* 1994) the same could apply for the blue tit.

We hypothesized that females engaging in EPCs may have increased sperm counts, since males are likely to transfer more sperm during EPCs, and social partners may attempt to ensure their paternity by increasing copulation rates and ejaculate sizes when their female has engaged in EPCs. Our data provided no evidence that eggs originating from a clutch containing EPO differed in sperm count compared to eggs from clutches without EPO. This may indicate that all females engage in EPCs which do not always result in EPO, that no effect exists of engaging in EPCs on sperm count or that the effect of EPCs is masked by other effects. Since we are unable to distinguish between the sperm of different males, we cannot distinguish between these explanations. Johnsen *et al.* (2011) assumed that a potential increase in sperm counts as a result of EPCs would be detectable only in the subsequent egg laid. Hence, they tested for a difference in sperm count on eggs that were fertilized by an extra-pair male versus eggs fertilized by the social partner in the blue tit, but found no effect. The same study did find such an effect in eggs from the bluethroat (*Luscinia svecica*), which seems surprising, since sperm can be stored in the SST for up to several weeks. Although passive sperm loss will occur, this would be proportional to the total amount of sperm in storage (Brillard and Antoine 1990). Hence, the effect of increased sperm numbers in the female reproductive tract should last for more than 24 hours. We would therefore have expected to find a between-clutch difference, rather than a difference between average sperm count on extra-pair and within-pair eggs in the population.

We found that sperm count was positively correlated to the tarsus length of the social partner. We can only offer tentative explanations for this correlation, since we cannot distinguish between the sperm of within- and extra-pair partners. Hence, several explanations for this result are possible. Since we know that larger males sire a larger proportion of offspring in their nest (Vedder *et al.* 2011), they possibly are more efficient mate guarders who are able to produce a large ejaculate or copulate often. In favour of this explanation, there is evidence that male body mass is correlated to testes size and that testis size is correlated to sperm production (Møller 1989). An equally likely explanation is that females may store more sperm of larger males as they are perceived as more attractive, irrespective of the number of sperm he inseminates. An alternative explanation is that there is a positive association between partner size and female body size/mass and that larger/heavier females have larger SST (although evidence for a correlation between female mass and sperm storage capacity was only found in a between-species comparison; Birkhead 1992). We found no evidence for this hypothesis, however, as male tarsus and female tarsus were not correlated in our dataset (for tarsus length:

$R^2 = 0.002$, $p = 0.79$, $n = 44$ pairs, for male tarsus/ female mass: $R^2 = 0.0004$, $p = 0.89$, $n = 44$ pairs). We therefore suggest that variation in sperm counts in the female reproductive tract represents copulation behaviour and/or post-copulatory tactics rather than differences in female sperm storage capacity. Johnsen *et al.* (2011) found no relationship between the number of copulations and the number of sperm on the eggs laid one or two days later in the tree swallow (*Tachycineta bicolour*), which may suggest that sperm numbers on the PVL reflect the sum of all copulations during the females' fertile period rather than only the copulations in the few days before laying of the egg (although they did not control for ejaculate sizes).

Conclusions

To conclude, this is one of the first studies showing significant (although indirect) between-individual differences in the number of sperm in the female tract of a wild living passerine. The fact that sperm number was related to a characteristic of the social male (i.e. tarsus length) suggests that sperm number is, at least in part, determined by copulation behaviour or post-copulatory tactics, rather than just maximum sperm storage capacity of the female. Future (experimental) studies (e.g. experimentally mimicking the copulation behaviour of wild birds in captivity and investigating the relationship between sperm number inseminated and sperm on the PVL) may provide more insight into the role of post-copulatory effects in passerines.

Acknowledgements

The animal experiments committee of the University of Groningen granted permission for all experimental procedures involving the handling of birds and eggs. We would further like to thank Bart Eggen and Loes Drenth form the Department of Devolopmental Genetics at the University of Groningen for letting me use their fluorescence microscope. The authors would further like to thank the Kraus Groeneveld Stichting for permission to work in de Vosbergen and Evelien Jongepier, Anna Harts, Daphne Niehoff, and Peter Wolfs for assistance with fieldwork. This research was financially supported by a Marie Curie International Incoming Fellowship to M.J.L.M. (CMIF1-CT-2005-008914) and grant number 028696 to J.K., both of which are components of the European Community's Sixth Framework Programme.

Box B

Volume of the cloacal protuberance as an indication of sexual activity in male blue tits (*Cyanistes caeruleus*)

Elske Schut, Michael J. L. Magrath, Kees van Oers and Jan Komdeur

Abstract

In male passerines, the accumulation of sperm in the sperm reserves causes the cloaca to swell up, forming the cloacal protuberance (CP). Since in most passerines, sperm production only occurs during a short period in spring, CP volume should be associated to stage of the breeding cycle, as was shown in the bearded tit (*Panurus biarmicus*). CP volume can be used for sex determination in sexually monochromatic species and as an indication that males are sexually active. Here, we show that CP volume was increased during the breeding season in captive male blue tits (*Cyanistes caeruleus*). Our results indicate that CP volume may be used as an indication of sexual activity for individual blue tits, although CP volume overlapped between sexually active and non-active individuals. Measuring CP volume at a single time point may, therefore, not in all cases allow the researcher to draw conclusions regarding the sexual status of an individual.

Submitted to Journal of Ornithology

Introduction

Many passerine species are only reproductively active during a short period in spring. At the onset of the breeding season, males will therefore start producing sperm. As soon as the breeding season is over, sperm production will cease again (Wright and Wright 1944; Selander and Hauser 1965; Partecke *et al.* 2004). In most passerines, males store produced sperm in their seminal glomerae (an extension of the ductus deferens) until it is ejaculated. Due to the accumulation of sperm in the seminal glomerae, the cloaca will swell up, forming the cloacal protuberance (CP; King and McLelland 1981; fig. 1). Previous studies have used the CP for sex determination (e.g. Mason 1938) or as an indication that males are sexually active (e.g. Wolfson 1952; Brouwer *et al.* 2009; Tonra *et al.* 2011). The association between CP volume and stage in the reproductive cycle has been described in the bearded tit (*Panurus biarmicus*). In the bearded tit, CP volume followed the reproductive cycle of the social partner, with CP volume reaching its peak on or close to the day of clutch initiation (Sax and Hoi 1998). In a comparison across passerine species, size of the CP was shown to be positively correlated to the size of the testes, number of sperm in storage, the length of sperm and copulation rate (Birkhead *et al.* 1993a).

Several (non-mutually exclusive) hypotheses have been proposed to explain the evolutionary function of the CP (reviewed in Birkhead *et al.* 1993a), including the "efficient copulation hypothesis" (proposing that the CP facilitates sperm transfer), the "spermatozoa size hypothesis" (larger CPs are needed to store similar numbers of longer sperm) and the "sperm competition hypothesis" (if sperm competition is strong and copulations are frequent, more sperm must be stored and the CP will be larger). Birkhead *et al.* (1993a) found supporting evidence for both the sperm competition hypothesis and the spermatozoa size hypothesis.

Here we investigate whether CP volume increases when males become sexually active in the blue tit (*Cyanistes caeruleus*). We are unaware of any previous study describing CP in the blue tit. Since blue tits lay an exceptionally large clutch and are known to copulate frequently during the breeding season (Kempenaers *et al.* 1995), we might expect males to store sperm in their seminal glomerae, to ensure the availability of adequate sperm. More specifically, the aims of our study are to determine (1) whether CP volume is increased in sexually active individuals and (2) whether sexually active and non-active individuals can be distinguished using a single measurement of CP volume.

Methods

We used 19 male captive blue tits to record copulation behaviour. All birds were hand reared and afterwards housed in aviaries (3 × 2 × 2 m; for details on the origin of the birds, see Vedder *et al.* 2010b). Birds were housed in individual cages (0.8 × 0.4 × 0.4 m) within an outdoor aviary. Hence, birds were kept at a natural light

schedule and in outside temperatures. Birds were fed *ad libitum* with a mixture of commercial bird food and beef hearth, supplemented with mealworms, wax moth larvae en caterpillars from the wild. Birds had *ad libitum* access to drinking water. Males were offered a live stimulus female with which they were free to interact every second day from March 30th until June 6th. A nest box and nesting material were provided. The male was allowed access to the female at 0830 (through removal of a sliding panel) and removed to his own cage at 1700h. Copulations were observed in six of the captive males; hence we used these six males for our CP analyses. Whether the other 13 males became sexually active or not is unclear, since the occurrence of copulations may be influenced by female behaviour. Since stimulating males to become sexually active may be problematic in captivity, males that were not observed copulating were excluded from the analyses.

We took three measurements for CP using sliding calipers (measurements were taken to the nearest 0.1 mm): height, width at base and width at opening. From these measures volume of the CP was calculated, based on a barrel-shape ($\pi \times$ radius$^2 \times$ height; e.g. Mulder and Cockburn 1993). CP was measured during the breeding season (on a day that the male was observed copulating in the morning, between May 8th and June 3rd 2008), and again when the breeding season was over and males no longer had access to females (June 16). In five out of the six males, copulations were recorded on two days and CP was measured twice. In those, the largest CP value was used. On June 16, all CP measures (height, width at base and width at opening) were taken twice immediately after each other for all 19 males, allowing us to calculate the repeatability following Lessells and Boag (1987).

Results

Our consecutive measurements on June 16 demonstrated that our measures of CP volume were repeatable ($R = 0.81 \pm 0.08$ SE, Fig. B.1). Furthermore, we found that the CP was significantly larger during the period in which males were sexually active than just after the breeding season ($p = 0.02$, Fig B.2). On average, CP had decreased after the breeding season by 24.98 mm^3 (range 5.8 – 53.8 mm^3; an average decrease of 54.4%). Between individuals there was overlap in CP values during and after the breeding season (range for sexually active males: 20.9 – 76.0 mm^3, range in values after season: 15.13 – 43.59 mm^3).

Discussion

In passerines, the storage of sperm in the seminal glomerae causes the cloaca to swell up, forming the cloacal protuberance (CP). The size of the CP may be used for sex determination in monochromatic species and to determine stage in the breeding cycle. Here, we investigated whether CP volume is increased in sexually active male blue tits.

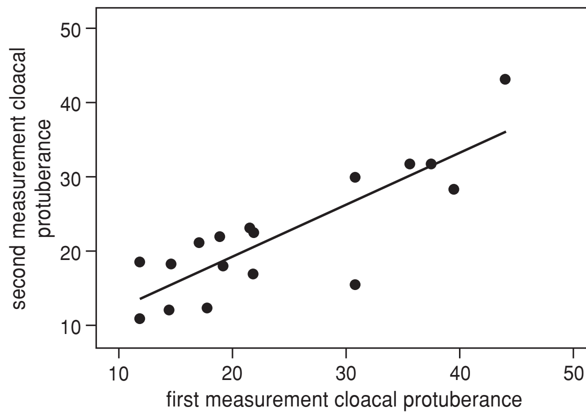


Figure B.1 Repeatability of two consecutive measurements of cloacal protuberance (mm^3); (n = 19 males, F = 9.75, $p < 0.001$, R = 0.81 ± 0.08 SE).

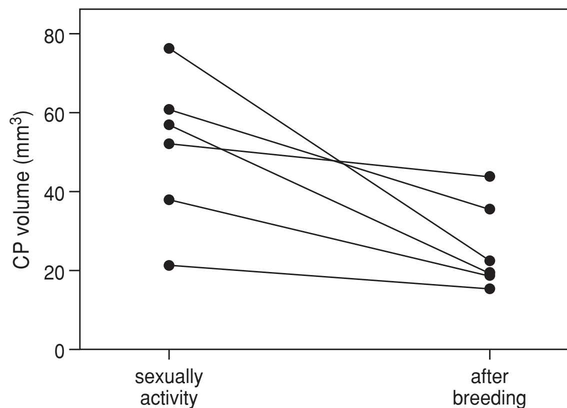


Figure B.2 Cloacal protuberance measurements for the six males that were known to copulate, comparing cloacal protuberance measurements after the breeding season and measures that were taken when males were sexually active. Lines connect the two CP values of the same male. (Paired T-test, t = 3.35, $p = 0.02$.)

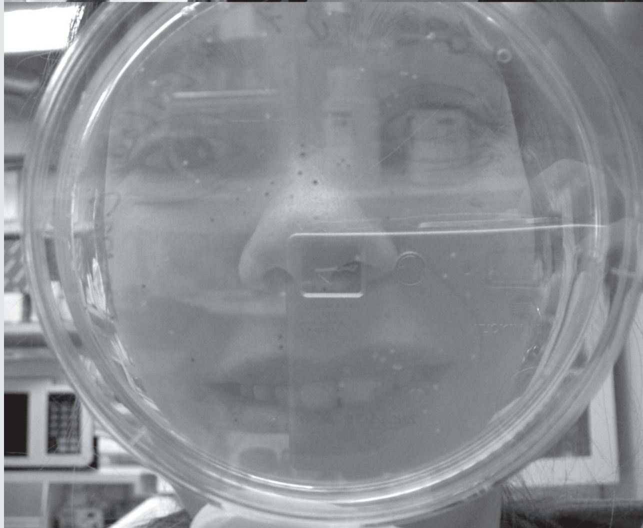
We found that CP volume was significantly larger during than after the period of sexual activity, indicating that CP volume could be used to determine whether a male is sexually active. CP volume overlapped between sexually active and non-active individuals, however. This overlap may in part be caused by the fact that we measured CP on a day that copulation was observed, rather than measuring CP of all males at regular intervals. Hence, we may have missed the real peak in CP volume. In two sexually active males, we found that CP was already decreasing the second time copulation was observed and CP was measured, indicating that CP had started to decrease while sperm was still available. Cases in which male passerines

produced sperm, but did not have a CP were previously recorded in other passerines (Quay 1986).

Our results indicate that blue tit males show visible signs of the storage of sperm in their seminal glomerae during the breeding season. However, CP volume may not in all cases give conclusive evidence of sexual activity when using a single measure for an individual (as we found overlap in CP volume between sexually active and non-active males). Future studies investigating the timing of peak CP volume relative to the peak in male sexual activity and the female breeding cycle may clarify how well CP volume in blue tits correlates with the males' peak period of sperm production.

Acknowledgements

The authors would like to thank Oscar Vedder, Anna Harts, Giuseppe Boncoraglio and Janne Ouwehand for practical assistance. ES received funding from the Schure- Beijerinck-Popping Stichting. The animal experiments committee of the University of Groningen granted permission to raise blue tits in captivity and for the experimental procedures (no. D4753A).



Part II

The Major Histocompatibility Complex in the blue tit

CHAPTER 5

Characterization of MHC-I in the Blue Tit (*Cyanistes caeruleus*) reveals low levels of genetic diversity and trans-population evolution across European populations

Elske Schut, Juan Rivero-de Aguilar, Santiago Merino, Michael. J. L. Magrath, Jan Komdeur and Helena Westerdahl

Abstract

The Major Histcompatibility Complex (MHC) is a vital component of the adaptive immune system in all vertebrates. This study is the first to characterize MHC class I (MHC-I) in blue tits (*Cyanistes caeruleus*), and we use MHC-I exon 3 sequence data from individuals originating from three locations across Europe; Spain, the Netherlands and Sweden. Our phylogeny of the 17 blue tit MHC-I alleles contains one allele cluster with low nucleotide diversity compared to the remaining more diverse alleles. We found significant evidence for balancing selection in the peptide binding region in the diverse allele group only. No separation according to geographic location was found in the phylogeny of alleles. Although the number of MHC-I loci of the blue tit is comparable to that of other passerine species, the nucleotide diversity of MHC-I appears to be much lower than that of other passerine species, including the closely related great tit (*Parus major*) and the severely inbred Seychelles warbler (*Acrocephalus sechellensis*). We believe that this initial MHC-I characterization in blue tits provides an important step towards understanding the mechanisms shaping MHC-I diversity in natural populations.

Immunogenetics 2011, 63(8): 531–542

Introduction

The Major Histocompatibility Complex (MHC) is a gene complex that encodes molecules of vital importance for the adaptive immune system of vertebrates (Klein 1986; Goldsby *et al.* 2000; Janeway *et al.* 2008). The MHC is known to be a remarkably diverse gene complex, containing many loci, a high level of polymorphism and high nucleotide diversity (Klein 1986; Nei and Hughes 1991). The high number of MHC loci is thought to have arisen by gene duplication, since many MHC genes are very similar in sequence (Beck *et al.* 1999; Hess and Edwards 2002). Balancing selection is thought to maintain MHC diversity and prolong the lifetime of MHC alleles (Potts and Wakeland 1990; Hedrick 2002). Several mechanisms of balancing selection have been proposed (Bodmer 1972; Potts and Wakeland 1990; Nei and Hughes 1991; Borghans *et al.* 2004; Oosterhout 2008), most of which belong to one of two categories: hypotheses involving pathogen driven selection and those involving mate choice. The pathogen driven selection hypotheses are based on the assumption that heterozygous individuals and/or individuals possessing rare alleles have increased survival probabilities and are more likely to produce offspring, e.g. due to unpredictable pathogen distributions. The mate choice hypotheses assume that selection is imposed by a female preference for mates with genotypes that increase the survival probabilities of their offspring. Selection of males with favourable genotypes can occur either pre- or post- copulatory (Potts and Wakeland 1990; Zeh and Zeh 1997).

The MHC can be divided into three classes: class I (MHC-I) and class II (MHC-II) genes are similar in sequence and are involved in the adaptive immune system, while class III genes encode molecules involved in the non-specific immune response (Goldsby *et al.* 2000; Janeway *et al.* 2008). Class I molecules present peptides that arise from proteins in the cytoplasm and contiguous structures like the nucleus (often intra-cellular pathogens), while class II molecules present peptides that arise in intra-cellular vesicles and extra-cellular space (often extra-cellular pathogens). However, there is also a phenomenon of "cross-presentation" where e.g. extra-cellular material is presented by class I molecules (Goldsby *et al.* 2000; Janeway *et al.* 2008). Specific regions of the MHC (exon 2 and 3 in MHC- I and exon 2 in MHC-II) encode the peptide binding regions (PBRs) that bind foreign peptides (Goldsby *et al.* 2000; Janeway *et al.* 2008) Since each MHC molecule can successfully bind a limited number of peptides, greater polymorphism at the PBRs should increase the number of pathogens that can be recognized by an individual, resulting in balancing selection (Hughes and Nei 1988; 1989).

The MHC of the chicken (*Gallus domesticus*) was the first to be characterized in a bird. The chicken MHC is smaller, with denser gene regions and smaller introns, compared to the human MHC, called Human Leukocyte Antigen complex (HLA). It is also less diverse than the HLA complex, with only two class I and two class II loci. The chicken is therefore said to have a “minimal essential MHC” (Kaufman *et al.* 1995; 1999). Another remarkable feature of the chicken MHC is that it has two

gene complexes (the classical BLB and non-classical YLB loci) that both contain MHC-I and II alleles, but these loci segregate independently (Miller *et al.* 1994; Hunt *et al.* 2006). At least one of the YLB loci is known to be expressed (Hunt *et al.* 2006). The BLB and YLB loci have also been reported in the black grouse (*Tetrao tetrix*) and the ring-necked pheasant (*Phasianus colchicus*) (Wittzell *et al.* 1995; Strand *et al.* 2007) and are thought to be a feature of galliform birds.

In general the MHC of passerine birds appears to be more diverse in terms of loci, polymorphism and the existence of pseudo genes than the MHC of galliform birds (with the exception of the Japanese quail; *Cortunix japonica*; Shiina *et al.* 1995; Westerdahl *et al.* 2000; Westerdahl 2007). In passerines, studies have mainly focused on MHC-II (i.e. Vincek *et al.* 1995; Westerdahl *et al.* 2000; Edwards *et al.* 2000; Freeman-Gallant *et al.* 2002; Jarvi *et al.* 2004; Miller and Lambert 2004; Richardson *et al.* 2005; Aguilar *et al.* 2006), while MHC-I has been investigated in only a few species (the house sparrow, *Passer domesticus*, Bonneaud *et al.* 2004, the Seychelles warbler, *Acrocephalus sechellensis*, Richardson and Westerdahl 2003, the great reed warbler, *Acrocephalus arundinaceus*, Westerdahl *et al.* 1999, and the scarlet rosefinch, *Carpodacus erythrinus*, Promerová *et al.* 2009). Pathogens are, as mentioned above, thought to maintain the MHC diversity and in the last ten years avian malaria has been investigated extensively in wild bird populations. There is a wide array of malaria strains that infect most bird species and they potentially exert a substantial selection pressure for MHC diversity. Avian malaria has both extra- and intracellular stages and hence both MHC-I and MHC-II are likely to be involved in the adaptive immune response against malaria parasites (Peirce 1981; Valkiunas 2005; Janeway *et al.* 2008).

Here, we investigate the MHC-I of the blue tit (*Cyanistes caeruleus*) across three populations in Europe. In blue tits, several species of intra-cellular parasites, including avian malaria, are known to negatively affect the survival and reproductive success of infected individuals (Merino *et al.* 2000; Cichon and Dubiec 2005; Tomás *et al.* 2007; Arriero *et al.* 2008; Knowles *et al.* 2010). Blue tits typically breed as socially monogamous pairs (del Hoyo *et al.* 2007), but studies in numerous populations revealed, that broods often contain offspring sired by males other than the social partner (extra-pair offspring; Kempenaers *et al.* 1997; Leech *et al.* 2001; Delhey *et al.* 2003; Brommer *et al.* 2006; Magrath *et al.* 2009). Consequently, the blue tit is an ideal species for investigating the potential role of MHC in both pre- and post-copulatory mate choice. Characterising MHC-I of the blue tit is an important first step towards future investigation into the role of pathogen mediated selection and mate choice in the maintenance of MHC diversity in passerines.

Specifically, this study aims to: (1) partly characterize the diversity of the MHC-I genes in blue tits, using individuals from three populations across their range, (2) compare the blue tit MHC-I diversity with other passerine species and (3) gain insights into the selection pressures acting on PBR and non-PBR regions within the blue tit MHC complex.

Methods

Study species

The blue tit is a common passerine species with a wide distribution; the *C. c. caeruleus* subspecies extends throughout Europe and Western and Northern Asia (del Hoyo *et al.* 2007). We defined birds as migratory when seasonal migration between breeding and wintering grounds occurs, since this may be of importance for the diversity of pathogens encountered during an individual's life time (Møller and Erritzøe 1998). Non-migratory individuals may still disperse. According to our definition, the blue tit is non-migratory across the majority of its distribution, although large-scale dispersal as well as partial migration occurs in the northern range of the distribution (i.e. Sweden; Smith and Nilsson 1987; Cramp and Perrins 1993).

Sample collection

We used DNA samples from populations in three countries: (I) The Netherlands (NL, *The Vosbergen* estate, near Groningen, 53°08′N, 06°35′E), (II) Spain (Sp, Valsain, central Spain, 40°49′N, 3°56′E) and (III) Sweden (Sw, 55°41′N, 13°26′E). From the Netherlands, we sequenced both gDNA and cDNA in three individuals, and only gDNA in an additional three individuals. From Spain and Sweden we sequenced seven individuals each (five gDNA, two cDNA; for practical reasons gDNA and cDNA samples were taken from different individuals in Spain and Sweden).

Birds were trapped in nest boxes and blood samples collected from the brachial vein. Blood samples for DNA analysis were stored in 99% ethanol. An ammonium-acetate or phenol/chloroform method was used for DNA extraction (Richardson *et al.* 2001; Sambrook *et al.* 2001). For RNA collection, 80–100 μL of blood was added to 100 μL K_2EDTA (0.2M) and 500 μl of Trizol-LS added immediately after (following Miller and Lambert 2003). All samples were then stored at 4°C.

Restriction Fragment Length Polymorphism

To get an estimate of the numbers of class I loci in the blue tit genome, a Restriction Fragment Length Polymorphism (RFLP) analysis was performed. A restriction cleavage and southern blot were performed on 7 μg genomic DNA using the restriction enzyme *Pvu II* and a radioactively labelled class I exon 3 clone (consisting of a purified 215 bp PCR product, see below; for details see Westerdahl *et al.* 1999).

Sequencing

Initially several primer combinations were used for gDNA sequencing (PcaH1-A23H3, PcaH1 grw-A23H3; Balakrishnan *et al.* 2010, PcaH2-A23H3 and A21B (Bonneaud *et al.* 2004)-A23H3, table 5.1 in supplementary material). The primer combination A21B-A23H3 was most successful and we continued with only this combination after initial testing. The primers sequenced the major part of exon 3 (215 bp, primers not included, figure 5.1 in supplementary material). The following

PCR protocol was used for DNA amplification: 94° for 2 min, then 35 cycles of (94°C for 30s, T_A for 30s, 72° C for 30s), then 72°C for 10 min. and, finally 4°C on a thermal cycler before the samples were stored at 4°C (see table 5.1 in supplementary material for T_A). Reagent concentrations: genomic DNA: 50 ng, primers: 0.5 μM, dNTP: 0.15 mM, 10× buffer, $MgCl_2$: 1.5 mM, Taq 5U, final volume: 40 μL (AmpliTaq DNA polymerase with GeneAmp, Applied Biosystems, US). A ligation reaction was performed, in which the PCR products were cloned into a bacterial vector (TOPO - TA cloning kit, Invitrogen, California, USA). Between 5 and 20 bacterial colonies per individual were amplified (using primers of the cloning kit, M13fw-M13rv) and sequenced on a capillary sequencer (ABI prism 3730, Applied Biosystems, California, USA) according to a standard big dye protocol (Big Dye Terminator mix V3.1, Applied Biosystems).

We extracted and cleaned the RNA samples using the RNeasy clean up-kit (Qiagen, Hilden, Germany). A two-step RT-PCR reaction was then performed using the Retroscript kit according to protocol (Ambion, Applied Biosystems) with the A21B-A23H3 primers and finally the obtained cDNA was amplified, ligated and sequenced (See above).

Definition of alleles

All sequences were blasted against previously published avian MHC-I sequences (NCBI GenBank) for confirmation and the MHC-I sequences were aligned using BioEdit (Hall 2009). Only completely identical sequences found in two independent PCR events (from either RNA or DNA) were defined as alleles. We refer to these alleles as verified, since the same sequence is unlikely to have arisen twice from independent amplification errors. Throughout this paper, sequences are reported without the primers. The word "allele" is used to indicate a 215 bp exon 3 sequence derived from cDNA and/or gDNA.

Table 5.1 Indicators of selection on MHC-I. Values for Tajima's D and the significance value of D, π (mean pair wise difference/ nucleotide diversity), S (the number of segregating sites), the number of sites included in the region and p_s (the number of segregating sites, divided by the total number of sites). The p-value of Tajima's D was obtained by testing against the assumption of neutrality

	All sequences			group 1			group 2		
	All sites	PBR	Non PBR	All sites	PBR	Non PBR	All sites	PBR	Non PBR
Tajima's D	0.56	0.62	0.48	–0.92	–0.69	–0.94	0.88	1.26	0.51
Significance D, p =	0.66	0.67	0.64	0.17	0.19	0.15	0.72	0.82	0.62
π	0.06	0.14	0.04	0.02	0.03	0.01	0.06	0.16	0.04
S	38	15	23	12	4	8	27	12	15
Number of sites	215	36	179	215	36	179	215	36	179
p_s	0.18	0.42	0.13	0.06	0.11	0.05	0.13	0.33	0.08

Analysis of sequences

A phylogeny of the identified alleles was derived in PAUP* v.4.0b10 (Ronquist and Huelsenbeck 2003), the model of nucleotide evolution was determined according to the Akaike Information Criterion (AIC) with MrModeltest2.3 (Nylander 2004). GTR was used as the substitution model, while across-site mutation rates were assumed to be gamma distributed. We analysed the dataset in MrBayes3.1.2 (Huelsenbeck and Ronquist 2001; 2003). We ran four Markov chains for 5,000,000 generations in two parallel replicates, with chain heating parameter set to 0.15. Trees were sampled at intervals of 1,000 generations, and posterior probabilities (PP) were calculated from 2,000 trees after excluding 3,000,000 generations as burn-in. As an outgroup, we used a great reed warbler (*Acrocephalus arundinaceus*) sequence.

The MHC-I sequences of the blue tit previously reported by Foerster *et al.* (2006) were added from NCBI GenBank (accession numbers: AM232710-14). These sequences grouped with our sequences in the phylogenetic tree of alleles (data not shown).

The amino acids that comprise the PBR were superimposed on the blue tit sequences using the great reed warbler sequences (Westerdahl *et al.* 1999). To determine whether the PBR has been under selection, we calculated values of d_N/d_S (Hughes and Nei 1989; Page and Holmes 1998) using the Nei-Gojobori method and performed a codon-based Z-test (Nej-Gojobori, Jukes Cantor) in MEGA 4.1 (Tamura *et al.* 2007) to determine whether there was evidence for the occurrence of selection on the PBR vs. non-PBR regions. Tajima's D was calculated in Arlequin (Excoffier *et al.* 2005; Tamura *et al.* 2007) as an additional indication of selection. The value of d_N/d_S is the ratio of non-synonymous mutations (i.e. mutations resulting in a change in the amino acid sequence) to synonymous mutations (i.e. mutations after which the amino acid sequence remains intact), while Tajima's D uses the number of polymorphic sites to calculate the divergence between sequences. A d_N/d_S ratio larger than 1 as well as positive Tajima's D values are indicative of balancing selection.

Conserved sites within exon 3 have previously been described in the chicken (Livant *et al.* 2004), the Japanese quail (Shiina *et al.* 1995), the duck (*Anas platyrhynchos*; Mesa *et al.* 2004), the great reed warbler (Westerdahl *et al.* 1999) and the scarlet rosefinch (Promerová *et al.* 2009). To investigate whether the same sites were conserved in the blue tit, the blue tit alleles were compared to previously published MHC-I sequences of the above mentioned species. These sequences were added from NCBI GenBank (for accession numbers see figure 5.1). Conserved sites were extrapolated following Kaufman *et al.* (1994).

Our verified alleles were added to NCBI GenBank (Accession numbers: JF742764-80). The blue tit was recently renamed *Cyanistes caeruleus*, but to name our sequences, we use *Parus caeruleus*, to ensure consistency with blue tit MHC-I sequences previously published in NCBI GenBank. To avoid confusion with blue tit MHC-I sequences previously published in GenBank, we numbered our alleles Paca UA*101-Paca UA*117.

Species comparison

In order to compare genetic diversity at MHC-I exon 3 between the different passerine species studied to date, we calculated nucleotide diversity of the alleles of each species, using Arlequin version 3.11 (Excoffier *et al.* 2005). Nucleotide sequences were obtained from NCBI GenBank. In the blue tit, the phylogenetic tree of alleles revealed a distinct cluster of alleles with very little sequence diversity. For the species comparison of nucleotide diversity, only the eight alleles outside this cluster were used, since these are the alleles with the highest sequence diversity and most likely to be under strong selection. This pattern of clustering was not found in any of the other species included in this analysis and we randomly selected eight alleles to allow for a comparison of sequence diversity.

Results

Diversity

POPULATION LEVEL

A total of 17 MHC-I alleles were verified in 234 sequences obtained from 20 individuals that originated from Sweden, Spain and the Netherlands (figure 1 in supplementary material). At least eight of these alleles were transcribed, as they were found in cDNA (figure 5.1, table 5.2 in supplementary material) and seven of these eight transcribed alleles translated into different amino acid sequences. In total 13 different amino acid sequences were found (figure 5.1). Alleles UA*101, UA*110, UA*106 and UA*113 have identical amino acid sequences, as do alleles UA*105 and UA*115 (figure 5.1). No gaps, shifts in reading frame or non-sense codons were detected. Transcribed alleles were found throughout the phylogenetic tree (figure 5.2).

Seven amino acid residues that are conserved in exon 3 of MHC-I across bird species have been described (named Y123, T143, K146, W147, Y159, L160 and Y171 in the chicken; Kaufman *et al.* 1994, see also Shum *et al.* 1999; Mesa *et al.* 2004). In the blue tit, five of these sites were also conserved (Y123, T143, W147, Y159 and Y171). K146 is polymorphic in the blue tit and has different amino acids compared to all other bird species, except for the scarlet rose finch. L160 is polymorphic in the blue tit, group 2 has the common leucin (L) while group 1 has glutamine (Q) (figure 5.1).

INDIVIDUAL LEVEL

There was evidence for at least four class I loci, because we found a maximum of seven alleles (215 bp sequences) per individual (individual R, table 5.2 in supplementary material). This finding was further supported by the RFLP analysis where each individual had between five and eight RFLP bands (out of a total number of 10 RFLP bands found in the two families), each RFLP band representing approximately one MHC allele. Four RFLP bands were non-variable and occurred in all individuals (band no. 1, 4, 8 and 10, figure 5.3).

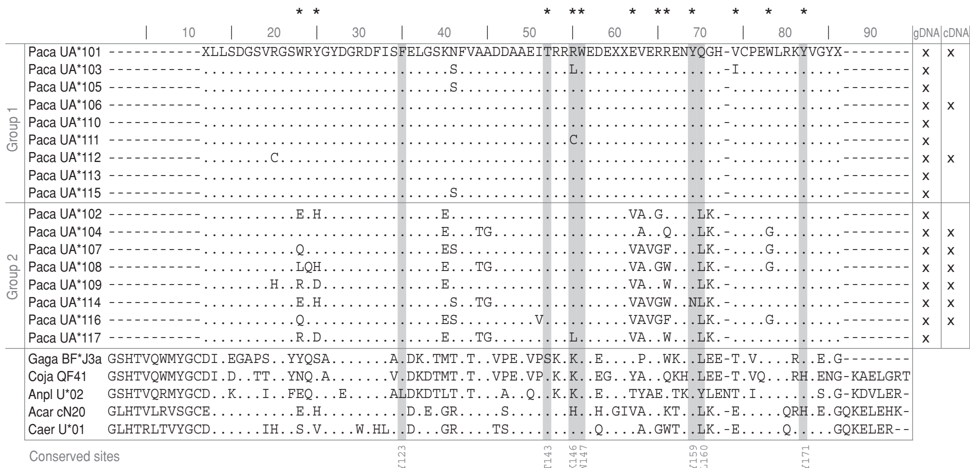


Figure 5.1 Amino acid sequences of exon 3 of the blue tit MHC-I, aligned with amino acid sequences of exon 3 of several other bird species: the chicken (*Gallus gallus*, Gaga BF*J3, accession number AY327148, alpha 2 region only, Livant *et al.* 2004), the Japanese quail (*Cortunix japonica* ,Coja, D29813Shiina *et al.* 1995), the duck (*Anas platyrhynchos*, Anpl, AY294416, Mesa *et al.* 2004), the great reed warbler (*Acrocephalus arundinaceus*, Acar cN20 exon 3 Westerdahl *et al.* 1999) and the Scarlet rosefinch (*Carpodacus erythrinus*, Caer U*01, FJ392762 Promerová *et al.* 2009), added from NCBI genbank. Asterisks mark the PBR, while shaded areas mark, and numbers below the figure, indicate the conserved sites as named in Kaufman *et al.* 1994. The columns on the right indicate whether the alleles were found in cDNA and gDNA.

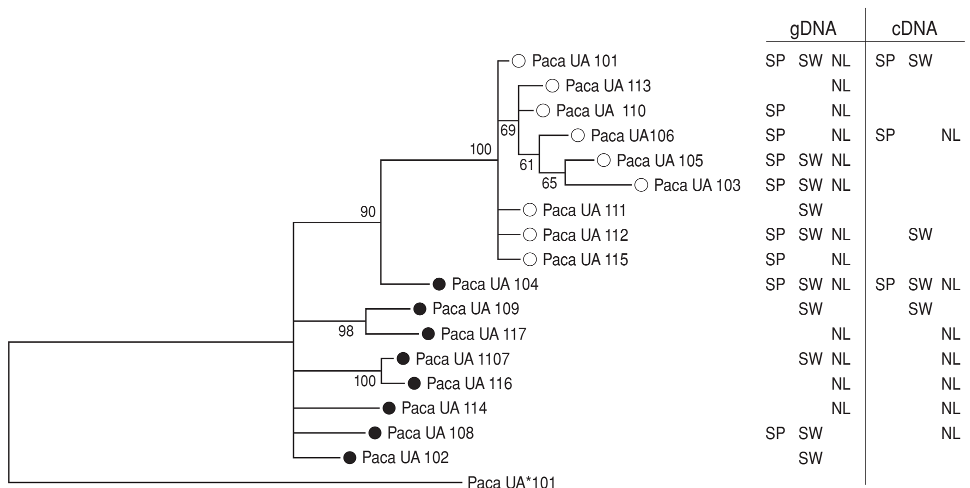


Figure 5.2 Phylogenetic tree for class I alleles in the blue tit. Numbers in the tree indicate the posterior probabilities expressed as a percentage (values below 50 not shown). The alleles of the allelic cluster with low diversity (group 1, supported by a posterior probability value of 100) are indicated by white dots, while all other alleles (group 2) have black dots. Abbreviations indicate from which population the DNA sample was taken (NL=the Netherlands, SW=Swedish, SP=Spanish).

Selection

PHYLOGENY

One distinct cluster containing nine MHC-I alleles was observed in the phylogenetic tree (supported by a posterior probability value of 100, figure 5.2). These nine alleles will be referred to as group 1, while the remaining eight alleles will be referred to as group 2. The clustering of the alleles in group 2 lacks phylogenetic support. The nucleotide diversity (π) and the number of segregating sites (S) within group 1 were significantly lower than in group 2 (Group1: π = 0.017 ± 0.011 SD, S = 12, group 2: π = 0.056, ± 0.033 SD, S = 27, table 5.1, $p < 0.05$, t-test). Five of the segregating sites contained the same polymorphism in group 1 and 2. In addition, four sites were monomorphic within group 1 and monomorphic in group 2, but differed between the groups (figure 5.1 in supplementary material). Overall the nucleotide diversity was 0.059, while there were 38 segregating sites.

There was no geographical separation of alleles across the phylogenetic tree, as alleles from all three populations (Spain, the Netherlands and Sweden) were distributed across the entire tree and frequently shared between sample locations (figure 2). Six out of the total of 17 alleles were found in all three locations (UA*101, 103, 104, 105, 108, 112), three that belong to group 1 and three to group 2. Some alleles were only found in one location (UA*102, 111, 113, 114, 117, figure 5.2). However, these alleles were highly similar to those found in other locations. Expressed alleles were found in all parts of the phylogenetic tree and in both group 1 and 2. We found evidence for at least four group 1 loci and two group 2 loci (table 5.2 in supplementary material).

SELECTION INDICES

We found significant balancing selection for the PBR of group 2, (d_N/d_S = 5.488, Z = 2.98, p = 0.02), but no evidence of any kind of selection (i.e. balancing or purifying) acting on the PBR of group 1 (d_N/d_S = 0.338, Z = –0.50, p = 0.62). None of the non-PBR regions were under selection (Group 1: d_N/d_S = 0.179, Z = –1.64, p = 0.10,

Table 5.2 Overview of the number of non-synonymous (d_N) and synonymous (d_S) mutations in the peptide binding region (PBR) and other regions (non-PBR) for class I. Value ± Standard error (bootstrap, n = 5000 replicates). p and Z values were obtained using a codon based Z-test.

	Group 1		Group 2	
	PBR	Non-PBR	PBR	Non-PBR
d_N	0.025 ±0.019	0.005 ±0.004	0.236±0.090	0.033 ±0.010
d_S	0.074 ±0.092	0.028 ±0.013	0.043± 0.039	0.041±0.023
d_N/d_S	0.338	0.179	5.488	0.805
p-value	0.62	0.10	0.02	0.74
Z	-0.50	-1.64	2.98	-0.33

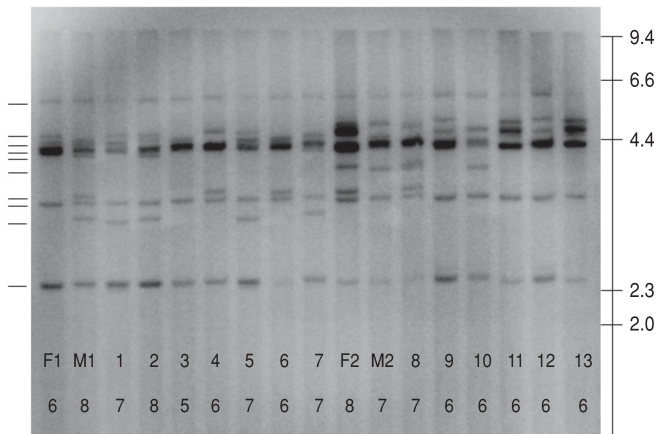


Figure 5.3 RFLP gel of two blue tit families. The top row of numbers indicates individuals: M1 and F1 are parents of individuals 1-7 (M = male, F = female) and M2 and F2 are parents of individuals 8–13. The second row indicates the total number of bands present for each individual. A size standard in Kb is shown on the right. The position of all RFLP bands found is indicated on the left of the gel. All RFLP bands were between 2.3 and 6.6 kb in length.

group 2: $d_N/d_S = 0.805$, Z = –0.33, $p = 0.74$; table 5.2; codon based Z-test, Nei-Gojobori, Jukes-Cantor).

The high values of Tajima's D in group 2 (D = 1.26, $p = 0.82$ and D = 0.51, $p = 0.62$ for PBR and non-PBR, respectively) indicate that alleles in this group have been maintained in the population for longer than expected under neutrality (as the phylogenetic tree indicates, figure 5.2). This effect was most pronounced in the PBR region. Both PBR and non-PBR regions of group 1 appear to be under purifying selection, as indicated by negative values of Tajima's D (D = –0.69, $p = 0.19$ and D = –0.94, $p = 0.15$ for PBR and non-PBR, respectively). None of the Tajima's D values provide significant evidence for a deviation from neutrality, however (table 5.1).

Species comparison

We found that the nucleotide diversity of blue tits was significantly lower than that of any of the other species, including the inbred Seychelles warbler ($p < 0.05$). The great reed warbler had the highest nucleotide diversity (table 5.3).

Table 5.3 Measures of genetic diversity (number of segregating sites (S), Tajima's D and the nucleotide diversity (π; $\pm$SD)) at the MHC-I for five species of passerine birds. See methods for selection of species and the MHC-I alleles from GenBank.

Species	GenBank accession numbers	Segregating sites (S)	Tajima's D	Nucleotide diversity (π) ± st. dev.
Great reed warbler (*Acrocephalus arundinaceus*)	AF449695 AF449696 AF449697 AF449699 AF449700 AF449701 AY306008 AY306009	64	0.23 (p = 0.61)	0.121 ± 0.067
Seychelles warbler (*Acrocephalus sechellensis*)	AJ557874 AJ557875 AJ557876 AJ557877 AJ557878 AJ557879 AJ557882 AJ557883	49	0.46 (p = 0.71)	0.096 ± 0.054
Great tit (*Parus major*	AF346821 AF346822 AF346823 AF346824 AF346825 AF346828 AF346829 AF346832	50	0.19 (p = 0.61)	0.111 ± 0.062
Blue tit (*Cyanistes caeruleus*)	Group 2	26	0.89 (p = 0.86)	0.056 ± 0.032
Scarlet rosefinch (*Carpodacus erythrinus*)	FJ392763 FJ392768 FJ392769 FJ392772 FJ392774 FJ392778 FJ392788 FJ392790	62	1.47 (p = 0.96)	0.149 ± 0.083

Discussion

Selection pressures

It is reasonable to expect that the prevalence and composition of pathogens vary between our three different blue tit populations, from Spain in the south to Sweden in the north, possibly reflecting climatic differences (i.e. Bensch and Akesson 2003; Merino and Møller 2010). Therefore, one may expect different selection pressures on the MHC resulting in population differentiation in MHC alleles. Our phylogenetic tree of alleles shows no obvious geographical relationship to the three source locations. One of the characteristic features of the MHC genes is that alleles can persist for a long time and that trans-species polymorphism (MHC alleles or lineages that are shared between diverged species) is common (Edwards and Hedrick 1998; Westerdahl 2007). In a phylogeny containing the MHC alleles of closely related species it is uncommon that the alleles will cluster in a species-specific manner. Most likely, a geographical relationship to the three source populations in the phylogenetic tree should only be expected when there are substantial differences in selection pressures operating. Hence, it may not be so surprising that we did not find evidence of population differentiation between our sample locations. In a study of population differentiation among three Belgian blue tit populations (on a much smaller spatial scale than our study), Verheyen *et al.* (1995) found significant population differentiation using selectively neutral markers. Ekblom *et al.* (2007) studied population differentiation in MHC-II in Scandinavian and East European populations of the great snipe (*Gallinago media*) and found significant geographical differentiation, but this was reflected in allele frequencies rather than in phylogeny. Therefore, we may expect to find a geographical structure in MHC-I when studying allele frequencies. Unfortunately, our present results do not allow us to test for differences in allele frequencies, since we sampled a limited number of individuals in each population.

The theory of pathogen driven selection predicts that pathogen distributions vary over time and in space and that selection acts on a species to keep up with its' changing environment (Bodmer 1972; Hedrick 2002). To recognize novel pathogens, the peptide binding regions (PBRs) of MHC molecules must be able to adapt rapidly and are expected to be under balancing selection (Hughes and Nei 1988; Hughes and Nei 1989). We found that the d_N/d_S ratio in the PBR of the alleles in group 2, the diverse allele group, was significantly higher than one. Furthermore, the positive (though non-significant) value of Tajima's D also indicated that group 2 has deeper branches than expected under neutrality. A positive Tajima's D value could be caused by demographic events in the population history (e.g. population expansion) or by the occurrence of balancing selection. If the demographic history was responsible for the positive Tajima's D value in the PBR of group 2, we would also expect to observe a positive value for the non-PBR regions, since they are regions within the same gene locus, but we did not. Therefore, balancing selection acting on the PBR of group 2 is the most likely explanation. It should be noted that we calculated d_N/d_S and Tajima's D from sequences derived from at least four loci, though ideally

it should be calculated within one locus. This may result in an overestimation of the number of synonymous substitutions (Hughes and Nei 1989). However, these numbers should be overestimated in both PBR and non-PBR regions, so it is safe to conclude that the PBR is under stronger positive selection than non-PBR regions in group 2.

The MHC-I alleles in group 1 have d_N/d_S ratios lower than one and negative Tajima's D values for the PBR as well as the non-PBR regions. These values are indicative of negative selection on group 1, though the evidence was not statistically significant. The alleles in group 1 also have remarkably little sequence variation, which could be explained by the alleles originating from a bottlenecked population (while the alleles of group 2 diversified under selection) or by strong negative selection. A bottleneck has occurred in the phylogenetic history of the blue tit (Kvist *et al.* 2004), but we also found some evidence for negative selection. Since the bottleneck in the blue tit population was not very severe, we cannot currently explain the lack of variation in group 1.

MHC-I compared to other passerines

We found evidence for the existence of four MHC-I loci in the blue tit. MHC-I has been described for a small number of passerine species: the Seychelles warbler, great reed warbler (Westerdahl *et al.* 1999; Richardson and Westerdahl 2003), scarlet rosefinch (Promerová *et al.* 2009), house sparrow (Bonneaud *et al.* 2004) and now the blue tit. These species represent phylogenetically very different groups within the passerines. Yet, the number of MHC-I loci is very similar in these species, with the exception of the great reed warbler (five loci in the house sparrow; Bonneaud *et al.* 2004; five in the scarlet rosefinch; Promerová *et al.* 2009; eight in the great reed warbler; Westerdahl *et al.* 1999). Preliminary RFLP-results suggests that the number of MHC-I loci in the Seychelles warbler also is higher, so the high number of MHC-I loci in the great reed warbler could be representative for this taxonomic group (Richardson and Westerdahl 2003).

It has previously been proposed that the MHC should be more diverse in migratory than in non-migratory species, since migratory species encounter more pathogen species and strains (Møller and Erritzøe 1998; Westerdahl *et al.* 2000). We did not find a consistent pattern when comparing the number of MHC-I loci or nucleotide diversity between migratory (great reed warbler, scarlet rosefinch) and non-migratory (house sparrow, Seychelles warbler, blue tit) passerines. Genetic diversity within species or populations may further be reflected in genetic polymorphism, a value difficult to compare between species, since it is likely to be correlated to the number of individuals sampled. Our inability to detect a difference in MHC-I diversity may be due to the very small number of species we compared. Hopefully, the characterisation of MHC-I for other passerine species will allow a more robust comparison in the near future.

The nucleotide diversity and the number of segregating sites in the blue tit MHC-I alleles were remarkably low compared to other passerine species, even

though only the variable blue tit alleles in group 2 were taken into account. The nucleotide diversity and number of segregating sites are also lower than those of the closely related great tit (*Parus major*). One explanation for the low diversity could be that the blue tit underwent a population bottleneck, after which the population expanded and spread throughout Europe (Kvist *et al.* 2004). Kvist *et al.* (2004) suggest that this bottleneck took place during the last ice age, when birds were left in two refuges, the Iberian Peninsula and the Balkan, resulting in the *C.c. caeruleus* and *C. c. ogliastrae* subspecies. Hence, the *C. c. caeruleus* subspecies arose from the refuge population in the Balkan and this bottleneck may explain the low MHC-I nucleotide diversity that we observed. The population history of the great tit is thought to be comparable to that of *C. c. caeruleus*, although the blue tit diverged more recently than the great tit (Kvist *et al.* 1999a; Kvist *et al.* 1999b), which may partly explain why the nucleotide diversity and number of segregating sites of the great tit is higher. In a species comparison of genetic variation among mitochondrial DNA, Kvist *et al.* (1999) found that levels of nucleotide diversity are comparable in the blue tit and the great tit (Kvist *et al.* 1999a). Surprisingly, the nucleotide diversity and number of segregating sites of MHC-I were even lower in the blue tit than in the Seychelles warbler, a species that underwent a severe recent (1920-approx. 1968) population bottleneck (Komdeur and Pels 2005). The bottleneck that the blue tit population went through is less recent and less severe than the bottleneck in the Seychelles warbler population and we would expect more genetic variation in the blue tit MHC. Another possible explanation for the low nucleotide diversity and number of segregating sites in the blue tit is the lack of diversifying selection, which could occur due to the absence of pathogens. However, there is evidence that blue tits are commonly infected with several species of blood parasites (such as avian malaria; Merino *et al.* 2000; Cichon and Dubiec 2005; Tomás *et al.* 2007; Arriero *et al.* 2008; Knowles *et al.* 2010) and we have no reason to believe that pathogen pressures are lower in blue tits compared to other passerine species. In case selection pressures from pathogens had relaxed during a longer period of the blue tits' population history, one would expect this to be reflected by a relatively low number of loci and loci becoming non-functional.

Our characterisation of MHC-I in the blue tit shows the existence of one phylogenetic cluster (group 1, versus the remaining alleles named group 2) with very low sequence diversity and indications of purifying rather than balancing selection, while the remaining alleles show the expected MHC characteristics. A non variable MHC-I allele cluster has previously been found in another passerine, the house sparrow (Bonneaud *et al.* 2004). For MHC-II, the division in two allele clusters that differ in nucleotide diversity has been described in Hawaiian honeycreepers (*Drepanidinae*) and Darwin's finches (*Geospizinae*) (Jarvi *et al.* 2004). The existence of two gene clusters that segregate independently (the classical BLB and non-classical YLB) is known in several galliform species (Miller *et al.* 1994; Wittzell *et al.* 1995; Hunt *et al.* 2006; Strand *et al.* 2007). Purifying selection is one of the defining characteristics of non-classical MHC alleles (Janeway *et al.* 2008) and a possible

explanation for the lack of polymorphism in the alleles in group 1 could be that these alleles are non-classical in origin, while the alleles in group 2 are classical. So far, we have too little background information on the blue tit MHC to determine whether the alleles we found are classical or non-classical.

We found evidence (although not statistically significant) that the alleles in group 1 are under purifying selection, which could explain the low nucleotide diversity found in this group of alleles. Is seems possible that the molecules derived from alleles in group 1 perform an essential function in immune recognition and selection acts to preserve them. Purifying selection on the alleles essential for immune recognition could have led to convergent evolution, leading to the existence of a non-variable gene cluster in several passerine species.

One could argue that the alleles in group 1 may be a radiation of recently originated alleles. If the alleles in group 1 were of recent origin, we would expect the values for d_N and d_S to be similar, since selection has not yet had a strong effect. When a phylogeny is drawn including only synonymous substitutions, the clustering in our phylogenetic tree completely disappears (data not shown) and we conclude that selection must be involved in creating this gene cluster. One may also argue that more clusters of highly similar alleles may exist in the blue tit and that the alleles in group 2 belong to one or more of these clusters, but that we simply did not find the alleles in the other clusters due to a limited sample size. In that case, we would erronously draw the conclusion that there is a large difference in diversity between group 1 and group 2, since we are comparing the group 1 alleles to alleles potentially belonging to several clusters. For a follow up project, we designed primers to specifically amplify the alleles of group 2 (data not shown), but we found no evidence for another gene cluster with low diversity.

Interestingly, one of the seven amino acid residues that are conserved in exon 3 of MHC-I across bird species differed between group 1 and 2 (L160). Group 1 had the common leucin (L160) changed to glutamine (Q160). Leucin and glutamine have different characteristics and such an amino acid change could therefore indicate different peptide binding abilities in the PBR of group 1 and 2.

An indication that the number of samples we obtained per individual is limited (for both cDNA and gDNA) may be that certain alleles were only revealed in the cDNA (and not in gDNA) in an individual. In order to reveal all alleles an individual possesses and all existing alleles across all blue tit populations, a very large number of sequences would have to be obtained. Importantly, however, it is unlikely that our main conclusions would be altered by increasing the sample sizes, since 1) the phylogenetic relationships would likely be maintained and 2) including rare alleles in our analysis is unlikely to increase the maximum number of alleles per individual, since our RFLP analysis confirmed our estimation of four loci.

Conclusions and future prospects

This study is the first to characterise Major Histocompatibility Complex class I in the blue tit and is among few published studies that have characterized MHC-I in

passerine species. Besides providing insight into the structure, diversity and selection acting on MHC-I, the characterisation of the blue tit MHC-I is a first step towards the development of high throughput methods for MHC-I screening. Such methods will make it feasible to explore the mechanisms imposing balancing selection on the PBR of MHC-I in natural blue tit populations. Among other insights, these future analyses may reveal why the blue tit MHC-I exhibits such low sequence diversity compared to other passerine species.

Acknowledgements

The authors are very grateful to the Molecular Ecology and Evolution lab at Lund University (Sweden) for allowing us to perform the molecular work. Staffan Bensch gave useful comments on the manuscript and the interpretation of the statistical analyses. Maria Karlsson and Kristin Scherman provided useful comments on earlier versions of the manuscript. ES received funding from the Dobberke Stichting in the Netherlands. HW received funding from the Swedish Research Council. SM and JRdA received financial support for this work from project CGL2009-09439 (Spanish Ministry of Science and Innovation MICINN). JK received funding by GEBACO (FP6/2002–2006, no. 28696) and INCORE (FP6–2005-NEST-Path, no. 043318). The authors would further like to thank Peter Korsten, Oscar Vedder, Mimi Lannefors, Eva Friman, Martin Stervander and Marco van der Velde for their assistance and Martin Stjernman, Lars Råberg and Bengt Hansson for generously sharing DNA from Swedish blue tits.

CHAPTER 6

MHC-I screening in blue tits (*Cyanistes caeruleus*) using Reference Strand mediated Conformation Analysis (RSCA)

Elske Schut, Juan Rivero-de Aguilar, Michael. J. L. Magrath, Jan Komdeur and Helena Westerdahl

Abstract

The Major Histocompatibility Complex (MHC) is a highly diverse gene complex, encoding proteins that are used in vertebrate immune recognition. Due to its diverse nature, the MHC is commonly studied by evolutionary biologists, as they strive to explain how this diversity is being maintained. To study the MHC an efficient genotyping method is required, but developing such a method, capable of dealing correctly with the high MHC polymorphism, while having limited knowledge about the genetic structure, is a challenge. This is the first study describing the use of Reference Strand-mediated Conformation Analysis (RSCA) in a passerine bird, the blue tit (*Cyanistes caeruleus*), to separate highly polymorphic MHC class I (MHC-I) alleles. The RSCA method is an accurate, cheap and high throughput method. Our results show that RSCA successfully screened MHC-I in blue tits and the working scheme outlined here has already been adapted for use in other passerines, e.g. great reed warblers (*Acrocephalus arundinaceus*) and house sparrows (*Passer domesticus*). We believe that RSCA is suitable for finding and verifying new MHC alleles and is a good complement to next generation sequencing (e.g. amplicon 454-sequencing). RSCA will aid in generating high quality MHC data in non-model organisms, data that should contribute to elucidating the mechanisms behind balancing selection, such as pathogen driven and sexual selection, in natural populations.

Introduction

The Major Histocompatibility Complex (MHC) genes are the most polymorphic genes known and encode proteins that are important for immune recognition in vertebrates. Molecules derived from MHC class I (MHC-I) are mainly involved in the recognition of peptides that arise from intracellular pathogens, while molecules derived from MHC class II are mostly involved in the recognition of extracellular pathogens (Klein 1986; Goldsby *et al.* 2000; Janeway *et al.* 2008). Selective forces are thought to maintain the high MHC diversity and different mechanisms have been proposed; the main hypotheses being pathogen driven selection and mate choice based on the MHC (Bodmer 1972; Nei and Hughes 1991; Hedrick 2002; Borghans *et al.* 2004; Piertney and Oliver 2006; Oosterhout 2008). Pathogen driven selection is an evolutionary arms race originating from host-pathogen interactions that results in an advantage for hosts with rare MHC alleles and/or MHC heterozygosity (Borghans *et al.* 2004). Mate choice based on the MHC potentially results in a higher reproductive success of individuals that mate disassortatively or with mates with heterozygous/ rare genotypes (see Jordan and Bruford 1998; Penn 2002 for a review). Despite the existence of much literature on the MHC and the potential selection pressures acting on it (see Edwards and Hedrick 1998 for a review), details on how MHC allelic diversity is maintained in natural populations are unknown. Furthermore, it is not understood why the number of MHC loci varies so much between species and how specific MHC alleles survive several speciation events (Westerdahl 2007; Spurgin and Richardson 2010).

In order to gain greater insight into the selective pressures acting on MHC in present time in natural populations, we wanted to screen MHC-I diversity in a passerine bird, the blue tit (*Cyanistes caeruleus*). The blue tit is a suitable study species because it is a commonly studied passerine (Kempenaers *et al.* 1997; Leech *et al.* 2001; Delhey *et al.* 2003; Brommer *et al.* 2007; Magrath *et al.* 2009) and hence, a range of selection pressures are well understood, including sexual and parasite selection (i.e. Kempenaers *et al.* 1997; Merino *et al.* 2000; Leech *et al.* 2001; Delhey *et al.* 2003; Brommer *et al.* 2007; Stjernman *et al.* 2008; Magrath *et al.* 2009). This prior knowledge will help place novel MHC results in context. For example, several parasites, including malaria, are known to severely affect survival and reproductive success in blue tits (Cichon and Dubiec 2005; Tomás *et al.* 2007; Arriero *et al.* 2008; Knowles *et al.* 2010) and we would like to know (amongst other questions) whether MHC-I characteristics affect the prevalence and infection intensity of avian malaria.

MHC-I has been studied in a handful of passerines to date and the number of loci differs considerably between species (between four and at least eight; Westerdahl *et al.* 1999; Richardson and Westerdahl 2003; Bonneaud *et al.* 2004; Promerová *et al.* 2009; Balakrishnan *et al.* 2010; Schut *et al.* 2011). Passerine MHC-I contains several homogenised loci, and a large number of alleles will therefore be amplified at the same time when screening for MHC-I diversity using a standard

PCR. These PCR products then require separation on base pair composition (rather than fragment length) and the most commonly used techniques for separating MHC alleles are Denaturing Gradient Gel Electrophoresis (DGGE; Knapp 2005), Single Strand Conformation Analysis (SSCP; Orita *et al.* 1989), Reference Strand mediated Conformation Analysis (RSCA; Argüello *et al.* 2007; Lenz *et al.* 2009) and amplicon 454-sequencing (Babik *et al.* 2009; for a review of different methods, see Babik 2010). Several earlier studies in birds have used DGGE to screen MHC genetic diversity (i.e Ekblom *et al.* 2004; Bonneaud *et al.* 2004; Westerdahl *et al.* 2004), but this method is laborious and requires hazardous chemicals. Recently the much less laborious technique of RSCA was successfully used to separate MHC alleles in red jungle fowl (*Gallus gallus*) and black grouse (*Tetrao tetrix*; Worley *et al.* 2008; Strand and Höglund 2011). Red jungle fowl and black grouse only have two MHC-I and two MHC-II loci, and at most four alleles need to be separated at the time. Passerines have more MHC-I loci so separating MHC-I alleles in passerines is more difficult. An important requirement for developing any molecular screening method for highly polymorphic genes in a non-model organism is an initial DNA sequencing protocol that gives an overview of the full molecular diversity, preferably even full-length cDNAs. Once such an overview of the molecular diversity is obtained, it is possible to develop primers that specifically amplify alleles of key interest (Westerdahl *et al.* 2004; Babik 2010).

In this study we use samples originating from blue tit populations right across their geographic distribution, Sweden, Spain and the Netherlands, to develop an RSCA screening protocol that is at least applicable to all European blue tits. We design specific primers, based on the partial characterization of the blue tit MHC-I, that preferentially amplify transcribed, highly polymorphic MHC-I alleles known to have been under balancing selection (Chapter 5). Finally, we present an RSCA screening protocol that separates these blue tit MHC-I alleles and an RSCA flow scheme that is applicable to any passerine species. We have also used this RSCA flow scheme for MHC-I successfully in great reed warblers *Acrocephalus arundinaceus* and house sparrows *Passer domesticus* (see below).

Methods

RSCA is a PCR based technique that uses a fluorescently labelled reference strand (FLR) that is hybridised to the PCR products to be screened. The FLR is obtained by amplifying the same DNA fragment used in the study species in related species (using identical primers). The hybridised fragments are then separated on e.g. a capillary sequencer and the degree of similarity between the FLR and the DNA fragment (PCR product) determines how well they hybridise and how fast these heteroduplexes migrate (Argüello *et al.* 2007). The different steps of the RSCA methodology in blue tits are summarized in Figure 6.1 and described in detail below.

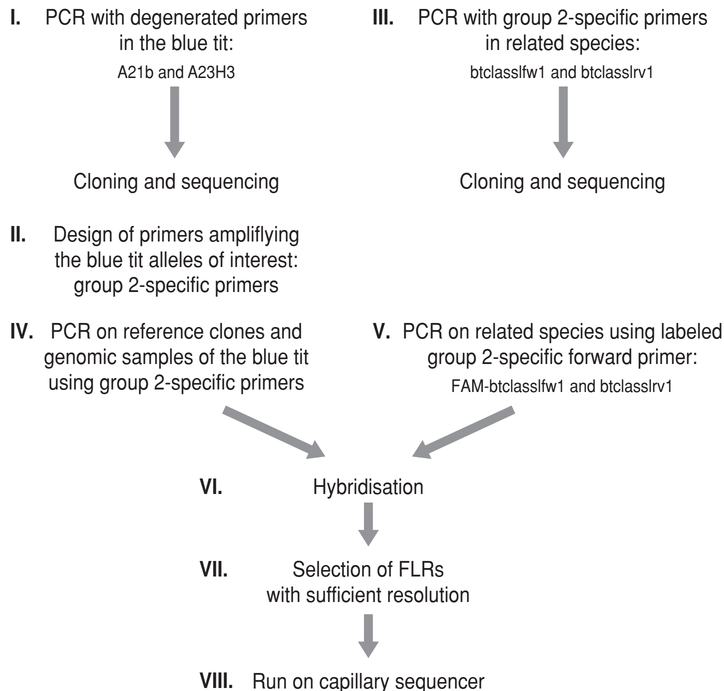


Figure 6.1 Flow scheme of the steps involved in development of the RSCA method. Numbers indicate the order in which steps were performed.

I. PCR with degenerated primers in the blue tit

In order to obtain background information on the genetic diversity of MHC-I in European blue tits, we used DNA and RNA samples from several populations (Chapter 5). We cloned and sequenced MHC-I exon 3 in 20 blue tit originating from three locations: Sweden (Revingehed, 55°41′N, 13°26′E, 20 km east of Lund, southern Sweden), Spain (Valsain, central Spain, 40°49′N, 3°56′E) and the Netherlands (*The Vosbergen* estate, near Groningen, 53°08′N, 06°35′E) using degenerate primers A21b-A23H3 (Bonneaud *et al.* 2004; Balakrishnan *et al.* 2010). These degenerated primers amplify most MHC-I alleles present in blue tits, since the results obtained using PCR and degenerated primers (maximum of 7 alleles per individual) correspond well to the diversity found in an RFLP analysis on unamplified genomic DNA (max. 8 alleles per individual, both the PCR and the RFLP results correspond to four MHC-I loci; Chapter 5). Therefore we are confident that we obtained a good overview of the MHC-I allelic complexity in the blue tit and that this background knowledge enables us to set up an RSCA protocol.

The blue tit MHC-I exon 3 sequences (215 bp fragments; *Paca* UA*101-117; GenBank accession numbers JF742764-80) will from here on be called alleles, although we are aware that they stem from several loci and are only partial frag-

ments of an allele. The MHC-I alleles form two groups in a phylogenetic tree, one of which is a distinct cluster with high bootstrap support ("group 1"; see Figure 2 in Chapter 5). The nucleotide diversity of the alleles in group 1 was significantly lower than that of the remaining alleles ("group 2"). Group 2 has a d_N/d_S ratio (number of synonymous substitutions/number of non-synonymous substitutions) larger than one in the peptide binding region (PBR) and a positive (although non-significant) value of Tajima's D, suggesting that balancing selection is acting on the PBR of group 2. In group 1 there were no indications of balancing selection (Chapter 5). Alleles from both group 1 and 2 were expressed and we did not find any evidence of non-functional genes.

In the present study, we added previously identified blue tit MHC-I sequences from German birds from NCBI GenBank (only those that include the entire 215 bp exon 3 sequences we found, parts outside this region were cut; accession numbers: AM232710-14). They align well with our blue tit sequences and share the group 1 and 2 polymorphism (Figure 6.2), although the statistical support for group 1 is low (Figure 6.3).The alleles in group 1 and 2 can be separated on polymorphic sites (187-188bp) in the 3'-end of the fragments (Figure 6.2).

II. Design of primers amplifying the alleles of key interest

Based on the phylogenetic grouping of alleles and the polymorphic sites separating groups 1 and 2 (Figure 6.2 in Chapter 5) we designed specific primers (btclassIfw1 and btclassIrv1, Figure 6.2) to amplify only the alleles of group 2. We chose to amplify the most variable alleles only, since they are expected to be under the strongest selection (as indicated by the d_N/d_S and Tajima's D values; Chapter 5). We used a touchdown PCR with the group 2-specific primers, starting with an annealing temperature (T_A) of 64°C, while T_A decreased by 0.8°C in each consecutive cycle during the first 9 cycles (i.e. 64–57.6°C), before the PCR continued with 26 cycles at 57°C (for other PCR conditions see above, reagent concentrations: DNA: 50 ng, Taq: 2.5U, Primers: 0.38 μM, 10× buffer, dNTP: 0.12 mM, $MgCl_2$: 1.44 mM, final volume: 26 μL).

III. PCR with group 2-specific primers in related species

To develop the FLRs that are to be hybridized to the blue tit alleles, we needed DNA fragments similar enough to hybridize with the blue tit alleles. To find such fragments, we tested our group 2-specific primers on genomic DNA from several passerine species: marsh tit (*Poecile palustris*), great tit (*Parus major*), great reed warbler, Basra reed warbler (*Acrocephalus griseldis*), zebra finch (*Taeniopygia guttata*), crow (*Corvus corone*) and house sparrow. Successful amplifications were obtained from all species except crow and zebra finch. We ligated and cloned the PCR product from a single great tit, marsh tit and house sparrow and then picked 12 colonies per individual (TOPO-cloning kit, Invitrogen, US, for details see Chapter 5). Then a standard PCR reaction with the group 2-specific primers (btclassIfw and btclassIrv1) was performed on each clone.

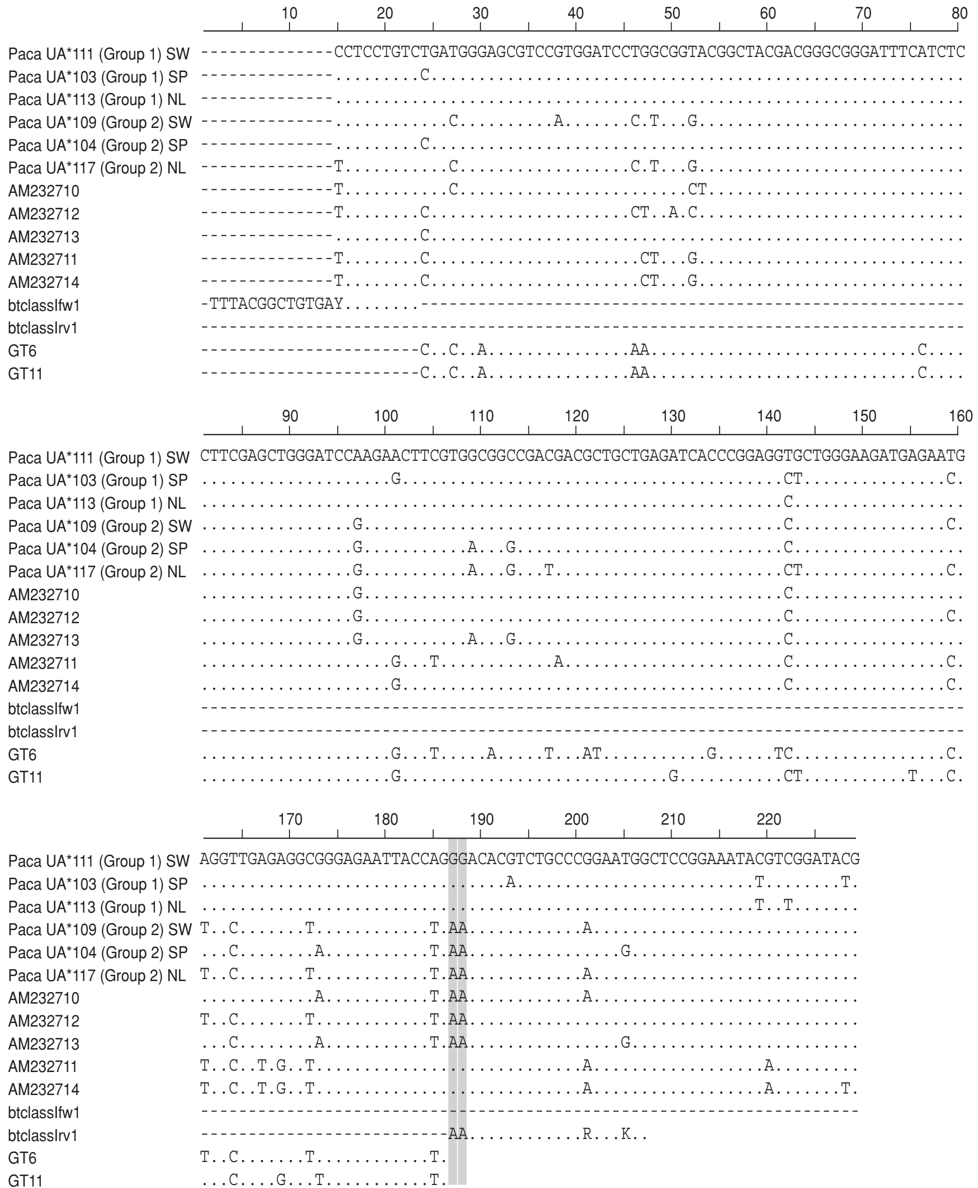


Figure 6.2 Sequences of alleles of group 1 and group 2 that were found in each of the populations (SW=Sweden, SP=Spain, NL= the Netherlands, alleles that are assigned to the Spanish populations, were found in all populations; Schut *et al.* 2011), the primers designed to amplify alleles of group 2 for the RSCA method (btclassIfw1 and btclassIrv1) and the FLRs. Also added are blue tit sequences from GenBank (AM232710-14). Dots indicate base pairs that are identical to allele UA*11. The polymorphic sites (187/188) that distinguish group 1 and group 2 are highlighted in grey.

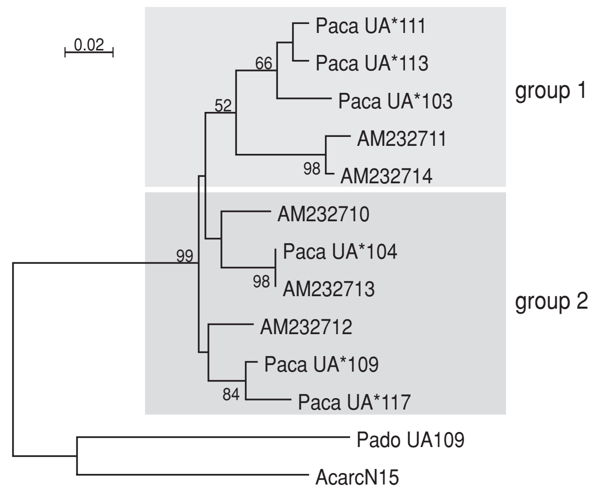


Figure 6.3 Phylogenetic tree for class I alleles in the blue tit. Included are the sequences found by Schut *et al.* 2011) (aca UA*103, UA*104, UA*109, UA*111, UA*113,UA*117) as well as the blue tit sequences previously deposited in NCBI GenBank (AM232710-14). We added a great reed warbler sequence (Westerdahl *et al.* 1999; AcarcN15; GenBank Accession number AJ005506) and a house sparrow sequence (Loiseau *et al.* 2011; Pado UA109; GenBank Accession number EU715816). The nucleotide analysis (Maximum likelihood, GTR, (G+I), 4 categories, bootstrap with1000 replications) was performed in Mega 5.03 (Tamura *et al.* 2011). The allele cluster with low diversity, group 1, is supported by a bootstrap of 52 and the remaining alleles, with more classical MHC characteristics, were assigned as group 2.

IV. PCR on blue tit reference clones and genomic samples

A PCR using the group 2-specific primers was performed on all eight blue tit group 2 MHC-I alleles (verified by cloning and sequencing, see Chapter 5) and also on genomic DNA from 14 Dutch blue tit families (105 offspring and 28 parents, collected between 2001-2003 from the population in *De Vosbergen*), using the PCR conditions described above (paragraph II). We also amplified MHC-I in two blue tit reference individuals, which were run along in every RSCA run, allowing us to correct for differences in running distance between alleles in consecutive runs.

V. PCR on related species using labelled group 2-specific forward primer

A PCR with unequal amounts of group 2-specific forward and reverse primers was performed on the cloned PCR products from great tit and marsh tit to create the FLRs (three clones per species). A fluorescent label (5′FAM) was attached to the forward primer (btclassIfw1). In this PCR we used 25 pmol of forward primer and 2.5 pmol of reverse primer. This results in an excess of labelled forward primer. For the blue tit (as well as the great reed warbler and the house sparrow) it worked well to have 10% reverse primer. However, this step should be optimized in every new species and a suitable range to test is between 5 and 20% reverse primer

(Reagent concentrations: genomic DNA: 50 ng, forward primer: 0.94 μM, reverse primer: 0.094 μM, dNTP: 0.18 mM , 10× PCR buffer, $MgCl_2$: 0.18 mM , Taq 3.25 U, final volume: 26.7 μL, the same PCR conditions as above apply, see II). The product of this PCR was then diluted 16× and this is the working concentration of the FLRs for blue tits. This step should be optimized for new FLRs and a range between 10× and 20× dilution is reasonable to test.

VI. Hybridisation of gDNA and single alleles

For the hybridisation reaction, 2 μL of the diluted (16×) fluorescently labelled great tit and marsh tit PCR product (FLR) was added to 3 μL of the concentrated blue tit genomic PCR product or 2 μL of the PCR product of a single allele (clone). During the hybridization, this cocktail was heated to 95°C for 10 min., to 55°C for 15 min. and then cooled to 4°C for 10 min. This concentrated hybridisation reaction can be stored at 4°C for at least five days and at –20°C for several weeks. Just before the RSCA is run ,15 μL of ddH_2O should be added to the reaction and then 11 μL of this diluted hybridisation reaction added to 10 μL ddH_2O with ROX 500 solution (to a final concentration of 0.09 μL ROX 500; Applied Biosystems, Foster City, California, US).

VII. Selection of FLRs with sufficient resolution

We tested great tit and marsh tit clones as FLRs by hybridizing the diluted product of their PCR (amplified with group 2-specific FAM forward primer) to the PCR product of blue tit clones. The two great tit clones ("GT6" and "GT11") together showed the best separation between allele peaks and together they gave sufficient resolution to detect all eight blue tit alleles (Figure 6.4). These two clones were therefore chosen as FLRs for blue tit MHC-I screening (in the great reed warbler we used FLRs from the Seychelles warbler *Acrocephalus seychellensis* and Marsh warbler *Acrocephalus palustris*, and in the house sparrow we used FLRs from tree sparrows *Passer montanus*). The PCR products from the two FLRs "GT6" and "GT11" were sequenced according to manufacturer's protocol (Big Dye Terminator mix V3.1, Applied Biosystems, US, for details see Chapter 5). The great tit sequences were highly similar to the blue tit alleles (89% average sequence similarity, compared to allele Cyca UA*1, Figure 6.2).

VIII. Running samples

The diluted hybridisation with ROX 500 size standard (VI, in total 21 μl; 11 μl diluted hybridisation plus 10 μl ddH_2O with ROX) was run on an ABI 310 capillary sequencer (Applied Biosystems), using a non-denaturing polymer. We used an injection time of 10s, an injection voltage of 15 kV, a running voltage of 13 kV, a running temperature of 30°C and a running time of 15 minutes. Each sample was run at least twice, once with each FLR. GeneMapper 4.0 software was used to visualize peak patterns.

RSCA results

Single alleles

All eight blue tit MHC-I alleles had a running distance between 200 and 300 (Figure 6.4). The first peak detected is always the homoduplex, the FLR that has hybridised with itself, which was detected at 212 and 222 for GT6 and GT11, respectively (Figure 6.5). Subsequently, the peaks of the different alleles were detected. The number of single nucleotide differences between the FLR and an allele was a good indicator of the running distance of the allele (GT6: $R^2 = 0.66$, $p = 0.015$, GT11: $R^2 = 0.60$, $p = 0.024$, Figure 6.6).

Using single alleles, we were able to distinguish between all allele combinations (known from earlier cloning and DNA sequencing) using the combined running distances of GT6 and GT11. FLR GT6 separated all eight alleles, although with less resolution for alleles that migrated short distances (237-244; UA*102, UA*107, UA*104, UA*116 and UA*109, Figure 6.4). FLR GT11 only separated five of the eight alleles (UA*102 and UA*107 have identical running distances (244) as well as UA*108, UA*109 and UA*117 (265); Figure 6.4). However, FLR GT11 is a good complement to FLR GT6, since it separates alleles where GT6 has a low resolution (UA*109 and UA*116, Figure 6.4). We estimated the number of MHC class I alleles possessed by an individual as the highest number of alleles identified using both FLRs.

Genomic samples

The individuals in the Dutch blue tit population had between two and eight MHC-I alleles per individual (range: 2-8 alleles in offspring, 3-7 among parents, average among parents: 4.5 ± 1.23 SE, see Figure 6.5 for example runs). We could only assign MHC haplotypes in four out of our 14 nests because the genotypic MHC

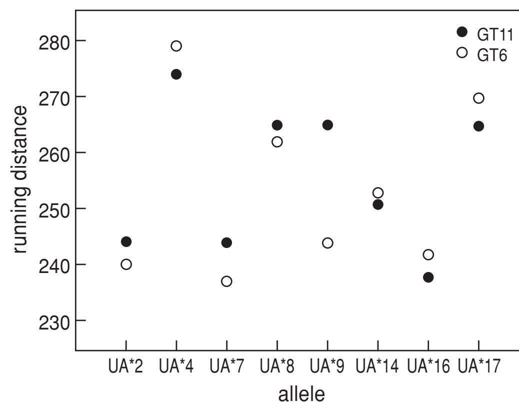


Figure 6.4 The running distances on the ABI310 capillary sequencer for each of the alleles in group 2 (Schut *et al.* 2010) for both FLRs.

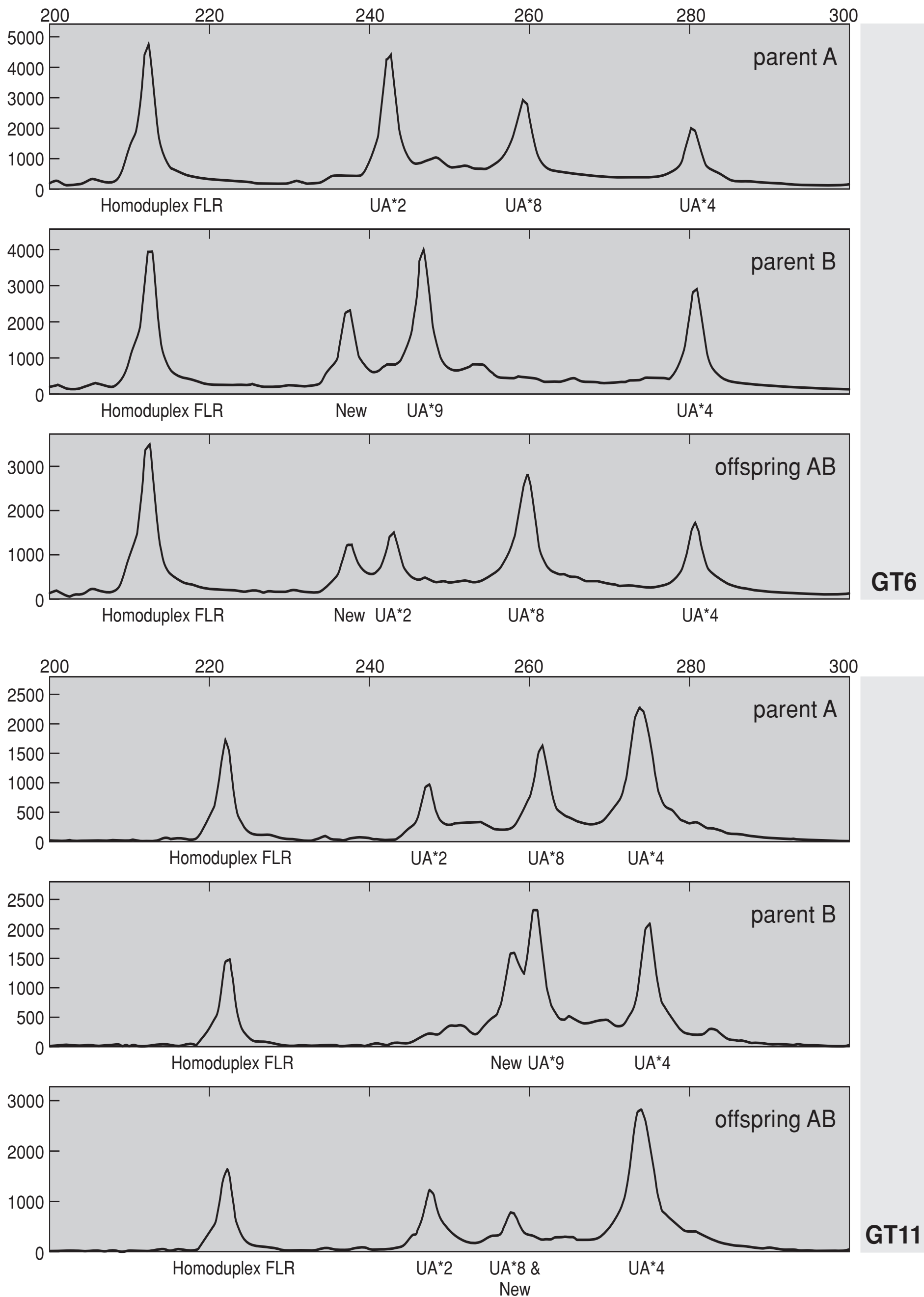


Figure 6.5 Examples of the sequencer runs of both parents and one offspring of a blue tit family for both FLRs. The X-axis denotes running distance on the sequencer, while the Y-axis denotes intensity of the signal. Underneath each peak is given the allele it belongs to. Note that a new allele (of which the sequence is unknown) was found in parent B.

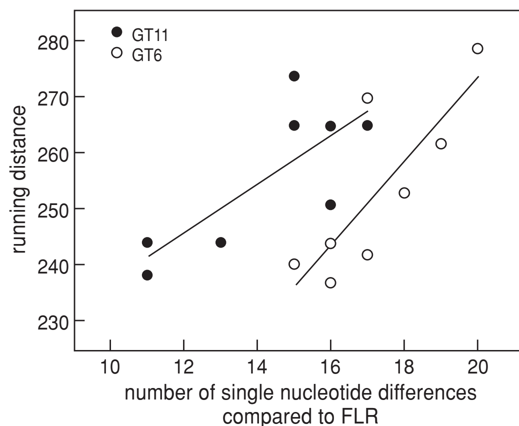


Figure 6.6 The correlations between the numbers of single nucleotide differences of each allele compared to the FLRs (GT6 open circles, GT11 filled circles) and the running distance of the heteroduplex on the sequencer.

similarity between parents was very high. The inheritance was consistent with each individual having two haplotypes that segregate independently. The allele sharing between parents varied between one and five alleles in the 14 nests (25–100% of the alleles were shared, average: 67.7%, SD: 18.86, expressed as the percentage of the average allele number of the parents). No allele peaks were found in offspring that were not also found in the parental genotypes. At least eight parents had a novel MHC-I allele and one parent even had two novel alleles. In total, nine different previously unidentified alleles were found, eight of which were detectable using both GT6 and GT11. However, one of the novel alleles was only seen with GT11.

Discussion

For scientists working with the highly variable MHC, it is a challenge to develop a protocol that efficiently genotypes individuals. Such a genotyping method needs to be able to distinguish between sequence variants, rather than fragments that differ in length. Within the bird MHC, loci are often highly similar due to gene conversion and crossing-over events and identical alleles may be derived from more than one locus (Wittzell *et al.* 1999; Westerdahl *et al.* 2004; Westerdahl 2007). When studying the MHC in passerines, primers therefore commonly amplify more than a single locus and hence the number of alleles to be separated may be high. In non-model vertebrates, like the blue tit, few if any details are known about the genetic background and it is important to use a molecular screening method that finds and separates also unknown alleles.

Previous studies on the MHC in birds have often used DGGE for genotyping (i.e. Ekblom *et al.* 2004; Bonneaud *et al.* 2004; Westerdahl *et al.* 2004). However, DGGE is laborious and uses hazardous chemicals. RSCA is a conformation-based method that only requires a successful PCR amplification of MHC in the study species and in a related species using identical primers. A further advantage of RSCA is that it can be run on an automated sequencer. RSCA separates previously unknown alleles and has successfully been used to genotype MHC in several species (i.e. humans; Argüello *et al.* 2007, cats; i.e Kennedy *et al.* 2003, stickleback; Lenz *et al.* 2009, red jungle fowl; Worley *et al.* 2008).

In this study, we adapted the RSCA method for use in the blue tit. One common problem when screening MHC diversity using PCR is that PCR artefacts will be created if too many allele copies are amplified at the same time (Babik 2010). Therefore it is advantageous to amplify a subset of alleles at the time. We narrowed down the number of alleles to be separated in blue tits by designing primers that only amplify the alleles subject to balancing selection (Figure 6.2).

An important validation of the RSCA technique is to evaluate how repeatable and stable it is. The series of peaks for an individual was repeatable across runs for blue tits, 34 out of 35 runs that were repeated gave an identical scoring in independent runs. In a single individual, the repeated runs did not correspond. This is most likely due to an error annotating this run, rather than a methodological problem. The RSCA results for five Dutch individuals were consistent with earlier results from cloning and DNA sequencing in these individuals (Chapter 5) and all alleles revealed using DNA sequencing were also found using RSCA.

However, the running distance of alleles did vary a little between runs and alleles in genomic samples often migrated a few units further or less far than when the sample was run from a clone (Figure 6.7). The running distances were slightly more variable in genomic samples than in clones and we were unable to distinguish between allele pair UA*102/UA*107 in genomic samples, even when using both FLRs. In several cases, we knew that offspring were homozygous for an allele (since they inherited it from both parents) and the two copies of the allele differed in their running distance, leading to an extra peak for one of the FLRs. Using both FLRs it was still possible to identify the two copies as the same allele.

In 8% of the individuals (11 individuals, belonging to four different nests) allele UA*114 was only found using one of the FLRs, but not in the other. In four cases allele UA*114 was only found using GT6, while in the remaining seven cases it was only found using GT11. Allele UA*114 is not abnormal compared to the other amplified alleles in the part of the sequence where the primer attaches, so there should not be an amplification problem with this allele. However, in several cases, this allele was only properly detected using one of the FLRs and we cannot be certain that allele UA*114 is always detected.

The MHC complex is a complicated gene complex to work with, due to its many loci, high polymorphism and high nucleotide diversity (Klein 1986; Nei and Hughes 1991). Since alleles derived from different loci are often highly similar or even iden-

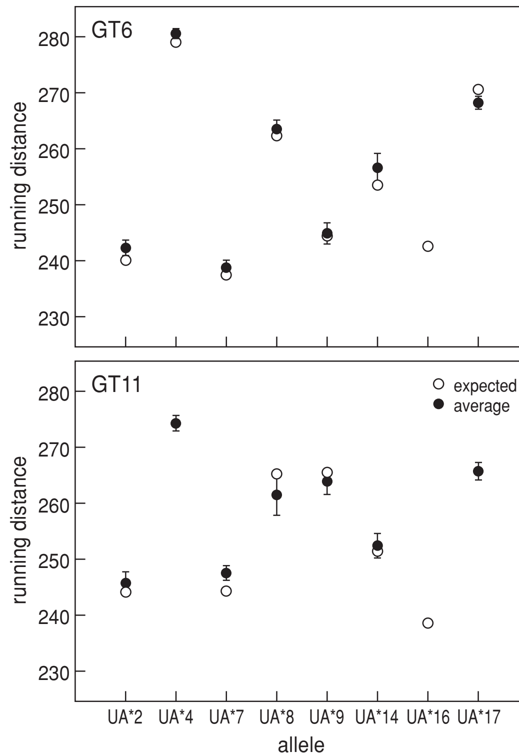


Figure 6.7 Average running distance, with standard deviation and the expected running distance (based on the single clones that were run) of each allele in group 2 for GT6 and GT11. No average running distance was obtained for allele UA*116, since it was not found in the data set. Note that the standard deviation of UA*114 is high, since it was found rarely (<25 times). For allele UA*104 and UA*117 the average and expected running distance overlap for GT11.

tical in sequence, it is often unclear how many loci are amplified concurrently, hence, it is almost impossible to develop a genotyping method with perfect resolution. RSCA does not provide 100% resolution of allelic diversity in our setup, although it is highly repeatable and gives a good estimate of MHC diversity, considering both nucleotide diversity and number of alleles per individual.

Using the RSCA method we successfully distinguished between 27 out of 28 possible allele pairs (from eight known blue tit MHC-I alleles). We did not find unexpected RSCA peaks in any of our 105 offspring in the Dutch blue tit families that would be indicative of false alleles (e.g. PCR artefacts) or gene conversion. Our results are consistent with each parent possessing two independently segregating haplotypes, as expected.

We started off with eight verified blue tit group-2 MHC-I alleles (Figure 6.3) and RSCA quickly led us to find another nine alleles in the Dutch sample (14 families), eight of which were detected using both FLRs. It is impossible for us to predict

whether all novel alleles will be detected using our selected FLRs. However, alleles that we might fail to detect are likely to be very similar to already verified alleles, because there is a strong correlation between number of single nucleotide differences between allele and FLR and the running distance (Figure 6). This RSCA setup for blue tits provides a good estimation of the MHC-I diversity within an individual and potentially what pathogen peptide diversity an individual is able to detect, even if it does not detect all alleles.

The RSCA protocol that we describe is a reliable method to separate MHC-I alleles in blue tits and we have successfully used a similar RSCA protocol in great reed warblers and house sparrows. The FLRs from closely related species seem to give the best resolution, in the blue tit we used FLRs from great tits (until recently the blue tit was classified in the same genera as the great tit), while in great reed warblers we used FLRs from two other *Acrocephalus* warblers and in house sparrows we used FLRs from another *Passer* species. Furthermore, it is possible to use the RSCA method that we describe on any capillary sequencer, for instance the ABI 3100 (which can run two 96 plates per day). Running the protocol on a different sequencer may require some fine tuning of the running conditions in order to achieve the resolution that we describe.

The amplicon 454 sequencing method is becoming a popular method for MHC genotyping. This method generates a million DNA sequences in a single run and amplicon data on a large number of individuals can be obtained within a single run (Babik *et al.* 2009). This large amount of data must then be "cleaned" so that only true MHC alleles remain and this can be difficult in a non-model organism. RSCA is a simple, straight forward method that gives an estimate of the number of alleles to expect per individual in a non-model organism. We believe that RSCA and amplicon 454-sequencing is a good combination to use for MHC genotyping, because RSCA estimates the number of alleles to expect per individual in an amplicon 454-run. Furthermore, amplicon 454-sequencing is still an expensive method and when detailed (sequence) information is not required for every individual in a database, the combined use of RSCA and 454 sequencing may be useful.

Conclusions and future prospects

The RSCA method can now be used to quickly and accurately screen MHC-I in blue tits. The method is set up to be effective for blue tits originating from locations across the entire Cyanistes caeruleus distribution. In future studies, the RSCA method (in combination with amplicon 454-sequencing) should help gain greater insight into the mechanisms behind balancing selection. Moreover, we show that RSCA is suitable for MHC-I genotyping in passerine species more generally and the RSCA flow scheme presented here has already been applied successfully in great reed warblers and house sparrows.

Acknowledgements

The authors are very grateful to the Molecular Population Biology lab at Lund University (Sweden) for allowing us to perform the molecular work and to Santiago Merino for supporting the work and providing useful comments to the manuscript. ES received funding from the Dobberke Stichting in the Netherlands. Peter Korsten, Bengt Hansson, Staffan Bensch, and Maria Karlsson kindly provided us with DNA samples. Mimi Lannefors and Maria Karlsson kindly allowed us to refer to their RSCA work. The authors would further like to thank Peter Korsten, Oscar Vedder, Mimi Lannefors, Eva Friman, Martin Stervander and Marco van der Velde for their assistance.

Data accessibility

Blue tit MHC-I exon 3 sequences; *Paca* UA*101-117: NCBI GenBank accession numbers: JF742764-80

German blue tit sequences: NCBI GenBank accession numbers: AM232710-14

Great reed warbler sequence AcarcN15 (used in fig. 6.2); NCBI GenBank Accession number AJ005506

House sparrow sequence Pado UA109 (used in fig. 6.2); NCBI GenBank Accession number EU715816

Primers btclassIfw1 and btclassIrv1: to be submitted to NCBI GenBank

Part III

MHC based mate choice

CHAPTER 7

Evidence for assortative social pairing according to MHC-I heterozygosity in the blue tit (*Cyanistes caeruleus*)

Elske Schut, Michael J. L. Magrath, Lyanne Brouwer, Marco van der Velde, Helena Westerdahl and Jan Komdeur

Abstract

Sexual selection theory predicts that females gain from mating with attractive partners by (amongst other possibilities) obtaining genetic benefits that confer their offspring with greater viability and/or attractiveness. According to the good genes hypothesis, females have a universally similar preference for male characteristics, while the genetic compatibility hypothesis proposes that the preferred genotype depends on the compatibility between the parental genotypes. The Major Histocompatibility Complex (MHC) is commonly hypothesised to play a key role in mate choice for genetic benefits, as the MHC has a central role in the immune system and is likely to affect individual survival. Here, we test whether MHC-I genotype is associated with social- and extra-pair mate choice in the blue tit (*Cyanistes caeruleus*). We found evidence for assortative social pairing according to MHC-I heterozygosity. Furthermore, there was no association between either MHC-I heterozygosity or MHC-I similarity of social partners and the likelihood that the social male was cuckolded. Our findings suggest the occurrence of mutual mate choice for mates with high MHC-I heterozygosity, most consistent with the good genes hypothesis.

Introduction

According to sexual selection theory, one way in which females benefit from mating with attractive partners is by gaining more viable or more attractive offspring (Andersson 1994). There are different theories on what makes an attractive partner. According to the good genes model of sexual selection (reviewed in Andersson 1994), the genetic benefits are additive and females have a universal preference for certain male characteristics that have a genetic basis. In contrast, the genetic compatibility hypothesis proposes that genetic benefits are non-additive and the quality of offspring will depend on compatibility between the parental genotypes (reviewed in Hettyey *et al.* 2010). Genetic incompatibilities between the parental genotypes can have serious deleterious effects for offspring fitness (survival and reproductive success), resulting in embryo mortality in extreme cases (reviewed in Zeh and Zeh 1996; Tregenza and Wedell 2000). Females should therefore avoid genetic incompatibilities and aim for a favourable combination of parental alleles (Hettyey *et al.* 2010).

Examples of mate choice for genetic benefits frequently involve the Major Histocompatibility Complex (MHC; Tregenza and Wedell 2000). The MHC is the gene complex encoding molecules involved in the immune system of vertebrates (Klein 1986). The MHC consists of several classes, of which class I (MHC-I) and class II (MHC-II) are directly involved in the adaptive immunity. MHC-I is mainly involved in the recognition of intra-cellular pathogens, while MHC-II is mainly responsible for the recognition of extra-cellular pathogens (Klein 1986; Janeway *et al.* 2008). Since MHC alleles are co-dominantly expressed, individuals possessing more alleles should recognize more pathogens (Janeway *et al.* 2008). Evidence for a fitness advantage of certain MHC genotypes has been demonstrated in several species e.g. in the Seychelles warbler (*Acrocephalus sechellensis*) where more MHC-I heterozygous individuals had increased survival probabilities; Brouwer *et al.* 2010). Since the MHC genotype is of great importance for offspring immunocompetence, female mating decisions may strongly influence offspring survival and reproductive success. Indeed, a role for pathogen resistance in mate choice was first suggested by Hamilton and Zuk (1982).

Evidence for MHC based mate choice has been found in several passerine species. In the Seychelles warbler (*Acrocephalus sechellensis*; Richardson *et al.* 2005) and the scarlet rosefinch (*Carpodacus erythrinus*; Promerová *et al.* 2011), MHC-I heterozygosity (e.g. the number of different alleles derived from several loci) was not associated with social mate choice, but more MHC diverse males were less likely to be cuckolded. In the Seychelles warbler, extra-pair offspring (EPO, offspring fathered by a male other than the social male) were also fathered by males more diverse than their social father (Brouwer *et al.* 2010). In the savannah sparrow (*Passerculus sandwichensis*; Freeman-Gallant *et al.* 2003) and the house sparrow (*Passer domesticus* Bonneaud *et al.* 2006a), females avoided pairing with MHC-similar males (MHC-II and MHC-I, respectively). Remarkably, savannah spar-

rows based their choice on the males' similarity rank compared to the other males in the population, rather than absolute similarity. MHC-II similarity rank also determined female fidelity (Freeman-Gallant *et al.* 2003). In the house sparrow, a positive correlation was revealed between male and female allele number (Bonneaud *et al.* 2006a). Hence, previous studies provide support for a role of MHC in mate choice among passerines and suggest that females may generally select partners to enhance the MHC diversity of their offspring (although not all studies found evidence for MHC based mate choice, e.g. Westerdahl 2004).

To gain further insight into the scope of mate choice for genetic benefits, we investigated the relationship between social- and extra-pair mating and MHC-I in a monogamously breeding passerine species, the blue tit (*Cyanistes caeruleus*). Blue tits have frequently been studied in the context of sexual selection, in part because of the relatively high rate of EPO in this species (11% EPO in 44% of broods in our population; Brommer *et al.* 2007; Vedder *et al.* 2011). A previous study on two Spanish blue tit populations found that microsatellite heterozygosity was positively correlated between social partners and suggested the occurrence of mutual mate choice (García-Navas *et al.* 2009). If blue tits also use mate choice to enhance offspring MHC diversity, as suggested by previous studies in passerines, we would expect to observe: (i) assortative social pairing for MHC-I heterozygosity, through mutual mate choice for high heterozygosity and/or (ii) a greater likelihood that broods contain EPO when the females' social partner has a low MHC-I heterozygosity or when MHC-I similarity between social partners is high.

Methods

Sample collection

Blood samples were collected from adult blue tits breeding in a nest box population in De Vosbergen estate, near Groningen, The Netherlands (53º08'N, 06º35'E). Immigrant breeders were frequently observed, suggesting that the population is outbred (Korsten 2006). A subset of 14 different pairs was sampled from the years 2001 (n = 4 pairs), 2002 (n = 6) and 2003 (n = 4; all samples were originally collected for use in another study). Adults were trapped in their nest box during nestling provisioning (6–14 days after hatching) and a small blood sample (ca. 10 μL) was taken from the brachial vein. All offspring were bled four days after hatching. Blood samples were stored at room temperature in 100% ethanol. An ammonium-acetate (Richardson *et al.* 2001) extraction method was used for DNA extraction.

Paternity analyses

Paternity of the chicks was determined using six microsatellite loci (Pca 3, Pca 7, Pca 8, Pca 9 (Dawson *et al.* 2000), Pocc 6 (Bensch *et al.* 1997) and Pdo 5 (Griffith *et al.* 1999) (see Magrath *et al.* 2009 for further details). Whether or not the social father was also the genetic father of the offspring was determined for all offspring

of our 14 pairs, although we did not attempt to assign extra-pair fathers. The mean exclusion probability was 0.99795 for the female and 0.99989 for the male (given that the genotype of the female was known). If a chick showed a mismatch with the social father for more than one deviating locus, it was assigned as extra-pair. We obtained blood samples from 129 nestlings, of which 11 nestlings in five nests were extra-pair.

Reference Strand mediated Conformation Analysis (RSCA)
The MHC-I genotype of all adults was determined using the RSCA method we described previously (Chapter 5). The RSCA protocol was run twice per individual, using FLRs GT6 and GT11. Using this protocol, blue tit MHC-I exon 3 sequences from four loci are amplified. Only the most polymorphic allele group is detected (Chapter 5). Henceforth, we will use the word "allele" to indicate exon 3 sequence variants. Since we previously showed that the RSCA method does not always distinguish between alleles UA*102 and UA*107 when an individual possesses one of these alleles (Chapter 5), we grouped these alleles in our band sharing analyses. For analyses based on allele number this does not change anything. Band sharing values are slightly exaggerated by this. However, since we know that alleles UA*102 and UA*107 are highly similar in sequence, this is justified. We define MHC-I heterozygosity as the total number of MHC-I alleles detected. Band sharing was defined as twice the number of shared bands divided by the sum of bands of both individuals: $D = 2F_{AB}/(F_A + F_B)$ (Following Wetton *et al.* 1987). Nine previously unidentified alleles were found. Running distances were compared to assess whether these alleles were the same or not.

Statistical analyses
To determine whether the observed social pairing differed significantly from random with respect to MHC-I genotype, a randomization test was performed, generating random pairs of using the 14 males and 14 females in our dataset (n = 5000 replications). To determine whether male heterozygosity or genetic similarity of the social partners determined the likelihood that the brood contained EPO, a logistic regression of male heterozygosity and band sharing of the social parents on presence/absence of EPO was performed in MLwiN 2.02 (Rasbash *et al.* 2005), using a binomial distribution with logit link function. A backwards elimination was used, starting with both factors in the model and eliminating non-significant terms. Significance was tested using the Wald statistic and two tailed probability testing was used.

Results

Adult blue tits had between 3 – 7 MHC-I alleles (median 4 alleles). The MHC-I heterozygosity (i.e. the number of different alleles) of the female was positively

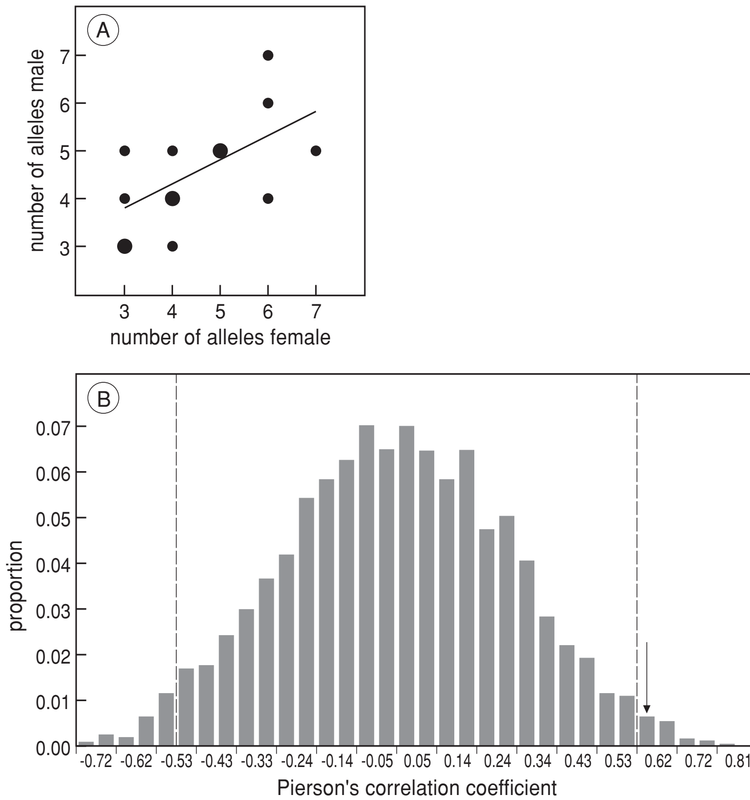


Figure 7.1 (A) Correlation between the number of MHC-I alleles possessed by the female and her social partner (R^2 = 0.38, r = 0.61, n = 14 pairs). Bigger dots indicate values that were observed twice. (B) Distribution of Pearson's correlation coefficients for the relationship between number of male and female alleles generated through 5000 randomizations. Labels on the X-axis show the upper bin limit. Note that rounding off of numbers makes it appear as though bins are unequal in size, while in fact they are not. The arrow indicates the observed value of r = 0.61. Dotted lines indicate the 95% confidence interval (between r = –0.52 and 0.57).

correlated to the MHC-I heterozygosity of her social partner (figure 7.1A). Our observed Pearson correlation coefficient of r = 0.61 lies outside the 95% confidence interval obtained by randomizations (figure 7.1B), indicating that social pairs in our population were not the results of random mating with respect to MHC-I heterozygosity. Average band sharing between social partners was 0.72 (range 0.25–1.00). We found no evidence for an effect of male heterozygosity or band sharing between the social parents on the likelihood that a nest contained EPO (male heterozygosity: effect:–0.445 ± 0.536 SE, df = 1, χ^2 = 0.69, p = 0.41; band sharing: effect: –9.739 ± 6.174 SE, df = 1, χ^2 = 2.488, p = 0.12).

Discussion

In line with our first expectation, we found evidence for assortative mating for MHC-I genotype, resulting in a positive correlation between the heterozygosity of the female and her social partner. Although it is widely accepted that in most species, females are the choosy sex (Andersson 1994), the most likely explanation for this positive correlation may be mutual mate choice for high MHC-I heterozygosity, or traits that are related to MHC-I heterozygosity (e.g. physical health). This is consistent with the previous study in blue tits that revealed mutual mate choice for microsatellite heterozygosity (García-Navas *et al.* 2009). Alternatively, our observed pattern of mate association may be explained by only a female preference for males that are comparable to them in heterozygosity, but we see no reason for such a preference, as none of the existing hypotheses on MHC based mate choice (i.e. choice for maximum offspring heterozygosity, Penn and Potts 1999, optimal offspring heterozygosity, Nowak *et al.* 1992; Milinski 2006, or offspring with beneficial alleles, e.g. Ekblom *et al.* 2004) predict it.

To obtain highly heterozygous offspring, females are likely to avoid pairing with individuals with low heterozygosity or individuals they share many alleles with, while outbreeding avoidance may lead to the avoidance of too dissimilar partners. In the house sparrow, female avoidance of highly dissimilar partners and partners with low heterozygosity resulted in a positive correlation between MHC-I heterozygosity (Bonneaud *et al.* 2006a), comparable to the correlation we found. We cannot determine whether females avoid paring with low heterozygous males/ males too (dis)similar to them (as was the case in the house sparrow; Bonneaud *et al.* 2006a), since we are unable to sample from unpaired individuals in our population, as adults were always caught in or near their nest box.

Contrary to our second prediction, we found no evidence that females attempted to increase offspring heterozygosity by obtaining extra-pair copulations when paired to a male with low heterozygosity or a male they shared many alleles with. Previous studies (using microsatellites) found no evidence that blue tits chose social- or extra-pair partners based on genetic relatedness, but did indicate that EPO in the blue tit are more heterozygous (using microsatellites) and healthier than their within-pair nest mates (Kempenaers *et al.* 1997; Foerster *et al.* 2003; Charmantier *et al.* 2004; Foerster *et al.* 2006). Hence, we had expected to find an association between male heterozygosity and/or band sharing of social partners and the likelihood the social male was cuckolded. Our small sample size and the fact that we did not identify extra-pair males may have limited our capacity to detect such relationships.

That we found a pattern of mate association based on MHC-I does not mean that MHC-I itself was the target of mate choice. Other loci (i.e. loci closely linked to the MHC; Jordan and Bruford 1998) may be the target of mate choice, resulting in

the pattern we detected. The observed pattern of mate association does not necessarily result from mate choice for genetic benefits, since more heterozygous individuals may well be healthier and therefore provide more direct benefits (i.e. high quality parental care).

In conclusion, we found evidence for assortative mate association according to MHC-I heterozygosity in the blue tit. A more extensive study is needed to test for other possible associations between MHC-I genotype and mate choice that our limited dataset did not allow us to examine, e.g. mate choice for specific, beneficial alleles. Moreover, it will be rewarding to evaluate the relationship between MHC genotypes and individual survival as these data become available.

Acknowledgements

The authors are grateful to the Molecular Population Biology lab at Lund University (Sweden) for allowing ES to perform the molecular work. The authors would like to thank Oscar Vedder and Peter Korsten for useful comments to the manuscript and Peter Korsten for fieldwork. ES received funding from the Dobberke Stichting in the Netherlands.

Box C

Do passerine birds use olfaction in MHC–recognition?

Elske Schut

Evidence for MHC based mate choice has been reported in a range of taxa, including mammals (e.g. mice (*Mus musculus domesticus*); Potts *et al.* 1991; Wedekind *et al.* 1996, human; Yamazaki *et al.* 1988; Wedekind *et al.* 1995 and grey mouse lemur (*Microcebus murinus*); Schwensow *et al.* 2008), birds (e.g. Seychelles warbler, (*Acrocephalus sechellensis*); Richardson *et al.* 2005, blue tit (*Cyanistes caeruleus*) (Chapter 7, this thesis) and the Great snipe (*Gallinago media*); Ekblom *et al.* 2004) and fish (e.g. stickleback (*Gasterosteus aculeatus*); Aeschlimann *et al.* 2003 and arctic charr (*Salvelinus alpines*); Skarstein *et al.* 2005). An important requirement for (pre-copulatory) mate choice on the basis of the MHC is that individuals are able to detect the MHC characteristics of potential partners. Hence, we would expect to find an association between MHC-genotype and phenotypic characteristics. In mammals (particularly mice and rats), MHC recognition is thought to occur through smell and various mechanisms have been proposed for MHC genotype to be reflected in odour (e.g. through MHC-derived molecules occurring in urine/sweat or because the MHC determines which micro-organisms are hosted by an individual; reviewed in Penn and Potts 1998). However, it has long been thought that birds are unable to smell.

Researchers most likely assumed that birds lack an olfactory ability, due to the position of the nostrils in birds (high up the bill, which does not allow them to probe for odours) and the fact that birds may not be capable of actively inhaling air through their nostrils (Roper 1999). Hence, researchers have been searching for associations between other phenotypic characteristics, such as conspicuous male ornaments and MHC-genotype. Such evidence for relationships between MHC characteristics and male ornament expression has been reported in several bird species. For instance, peacock (*Pavo cristatus*) males were found to have longer trains if their MHC class II diversity was higher (Hale *et al.* 2009) while male spur length was related to MHC genotype (of both class I and II) in the ring-necked pheasant (*Phasianus colchicus*; Schantz *et al.* 1996). More recently, however, researchers have come to realise that birds do posses the ability to smell. The anatomy of the olfactory system in birds is similar to that of mammals, although there is substantial variation in size of the different components between bird species (Balthazart and Taziaux 2009). In addition, several bird species have been demonstrated to possess functional olfactory receptor genes (genes expressed in neurons in the olfactory epithelium; Steiger *et al.* 2008). Together, this would indicate that birds have a functional olfactory system. Indeed, evidence that birds are able to smell has been found in several taxa (reviewed in Balthazart and Taziaux 2009).

Odour perception in passerines

Passerines have a relatively small olfactory bulb (the part of the brain involved in olfaction), compared to other bird orders (Bang and Cobb 1968). Hence, passerines have been assumed to have a poor olfactory ability, even after evidence in other

bird orders demonstrated that birds perceive odours. Yet, numerous studies have found evidence that passerines are able to detect odours (Table C.1). Early studies generally used physiological indications, such as olfactory bulb electrical activity, cardiac rate- and respiratory responses to olfactory stimuli or surgically removed olfactory function to experimentally demonstrate that birds can perceive smell. Physiological indications were used in combination with conditioning; birds were trained to respond to an olfactory stimulus by combining the stimulus with an electric shock. Birds increase their cardiac/respiratory rate in response to the electric shock. After an initial training period, only the odour stimulus is given and cardiac/respiratory rate is recorded (i.e. Snyder and Peterson 1979; Clark and Mason 1989; Clark 1991; Table C.1). These studies demonstrate the ability to detect odour, but do not necessarily mean that these bird species respond to odour, or use olfaction in an ecological context. Recently, studies have focussed on the use of olfaction in more ecological contexts (see Table C.1). These ecological contexts (and examples of experimental evidence) include:

(i) Predator recognition; blue tits (*Cyanistes caeruleus*) avoided entering and spent less time inside their nest box when mustelid odour (e.g. the odour that members of the weasel family produce) was artificially added (Amo *et al.* 2008).
(ii) Food location; e.g. common ravens (*Corvus corax*) were able to determine, based on olfaction, which of a pair of buried containers contained food (Harriman and Berger 1986).
(iii) Nest building; several passerine species are known to incorporate odorous plants in their nest. The function of odorous plants as nest material is still debated, but it is possible that odorous plants are used to repel parasites (e.g. Gwinner 1997; Petit *et al.* 2002). Female blue tits were shown to use odour cues to determine when the odorous plants in their nest required replenishing (Petit *et al.* 2002).
(iv) Recognition of food items and avoidance of others; e.g. adult zebra finches (*Taeniopygia guttata*) took longer to consume their food if it was treated with a combination of a novel odour and a novel colour, rather than only a novel colour (Kelly and Marples 2004). Studies on the role of olfaction in food recognition commonly find that odour is not the only cue used for food recognition, but that a combination with visual cues is important (Avery and Nelms 1990; Kelly and Marples 2004; Siddall and Marples 2011).
(v) Navigation; European starlings (*Sturnus vulgaris*) were translocated experimentally from their nest box. Individuals in which the olfactory nerve was dissected were less likely to return after translocation over long distances (Wallraff *et al.* 1995; although dissection may have damaged other structures needed for navigation).
(vi) Nest recognition; juvenile zebra finches were able to identify their natal-nest box based on olfaction when given the choice between two nest boxes (Caspers and Krause 2011).

Table C.1 Overview of physiological and ecological studies demonstrating the olfactory ability of passerine birds

Physiological studies

Species	Response	Reference
Canary (*Serinus canaria*)	Cardiac response rate & respiratory response	Avery and Nelms (1990)
European starling (*Sturnus vulgaris*)	Electrical activity of olfactory nerves & olfactory nerves section	Clark and Mason (1987)
Gray catbird (*Dumetella carolinensis*)	Cardiac response rate	Clark *et al.* (1993)
European goldfinch (*Carduelis carduelis*)	Cardiac response rate	Clark *et al.* (1993)
Eastern phoebe (*Sayornis phoebe*)	Cardiac response rate	Clark *et al.* (1993)
Great tit (*Parus major*)	Cardiac response rate	Clark *et al.* (1993)
Black capped chickadee (*Parus atricapillus*)	Cardiac response rate	Clark *et al.* (1993)
Cedar waxwing (*Bombycilla cedrorum*)	Cardiac response rate	Clark (1991)
Tree swallow (*Tachycineta bicolor*)	Cardiac response rate	Clark (1991)
Brown-headed cowbird (*Molothrus ater*)	Cardiac response rate	Clark and Mason (1989)
Black-billed magpie (*Pica pica*)	Respiratory response	Snyder and Peterson (1979)

Ecological studies

Species	Context	Reference
Blue tit (*Cyanistes caeruleus*)	Predator recognition Locating food	Amo *et al.* (2008) Anisimov *et al.* (2004)
(*C. caeruleus ogliastrae*)	Locating food Nest building	Mennerat *et al.* (2005) Petit *et al.* (2002)
Great tit (*Parus major*)	Locating food	Anisimov *et al.* (2004)
Zebra finch (*Taeniopygia guttata*)	Food recognition/avoidance Nest recognition by juveniles	Kelly and Marples (2004) Caspers and Krause (2011)
European starling (*Sturnus vulgaris*)	Food recognition/avoidance Navigation to the nest box, after olfactory nerve section Nest building	Clark and Mason (1987) Wallraff *et al.* (1995) Gwinner and Berger (2007)
Blackbird (*Turdus merula*)	Food recognition/avoidance	Saxton *et al.* (2004)
Silvereye (*Zosterops lateralis*)	Food recognition/avoidance	Saxton *et al.* (2004)
Red-winged blackbird (*Agelaius phoeniceus*)	Food recognition/avoidance	Avery and Nelms (1990)
Black-billed magpie (*Pica pica*)	Locating food	Buitron and Nuechterlein (1985)
Common raven (*Corvus corax*)	Locating food	Harriman and Berger (1986)
European robin (*Erithacus rubecula*)	Food recognition/avoidance	Siddall and Marples (2011)

In conclusion, there is extensive evidence that passerines have the ability to perceive odours and use it in a range of ecological contexts (including several studies on the blue tit). Whether olfaction is used for MHC-recognition in passerines remains to be determined. Studies on other bird species demonstrate that odour may be used in individual recognition (e.g. Antarctic prions, *Pachiptila desolata*, that are able to recognize their partner; Bonadonna and Nevitt 2004), and it seems plausible that passerines use olfaction in a similar way. It is still unclear how MHC-genotype is associated with bird odour, although some of the above mentioned hypotheses proposed for mammals (reviewed by Penn and Potts 1998) may apply to birds. Several possible sources of odorous substances are known in birds, including the uropygial gland and anal gland (reviewed in Hagelin and Jones 2007). Uropygial gland secretions are promising as a potential source of MHC-related individual differences in odour. The uropygial gland produces wax-like substances that birds use to maintain their feathers and production of the secretions may differ seasonally, with sex, age and diet (reviewed in Hagelin and Jones 2007). There is evidence in a passerine species, the dark-eyed junco (*Junco hyemalis*), that the composition of uropygial gland secretions was repeatable within individuals and differed between the sexes and population of origin. The authors suggest that the composition of uropygial gland secretion has a genetic basis and might be used in mate recognition (Whittaker *et al.* 2010). It seems quite plausible that MHC-genotype is associated with the composition of uropygial gland secretions. No doubt, future studies will elucidate the role of olfaction in MHC-recognition in (passerine) birds.

Heritability of MHC-I heterozygosity in the blue tit

Elske Schut

Several of the theories on the MHC characteristics that a female should prefer in a mate state that females should choose a mate based on MHC allele number (i.e. mate choice for maximal or optimal MHC heterozygous offspring (reviewed in Penn and Potts 1999; Milinski 2006). An important assumption of these theories is that MHC heterozygosity is heritable, yet this assumption has seldom been tested. Heterozygosity according to the general definition (i.e. that used for most gene regions, but not the MHC, see box A) is defined as the proportion of loci at which an individual is heterozygous. Since during the production of gametes (meiosis) the different chromosome copies obtained from the father and the mother are separated, offspring do not directly inherit heterozygosity according to this definition. Instead, whether or not offspring heterozygosity is comparable to parental heterozygosity is dependent on the frequency of different alleles in the population: if allele A is frequent in the population but B is not, individuals with allele B are likely to be heterozygous themselves and at least the offspring inheriting allele B (50% of their offspring) will be heterozygous. Hence, individuals heterozygous for loci in which allele frequencies are unequal (e.g. population frequency of allele A: 0.9, frequency of allele B: 0.1) will obtain (on average) more heterozygous offspring. If allele frequencies are (almost) equal in the population (e.g. population frequency of allele A: 0.5, frequency of allele B: 0.5), however, parental and offspring heterozygosity are not related (Mitton *et al.* 1993). Evidence for the heritability of heterozygosity was previously presented, e.g. by Mitton *et al.* (1993) for several functional loci in domesticated horses (*Equus ferus caballus*) and by García-Navas *et al.* (2009) for microsatellite loci in the blue tit.

For the MHC, however, another mechanism also contributes to the heritability of heterozygosity, due to the different use of the term heterozygosity. Since the same allele can be present at several loci and researchers cannot determine which allele is derived from which locus, the number of loci at which an individual is heterozygous is estimated as the number of different alleles found in an individual. Since the different MHC loci are inherited together, MHC heterozygosity is expected to be heritable as the haplotypes passed on by more heterozygous individuals contain more different alleles, independent of allele frequencies in the population (see Box A).

Here, we provide evidence that MHC-I heterozygosity is (as expected) heritable in the blue tit (*Cyanistes caeruleus*). To determine whether there is a correlation between parental and offspring allele numbers, MHC-I heterozygosity was determined for 14 blue tit pairs and their offspring from the population in *De Vosbergen*, using RSCA (Chapter 6, this thesis). Paternity of all offspring was determined and only within-pair offspring were used in this analysis (this thesis, Chapter 5). Since the number of alleles in the two haplotypes of an individual is variable, offspring allele number varies within a brood. Hence, we investigated not only whether a correlation between the average of the allele number of the parents and average allele number of all offspring exists, but we also tested for a correlation with the lowest number of alleles found in an individual in the brood (minimum offspring

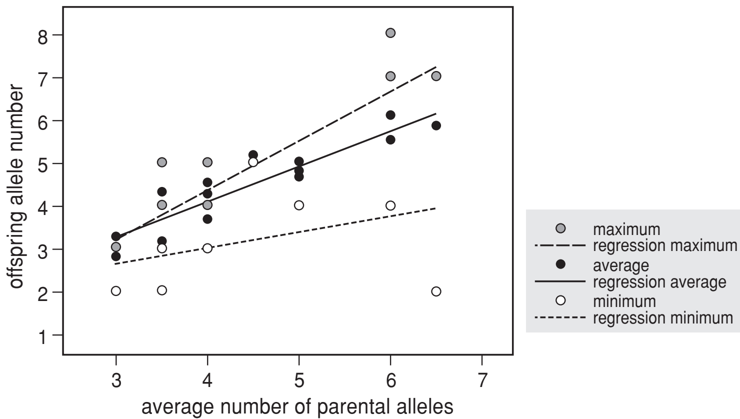


Figure D.1 Heritability of heterozygosity in our blue tit population. Positive correlations between the average allele number of the two parents and maximum allele number in the brood ($R^2 = 0.76$, $p < 0.001$), average offspring allele number ($R^2 = 0.85$, $p < 0.001$) and the minimum allele number in the brood ($R^2 = 0.46$, $p = 0.101$) were found. For minimum allele number, this effect was non-significant.

allele number) and the highest number of alleles found in an individual in the brood (maximum offspring allele number). We found that average allele number of the parents was positively correlated with average, maximum and minimum offspring allele number (although the correlation with minimum offspring allele number was not significant; Fig. D.1) Furthermore, there were positive correlations between both female allele number and male allele number and average offspring allele number ($R^2 = 0.71$, $p < 0.001$ and $R^2 = 0.63$, $p < 0.001$, respectively). Our data indicate that, on average more heterozygous parents have more heterozygous offspring, but the difference between the nestling with the highest heterozygosity and the nestling with the lowest heterozygosity is largest for the most heterozygous parents in our dataset. This indicates that having heterozygous parents is beneficial for some of the offspring, but not all offspring may share this benefit (as predicted by Partridge 1983). The large difference in heterozygosity within a brood is most likely caused by large differences in the number of alleles in the different parental haplotypes. In case the two haplotypes of an individual differ largely in allele number, and since one would expect the random segregation of haplotypes, individuals with few alleles would be equally likely to occur as heterozygous individuals. In our population, assortative mate association on the basis of MHC-I allele number is known to occur (i.e. individuals with few alleles have a partner with few alleles, while individuals with many alleles have a partner with many alleles; Chapter 7, this thesis). This pattern of mate association is likely to enhance the effect that offspring from parents with a higher total heterozygosity are on average more heterozygous, as in our population both parent have a higher allele number. Hence, some individuals will inherit haplotypes with many alleles from both parents. In

the absence of assortative mate association, the male and female parent may differ in heterozygosity by several alleles (in our population, allele numbers ranged between 2 and 7; this thesis, Chapter 5) and offspring are more likely to have allele numbers intermediate between the parents. Another important determinant of offspring allele number that is not taken into account in this analysis is the number of alleles that is shared between the parents. If parents do not share any alleles, the number of alleles of an individual offspring is simply the sum of the alleles in his maternal and paternal haplotypes. Alleles shared between the parents may be inherited in both the maternal and the paternal haplotype and complicate the relationship between parental- and offspring allele number. One may speculate that more heterozygous parents are more likely to have alleles in common, resulting in a non-linear relationship between parental- and offspring allele number. In our dataset, there is no correlation between heterozygosity and the number of alleles shared between the parents, however ($R = 0.03$, $p = 0.53$; for details on the calculation of band sharing, see chapter 7).

CHAPTER 8

Synthesis

Elske Schut

Both the good genes and genotypic compatibility hypotheses of sexual selection predict that females benefit from mating with their preferred partner by gaining genetic benefits that improve the quality (i.e. survival and reproductive success) of their offspring (Andersson 1994). Due to rapid developments in the field of molecular biology, scientists are starting to acquire the tools they need to determine whether individuals choose partners based on genetic characteristics, such as good genes or compatibility with the own genotype (i.e. whether additive or non-additive effects are important). Recent investigations into the genetic benefits of mate choice commonly examine the potential role of MHC genes (i.e. the gene complex involved in adaptive immunity). Due to its role in adaptive immunity, the MHC is likely to be an important determinant of individual fitness (Goldsby *et al.* 2000; Janeway *et al.* 2008) and has been implicated in mate choice in a range of species (e.g. Yamazaki *et al.* 1988; Potts *et al.* 1991; Aeschlimann *et al.* 2003; Richardson *et al.* 2005).

Advances in molecular biology also provided essential new insights into avian mating behaviour. Due to the development of molecular parentage assignment, it became obvious that even in socially monogamous species, females commonly mate with multiple males within a single breeding cycle (Birkhead and Møller 1992; Westneat and Webster 1994; Griffith *et al.* 2002). Therefore, the potential exists for females to select the genetically favourable father for their offspring through post-copulatory sexual selection ('the genetically loaded raffle'; Griffith and Immler 2009). Several studies have provided evidence for an association between male genotype and fertilization success (Birkhead 2010), but the physiological mechanisms involved remain unclear.

The aim of this thesis was to gain insight into the process of sexual selection in the blue tit (*Cyanistes caeruleus*). This chapter discusses the main findings of my thesis and their ecological context. Part I of this thesis deals with the number of sperm in the female reproductive tract and the factors influencing differences between individuals. Part II describes the gene structure of MHC-I in the blue tit and the development of a method to quickly genotype individuals for MHC-I. The background information provided on MHC-I and a new screening method developed in this study now enables us to investigate mate choice based on MHC-I in the blue tit. Part III then describes patterns of MHC-I based mate choice in a blue tit population in the north-east of the Netherlands (*De Vosbergen*).

Mate choice for genetic benefits

While the role of genetic benefits in mate choice has received much recent attention, the issue is far from resolved. An important requirement for the evolution of mate choice for genetic benefits is that heritable variation in fitness exists, but studies investigating the heritability of fitness have yielded mixed results (e.g. Kruuk *et al.* 2000; Connallon 2010). In a meta-analysis, Møller and Alatalo (1999)

demonstrated that in general, variance in a preferred male trait only explains a small proportion of the variance in offspring viability. There are patterns of mate choice that cannot be explained by the alternative options of mate choice for direct benefits or male-male competition, however. In particular mate choice for genetic compatibility (Neff and Pitcher 2005) is hard to explain without the theory of genetic benefits, since there are no intrinsic quality differences between compatible and non-compatible males. This would suggest that heritable variation in fitness does exist, at least in species in which mate choice for genetic compatibility has been described.

The function of extra-pair copulations

One of the main unresolved questions in post-copulatory sexual selection is the evolutionary function of multiple mating in females (Griffith *et al.* 2002; Birkhead 2010). The hypotheses to explain why engaging in extra-pair copulations could be adaptive for females can be divided into three main categories: (i) hypotheses posing that females engage in extra-pair copulations to obtain genetic benefits for their offspring (e.g. increased genetic diversity of the brood, good genes), (ii) hypotheses posing that females use extra-pair copulations as an insurance against the possible infertility of their social partner and, (iii) hypotheses posing that females obtain other non-genetic benefits from engaging in extra-pair copulations (Birkhead and Møller 1992; Møller 1998). Another possible explanation for the occurrence of extra-pair copulations is that females do not engage in them voluntarily, but that extra-pair copulations result from male coercion (McKinney and Evarts 1998). Kempenaers *et al.* (1992) provided evidence that female blue tits actively seek extra-pair copulations from neighbouring males. Kempenaers *et al.* (1995) described that females were harassed by neighbouring males when their social partner was removed, but few forced copulations were successful. Together, these findings suggest that extra-pair offspring do not result from male coercion in the blue tit. In the blue tit, interactions between the female and her extra-pair partner(s) are brief and females do not obtain direct benefits, such as parental care, from extra-pair partners. Hence, the most likely explanations are that female blue tits engage in extra-pair copulations to obtain genetic benefits or as an insurance against the potential infertility of their social partner.

Vedder *et al.* (2011) suggested that the most likely function of extra-pair copulations in *De Vosbergen* blue tit population was fertility insurance. In chapter 3 we investigated whether female blue tits generally store enough sperm to fertilize their entire clutch. We hypothesized that, since in most passerines copulation ceases or declines markedly in frequency after the onset of egg laying and the blue tit lays an extraordinarily large clutch (Cramp and Perrins 1993), female blue tits may be sperm depleted towards the end of the laying sequence. Hence, we expected unhatched eggs, caused by insufficient sperm reserves, to occur towards the end of

the laying sequence and to be more frequent in larger clutches. Based on a three year dataset, we found no evidence that hatching failure is more likely to occur towards the end of the laying sequence or that sperm numbers decrease over the laying sequence. In addition, we found no evidence that the likelihood of eggs failing to hatch was associated with sperm numbers in the female reproductive tract. These findings suggest that females continue to copulate throughout their laying sequence, since passive sperm loss would otherwise lead to a decrease in sperm number in the female reproductive tract (Wishart 1987; Birkhead *et al.* 1993). There was no evidence that sperm are generally in short supply and that fertility insurance is needed (unless, of course the social male would be infertile or incompatibilities exist between male and female genotype).

It is likely that females are often constrained in their choice of a social mate (Gowaty 1996; e.g. by the need to obtain paternal care) and therefore obtain a partner who is not genetically optimal. Since extra-pair males do not contribute parental care, females are less constrained in their choice of an extra-pair partner (except perhaps by the mate guarding behaviour of their partner). Hence, females have the potential to improve the genetic quality of their offspring by engaging in extra-pair copulations. Indeed, in a Belgian blue tit population, cuckolded males had a lower survival probability than males who were not cuckolded (Kempenaers *et al.* 1992; 1997), suggesting that females paired to a low quality male should seek extra-pair copulations. In chapter 7, we tested whether the likelihood that a brood contained extra-pair offspring was associated with the MHC-I heterozygosity of the social male or the genetic similarity of social partners but found no effect. Hence, we found no evidence that females engage in extra-pair copulations to obtain genetic benefits with regard to MHC-I.

Recently, the hypothesis that female extra-pair behaviour evolved as a by-product of male promiscuous behaviour was proposed. According to this hypothesis, male and female mating behaviour are genetically correlated and female promiscuity evolved as a result of the benefits males received from their promiscuous behaviour. In the zebra finch (*Taeniopygia guttata*), evidence for such a genetic correlation between male and female promiscuous behaviour has indeed been found (Forstmeier *et al.* 2011). We cannot exclude the possibility that a similar correlation exists in blue tits and that females receive no benefits from their extra-pair behaviour.

Post-copulatory sexual selection

Since extra-pair offspring were common in our blue tit population (11% of offspring in 44% of nests; Brommer *et al.* 2007; Vedder *et al.* 2011), we know that female blue tits often mate with more than one male within a reproductive cycle. Hence, post-copulatory processes may form an important determinant of reproductive success for both males and females. There are several ways in which both sexes may influ-

ence offspring paternity. Amongst other possibilities, males can influence the number of their sperm that reach the female sperm storage tubules (SST). To do so, males may adjust their ejaculate size to the likelihood of sperm competition (i.e. if they have information on the copulation behaviour of their partner) and by adjusting the timing of copulation relative to the females' reproductive cycle. Females may (amongst other possibilities) influence their chances of being fertilized by their preferred male by the selective ejection of sperm after copulation or the selective uptake and release of sperm from the SST. Evidence has been found for a role of the male genotype in fertilization success (reviewed in Birkhead 2010) and male-female interactions at the cellular level (explained in more detail in the introduction) may be responsible for that. Several of the mechanisms for post-copulatory sexual selection mentioned above potentially result in a difference in the number of sperm in the reproductive tracts of different females. In chapter 4 we tested whether between-individual differences in the number of sperm the female has in storage exist and whether between-individual differences in sperm number in the female reproductive tract are associated with characteristics of the females' social partner. We collected freshly laid eggs from our population in *De Vosbergen* to dissect the perivitelline layers around the yolk and count the number of sperm trapped between the layers to investigate the relationship between sperm count and characteristics of the social male. Previous studies show that the number of sperm on the perivitelline layers provides an index of the number of sperm the female has stored in her SST (Birkhead and Fletcher 1994). We chose to use this indirect method to determine the number of sperm in the female tract, rather than the more direct method of sacrificing the female and dissecting the SST, since sacrificing a large number of females is not desirable. The choice to remove eggs allowed us to get an indication of sperm numbers in the female tract of 68 female blue tits. Our data not only indicate the existence of between-individual differences in sperm counts, but also that females paired to a social partner with a longer tarsus had more sperm in storage. An important limitation of this method is that we cannot distinguish between the sperm of different males. Therefore, we can only speculate on the actual causes of between-individual differences. There are several possible explanations for the positive correlation between tarsus length of the social partner and sperm count: (i) females may store more sperm of larger males, since they are more attractive, (ii) larger males may produce more sperm, resulting in more sperm per ejaculate or (iii) larger males may use increased copulation rates as a mechanism to ensure paternity. Several previous studies reported that extra-pair males in the blue tit had longer tarsi (Kempenaers *et al.* 1997; Foerster and Kempenaers 2004) and in our study population, polygynous blue tit males lost less paternity in their nests when they had longer tarsi (Vedder *et al.* 2011). These results would be in line with all above-mentioned hypotheses.

Since within-pair copulations in the blue tit are frequent (7-–28 copulations per day during the fertile period; Kempenaers *et al.* 1992), one may expect that the female sperm storage tubules would simply be saturated with sperm. The fact that

we found between-individual differences in the number of sperm associated with a male characteristic indicates that between-individual differences in the number of sperm in the reproductive tract reflect copulation behaviour, rather than simple differences in female sperm storage capacity. Contrary to this conclusion, Johnsen *et al.* (2011) found no relationship between the number of copulations and the number of sperm on the eggs laid one or two days later in the tree swallow (*Tachycineta bicolour*). This finding may suggest that sperm numbers on the PVL reflect the sum of all copulations during the females' fertile period rather than only the copulations in the few days before laying of the egg. Our study is the first to demonstrate an association between male characteristics and the number of sperm a female has in storage in a wild living passerine species.

Evolution of the Major Histocompatibility Complex

The MHC is a complex gene region that is more diverse than other gene regions at several levels: the high number of gene loci, high sequence diversity and high genetic polymorphism (Klein 1986; Nei and Hughes 1991). Due to its high genetic diversity, the MHC has been subject to many studies investigating the mechanisms creating and maintaining genetic diversity. The MHC is common to all vertebrates and must have arisen hundreds of millions of years ago (see Danchin *et al.* 2004 for details). The high number of gene loci is thought to have arisen through the duplication of existing genes, which diverged over its long existence time (Beck *et al.* 1999). The high genetic polymorphism is thought to be maintained by balancing selection (i.e. selection prolonging the lifetime of alleles in the population; Potts and Wakeland 1990; Hedrick 2002) and MHC alleles are known to have a long lifetime compared to other alleles. In line with this idea, allelic lineages (i.e. lineages of highly similar alleles) within the MHC are often found in several species, indicating that the lineages already existed before the species diverged (i.e. trans-species evolution; Edwards and Hedrick 1998; Westerdahl 2007). Within the MHC, sequence variants derived from different loci can be highly similar. This similarity is caused by the process of gene conversion. During gene conversion (also called segmental mutation), sequence fragments are transferred from one locus to another. When long sequence fragments are transferred in this way, it will decrease sequence variation between loci (Högstand and Böhme 1999). A prerequisite for the occurrence of gene conversion is the presence of several (closely linked) loci with partial sequence similarities (Högstand and Böhme 1999). Although MHC loci are likely to have diverged over time, their common origin (due to gene duplication; see above) is still reflected in parts of their sequence (Beck *et al.* 1999). Therefore, gene conversion may be more likely to occur in the MHC region than elsewhere in the genome.

Based on differences between bird orders, inferences on MHC evolution can be made. Hence, the characterization of the MHC of additional bird species may in

future help us understand the evolutionary processes shaping the avian MHC (reviewed in Hess and Edwards 2002; Westerdahl 2007). The characterization of the blue tit MHC therefore not only provides insight into the evolutionary processes shaping the blue tit MHC, but may also contribute to our understanding of the evolution of the avian MHC. The first bird species in which the MHC was characterized was the chicken (*Gallus domesticus*; Kaufman *et al.* 1995; Kaufman *et al.* 1999). Since then, the MHC has been characterized in species belonging to several bird orders, including passerines (e.g. Westerdahl *et al.* 1999; Westerdahl *et al.* 2000). An important difference between the MHC of galliform and passerine birds is that in general, the passerine MHC contains a higher number of loci (Westerdahl 2007; with the exception of the Japanese quail, *Cortunix japonica*; Shiina *et al.* 1995). To date the MHC has been characterized in too few bird species to be able to determine whether this is a phylogenetic effect (i.e. caused by differences in the history of different bird orders) or an effect caused by differences in selection imposed by the pathogen environment. Within passerines, a difference in the number of MHC alleles in migratory and non-migratory species has been suggested, since migratory species are likely to encounter more different pathogen environments (Møller and Erritzøe 1998; Westerdahl *et al.* 2000). In chapter 5, we compare the MHC-I nucleotide diversity (a measure of the amount of sequence variation observed when comparing between different sequences) and the number of loci detected between the migratory (great reed warbler, *Acrocephalus arundinaceus*, scarlet rosefinch, *Carpodacus erythrinus*) and non-migratory (house sparrow, *Passer domesticus*, Seychelles warbler, blue tit) passerines for which MHC-I was characterized. We found no evidence for a higher genetic diversity of MHC-I in migratory species than in non-migratory species, but the small number of passerine species in which MHC-I has been characterized is an important limitation. A remarkable result of our between-species comparison of nucleotide diversity is that the blue tit MHC-I nucleotide diversity was significantly lower than that of other passerine species, a result that we cannot sufficiently explain based on selection induced through pathogen induced selection (i.e. selection induced by the evolutionary arms race between pathogen and host) and the blue tits' demographic history. It is important to note that the comparison of the nucleotide diversity between species is complicated by differences in the number of MHC-I loci a species possesses, the genetic polymorphism and the sampling of individuals, however. In species with more (non-functional) loci, one may expect to see a higher nucleotide diversity, due to more differentiation between alleles. When samples are derived from related individuals, nucleotide diversity will be lower than when studying non-related individuals. Hence, the comparison between nucleotide diversities of different species may not be as straightforward as at appears at first sight (see chapter 5).

MHC sequence characteristics can also be used to make inferences about the selection processes working on the MHC within a species. Parts of the sequences derived from MHC-I exon 2 and 3 encode the peptide binding region of the MHC

molecule. The amino acids constituting the peptide binding region determine which pathogens can be recognized by the specific immune system. More heterozygous individuals should be able to recognize more different pathogens and we would expect that the peptide binding region is subject to balancing selection (Potts and Wakeland 1990; Hedrick 2002). Several measures based on the MHC sequence provide evidence of balancing selection. The ratio of the number of nonsynonymous mutations (i.e. mutations resulting in a change in the amino acid sequence) to synonymous mutations (i.e. mutations that leave the amino acid sequence intact; called the dN/dS ratio), as well as the value of Tajima's D (a measure that uses the number of polymorphic sites to calculate divergence between sequences) can be calculated when comparing several sequences (which we did in Chapter 5; Page and Holmes 1998).

Cloning and sequencing of the genomic DNA of 20 blue tit individuals originating from three locations (Spain, the Netherlands and Sweden) yielded 17 MHC-I allelic variants. Most likely this number will be increased if more individuals are sequenced, but our characterization provides a good starting point to assess the blue tit MHC-I genetic structure. To gain insight into the genetic structure of the blue tit MHC-I, we built a phylogeny based on allelic diversity. Nine of the blue tit MHC-I alleles form a cluster with low diversity compared to the other eight alleles. Evidence for a non-diverse allele cluster in MHC-I, like that of the blue tit, was previously found in the house sparrow (Bonneaud *et al.* 2004) and this clustering resembles the division between classical and non-classical alleles found in *galliform* birds (Wittzell *et al.* 1995; Miller and Lambert 2004; Hunt *et al.* 2006; Strand *et al.* 2007). We currently have too little background information on the blue tit MHC-I alleles (e.g. segregation patterns) to conclude whether the classical/non-classical division is found in passerines, however. We found evidence for balancing selection on the peptide binding region of the eight diverse alleles only. Non-PBR regions are part of the structural part of the MHC molecule and may be under strict selection against mutations. We can only speculate about the reason that we found no evidence for selection acting on the non-diverse allele, since we have little information about the origin of the two allele groups. It is possible that the non-diverse allele cluster represents a non-functional gene region.

To detect selection pressures currently working in a population, one may use differences in allele frequencies between years (e.g. Westerdahl *et al.* 2004). For the blue tit, such data are not yet available. To answer questions regarding the mechanisms currently imposing selection on MHC-I in the blue tit, a method to quickly and accurately genotype individuals was required. Chapter 6 describes how we adapted the existing method Reference Strand mediated Conformation Analysis for use in the blue tit. As far as we know, this is the first study describing the use of RSCA in a passerine, although RSCA was described for several galliform species (Worley *et al.* 2008; Strand and Höglund 2011). Screening the MHC in passerines is potentially more difficult that in galliform species, due to the higher number of loci in passerines. We show that RSCA can successfully be used to screen the passerine

MHC. The RSCA method allows us to screen individuals for the eight blue tit MHC-I alleles under selection, and allows us to find and verify previously unidentified alleles (i.e. not yet sequenced). In addition, we describe a working scheme that can be used to adapt RSCA for the use in other passerines. In chapter 7, the RSCA method was used to investigate the occurrence of MHC based mate choice in our blue tit population.

Mate choice based on the MHC

The genetic polymorphism at the MHC is thought to be maintained by balancing selection (see above). There are different theories on what causes balancing selection, the leading hypotheses are pathogen driven selection and mate choice based on MHC genotype (Bodmer 1972; Potts and Wakeland 1990; Nei and Hughes 1991; Borghans *et al.* 2004; Oosterhout 2008). According to the pathogen driven selection theory, balancing selection is caused purely by an evolutionary arms race occurring between pathogens and their host. The host species needs to keep adapting to the constantly changing pathogen environment. According to the mate choice theory, the same arms race occurs, but individuals choose a partner based on MHC genotype in order to ensure that their offspring are optimally adapted to the pathogen environment they face. Theoretically, females have several options that are advantageous for their offspring; they should aim for: (i) heterozygous offspring (by choosing highly heterozygous or dissimilar mates), (ii) offspring with certain (most likely rare) alleles that enable them to recognize prevalent (harmful) infections (by choosing a mate with those alleles), (iii) optimally heterozygous offspring, because too highly heterozygous offspring may suffer from autoimmune reactions or from negative effects of outbreeding (by matching the heterozygosity of their mate to their own heterozygosity; highly heterozygous females should choose a mate with lower heterozygosity and low heterozygous females should choose a mate with high heterozygosity). In addition, females should avoid mating with a partner whose genotype is incompatible with their own genotype, since genotypic incompatibilities may reduce the survival probabilities of their offspring (Zeh and Zeh 1996). The different forms of mate choice will result in balancing selection, as they increase the likelihood that alleles will be maintained in the population, rather than being lost. A preference for highly heterozygous mates ensures that heterozygous haplotypes remain in the population. An additional advantage of choosing highly heterozygous males is that they are more likely to possess rare alleles. If females aim for optimally heterozygous offspring, there will be a preference for highly heterozygous partners in part of the population (i.e. in females with low heterozygosity). A preference for rare alleles allows rare alleles to initially quickly spread in the population and remain present, rather than going to extinction. Rare alleles are more likely to be beneficial than common alleles, since pathogens are likely to have adapted in such a way that individuals with common alleles are unable to recognize them. A

preference for dissimilar males also promotes allelic diversity in the population.

To test whether female blue tits in our population prefer more MHC-I heterozygous mates, we used the RSCA method (mentioned above) to screen the MHC genotypes of 14 blue tit social pairs (chapter 7). Our study provides evidence that mate choice in the blue tit is assortative according to MHC-I heterozygosity, but found no evidence that the likelihood that a brood contained extra-pair offspring was influenced by MHC-I heterozygosity of the social partner or genetic similarity of the social mates. We performed a randomization test with our 14 males and 14 females (i.e. creating pairs at random using our individuals) to determine whether the correlation between the MHC-I allele number of the female and her social male originates from non-random mating. The correlation between female and social male heterozygosity is best explained by mutual mate choice (i.e. mate choice by both male and female) for high MHC-I heterozygosity. In chapter 5, we found evidence for balancing selection on the PBR of the blue tit alleles in the diverse allele group. Mutual mate choice for MHC-I heterozygosity could enforce balancing selection on the blue tit MHC. Assuming that blue tits obtain genetic benefits from their mate choice, our results are best explained by the hypothesis that individuals select a partner on the basis of good genes, rather than compatibility of the parental genotypes, since individuals do not base their mate choice on the compatibility with their own genotype.

Vedder *et al.* (2011) found evidence that the proportion of within-pair paternity that a polygynous male lost was positively correlated among primary and secondary females and among nests of the same male in different years (even when the male paired with a different female), which also supports the good genes hypothesis. Under the genetic compatibility hypothesis, one would expect that the proportion of within-pair paternity lost depended on the compatibility between parental genotypes and therefore would differ between females. Evidence of the repeatability of within-pair paternity loss of males between different years was also found in other blue tit populations (Kempenaers *et al.* 1997; Leech *et al.* 2001). The repeatability of the proportion of within-pair offspring lost may alternatively be explained by between-male differences in the efficiency of mate guarding. Since polygygnous males are unlikely to mate guard at both nests, this could not explain the within-year repeatability. Another alternative explanation could be that some males produce larger ejaculates and are therefore more successful in competition with the ejaculates of other males.

We only found evidence in line with mate choice according to the good genes model for the choice of a social partner in our blue tit population. We have not performed rigorous testing to distinguish between the two hypotheses, however. Furthermore, we cannot exclude the possibility that the observed correlation between male and female genotype is caused by mate choice for direct benefits (Andersson 1994). In blue tits, both sexes provide parental care, females incubate and males defend the breeding territory. Hence, if more MHC-I heterozygous individuals are healthier, this may improve offspring survival, since healthier males

may be able to obtain a better territory. Healthier females may incubate more efficiently and both sexes may be better at offspring provisioning when they are healthier. Under this scenario, mutual mate preferences for a healthy partner may lead to the correlation between male and female MHC-I heterozygosity that we observed.

Conclusions and future perspectives

The results of my project demonstrate that, in our population, the fertility of blue tit eggs is independent of the number of sperm females have in storage. It is possible that sperm numbers are important, but that all females in our population have sperm in sufficient supply. Since the number of sperm does not decrease between the 2nd and 10th egg within a clutch, we conclude that in blue tits, copulation continues throughout the laying sequence. Our results further demonstrate that the number of sperm in the female sperm storage tubules is associated with copulation behaviour and/or post-copulatory sexual selection, rather than merely female sperm storage capacity. Hence, my research provides a first step towards understanding the factors determining the number of sperm in the female reproductive tract. Furthermore, my thesis provides the first description of the MHC-I alleles in the blue tit. We succeeded in developing a method to quickly screen MHC genotypes based on our sequencing results and were the first to describe RSCA in passerines. My data indicate that MHC-I allele number is positively correlated between social partners in our blue tit population. This could indicate mutual mate choice for maximal MHC-I heterozygosity.

Demonstrating genetic benefits of mate choice is extremely complicated in free-living species, because it is difficult to determine to what extent differences in survival and reproductive success are due to genetic differences. Based on the assumption that extra-pair offspring are generally the result of preferred matings (since females are free in their choice of an extra-pair partner, see above), differences between within- and extra-pair offspring are frequently used as evidence for mate choice for genetic benefits, e.g. in blue tits, several studies found that extra-pair offspring had improved genetic quality and survival probabilities (Kempenaers *et al.* 1997; Foerster *et al.* 2003; Dreiss *et al.* 2008). Not all studies report an advantage of being extra-pair, however (Sardell *et al.* 2011; Reid and Sardell 2012). In our study population, there were also quality differences between within- and extra-pair offspring, but this difference largely resulted from hatching order effects, since extra-pair offspring were mainly found early in the laying sequence (Magrath *et al.* 2009). Hence, the comparison between within-pair and extra-pair paternity does not solely reflect genetic differences in offspring quality. Even when using experimental pairings or cross-fostering, maternal effects (e.g. the amount of resources that females invest in their eggs; e.g. McFarlane *et al.* 2010) cannot be completely excluded. The use of animal models (i.e. by determining the amount of variation in

offspring quality that is explained by genotype based on a pedigree) may in future provide greater insight into the genetic benefits gained from mating with the preferred partner.

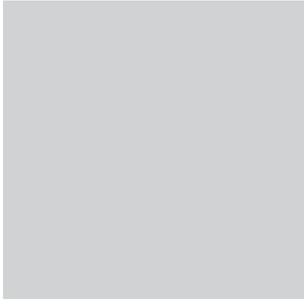
While researchers have commonly studied the outcome of mate choice, details on the actual process of pair formation are lacking in wild blue tits. This information is of great importance for our understanding of mate choice, however. In our field population, breeding attempts by blue tits are first observed by the end of March, when females start building the nest. It is unclear exactly when and how pair formation takes place. During winter, blue tits form loose foraging flocks of mixed sex, that remain in the breeding area (Colquhoun 1942; Ekman 1989). Individuals are known to roost in a nest box close to the one in which they bred during the previous breeding season or will breed during the following season (Colquhoun 1942, Korsten and Komdeur, unpublished data). If individuals that bred during the previous breeding season are still around during early spring in the following year, unoccupied nest boxes may be hard to come by. Female blue tits may in particular be limited in their mate choice by the need to obtain a breeding territory. Breeding territories are defended by the male and females may be forced to pair with a non-preferred social partner if they are to breed at all. In addition, interactions during winter may in part determine pair formation. Our limited knowledge of the details of pair formation makes it difficult to interpret results on social mate choice, like those we found in chapter 7. Hopefully, future studies using radio-tracking may clarify the social interactions between wintering blue tits and provide important insights into the process of pair formation.

Advances in the field of molecular biology (e.g. the development of 454-sequencing, Babik *et al.* 2009) are extremely rapid and determining the genotypes of a large number of individuals is becoming easier and cheaper. This will enable us to elucidate the association between genotype and fitness, e.g. the link between MHC alleles and immunocompetence. Studies on MHC based mate choice commonly examine only one class of MHC alleles, while details on both MHC class I and II are required to obtain a complete impression of immunocompetence. In future, the availability of genotyping methods like RSCA and the development of 454-sequencing may enable us to use combined characterizations of MHC class I and II.

It has long been assumed that birds did not have a strongly developed sense of smell. Recently, it has become clear that birds use olfaction in several contexts. Hence, determining whether blue tits use olfaction in individual recognition and whether MHC genotype might determine individual odour is an interesting avenue for further research (Box C). The use of gas-chromatography may provide insight into individual differences in body odour (e.g. Whittaker *et al.* 2010) and may aid in the reproduction of individual body odours.

While the role of MHC genotype in pre-copulatory sexual selection is commonly studied, evidence of a role for the MHC in post-copulatory sexual selection is lacking in passerines. There is evidence in birds that male genotype is associated

with fertilization success and the immune response in the female reproductive tract could potentially play a role in that. Unfortunately, invasive techniques are required to study the processes occurring in the female reproductive tract. However, technological advances (such as the possibility to use different fluorescent labels for the sperm of different males; Manier *et al.* 2010) may make the study of post-copulatory sexual selection easier in future.



References

A

Ackerman, KJ, Reinecke, AJ, Els, HJ, Grobler, DG & Reinecke, SA (1999). Sperm abnormalities associated with high copper levels in Impala (*Aepyceros melampus*) in the Kruger National Park, South Africa. Ecotoxology and Environmental Safety 43: 261–266.

Aeschlimann PB, Häberli MA, Reusch TBH, Boehm T, Milinski M (2003) Female sticklebacks G*asterosteus aculeatus* use self-reference to optimize MHC allele number during mate selection. Behaviour Ecology and Sociobiology 54: 119–126

Aguilar A, Edwards SV, Smith TB, Wayne RK (2006) Patterns of variation in MHC class II beta loci of the little greenbul (*Andropadus virens*) with comments on MHC evolution in birds. Journal of Heredity 97: 133–142

Alonso-Alvarez C, Doutrelant C, Sorci G (2004) Ultraviolet reflectance affects male-male interactions in the blue tit (*Parus caeruleus ultramarinus*). Behavioral Ecology 15: 805–809

Amo L, Galván I, Tomás G, Sanz JJ (2008) Predator odour recognition and avoidance in a songbird. Functional Ecology 22: 289–293

Andersson M (1994) Sexual Selection. Princeton University Press, Princeton, New Jersey

Andersson S, Ornborg J, Andersson M (1998) Ultraviolet sexual dimorphism and assortative mating in blue tits. Proceedings of the Royal Society in London Series B 265: 445–450

Anisimov VD, Barsova LI, Popovkina AB (2004) Olfactory reception in food search in tits of the genus Parus (Passeriformes, Paridae). Zoologichesky zhurnal 83: 1138–1147

Argüello, JR, Soteriou, B, McWhinnie, A, Garcia, C, Perez-Rodriguez, M, Corell, A, Marsh, SGE, Little, AM, and Madrigal, JA Typing for HLA alleles by Reference Strand Mediated Conformation Analysis (RSCA). International Histocompatibility Working Group 20: 1–17. 2007.

Arriero E, Moreno J, Merino S, Martínez J (2008) Habitat effects on physiological stress response in nestling blue tits are mediated through parasitism. Physiological and Biochemical Zoology 81: 195–203

Avery ML, Nelms CO (1990) Food avoidance by red-winged blackbirds conditioned with a pyrazine odour. The Auk 107: 544–549

B

Babik W (2010) Methods for MHC genotyping in non-model vertebrates. Molecular Ecology Resources 10: 237–251

Babik W, Taberlet P, Ejsmond MJ, Radwan J (2009) New generation sequencers as a tool for genotyping of highly polymorphic multilocus MHC system. Molecular Ecology Resources 9: 713–719

Bakst MR (1987) Anatomical basis of sperm-storage in the avian oviduct. Scanning Microscopy 1: 1257–1266

Bakst, MR & Akuffo, V (2002) Impact of egg storage on embryo development. Avian and Poultry Biology Reviews 13: 125–131

Bakst, MR & Cecil, HC. (1997) Techniques for semen evalua-tion, Semen Storage, and Fertility Determination. Savoy, Illinois: Poultry Science Association

Bakst, MR, Gupta, SK & Akuffo, V (1997) Comparative development of the Turkey and Chicken embryo from cleav-age through hypoblast formation. Poultry Science 76: 83–90

Bakst, MR, Gupta, SK, Potts, W & Akuffo, V (1998) Gross appearance of the Turkey blastoderm at oviposition. Poultry Science 77: 1228–1233

Bakst, MR & Wishart, GJ (1994) Proceedings: First Interna-tional Symposium on the Artificial Insemination of Poultry. Savoy, Illinois: Poultry Science Association

Bakst MR, Wishart G, Brillard JP (1994) Oviducal sperm selection, transport, and storage in poultry. Poultry Science Review 5: 117–143

Balakrishnan C, Ekblom R, Volker M, Westerdahl H, Godinez R, Kotkiewicz H, Burt D, Graves T, Griffin D, Warren W, Edwards S (2010) Gene duplication and fragmentation in the zebra finch major histocompatibility complex. BMC Biology 8: 29

Balthazart J, Taziaux M (2009) The underestimated role of olfaction in avian reproduction? Behavioural Brain Research 200: 248–259

Bang BG, Cobb S (1968) The size of the olfactory bulb in 108 species of birds. Auk 85: 55–61
Barbato GF, Cramer PG, Hammerstedt RH (1998) A practical *in vitro* sperm-egg binding assay that detects subfertile males. Biology of reproduction 58: 686–699
Beck S, Geraghty D, Inoko H, Rowen L (1999) Complete sequence and gene map of a human major histocompatibility complex. Nature 401: 921–923
Beissinger, SR, Cook, MI & Arendt, WJ (2005) The shelf life of bird eggs: testing egg viability using a tropical climate gradient. Ecology 86: 2164–2175
Bensch S, Akesson A (2003) Temporal and spatial variation of hematozoans in Scandinavian willow warblers. Journal of Parasitology 89: 388–391
Bensch, S, Hasselquist, D & von Schantz, T (1994) Genetic similarity between parents predicts hatching failure: non-incestuous inbreeding in the Great Reed Warbler? Evolution 48: 317–326
Bensch S, Price T, Kohn J (1997) Isolation and characterization of microsatellite loci in a Phylloscopus warbler. Molecular Ecology 6: 91–92
Bijnens L (1988) Blue Tit *Parus caeruleus* song in relation to survival, reproduction and biometry. Bird Study 35: 61–67
Birkhead TR (1992) Numbers and size of sperm storage tubules and the duration of sperm storage in birds: a comparative study. Biological Journal of the Linnean Society 45: 363–372
Birkhead TR (1998a) Sperm competition in birds. Reviews of reproduction 3: 123–129
Birkhead TR (1998b) Cryptic female choice: Criteria for establishing female sperm choice. Evolution 52: 1212–1218
Birkhead TR (2000) Defining and demonstrating postcopulatory female choice- Again. Evolution 54: 1057–1060
Birkhead TR (2010) How stupid not to have thought of that: post-copulatory sexual selection. Journal of Zoology 281: 78–93
Birkhead TR, Atkin L, Møller AP (1987) Copulation behaviour of birds. Behaviour 101: 101–138
Birkhead TR, Brillard JP (2007) Reproductive isolation in birds: postcopulatory prezygotic barriers. Trends in Ecology and Evolution 22: 266–272
Birkhead TR, Briskie JV, Lifjeld JT, Slagsvold T (1997) Breeding-cycle patterns of sperm storage in the Pied Flycatcher (*Ficedula hypoleuca*). The Auk 114: 792–796
Birkhead TR, Briskie JV, Møller AP (1993a) Male sperm reserves and copulation frequency in birds. Behavioral Ecology and Sociobiology 32: 85–93
Birkhead TR, Fletcher F (1994) Numbers of spermatozoa attached to and penetrating perivitelline layers of Japanese quail eggs. The Auk 111: 997–1000
Birkhead TR, Fletcher F (1998) Sperm transport in the reproductive tract of female zebra finches (*Taeniopygia guttata*). Journal of Reproduction and Fertility 114: 141–145
Birkhead TR, Hall J, Schut E, Hemmings N (2008) Unhatched eggs: methods for discriminating between infertility and early embryo mortality. Ibis 150: 508–517
Birkhead, TR, Hunter, FM & Pellatt, JE (1989) Sperm competition in the zebra finch, *Taeniopygia guttata*. Animal Behaviour 38: 935–950
Birkhead TR, Martinez JG, Burke T, Froman DP (1999) Sperm mobility determines the outcome of sperm competition in the domestic fowl. Proceedings of the Royal Society of London Series B 266: 1759–1764
Birkhead TR, Møller AP (1992) Sperm competition in birds. Academic Press, San Diego
Birkhead TR, Møller AP (1998) Sperm competition and sexual selection. Academic press, San Diego
Birkhead TR, Pellatt EJ, Fletcher F (1993b) Selection and utilization of spermatozoa in the reproductive tract of the female zebra finch *Taeniopygia guttata*. Journal of Reproduction and Fertility 99: 593–600
Birkhead TR, Pellatt EJ, Hunter FM (1988) Extra-pair copulation and sperm competition in the zebra finch. Nature 334: 60–62
Birkhead, TR, Pellatt, EJ, Matthews, IR, Roddis, NJ, Hunter, FM, McPhie, F & Castillo-Juarez, H (2006) Genic capture and the genetic basis of sexually selected traits in the Zebra Finch. Evolution 60: 2389–2398

Birkhead TR, Sheldon BC, Fletcher F (1994a) A comparative study of sperm-egg interactions in birds. Journal of reprodcution and fertility 101: 353–361

Birkhead, TR, Veiga, JP & Fletcher, F (1995) Sperm competition and unhatched eggs in the House Sparrow. Journal of Avian Biology 26: 342–345

Birkhead TR, Veiga JP, Møller AP (1994b) Male sperm reserves and copulation behaviour in the house sparrow, *Passer domesticus*. Proceedings of the Royal Society of London Series B 256: 247–251

Bodmer WF (1972) Evolutionary significance of the HL-A system. Nature 237: 139–146

Bonadonna F, Nevitt GA (2004) Partner-specific odor recognition in an Antarctic seabird. Science 306: 835

Bonneaud C, Chastel O, Federici P, Westerdahl H, Sorci G (2006a) Complex *Mhc*-based mate choice in a wild passerine. Proceedings of the Royal Society in London Series B 273: 1111–1116

Bonneaud C, Pérez-Tris J, Federici P, Chastel O, Sorci G (2006b) Major histocompatibility alleles associated with local resistance to malaria in a passerine. Evolution 60: 383–389

Bonneaud C, Sorci G, Morin V, Westerdahl H, Zoorob R, Wittzell H (2004) Diversity of Mhc class I and IIB genes in house sparrows (*Passer domesticus*). Immunogenetics 55: 855–865

Borghans JAM, Beltman JB, de Boer RJ (2004) MHC polymorphism under host-pathogen coevolution. Immunogenetics 55: 732–739

Bramwell, RK, Marks, HL & Howarth, B (1995) Quantitative determination of spermatozoa penetration of the perivitelline layer of the hen's ovum as assessed on oviposited eggs. Poultry Science 74:1875–83.

Brillard, JP (1990). Control of fertility in birds. Paris: INRA.

Brillard JP (1993) Sperm storage and transport following natural mating and artificial insemination. Poultry Science 72: 923–928

Brillard JP, Antoine H (1990) Storage of sperm in the uterovaginal junction and its incidence on the numbers of spermatozoa present in the perivitelline layers of hens eggs. British Poultry Science 31: 635–644

Brillard JP, Bakst MR (1990) Quantification of spermatozoa in the sperm-storage tubules of turkey hens and the relation to sperm numbers in the perivitelline layer of eggs. Biology of Reproduction 43: 271–275

Brillard, JP, Beaumont, C & Scheller, MF (1998) Physiological responses of hens divergently selected on the number of chicks obtained from a single insemination. Journal of Reproduction and Fertility 114: 111–117

Briskie JV (1994) Seasonal patterns of sperm storage in the Yellow-headed Blackbird *Xanthocephalus xanthocephalus*. Ibis 136: 323–330

Briskie, JV & Mackintosh, M (2004) Hatching failure increases with severity of population bottlenecks in birds. Proceedings of the National Academy of Sciences USA 101: 558–561

Brommer JE, Korsten P, Bouwman KA, Berg ML, Komdeur J (2007) Is extrapair mating random? On the probability distribution of extrapair young in avian broods. Behavioral Ecology 18: 895–904

Brouwer L, Barr I, van de Pol M, Burke T, Komdeur J, Richardson DS (2010) MHC-dependent survival in a wild population: evidence for hidden genetic benefits gained through extra-pair fertilizations. Molecular Ecology 19: 3444–3455

Brouwer L, Groothuis TGG, Vedder O, Eikenaar C, Richardson DS, Komdeur J (2009) Do primary males physiologically suppress subordinate males? An Experiment in a Cooperatively Breeding Passerine. Ethology 115: 576–587

Brun, JM, Sellier, N, Richard, MM, Battellier, F & Brillard, JP (2004) Effects of in vivo sperm storage duration and age of the Common Duck (*Anas platyrhynchos*) on fertility and embryonic survival in pure- and cross-breeding with Muscovy ducks (*Cairina moschata*). Proc. XXII World Poultry Congress. Istanbul

Buitron D, Nuechterlein GL (1985) Experiments on olfactory detection of food caches by black-billed magpies. The Condor 87: 92–95

C

Cabezas-Díaz S, Virgós E (2007) Adaptive and non-adaptive explanations for hatching failure in eggs of the Red-legged Partridge *Alectoris rufa*. Ardea 95: 55–63

Caspers BA, Krause ET (2011) Odour-based natal nest recognition in the zebra finch (*Taeniopygia guttata*), a colony-breeding songbird. Biology Letters 7: 184–186

Catchpole CK, Slater PJB (2008) Bird song: Biological Themes and Variation. Cambridge University Press, Cambridge, UK

Charmantier A, Blondel J, Perret P, Lambrechts MM (2004) Do extra-pair paternities provide genetic benefits for female blue tits *Parus caeruleus*? Journal of Avian Biology 35: 524–532

Christensen, VL (2001) Factors associated with early embryo mortality. World's Poultry Science Journal 57: 359–372

Christians JK, Williams TD (2001) Interindividual variation in yolk mass and the rate of growth of ovarian follicles in the zebra finch (*Taeniopygia guttata*). Journal of Comparative Physiology B-Biochemical Systemic and Environmental Physiology 171: 255–261

Cichon M, Dubiec A (2005) Cell-mediated immunity predicts the probability of local recruitment in nestling blue tits. Journal of Evolutionary Biology 18: 962–966

Clark L (1991) Odor detection thresholds in tree swallows and cedar waxwings. The Auk 108: 177–180

Clark L, Avilova KV, Bean NJ (1993) Odor thresholds in passerines. Comparative biochemistry and physiology 104A: 305–312

Clark L, Mason JR (1989) Sensitivity of brown-headed cowbirds to volatiles. The Condor 91: 922–932

Clark L, Mason JR (1987) Olfactory discrimination of plant volatiles by the European starling. Animal Behaviour 35: 227–235

Clutton-Brock T (2009) Sexual selection in females. Animal Behaviour 77: 3–11

Colquhoun MK (1942) Notes on the social behaviour of blue tits. British Birds 35: 234–240

Connallon T (2010) Genic capture, sex linkage, and the heritability of fitness. The American Naturalist 175: 564–576

Cook, MI, Beissinger, SR, Toranzos, GA, Rodriguez, RA & Arendt, WJ (2003) Trans-shell infection by pathogenic microorganisms reduces the shelf life of unincubated bird eggs: a constraint on the onset of incubation? Proceedings of the Royal Society of London Series B 270: 2233–2240

Cook, MI, Beissinger, SR, Toranzos, GA, Rodriguez, RA & Arendt, WJ (2005) Microbial infection affects egg viability and incubation behavior in a tropical passerine. Behavioral Ecology 16: 30–36

Cordero PJ, Aparicio JM, Veiga JP (2004) Parental genetic characteristics and hatching success in the spotless starling, *Sturnus unicolor*. Animal Behaviour 67: 637–642

Cordero PJ, Wetton JH, Parkin DT (1999) Within-clutch patterns of egg viability and paternity in the House Sparrow. Journal of avian biology 30: 103–107

Cramp S, Perrins CM (1993) Birds of the Western Palearctic. Oxford University Press, Oxford

D

Danchin E, Vitiello V, Vienne A, Richard O, Gouret P, McDermott MF, Pontarotti P (2004) The major histocompatibility complex origin. Immunological Reviews 198: 216–232

Darwin CR (1871) The descent of man, and selection in relation to sex. The Modern Library, London

Davies NB (1983) Polyandry, cloaca-pecking and sperm competition in dunnocks. Nature 302: 334–336

Dawson DA, Hanotte O, Greig C, Stewart IRK, Burke T (2000) Polymorphic microsatellites in the blue tit *Parus caeruleus* and their cross-species utility in 20 songbird families. Molecular Ecology 9: 1941–1944

del Hoyo, J., Elliot, A., and Bowles, W. Handbook of the birds of the world. [12]. 2007. Barcelona, Lynx Edicions.

Delhey K, Johnsen A, Peters A, Andersson A, Kempenaers B (2003) Paternity analysis reveals opposing selection pressures on crown coloration in the blue tit (*Parus caeruleus*). Proceedings of the Royal Society in London Series B 270: 2057–2063

Delhey K, Peters A, Johnsen A, Kempenaers B (2006) Seasonal changes in blue tit crown color: do they signal individual quality? Behavioral Ecology 17: 790–798

Delhey K, Peters A, Johnsen A, Kempenaers B (2007) Brood sex ratio and male UV ornamentation in blue tits (*Cyanistes caeruleus*): correlational evidence and an experimental test. Behavioral Ecology and Sociobiology 61: 853–862

Dreiss A, Richard M, Moyen F, White J, Møller AP, Danchin E (2005) Sex ratio and male sexual characters in a population of blue tits, *Parus caeruleus*. Behavioral Ecology 17: 13–19

Dreiss AN, Silva N, Richard M, Moyen F, Théry M, Møller AP, Danchin É (2008) Condition-dependent genetic benefits of extrapair fertilization in female blue tits *Cyanistes caeruleus*. Journal of Evolutionary Biology 21: 1814–1822

E

Eberhard WG (1996) Female control: Sexual selection by cryptic female choice. Princeton, New Jersey

Edwards SV, Gasper J, Garrigan D, Martindale D, Koop BF (2000) A 39-kb sequence around a blackbird Mhc class II gene: Ghost of selection past and songbird genome architecture. Molecular Biology and Evolution 17: 1384–1395

Edwards SV, Hedrick PW (1998) Evolution and ecology of MHC molecules: from genomics to sexual selection. Trends in Ecology and Evolution 13: 305–311

Ekblom R, Sæther SA, Grahn M, Fiske P, Kålås JA, Höglund J (2004) Major histocompatibilty complex variation and mate choice in a lekking bird, the great snipe (*Gallinago media*). Molecular Ecology 13: 3821–3828

Ekblom, R, Sæther, SA, Jacobsson, P, Fiske, P, Sahlman, T, Grahn, M, Kålås, JA, and Höglund, J (2007) Spatial pattern of MHC class II variation in the great snipe (*Gallinago media*). Molecular Ecology 16(7): 1439–1451

Ekman J (1989) Ecology of non-breeding social systems of *Parus*. Wilson Bulletin 101: 263–288

Etches, RJ (1996) Reproduction in Poultry. Wallingford, Oxford: CAB International

Excoffier L, Laval G, Schneider S (2005) Arlequin (version 3.0): an integrated software package for population genetics data analysis. Evolutionary Bioinformatics 1: 47–50

Eyal-Giladi, H & Kochav, S (1976) From cleavage to primitive streak formation: A complementary normal table and a new look at the first stages of the development of the chick. Developmental Biology 49: 321–337

F

Foerster K, Delhey K, Johnsen A, Lifjeld JT, Kempenaers B (2003) Females increase offspring heterozygosity and fitness through extra-pair matings. Nature 425: 714–717

Foerster K, Kempenaers B (2004) Experimentally elevated plasma levels of testosterone do not increase male reproductive success in blue tits. Behavioral Ecology and Sociobiology 56: 482–490

Foerster K, Valcu M, Johnsen A, Kempenaers B (2006) A spatial genetic structure and effects of relatedness on mate choice in a wild bird population. Molecular Ecology 15: 4555–4567

Foerster, K., Westerdahl, H., von Schantz, T., and Kempenaers, B. (2006) Typing MHC class I genes in blue tits (*Parus caeruleus*), using motif-specific PCR and denaturant gradient gel electrophoresis. Unpublished

Folstad I, Karter AJ (1992) Parasites, bright males, and the immunocompetence handicap. The American Naturalist 139: 603–622

Forstmeier W, Martin K, Bolund E, Schielzeth H, Kempenaers B (2011) Female extrapair mating behavior can evolve via indirect selection on males. Proceedings of the National Academy of Sciences of the United States of America 108: 10608–10613

Franklin, L.E. (1970). Fertilization and the role of the acrosomal region in non-mammals. Biology of Reproduction 2: 159–176

Freeland JM (2005) Molecular Ecology. Wiley, West Sussex, UK
Freeman-Gallant CR, Johnson EM, Saponara F, Stanger M (2002) Variation at the major histocompatibility complex in Savannah sparrows. Molecular Ecology 11: 1125–1130
Freeman-Gallant CR, Meguerdichian M, Wheelwright NT, Sollecito SV (2003) Social pairing and female mating fidelity predicted by restriction fragment length polymorphism similarity at the major histocompatibility complex in a songbird. Molecular Ecology 12: 3077–3083

G

García-Navas V, Ortego J, Sanz JJ (2009) Heterozygosity-based assortative mating in blue tits (*Cyanistes caeruleus*): implications for the evolution of mate choice. Proceedings of the Royal Society of London Series B 276: 2931–2940
Gee, GF (1983) Avian artificial insemination and semen preservation. In Risser, A.C. Jr & Todd, F.S. (eds) IFCB Symposium on Breeding Birds in Captivity: 375–398. North Hollywood, CA: International Foundation for the Conservation of Birds
Goldsby RA, Kindt TJ, Osborne BA (2000) Immunology. W.H. Freeman and company, New York
Gowaty PA (1996) Battles of the sexes and origins of monogamy. In: Partnerships in Birds: The Study of Monogamy. Oxford university press, Oxford, pp 21–52
Griffith SC, Immler S (2009) Female infidelity and genetic compatibility in birds: the role of the genetically loaded raffle in understanding the function of extrapair paternity. Journal of Avian Biology 40: 97–101
Griffith SC, Owens IPF, Thuman KA (2002) Extra pair paternity in birds: a review of interspecific variation and adaptive function. Molecular Ecology 11: 2195–2212
Griffith SC, Stewart IRK, Dawson DA, Owens IPF, Burke T (1999) Contrasting levels of extra-pair paternity in mainland and island populations of the house sparrow (*Passer domesticus*): is there an 'island effect'? Biological Journal of the Linnean Society 68: 303–316
Gupta, SK & Bakst, MR (1993) Turkey embryo staging from cleavage through hypoblast formation. Journal of Morphology 217: 313– 325
Gwinner H (1997) The function of green plants in nests of European starlings (*Sturnus vulgaris*). Behaviour 134: 337–351
Gwinner H, Berger S (2007) Starling males select green nest material by olfaction using experience-independent and experience-dependent cues. Animal Behaviour 75: 971–976

H

Hagelin JC, Jones IL (2007) Bird odors and other chemical substances: a defense mechanism or overlooked mode of intraspecific communication. The Auk 124: 741–761
Hale ML, Verduijn MH, Møller AP, Wolff K, Petrie M (2009) Is the peacock's train an honest signal of genetic quality at the major histocompatibility complex? Journal of Evolutionary Biology 22: 1284–1294
Hall TA (2009) BioEdit: a user-friendly biological sequence allignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symposium Series 41: 95–98
Hamburger, V & Hamilton, HL (1951) A series of normal stages in the development of the chick embryo. Journal of Morphology 88: 49–92
Hamilton WD, Zuk M (1982) Heritable true fitness and bright birds: A role for parasites? Science 218: 384–387
Hammerstedt RH, Cramer PG, Barbato GF, Armann RP, O'Brien JS, Griswold MD (2001) A fragment of prosaposin (SGP-1) from rooster sperm promotes sperm-egg binding and improves fertility in chickens. Journal of andrology 22: 361–375
Hansson B (2004) Marker-based relatedness predicts egg-hatching failure in great reed warblers. Conservation Genetics 5: 339–348
Harriman AE, Berger RH (1986) Olfactory Acuity in the Common Raven (*Corvus corax*). Physiology and Behaviour 36: 257–262
Hedrick PW (2002) Pathogen resistance and genetic variation at MHC loci. Evolution 56: 1902–1908

Hess CM, Edwards SV (2002) The evolution of the Major Histocompatibility Complex in birds. BioScience 52: 423–431

Hettyey A, Hegyi G, Puurtinen M, Hoi H, Török J, Penn DJ (2010) Mate choice for genetic benefits: time to put the pieces together. Ethology 116: 1–9

Högstand K, Böhme J (1999) Gene conversion can create new MHC alleles. Immunological Reviews 167: 305–317

Hoyt DF (1979) practical methods of estimating volume and fresh weight of bird eggs. Auk 96: 73–77

Huelsenbeck JP, Ronquist F (2001) MRBAYES: Bayesian inference of phylogenetic trees. Bioinformatics 17: 754–755

Hughes AL, Nei M (1988) Pattern of nucleotide substitution at major histocompatibility complex class I loci reveals overdominant selection. Nature 335: 167–170

Hughes AL, Nei M (1989) Nucleotide substitution at major histocompatibility complex class II loci: Evidence for overdominant selection. Proceedings of the National Academy of Sciences of the United States of America 86: 958–962

Hunt HD, Goto RM, Foster DN, Bacon LD, Miller MM (2006) At least one YMHCI molecule in the chicken is alloimmunogenic and dynamically expressed on spleen cells during development. Immunogenetics 58: 297–307

Hunt S, Bennett ATD, Cuthill IC, Griffiths R (1998a) Blue tits are ultraviolet tits. Proceedings of the Royal Society in London Series B 265: 451–455

Hunt S, Cuthill IC, Bennett ATD, Griffiths R (1998b) Preferences for ultraviolet partners in the blue tit. Animal Behaviour 58: 809–815

J

Jamieson, IG & Ryan, CJ (2000) Increased egg infertility associated with translocating inbred Takahe (*Porphyrio hochstetteri*) to island refuges in New Zealand. Biological Conservation 94: 107–114

Janeway CA, Murphy K, Travers P, Ehrenstein M (2008) Janeway's immunobiology. Garland Science, New York

Jarvi SI, Tarr CL, McIntosh CE, Atkinson CT, Fleischer RC (2004) Natural selection of the major histocompatibility complex (Mhc) in Hawaiian honeycreepers (Drepanidinae). Molecular Ecology 13: 2157–2168

Jennions MD, Petrie M (2000) Why do females mate multiply? A review of the genetic benefits. Biological reviews 75: 21–64

Johnsen A, Carter KL, Delhey K, Lifjeld JT, Robertson RJKB (2011) Laying-order effects on sperm numbers and on paternity: comparing three passerine birds with different life histories. Behavioral Ecology and Sociobiology Online early:

Jordan WC, Bruford MW (1998) New perspectives on mate choice and the MHC. Heredity 81: 239–245

K

Kalinowski ST, Taper ML, Marshall TC (2007) Revising how the computer program CERVUS accommodates genotyping error increases success in paternity assignment. Molecular Ecology 16: 1099–1106

Kaufman J, Milne S, Göbel TWF, Walker BA, Jacob JP, Auffray C, Zoorob R, Beck S (1999) The chicken B locus is a minimal essential major histocompatibilty complex. Nature 401: 923–925

Kaufman J, Salomonsen J, Flajnik M (1994) Evolutionary conservation of MHC class I and class II molecules-different yet the same. Seminars in Immunology 6: 411–424

Kaufman J, Völk H, Wallny HJ (1995) A "minimal essential Mhc"and an "unrecognized Mhc": Two extremes in selection for polymorphism. Immunological Reviews 143: 63–88

Kelly DJ, Marples NM (2004) The effects of novel odour and colour cues on food acceptance by the zebra finch, *Taeniopygia guttata*. Animal Behaviour 68: 1049–1054

Kempenaers B, Adriaensen F, Noordwijk AJv, Dhondt AA (1996) Genetic similarity, inbreeding and hatching failure in blue tits: Are unhatched eggs infertile? Proceedings of the Royal Society in London Series B 263: 179–185

Kempenaers, B, Congon, B, Boag, P & Robertson, R (1999) Extrapair paternity and egg hatchability in tree swallows: evidence for the genetic incompatibility hypothesis? Behavioral Ecology 10: 304–311

Kempenaers B, Plompen W, Briskie JV (1994) Patterns of sperm storage in two species of tits. Ardea 82: 185–192

Kempenaers B, Verheyen GR, Broeck Mvd, Burke T, Broeckhoven Cv, Dhondt AA (1992) Extra-pair paternity results from female preference for high-quality males in the blue tit. Nature 357: 494–496

Kempenaers B, Verheyen GR, Dhondt AA (1995) Mate guarding and copulation behaviour in monogamous and polygynous blue tits: do males follow a best-of-a-bad-job strategy? Behavioural Ecology and Sociobiology 36: 33–42

Kempenaers B, Verheyen GR, Dhondt AA (1997) Extra-pair paternity in the blue tit (*Parus caeruleus*): female choice, male characteristics, and offspring quality. Behavioural Ecology 8: 481–492

Kennedy LJ, Ryvar R, Brown JJ, Ollier WER, Radford AD (2003) Resolution of complex feline leukocyte antigen DRB loci by reference strand-mediated conformational analysis (RSCA). Tissue Antigens 62: 313–323

King, AS and McLelland, J Form and function in birds. 1981. New York, Academic press inc.

Kingma, SA, Komdeur, J, Vedder, O, Engelhardt, N von, Korsten, P, and Groothuis, TGG. Rapid changes in maternal yolk androgen deposition in response to manipulated male attractiveness in wild blue tits: correlational versus experimental evidence. 2007.

Kirby, JD & Froman, DP (1990) Analysis of poultry fertility data. Poultry Science 69: 1764–1768

Kirby, JD & Froman, DP (1991) Analysis of poultry fertility data. Poultry Science 70: 1986–1990

Klein J (1986) Natural history of the major histocompatibility complex. Wiley, New york

Knape J, Sköld M, Jonzén N, Åkesson M, Bensch S, Hansson B, Hasselquist D (2008) An analysis of hatching success in the great reed warbler *Acrocephalus arundinaceus*. Oikos 117: 430–438

Knapp LA (2005) Denaturing gradient gel electrophoresis and its use in the detection of major histocompatibility complex polymorphism. Tissue Antigens 65: 211–219

Knowles SCL, Palinauskas V, Sheldon BC (2010) Chronic malaria infections increase family inequalities and reduce parental fitness: experimental evidence from a wild bird population. Journal of Evolutionary Biology 23: 557–569

Kochav, S, Ginsburg, M & Eyal-Giladi, H (1980) From cleavage to primitive streak formation: A complementary normal table and a new look at the first stages of the development of the chick. Developmental Biology 79: 296–308

Koenig WD (1982) Ecological and social factors affecting hatchability of eggs. The Auk 99: 526–536

Komdeur J, Pels MD (2005) Rescue of the Seychelles warbler on Cousin Island, Seychelles: The role of habitat restoration. Biological Conservation 124: 15–26

Korsten, P. Avian Sex Allocation and Ornamental Coloration: A study on blue tits. 2006. Groningen, The Netherlands, Rijksuniversiteit Groningen.

Korsten P, Lessells CM, Mateman AC, Velde Mvd, Komdeur J (2006) Primary sex ratio adjustment to experimentally reduced male UV attractiveness. Behavioral Ecology 17: 539–546

Korsten P, Vedder O, Szentirmai I, Komdeur J (2007) Absence of status signalling by structurally based ultraviolet plumage in wintering blue tits (*Parus caeruleus*). Behavioral Ecology and Sociobiology 61: 1933–1943

Kosin, IL (1944) Macro- and microscopic methods of detecting fertility in unincubated hen's eggs. Poultry Science 23: 266–269.

Kosin, IL (1945) Abortive parthenogenesis in the domestic chicken. Anatomical Record 91: 245–251

Krokene C, Rigstad K, Dale M, Lifjeld JT (2004) The function of extrapair paternity in blue tits and great tits: good genes or fertility insurance? Behavioral Ecology 9: 649–656

Kruuk LEB, Clutton-Brock TH, Pemberton JM, Brotherstone S, Guinness FE (2000) Heritability of fitness in a wild mammal population. Proceedings of the National Academy of Sciences of the United States of America 97: 698–703

Kruuk LEB, Sheldon BC, Merilä J (2002) Severe inbreeding depression in collared flycatchers (*Ficedula albicollis*). Proceedings of the Royal Society of London Series B 269: 1581–1589

Kvist L, Ruokonen M, Lumme J, Orell M (1999a) Different population structures in northern and southern populations of the European blue tit (*Parus caeruleus*). Journal of Evolutionary Biology 12: 798–805

Kvist L, Ruokonen M, Lumme J, Orell M (1999b) The colonization history and present-day population structure of the European great tit (Parus major major). Heredity 82: 495–502

Kvist L, Viiri K, Dias PC, Rytkönen S, Orell M (2004) Glacial history and colonization of Europe by the blue tit *Parus caeruleus*. Journal of Evolutionary Biology 35: 352–359

L

Lambrechts M, Dhondt AA (1988) The anti-exhaustion hypothesis: a new hypothesis to explain song performance and song switching in the great tit. Animal Behaviour 36: 327–334

Leech DI, Hartley IR, Stewart IRK, Griffith SC, Burke T (2001) No effect of parental quality or quality of extrapair paternity on brood sex ration in the blue tit (*Parus caeruleus*). Behavioral Ecology 12: 674–680

Lenz TL, Eizaguirre C, Becker S, Reusch TBH (2009) RSCA genotyping of MHC for high-throughput evolutionary studies in the model organism three-spined stickleback *Gasterosteus aculeatus*. BMC Evolutionary Biology 9:

Lessells CM, Boag PT (1987) Unrepeatable repeatabilities: A common mistake. The Auk 104: 116–121

Lifjeld JT, Aas CK, Birkhead TR, Fletcher F, Krokene C, Rigstad K (2000) Variation in the number of spermatozoa in blue tit and great tit eggs. The Auk 117: 246–249

Liptói, K, Varga, Á, Hidas, A & Barna, J (2004) Determination of the rate of true fertility in duck breeds by the combination of two *in vitro* methods. Acta Veterinaria Hungaria 52: 227–233

Livant EJ, Brigati JR, Ewald SJ (2004) Diversity and locus specificity of chicken *MHC B* class I sequences. Animal Genetics 35: 18–27

Lodge, JR, Fechheimer, NS & Jaap, RG (1971) The relationship of *in vivo* sperm storage interval to fertility and embryonic survival in the chicken. Biology of Reproduction 5: 252–257

Loiseau C, Zoorob R, Garnier S, Birard J, Federici P, Julliard R, Sorci G (2008) Antagonistic effects of a *Mhc* class I allele on malaria-infected house sparrows. Ecology Letters 11: 258–265

Loiseau C, Zoorob R, Robert A, Chastel O, Julliard R, Sorci G (2011) *Plasmodium relictum* infection and MHC diversity in the house sparrow (*Passer domesticus*). Proceedings of the Royal Society B-Biological Sciences 278: 1264–1272

M

Magrath MJL, Vedder O, Velde Mvd, Komdeur J (2009) Maternal effects contribute to the superior performance of extra-pair offspring. Current Biology 19: 792–797

Manier MK, Belote JM, Berben KS, Novikov D, Stuart WT, Pitnick S (2010) Resolving Mechanisms of Competitive Fertilization Success in *Drosophila melanogaster*. Science 328: 354–357

Mason EA (1938) Determining sex in breeding birds. Bird-banding 9: 46–48

Mays HL, Hill GE (2004) Choosing mates: good genes versus genes that are a good fit. TRENDS in Ecology and Evolution 19: 554–559

McFarlane ML, Cherry MI, Evans MR (2010) Female cape sugarbirds (*Promerops cafer*) modify egg investment both for extra-pair mates and for male tail length. Journal of Evolutionary Biology 23: 1998–2003

McKinney F, Evarts S (1998) Sexual coercion in waterfowl and other birds. In: Avian reproductive tactics: female and male perspectives. American Ornithologists' Union, Washington, pp 163-195

Meijerhof, R 1992. Pre-incubation holding of hatching eggs. World's Poultry Science Journal 48: 57–68

Mennerat A, Bonadonna F, Perret P, Lambrechts MM (2005) Olfactory conditioning experiments in a food-searching passerine bird in semi-natural conditions. Behavioural Processes 70: 264–270

Merino S, Møller AP (2010) Host-parasite interactions and climate change. In: MøllerAP, FiedlerW, Berthold P (eds) Effects of climate change on birds. Oxford University Press, New York, pp 213–226

Merino S, Moreno J, Sanz JJ, Arriero E (2000) Are avian blood parasites pathogenic in the wild? A medication experiment in blue tits (*Parus caeruleus*). Proceedings of the Royal Society of London Series B 267: 2507–2510

Mesa CM, Thulien KJ, Moon DA, Veniamin SM, Magor KE (2004) The dominant MHC class I gene is adjacent to the polymorphic TAP2 gene in the duck, *Anas platyrhynchos*. Immunogenetics 56: 194–203

Milinski M (2006) The Major Histocompatibility Complex, sexual selection and mate choice. Annual Review of Ecology, Evolution and Systematics 37: 159–186

Miller HC, Lambert DM (2003) An evaluation of methods of blood preservation for TR-PCR from endangered species. Convervation Genetics 4: 651–654

Miller HC, Lambert DM (2004) Genetic drift outweighs balancing selection in shaping post-bottleneck major histocompatibility complex variation in New Zealand robins (Petroicidae). Molecular Ecology 13: 3709–3721

Miller MM, Goto R, Bernot A, Zoorob R, Auffray C, Bumstead N, Briles WE (1994) Two Mhc class I and two Mhc class II genes map to the chicken Rfp-Y system outside the B complex. Proceedings of the National Academy of Sciences of the United States of America 91: 4397–4401

Mitton JB, Schuster WSF, Cothran EG, de Fries JC (1993) Correlation between the individual heterozygosity of parents and their offspring. Heredity 71: 59–63

Morrow, EH, Arnqvist, G & Pitcher, TE (2002) The evolution of infertility: does hatching rate in birds co-evolve with female polyandry? Journal of Evolutionary Biology 15: 702–709

Møller AP (1989) Ejaculate quality, testis size and sperm production in mammals. Functional Ecology 3: 91–96

Møller AP (1998) Sperm competition and sexual selection. In: Birkhead, T. and Møller, A.P. (eds). Sperm competition and sexual selection. Academic press, San Diego

Møller AP, Alatalo RV (1999) Good genes effects in sexual selection. Proceedings of the Royal Society in London Series B 266: 85–91

Møller AP, Erritzøe J (1998) Host immune defence and migration in birds. Evolutionary Ecology 12: 945–953

Mulder RA, Cockburn A (1993) Sperm competition and the reproductive anatomy of male superb fairy-wrens. Auk 110: 588–593

N

Naz RK, Menge AC (1994) Antisperm antibodies: orgin, regulation, and sperm reactivity in human infertility. Fertility and Sterility 61: 1001–1013

Neff BD, Pitcher TE (2005) Genetic quality and sexual selection: an integrated framework for good genes and compatible genes. Molecular Ecology 14: 19–38

Nei M, Hughes AL (1991) Polymorphism and evolution of the Major Histocompatibility Complex loci in mammals. In: Evolution at the Molecular Level. Sinauer associates, Sunderland, pp 222–247

Nowak MA, Tarczy-Hornoch K, Austyn JM (1992) The optimal number of major histocompatibility complex molecules in an individual. Immunology 89: 10896–10899

Nylander, J. A. A. MrModeltest v2. Program distributed by the author. Evolutionary Biology Centre, Uppsala University. 2004.

O

Oberweger K, Goller F (2001) The metabolic cost of birdsong production. The journal of experimental biology 204: 3379–3388

Olano-Marin J, Mueller JC, Kempenaers B (2011a) Heterozygosity and survival in blue tits (*Cyanistes caeruleus*): contrasting effects of presumably functional and neutral loci. Molecular Ecology 20: 4028–4041

Olano-Marin J, Mueller JC, Kempenaers B (2011b) Correlations between heterozygosity and reproductive success in the blue tit (*Cyanistes caeruleus*): an analysis of inbreeding and single locus effects. Evolution 65 (11): 3175–3194

Olsen, MW & Neher, BH (1948) The site of fertilization in the domestic fowl. Journal of Experimental Zoology 109: 355–366.

Oosterhout vC (2008) A new theory of MHC evolution: beyond selection on the immune genes. Proceedings of the Royal Society in London Series B 276: 657–665

Orita M, Iwahana H, Kanazawa H, Hayashi K, Sekiya T (1989) Detection of polymorphisms of human DNA by gel electrophoresis as single-strand conformation polymorphisms. Proceedings of the National Academy of Science 86: 2766–2770

Örnborg J, Andersson S, Griffith SC, Sheldon BC (2002) Seasonal changes in a ultraviolet structural colour signal in blue tits, *Parus caeruleus*. Biological Journal of the Linnean Society 76: 237–245

P

Page RDM, Holmes EC (1998) Molecular Evolution: A Phylogentic Approach. Blackwell Science,

Parker GA (1998) Sperm competition and the evolution of ejaculates: toward a theory base. In: Sperm competition and sexual selection. Academic Press, pp 3-54

Parker GA (1970) Sperm competition and its evolutionary consequences in the insects. Biological Review 45: 525–567

Partecke J, van 't Hof T, Gwinner E (2004) Differences in the timing of reproduction between urban and forest European blackbirds (*Turdus merula*): result of phenotypic flexibility or genetic differences? Proceedings of the Royal Society of London Series B 271: 1995–2001

Partridge L (1983) Non-random mating and offspring fitness. In: Bateson, P. (Ed). Mate choice. Cambridge university press, Cambridge, UK

Paterson S, Pemberton PM (1997) No evidence for major histocompatibility complex-dependent mating patterns in a free-living ruminant population. Proceedings of the Royal Society of London Series B 264: 1813–1819

Peirce MA (1981) Distribution and host-parasite check-list of the haematozoa of birds in Western Europe. Journal of Natural History 15: 419–458

Pellat EJ (1997) Changes in the size and content of sperm storage tubules during the breeding cycle of the Zebra Finch *Taeniopygia guttata*. Ibis 140: 528–530

Penn D, Potts W (1998) How do Major Histocompatibility Complex genes influence odor and mating preferences? Advances in Immunology 69: 411–436

Penn DJ (2002) The scent of genetic compatibility: sexual selection and the Major Histocompatibility complex. Ethology 108: 1–21

Penn DJ, Potts WK (1999) The evolution of mating preferences and Major Histocompatibility Complex genes. The American Naturalist 153: 145–164

Petit C, Hossaert-McKey M, Perret P, Blondel J, Lambrechts MM (2002) Blue tits use selected plants and olfaction to maintain an aromatic environment for nestlings. Ecology Letters 5: 585–589

Piertney SB, Oliver MK (2006) The evolutionary ecology of the major histocompatibility complex. Heredity 96: 7–21

Pizzari T, Birkhead TR (2000) Female feral fowl eject sperm of subdominant males. NATURE 405: 787–789

Pletschet, SM & Kelly, JF (1990). Breeding biology and nesting success of palila. The Condor 92: 1012–1021

Poesel A, Foerster K, Kempenaers B (2001) The dawn song of the blue tit *Parus caeruleus* and its role in sexual selection. Ethology 107: 521–531

Potti J, Merino S (1996) Causes of hatching failure in the pied flycatcher. The Condor 98: 328–336

Potts WK, Manning CJ, Wakeland EK (1991) Mating patterns in seminatural populations of mice influenced by MHC genotype. Nature 352: 619–621

Potts WK, Wakeland EK (1990) Evolution of diversity at the Major Histocompatibility Complex. Trends in Ecology & Evolution 5: 181–187

Promerová M, Albrecht T, Bryja j (2009) Extremely high MHC class I variation in a population of a long-distant migrant, the Scarlet Rosefinch (*Carpodacus erythrinus*). Immunogenetics 61: 451–461

Promerová M, Vinkler M, Bryja J, Poláková R, Schnitzer J, Munclinger P, Albrecht T (2011) Occurence of extra-pair paternity is connected to social male's MHC-variability in the scarlet rose finch *Carpodacus erythrinus*. Journal of Avian Biology 42: 5–10

Q

Quay WB (1986) Cloacal protuberance and cloacal sperm in passerine birds: Comparative-Study of quantitative relations. Condor 88: 160–168

R

Rasbash, J, Browne, W, Healy, M, Cameron, B, and Charlton, C Multilevel Models Project. 2005. London, Institute of Education, University of London

Reid JM, Sardell RJ (2012) Indirect selection on female extra-pair reproduction? Comparing the additive genetic value of maternal half-sib extra-pair and within-pair offspring. Proceedings of the Royal Society of London Series B In press

Richardson DS, Jury FJ, Blaakmeer K, Komdeur J, Burke T (2001) Parentage assignment and extra-group paternity in a cooperative breeder: the seychelles warbler (*Acrocephalus seychellensis*). Molecular Ecology 10: 2263–2273

Richardson DS, Komdeur J, Burke T, von Schantz T (2005) MHC-based patterns of social and extra-pair mate choice in the Seychelles warbler. Proceedings of the Royal Society in London Series B 272: 759–767

Richardson DS, Westerdahl H (2003) MHC diversity in two *Acrocephalus* species: the outbred Great reed warbler and the inbred Seychelles warbler. Molecular Ecology 12: 3523–3529

Romanoff, AL (1960) The Avian Embryo. New York: The Macmillan Company

Ronquist F, Huelsenbeck JP (2003) MrBayes 3: Bayesian phylogenetic inference under mixed models. Bioinformatics 19: 1572–1574

Roper TJ (1999) Olfaction in birds. Advances in the study of Behaviour 28: 247–332

S

Sambrook J, Russell DW, Irwin N (2001) Molecular cloning: a laboratory manual. Cold spring harbor laboratory press, New york

Sardell RJ, Arcese P, Keller LF, Reid JM (2011) Sex-specific differential survival of extra-pair and within-pair offspring in song sparrows, *Melospiza melodia*. Proceedings of the Royal Society of London Series B 278: 3251–3259

Sax A, Hoi H (1998) Individual and temporal variation in cloacal protuberance size of male Bearded Tits (*Panurus biarmicus*). Auk 115: 964–969

Sax A, Hoi H, Birkhead TR (1998) Copulation rate and sperm use by female bearded tits, *Panurus biarmicus*. Animal Behaviour 56: 1199–1204

Saxton VP, Creasy GL, Paterson AM, Throught MCT (2004) Response of blackbirds (*Turdus merula*) and sivereyes (*Zosterops lateralis*) to geraniol and 2-methoxy-3-isobutylpyrazine. American Journal of Enology and Viticulture 55: 292–294

Schantz Tv, Wittzell H, Göransson G, Grahn M, Persson K (1996) MHC genotype and male ornamentation: genetic evidence for the Hamilton-Zuk model. Proceedings of the Royal Society in London Series B 263: 265–271

Schut, E, Hemmings, N & Birkhead, TR (2008) Partheno-genesis in a passerine bird, the Zebra Finch *Taeniopygia guttata*. Ibis 150: 197–199

Schwensow N, Eberle M, Sommer S (2008) Compatibility counts: MHC-associated mate choice in a wild promiscuous primate. Proceedings of the Royal Society in London Series B 275: 555–564

Selander RK, Hauser RJ (1965) Gonadal and behavioral cycles in the great-tailed grackle. Condor 67: 157–182

Sellier, N, Brun, JM, Richard, M-M, Batellier, F, Dupuy, V & Brillard, JP (2005) Comparison of fertility and embryo mortality following artificial insemination of Common Duck females (*Anas platyrhynchos*) with semen from Common or Muscovey (*Cairina moschata*) Drakes. Theriogenology 64: 429–439

Sellier, N, Brillard, J-P, Dupuy, V & Bakst, MR (2006) Comparative staging of embryo development in chicken, turkey, duck, goose, guinea fowl, and Japanese quail assessed from five hours after fertilization through seventy-two hours of incubation. Journal of Applied Poultry Research 15: 219–228

Sheldon BC, Andersson S, Griffith SC, Ornborg J, Sendecka J (1999) Ultraviolet colour variation influences blue tit sex ratios. NATURE 402: 874–877

Shiina T, Ando A, Imanishi T, Kawata H, Hanzawa K, Gojobori T, Inoko H, Watanabe S (1995) Isolation and characterization of cDNA clones for Japanese quail (*Coturnix japonica*) major histocompatibilty complex (*MhcCoja*) class I molecules. Immunogenetics 42: 213–216

Shum BP, Rajalingam R, Magor KE, Azumi K, Carr WH, Dixon B, Stet RJM, Adkison MA, Hedrick RP, Parham P (1999) A divergent non-classical class I gene conserved in salmonids. Immunogenetics 49: 479–490

Siddall EC, Marples NM (2011) The effect of pyrazine odor on avoidance learning and memory in wild robins Erithacus rubecula. Current Zoology 52: 208–214

Siva-Jothy MT, Skarstein F (1998) Towards a functional understanding of "good genes". Ecology Letters 1: 178–185

Skarstein F, Folstad I, Liljedal S, Grahn M (2005) MHC and fertilization success in the Arctic charr (*Salvelinus alpinus*). Behavioural Ecology and Sociobiology 57: 374–380

Small, AO, Schlusser, K, Ryan, CJ & Jamieson, IG (2000) Detecting sperm on the perivitelline membrane of incubated turkey eggs and its implications for research on fertility problems in endangered species. Wildlife Research 27: 635–637

Smith HG, Nilsson JÅ (1987) Intraspecific variation in migratory pattern of a partial migrant, the blue tit (*Parus caeruleus*): an evaluation of different hypotheses. The Auk 104: 109–115

Snyder GK, Peterson TT (1979) Olfactory sensitivity in the black-billed magpie and in the pigeon. Comparative biochemistry and physiology 62A: 921–925

Spottiswoode C, Møller AP (2004) Genetic similarity and hatching success in birds. Proceedings of the Royal Society of London Series B 271: 267–272

Spurgin LG, Richardson DS (2010) How pathogens drive genetic diversity: MHC, mechanisms and misunderstandings. Proceedings of the Royal Society B-Biological Sciences 277: 979–988

Steele, MG, Meldrum, W, Brillard, JP & Wishart, GJ (1994) The interaction of avian spermatozoa with the perivitelline layer *in vitro* and *in vivo*. Journal of Reproduction and Fertility 101: 599–603

Steele MG, Wishart GJ (1992) Evidence for a species-specific barrier to sperm transport within the vagina of the chicken hen. Theriogenology 38: 1107–1114

Steiger SS, Fidler AE, Valcu M, Kempenaers B (2008) Avian olfactory reception repertoires: evidence for a well-developed sense of smell in birds? Proceedings of the Royal Society of London Series B 275: 2309–2317

Stepinska, U & Bakst, MR (2006) Fertilization. In Jamieson, B.G.M. (ed.) Reproductive Biology and Phylogeny of Aves: 533–587. Enfield, New Hampshire, USA & Plymouth UK: Science Publishers Inc.

Stjernman M, Raberg L, Nilsson JA (2008) Maximum Host Survival at Intermediate Parasite Infection Intensities. Plos One 3:

Stjernman M, Råberg L, Nilsson JÅ (2004) Survival costs of reproduction in the blue tit (*Parus caeruleus*) : a role for blood parasites? Proceedings of the Royal Society in London Series B 271: 2387–2394

Strand T, Westerdahl H, Höglund J, Alatalo RV, Siitari H (2007) The *Mhc* class II of the Black grouse (*Tetrao tetrix*) consists of low numbers of B and Y genes with variable diversity and expression. Immunogenetics 59: 725–734

Strand TM, Höglund J (2011) Genotyping of black grouse MHC class II B using Reference Strand-Mediated Conformational Analysis (RSCA). BMC research notes 4: 183

Stockley P (1999) Sperm selection and genetic incompatibility: does relatedness of mates affect male success in sperm competition. Proceedings of the Royal Society in London Series B 266: 1663–1669

Strand TM, Höglund J (2011) Genotyping of black grouse *MHC* class II B using Reference Strand-Mediated Conformational Analysis (RSCA). BMC research notes 4:

Svensson L (1992) Identification guide to European passerines. Märstatryck, Stockholm

Svensson, M, Rintamäki, PT, Birkhead, TR, Griffith, SC & Lundberg, A (2007) Impaired hatching success and male-biased embryo mortality in Tree Sparrows. Journal of ornithology 148: 117–122

Summers-Smith, JD (1995) The Tree Sparrow. Guisborough, Cleveland: J. Denis Summers-Smith.

T

Tamura K, Dudley J, Nei M, Kumar S (2007) MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) Software Version 4.0. Molecular Biology and Evolution 24: 1596–1599

Tomás G, Merino S, Moreno J, Morales J (2007) Consequences of nest reuse for parasite burden and female health and condition in blue tits, *Cyanistes caeruleus*. Animal Behaviour 73: 805–814

Tonra CM, Marra PP, Holberton RL (2011) Early elevation of testosterone advances migratory preparation in a songbird. The Journal of Experimental Biology 214: 2761–2767

Tregenza T, Wedell N (2000) Genetic compatibility, mate choice and patterns of parentage: Invited Review. Molecular Ecology 9: 1013–1027

Tripet F, Glaser M, Richner H (2002) Behavioural responses to ectoparasites: time-budget adjustments and what matters to Blue Tits *Parus caeruleus* infested by fleas. Ibis 144: 461–469

Trivers RL (1972) Parental investment and sexual selection. In: Sexual selection and the descent of man, 1871–1971. Aldine-Atherton, Chicago, pp 136-179

V

Valkiunas G (2005) Avian Malaria Parasites and Other Haemosporidia. CRC Press, Boca Raton, Florida, USA

van Noordwijk AJ, Scharloo W (1981) Inbreeding in an island population of the great tit. Evolution 35: 674–688

Vedder, O, All's fair in love and war. (2011) Dissertation. The University of Groningen, the Netherlands.

Vedder O, Komdeur J, van der Velde M, Schut E, Magrath MJL (2011) Polygyny and extra-pair paternity enhance the opportunity for sexual selection in blue tits. Behavioural Ecology and Sociobiology 65: 741–752

Vedder O, Korsten P, Magrath MJL, Komdeur J (2008) Ultraviolet plumage does not signal social status in free-living blue tits; an experimental test. Behavioral Ecology 19: 410–416

Vedder O, Magrath MJL, Harts AMF, Schut E, van der Velde M, Komdeur J (2010a) Reduced extra-pair paternity in response to experimental stimulation of earlier incubation onset in blue tits. Behavioral Ecology

Vedder O, Schut E, Magrath MJL, Komdeur J (2010b) Ultraviolet crown colouration affects contest outcomes among male blue tits, but only in the absence of prior encounters. Functional Ecology 24: 417–425

Verheyen GR, Kempenaers B, Adriaensen F, van den Broeck M, Matthysen E, van Broeckhoven C, Dhondt AA (1995) The genetic structure of *Parus caeruleus* (blue tit) populations as revealed by minisatellite single locus probes. Heredity 75: 571–577

Vincek V, Klein D, Graser RT, Figueroa F, O'hUigin C, Klein J (1995) Molecular cloning of major histocompatibilty complex class II B gene cDNA from the Bengalese finch *Lonchura striata*. Immunogenetics 42: 262–267

W

Wallraff HG, Kiepenheuer J, Neumann MF, Streng A (1995) Homing experiments with starlings deprived of the sense of smell. The Condor 97: 20–26

Walsh PS, Metzger DA, Higuchi R (1991) Chelex-100 as a medium for simple extraction of DNA for PCR-based typing from forensic material. Biotechniques 10: 506–513

Watt, J.M., Petitte, J.N. & Etches, R.J. (1993) Early develop-ment of the chick embryo. Journal of Morphology 215: 165–182

Weaver RF, Hedrick PW (1995) Basic Genetics. Wm. C. Brown Publishers,

Wedekind C, Chapuisat M, Macas E, Rülicke T (1996) Non-random fertilization in mice correlates with the MHC and something else. Heredity 77: 400–409

Wedekind C, Seebeck T, Bettens F, Paepke AJ (1995) MHC-dependent mate preferences in humans. Proceedings of the Royal Society in London Series B 260: 245–249

Westerdahl, H Avian MHC: variation and selection in the wild. 2003. Dissertation. Lund University, Sweden.

Westerdahl H (2007) Passerine MHC: genetic variation and disease resistance in the wild. Journal of Ornithology 148: s469–s477

Westerdahl H (2004) No evidence of an MHC-based female mating preference in great read warblers. Molecular Ecology 13: 2465–2470

Westerdahl H, Hansson B, Bensch S, Hasselquist D (2004) Between-year variation of MHC allele frequencies in great reed warblers: selection or drift? Journal of Evolutionary Biology 17: 485–492

Westerdahl H, Waldenström J, Hansson B, Hasselquist D, Schantz Tv, Bensch S (2005) Associations between malaria and MHC genes in a migratory songbird. Proceedings of the Royal Society in London Series B 272: 1511–1518

Westerdahl H, Wittzell H, Schantz Tv (1999) Polymorphism and transcription of *Mhc* class I genes in a passerine bird, the great reed warbler. Immunogenetics 49: 158–170

Westerdahl H, Wittzell H, Schantz Tv (2000) *Mhc* diversity in two passerine birds: no evidence for a minimal essential Mhc. Immunogenetics 52: 92–100

Westerdahl H, Wittzell H, von Schantz T, Bensch S (2004) MHC class I typing in a songbird with numerous loci and high polymorphism using motif-specific PCR and DGGE. Heredity 91: 534–542

Westneat DF, Birkhead TR (1998) Alternative hypotheses linking the immune system and mate choice for good genes. Proceedings of the Royal Society in London Series B 265: 1065–1073

Westneat DF, Sherman PW, Morton ML (1990) The ecology and evolution of extra-pair copulations in birds. Current ornithology 7: 331–369

Westneat DF, Webster MS (1994) Molecular analysis of kinship in birds: interesting questions and useful techniques. In: Molecular Ecology and Evolution: Approaches and Applications. Birhauser Verlag, Basel, pp 91–126

Wetton JH, Carter RE, Parkin DT, Walters D (1987) Demographic study of a wild house sparrow population by DNA fingerprinting. NATURE 327: 147–149

Wetton JH, Parkin DT (1991) An association between fertility and cuckoldry in the house sparrow, *Passer domesticus*. Proceedings of the Royal Society of London Series B 245: 227–233

Whittaker DJ, Soini HA, Atwell JW, Hollars C, Novotny MV, Ketterson ED (2010) Songbird chemosignals: volatile compounds in preen gland secretions vary among individuals, sexes, and populations. Behavioral Ecology 21: 608–614

Wilson, J (1994) Breakout fertility – what to look for and what to expect. In Bakst, M.R. & Wishart, G.J. (eds) Proceedings of the First International Symposium on the Artificial Insemination of Poultry: 197–206. Maryland: United States Department of Agriculture

Wishart GJ (1987) Regulation of the length of the fertile period in the domestic fowl by numbers of oviducal spermatozoa, as reflected by those trapped in laid eggs. Journal of Reproduction and Fertility 80: 498

Wishart, GJ (1997) Quantitative aspects of sperm:egg interaction in chickens and turkeys. Animal Reproductive Science 48: 81–92

Wittzell H, Bernot A, Auffray C, Zoorob R (1999) Concerted evolution of two Mhc class II B loci in pheasants and domestic chickens. Molecular Biology and Evolution 16: 479–490

Wittzell H, Schantz Tv, Zoorob R, Auffray C (1995) Rfp-Y-like sequences assort independently of pheasant Mhc genes. Immunogenetics 42: 68–71

Wolfson A (1952) The cloacal protuberance- a means for determining breeding condition in live male passerines. Bird-banding 23: 159–165

Worley K, Gillingham M, Jensen P, Kennedy LJ, Pizzari T, Kaufman J, Richardson DS (2008) Single locus typing of MHC class I and class II B loci in a population of red jungle fowl. Immunogenetics 60: 233–247

Wright PL, Wright MH (1944) Reproductive cycle of the male red-winged blackbird. Condor 46: 46–59

Y

Yamazaki K, Beauchamp GK, Kupniewski D, Bard J, Thomas L, Boyse EA (1988) Familial Imprinting Determines H-2 Selective Mating Preferences. Science 240: 1331–1332

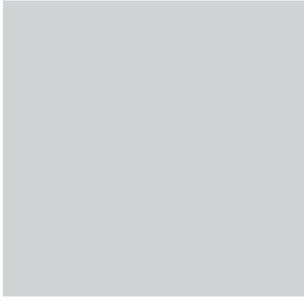
Yamazaki K, Yamaguchi M, Andrews PW (1978) Mating preferences of F2 segregants of crosses between MHC-congenic mouse strains. Immunogenetics 6 (3): 253–259

Z

Zahavi A (1975) Mate selection - Selection for a handicap. Journal of theoretical Biology 53: 205–214

Zeh JA, Zeh DW (1996) The evolution of polyandry I: intragenomic conflict and genetic incompatibility. Proceedings of the Royal Society in London Series B 263: 1711–1717

Zeh JA, Zeh DW (1997) The evolution of polyandry II: post-copulatory defences against genetic incompatibility. Proceedings of the Royal Society in London Series B 264: 69–75



Nederlandse samenvatting

Seksuele selectie in de pimpelmees: de rol van de MHC-genen en effecten die optreden na copulatie

Seksuele selectie

De evolutietheorie van Darwin (1859) stelt dat soorten veranderen doordat de individuen die het best zijn aangepast aan hun omgeving het langst leven en voor de meeste nakomelingen zorgen. Genen die zorgen dat individuen beter zijn aangepast dan hun soortgenoten zullen daardoor toenemen in de populatie en mogelijk de oorspronkelijke genen vervangen. Seksuele selectie is een vorm van natuurlijke selectie, die werkt op het vermogen van individuen om een partner te vinden en nakomelingen te krijgen. Seksuele selectie verklaart de ontwikkeling van de opvallende ornamenten die de mannetjes van veel diersoorten dragen. Een bekend voorbeeld van zo'n ornament is de opvallende staart van de pauw. Dergelijke ornamenten zijn lastig te verklaren op basis van andere vormen van natuurlijke selectie. Ornamenten verminderen namelijk vaak de overlevingskansen van het individu, bijvoorbeeld doordat de ornamenten goed zichtbaar zijn voor roofdieren. Volgens de theorie van seksuele selectie spelen de ornamenten een rol in het vermogen van individuen om nakomelingen te krijgen. Doordat individuen met een ornament meer nakomelingen krijgen neemt het ornament toe in frequentie in de populatie en zullen na verloop van tijd alle mannen het ornament bezitten. Een belangrijke voorwaarde voor dit scenario is dat het al dan niet hebben van het ornament erfelijk is. Seksuele selectie kent twee vormen: selectie door concurrentie tussen individuen van hetzelfde geslacht voor toegang tot de andere sekse en selectie opgelegd doordat de andere sekse kieskeurig is in zijn partnerkeuze. In mijn proefschrift ligt de focus op de partnerkeuze.

In de meeste diersoorten investeren vrouwtjes relatief veel in hun nakomelingen en kunnen zij daarom weinig nakomelingen produceren in vergelijking tot mannetjes (hoewel er uitzonderingen zijn waarin het andersom is). Voor vrouwtjes is het daarom extra belangrijk om selectief te zijn in de keuze van een partner, omdat zij ervoor moeten zorgen dat de nakomelingen die zij produceren van hoge kwaliteit zijn. De vrouwelijke sekse wordt daarom in het algemeen gezien als de meest kieskeurige sekse in de partnerkeuze. Dat wil zeggen dat vrouwtjes proberen om een zo goed mogelijke partner te krijgen en alleen paren met een minder goede man als ze geen andere optie hebben. Vrouwtjes ondervinden op verschillende manieren voordeel van het kiezen van een goede partner. Vrouwtjes kunnen 'directe' voordelen ondervinden, waardoor ze meer nakomelingen bijdragen aan de populatie. Een voorbeeld van een direct voordeel is een goede overleving van de nakomelingen door goede ouderzorg door de vader. Daarnaast kunnen vrouwtjes 'indirecte voordelen' ondervinden, door het krijgen van nakomelingen van goede kwaliteit, die beter overleven en op hun beurt meer nakomelingen krijgen. Door indirecte voordelen dragen vrouwtjes verder in de toekomst meer nageslacht bij aan de populatie.

Er zijn verschillende theorieën over de mannelijke eigenschappen die nakomelingen van goede kwaliteit opleveren. De 'goede genen' theorie stelt dat vrouwtjes een universele voorkeur hebben voor bepaalde mannelijke kenmerken (bijvoorbeeld een zo groot mogelijk ornament) en dat paren met een man met die kenmerken goede nakomelingen oplevert. De theorie van 'genetische compatibiliteit' stelt

echter dat vrouwelijke voorkeuren niet universeel zijn, maar afhangen van de genen van de vrouw. Hoe aantrekkelijk een man is, wordt bepaald door de match tussen de mannelijke en het vrouwelijke genen.

Seksuele selectie na copulatie

Seksuele selectie houdt niet op bij de keuze van een partner. Ook na het initiëren van een copulatie kunnen zowel de man als de vrouw invloed uitoefenen op de uiteindelijke bevruchting (alles wat na het initiëren van een copulatie plaatsvindt wordt onder de noemer van 'seksuele selectie na copulatie' geschaard). In zangvogels zijn buitenechtelijke nakomelingen een bekend fenomeen. Daardoor is ook duidelijk dat vrouwtjes regelmatig binnen één voortplantingscyclus copulaties hebben met meerdere mannen. Vrouwtjes kunnen bijvoorbeeld proberen om buitenechtelijke nakomelingen te krijgen op het moment dat ze gepaard zijn met een man die niet hun voorkeur had, maar wel nakomelingen willen krijgen van goede kwaliteit. Dit kan bijvoorbeeld voorkomen als de meer aantrekkelijke mannen al gepaard waren, of omdat de huidige partner genetisch gezien niet de beste keuze was, maar wel de meeste ouderzorg levert.

Doordat vrouwtjesvogels sperma kunnen opslaan in spermaopslagorgaantjes (SOO) blijft het sperma langer goed. De kans dat een vrouwtje levensvatbaar sperma van meerdere mannen tegelijkertijd bezit is daardoor groter. Doordat spermacellen na de inseminatie een lange weg moeten afleggen tot de plek waar bevruchting plaatsvindt, is er de mogelijkheid voor zowel de vrouw als de man om de uiteindelijke vaderschap nog na de copulatie te beïnvloeden. De vrouw zal ernaar streven om de eicellen te laten bevruchten door de beste man, terwijl de man zal proberen ervoor te zorgen dat hij zelf zoveel mogelijk eicellen bevrucht. Doordat seksuele selectie na copulatie grotendeels binnen het vrouwelijk lichaam plaatsvindt, zijn details van de mechanismen van seksuele selectie na copulatie deels onbekend. Theoretisch gezien zijn er echter verschillende mogelijkheden voor zowel man als vrouw om de bevruchting te beïnvloeden. Bekend is dat vrouwen maar een klein deel van al het sperma opnemen en hierin selectief kunnen zijn. Mannen kunnen de hoeveelheid sperma die ze insemineren aanpassen afhankelijk van hun bevruchtingskansen. Als duidelijk is dat de vrouw waarmee hij paart al sperma van een andere man bezit, kan een man zijn bevruchtingskansen vergroten door meer sperma te insemineren dan hij normaal zou doen. Daarnaast is bekend dat de genen van de man een rol kunnen spelen in zijn bevruchtingssucces (bijvoorbeeld de genen van het MHC, die ik later zal beschrijven). Dit wordt waarschijnlijk veroorzaakt doordat interacties plaatsvinden tussen het sperma of de zaadvloeistof en (cellen in) het voortplantingssysteem van de vrouw (bijvoorbeeld cellen van het vrouwelijk immuunsysteem). Uiteindelijk zal het de combinatie van mannelijke en vrouwelijke invloeden zijn die bepaalt welke man de eicellen bevrucht en zijn mannelijke en vrouwelijke effecten lastig van elkaar te onderscheiden.

Het Major Histocompatibility Complex

De genen van het Major Histocompatibility Complex (MHC) spelen een belangrijke rol in het immuunsysteem. Het MHC kan worden verdeeld in verschillende klasses; klasse I (MHC-I) en II (MHC-II) zijn bepalend voor de herkenning van ziekteverwekkers door het adaptieve immuunsysteem, terwijl klasse III een rol speelt in het niet-specifieke immuunsysteem dat voorkomt dat ziekteverwekkers het lichaam binnendringen. Een gen komt tot expressie doordat op basis van de genetische code een eiwit gevormd wordt dat een bepaalde rol vervult. De eiwitten gevormd op basis van de MHC-I en -II genen binden eiwitten afkomstig van ziekteverwekkers en presenteren die aan een type witte bloedcel (T-cellen), waardoor een immuunreactie in gang gezet wordt. MHC-I moleculen binden voornamelijk ziekteverwekkers die de cel binnendringen, terwijl MHC-II moleculen voornamelijk ziekteverwekkers afkomstig uit de bloedbaan binden. Door verschil in de MHC-genen, verschillen ook de typen ziekteverwekkers en het aantal ziekteverwekkers dat het immuunsysteem herkent tussen individuen. De MHC-genen kunnen namelijk behoorlijk verschillen in diversiteit. Een individu heeft normaal gesproken van elk gen twee kopieën (één gekregen van de vader en één gekregen van de moeder) die in genetische code iets verschillen. De verschillende kopieën worden allelen genoemd. Hoewel elk individu per gen maar twee allelen bezit kunnen er meer dan twee verschillende allelen aanwezig zijn in de populatie. Bij het MHC is het zo dat dezelfde allelen op verschillende 'genlocaties' (loci) aanwezig kunnen zijn. Daardoor kan het aantal allelen dat een individu heeft en dus het aantal verschillende ziekteverwekkers dat een individu kan herkennen sterk verschillen. De MHC-genen spelen daarom in potentie een belangrijke rol in de overleving van individuen.

Een opvallend kenmerk van het MHC is de hoge diversiteit. Het MHC heeft relatief veel genloci, veel diversiteit in de genetische code en veel allelen. De hoge diversiteit van het MHC is de reden dat het zo interessant is om te bestuderen. Doordat selectie normaal gesproken leidt tot vermindering van het aantal allelen in de populatie vermoedt men dat in het geval van de MHC-genen een speciale vorm van selectie (zogenaamde 'balancerende selectie') zorgt dat dit grote aantal allelen in stand gehouden wordt.

De rol van het MHC in de partnerkeuze

Eén van de theorieën over de manier waarop balancerende selectie tot stand komt, stelt dat partnerkeuze op basis van de MHC-genen het grote aantal MHC-allelen in stand houdt. Doordat de MHC-genen van groot belang zijn voor de resistentie tegen ziekteverwekkers hebben zij een grote invloed op de overleving. Daarom is het voor ouders van belang dat hun nakomelingen goede MHC-genen hebben. Er zijn verschillende theorieën over wat voordelig is voor de overleving: zo veel mogelijk allelen, zodat zo veel mogelijk verschillende ziekteverwekkers bestreden kunnen

worden, een optimaal aantal allelen om auto-immuun reacties te voorkomen of specifieke allelen die weerstand bieden tegen een veel voorkomende ziekteverwekker.

Verschillende eerdere studies hebben aangetoond dat het MHC een rol speelt in de partnerkeuze, onder andere in zangvogels. In de Savannah gors en de huismus vermeden vrouwtjes mannetjes wiens MHC erg op dat van henzelf leek. In de Seychellen zanger en de roodmus bleken vrouwtjes hun sociale partner niet te selecteren op basis van zijn MHC, maar was het risico dat de sociale partner bedrogen werd wel groter als de sociale partner een lage MHC diversiteit had.

Doelstelling

Het doel van mijn project was tweeledig. Mijn eerste doel was om inzicht te krijgen in het optreden van seksuele selectie na de copulatie in de pimpelmees. Om copulatie gedrag en processen die optreden na copulatie beter te begrijpen, bestudeer ik in deel I van dit proefschrift de beschikbaarheid van sperma in de pimpelmees. Mijn tweede doel was om uit te vinden of de MHC-genen een rol spelen in de partnerkeuze van de pimpelmees. De MHC-genen waren al wel beschreven in verschillende andere zangvogel soorten, maar nog niet in de pimpelmees. Deel II van mijn proefschrift beschrijft de MHC-I genen van de pimpelmees, terwijl in deel III de rol van de MHC-I genen in de partnerkeuze wordt beschreven.

Dit onderzoek werd uitgevoerd in een pimpelmeespopulatie op landgoed *De Vosbergen* (in de buurt van Groningen), die sinds 2001 bestudeerd wordt. Het gebied bestaat uit een mengeling van loof- en naaldbos dat wordt afgewisseld met weiland. In het gebied hangen in totaal 189 pimpelmees nestkasten, waarvan ieder broedseizoen ongeveer de helft bezet wordt door broedende pimpelmezen. Door dagelijks de nestkasten te controleren tussen eind maart en half juni werd de voortgang van het broedproces in de gaten gehouden. Jongen werden uit de nestkast gehaald om ze op te meten, van een metalen ring te voorzien en een bloedmonster te nemen. Adulten werden in de nestkast gevangen (door middel van een klep die dichtvalt als de vogel in de kast gaat) voor het meten, ringen en verzamelen van een bloedmonster. Bloedmonsters werden gebruikt voor het bepalen van de genetische vaderschap. In deze pimpelmees populatie is 11% van alle jongen buitenechtelijk en komen buitenechtelijke jongen voor in 44% van de nesten.

De beschikbaarheid van sperma in de pimpelmees

Om seksuele selectie na de copulatie te begrijpen is het van belang om basisinformatie te hebben over het copulatie gedrag van de pimpelmees, zoals de timing van copulaties in relatie tot de vruchtbare periode van de vrouw. Daarnaast is het interessant om te weten hoeveel spermacellen pimplemeesvrouwtjes beschikbaar

hebben. **Hoofdstuk 2** beschrijft een methode om te bepalen of eieren die niet uitkomen bevrucht zijn of om een andere reden (bijv. incompatibiliteit tussen mannelijk- en vrouwelijk genotype) niet levensvatbaar zijn. In **hoofdstuk 3** pas ik een deel van deze methodologie toe om te bepalen of pimpelmeesvrouwen genoeg sperma beschikbaar hebben voor het bevruchten van hun volledige legsel. De pimpelmees heeft een bijzonder groot legsel vergeleken bij andere zangvogel soorten. Omdat bekend is dat in zangvogels copulaties vaak ophouden of sterk verminderen in frequentie na het begin van de eileg, zou het grote legsel van de pimpelmees ertoe kunnen leiden dat de laatst gelegde eieren onbevrucht blijven. Of het paargedrag van pimpelmezen ook afneemt na aanvang van de eileg is onduidelijk, omdat het moeilijk is om copulaties te observeren in wilde vogels. Om te onderzoeken of de laatste eieren in pimpelmees legsels vaker onbevrucht blijven, werden pas gelegde eieren van in het wild broedende pimpelmezen verzameld. Deze eieren werden vervolgens opengebroken, zodat de spermacellen op de vliezen rond de eidooier gevisualiseerd en geteld konden worden. Op die vliezen vindt men de spermacellen die aanwezig waren op de plaats van bevruchting, maar de eicel niet zijn binnengedrongen. Het aantal spermacellen op de vliezen geeft een indicatie van het aantal spermacellen dat het pimpelmeesvrouwtje in haar voortplantingssysteem had. We vonden geen bewijs voor de theorie dat de laatste eieren in het broedsel vaker onbevrucht waren en eieren uit een groter legsel hadden niet meer kans om onbevrucht te zijn. Er was ook geen relatie tussen het aantal bevruchte eieren in een nest en het aantal spermacellen dat het vrouwtje beschikbaar had. Daarnaast nam het aantal spermacellen niet af tussen het tweede en het tiende ei van hetzelfde legsel. Het lijkt er daarom op dat pimpelmezen, in tegenstelling tot andere zangvogel soorten, doorgaan met copuleren tot het leggen van het laatste ei.

Om meer te weten te komen over seksuele selectie na copulatie bestudeer ik in **hoofdstuk 4** de mannelijke kenmerken die de sperma-aantallen in het vrouwelijke de SOO beïnvloeden. Het zou kunnen dat processen na de copulatie de sperma-aantallen in de SOO beïnvloeden en dat dit samenhangt met mannelijke kenmerken, bijvoorbeeld doordat vrouwtjes meer sperma opnemen van aantrekkelijke mannen. Onze resultaten laten zien dat sperma-aantallen enkel samenhangen met het lichaamsformaat van de sociale partner van de vrouw; een grotere sociale partner betekent meer sperma. Voor dit verband zijn verschillende verklaringen mogelijk. Een mogelijke verklaring is dat grotere mannen simpelweg meer sperma produceren en hun vrouwtjes daarom meer sperma hebben opgeslagen. Een alternatieve verklaring zou kunnen zijn dat vrouwtjes grotere mannen zien als mannen met goede genen en daarom veel van hun sperma opslaan. Omdat we geen informatie hebben over paargedrag en de aantallen spermacellen die verschillende mannen hebben geïnsimineerd kunnen wij die verklaringen helaas niet van elkaar onderscheiden. Omdat sperma-aantallen samen blijken te hangen met een mannelijk kenmerk, concluderen we dat sperma-aantallen onder andere een reflectie zijn van het copulatie gedrag en/of effecten na de copulatie en niet enkel van de vrouwelijke opslag capaciteit.

Het MHC van de pimpelmees

In **hoofdstuk 5** wordt de genetische structuur van MHC-I in de pimpelmees beschreven. In bloedmonsters van 20 pimpelmezen afkomstig uit Spanje, Zweden en Nederland werden 17 verschillende MHC-I allelen gevonden. De gevonden allelen bleken afkomstig van 4 loci, waardoor het aantal allelen per individu varieerde tussen 2 en 7. Uit onze resultaten blijkt dat MHC-I in de pimpelmees bestaat uit twee groepen allelen die behoorlijk van elkaar verschillen in de genetische code. Daarnaast bestaat één van de groepen uit allelen die in genetische code veel meer van elkaar verschillen dan de allelen uit de andere groep. Dit zou erop kunnen duiden dat de twee groepen MHC-I genen onafhankelijk van elkaar overerven.

Het beschrijven van de MHC-I genen was de eerste stap naar het ontwikkelen van een methode om snel een beeld te krijgen van de MHC-I allelen die een individu bezit. Het beschrijven van de genetische code van een bepaald gen (het zogenaamde sequencen) is namelijk veel werk en is erg kostbaar. Daarom was het wenselijk om een methode te ontwikkelen waarmee verschillende alellen op een snellere en goedkopere manier herkend worden. **Hoofdstuk 6** beschrijft de ontwikkeling van deze methode. We hebben daarvoor Reference Strand mediated Conformation Analysis (RSCA) gebruikt. Tijdens RSCA werden onze pimpelmees allelen gehybridizeerd aan een specifiek koolmees allel (afkomstig van hetzelfde gen en daardoor sterk gelijkend op, maar nooit identiek aan de pimpelmees allelen). Dit koolmees allel wordt een FLR genoemd. Doordat de pimpelmees allelen in verschillende mate in genetische code overeenkomen met de FLR vormt het product van de hybridizatie een molecuul waarvan de vorm verschillend is voor elk pimpelmees allel. Door deze moleculen vervolgens met behulp van elektroforese door een gel te laten migreren in een capillaire sequencer en de FLR een fluorescent label te geven, kan bepaald worden welke allelen in het DNA van elk individu aanwezig waren. Onze methode gebruikt twee van deze FLRs om alle allelen te kunnen onderscheiden. RSCA was al eerder toegepast voor de MHC-genen van vogels uit de orde *Galliformes* (kippen, kalkoenen, fazanten, hoenders etc.) maar nog nooit voor zangvogels. Omdat het MHC van zangvogels in het algemeen veel ingewikkelder is dan dat van vogels uit de orde *Galliformes* is ook het toepassen van RSCA in zangvogels ingewikkelder. Uit onze resultaten blijkt desondanks dat RSCA ook in zangvogels succesvol kan worden toegepast.

In **hoofdstuk 7** onderzocht ik of de MHC-I genen een rol spelen in de keuze van een sociale- en buitenechtelijke partner in de pimpelmees. In pimpelmezen blijken de aantallen MHC-I allelen van de vrouw positief te correleren met de aantallen MHC-I allelen van haar sociale partner. Er was geen bewijs dat pimpelmeesvrouwen meer geneigd waren om een buitenechtelijke partner te zoeken als de sociale partner minder diverse MHC-I genen had of meer overeenkwam met haar eigen genen. De gevonden correlatie zou verklaard kunnen worden door een wederzijdse voorkeur (door zowel man als vrouw) voor een partner met diverse MHC-I genen.

Conclusie

De resultaten van mijn onderzoek tonen aan dat het uitkomstsucces van pimpelmeeseieren onafhankelijk is van het aantal spermacellen dat de vrouw in haar SOO heeft. Het is mogelijk dat sperma-aantallen wel een effect hebben, maar dat alle vrouwen in onze studie ruim voldoende spermacellen beschikbaar hadden. Uit het feit dat sperma-aantallen niet verschillen tussen tweede en tiende eieren van hetzelfde legsel concluderen wij dat pimpelmezen gedurende de eileg doorgaan met copuleren. Het aantal spermacellen in de SOO blijkt niet alleen af te hangen van de sperma-opslagcapaciteit van de vrouw, maar ook een reflectie te zijn van het copulatiegedrag en/of seksuele selectie na copulatie. Vrouwen gepaard met een grotere man hebben meer sperma in opslag. Mijn onderzoek heeft daarmee een eerste stap gezet richting het ontrafelen van de factoren die sperma-aantallen beïnvloeden.

Daarnaast geeft mijn proefschrift de eerste beschrijving van de MHC-I genen van de pimpelmees. Op basis van deze beschrijving is het gelukt om een methode te ontwikkelen om op een gemakkelijke en snelle manier te bepalen welke MHC-I allelen een individu bezit. Daarmee waren wij ook de eersten die de toepassing van RSCA in zangvogels beschreven. Het aantal MHC-I allelen van pimpelmeesvrouwtjes bleek positief te correleren met het aantal MHC-I allelen van hun sociale partner. Dit verband zou veroorzaakt kunnen worden door wederzijdse partnerkeuze voor een maximaal aantal allelen. Onze bevindingen zijn dus in overeenstemming met de theorie dat partnerkeuze voor goede of compatibele genen plaatsvindt in de pimpelmees.

Verschillende studies tonen aan dat partnerkeuze op basis van de MHC-genen plaatsvindt in zangvogels. Een belangrijke voorwaarde voor partnerkeuze op basis van het MHC is dat vogels kunnen herkennen welke MHC-allelen een potentiële partner bezit. Tot nu toe is echter onduidelijk hoe vogels MHC-allelen herkennen. Het is bekend dat geurperceptie een belangrijke rol speelt in MHC-herkenning in zoogdieren. Voor lange tijd werd echter aangenomen dat vogels niet konden ruiken. Het tegendeel blijkt waar te zijn. Hopelijk zal toekomstig onderzoek uitwijzen of vogels hun reukzin gebruiken voor het herkennen van de MHC-allelen van potentiële partners. Het zou daarnaast interessant zijn om de twee doelen van mijn project bij elkaar te brengen; ook om duidelijk te maken of de MHC-genen een rol spelen in de seksuele selectie na copulatie in vogels vertrouw ik op toekomstige onderzoeksresultaten.

Addresses of co-authors

Tim. R. Birkhead and Nicola Hemmings
Department of Animal and Plant Sciences, The University of Sheffield,
Sheffield S10 2TN, UK

Lyanne Brouwer
Evolution, Ecology and Genetics, Research School of Biology, The Australian National University, Canberra, ACT 0200, Australia

Joanna Hall
School of Experimental Psychology, University of Bristol, 12a, Priory Road
Bristol BS8 1TU, United Kingdom

Jan Komdeur
Behavioural Ecology and Self-organization, Centre for Ecological and Evolutionary Studies, University of Groningen, PO Box 11103, 9700 CC Groningen, The Netherlands

Michael J. L. Magrath
Department of Wildlife Conservation and Science, Zoos Victoria, PO Box 74,
Victoria 3052, Australia

Kees van Oers
Netherlands Institute of Ecology, P.O. Box 50, 6700 AB Wageningen, The Netherlands

Juan Rivero-de Aguilar and Santiago Merino
Departamento de Ecología Evolutiva, Museo Nacional de Ciencias Naturales (CSIC),
C/José Gutiérrez Abascal 2, Madrid, Spain.

Oscar Vedder
Edward Grey Institute, Department of Zoology, University of Oxford,
South Parks Road, Oxford, OX1 3PS, UK

Helena Westerdahl
Molecular Ecology and Evolution Lab, Department of Biology, Lund University,
SE-223 62 Lund, Sweden