Female choice and male humoral immune response in the lekking great snipe (*Gallinago media*)

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Parasites and diseases constitute major evolutionary forces in many natural populations, and thus having an efficient immune defense to resist infections is crucial for many organisms. Properties of the immune response may also influence mate choice decisions in many animals. Theory predicts several advantages for females when choosing males with superior immune systems. These benefits can be both direct (e.g. increased paternal care and reduced disease transmission) and indirect (good genes). We have investigated female choice with respect to antibody response to two novel antigens in males of a lekking bird, the great snipe (*Gallinago media*). Because of the lek mating system, female choice probably mainly incurs indirect (genetic) rather than direct benefits. Males responded to vaccination with diphtheria and tetanus toxoids by producing specific antibodies to both antigens. Triggering the immune system had no negative impact on display activities or survival. Males that were chosen by females as mates had on average higher antibody response to the tetanus antigen than their neighbors. We did not, however, find any covariance between the strength of the antibody response and male mating success. *Key words:* bird, ELISA, humoral immunocompetence, lek, mate choice. *[Behav Ecol 16:346–351 (2005)]*

L ekking species are well suited as models for studying mate choice and sexual selection. Because females in these species do not gain any material benefits from choosing a certain male, and mating success often varies substantially between males, lek systems have long been regarded as promising for studying the genetic benefits of mate choice (Andersson, 1994; Höglund and Alatalo, 1995). Although females in lekking species appear to be choosy about whom they mate with (Höglund and Alatalo, 1995; Sæther et al., 1999), and some studies have found the males that are preferentially chosen survive better (Alatalo et al., 1991; Petrie, 1992), it is still not clear exactly which benefits females accrue by exerting mate choice.

It has been suggested that the ability to mount an immune response is important in mate choice (Folstad and Karter, 1992; Hamilton and Zuk, 1982; Møller et al., 1999). By choosing males with superior immune response, females could obtain both direct and indirect benefits. Direct benefits include avoidance of sexually transmitted diseases (Sheldon, 1993), access to better territories, and more parental care (Møller et al., 1999). On the other hand, females accrue indirect benefits if there is an increased possibility that the offspring will inherit genes coding for a high quality immune system (Møller and Alatalo, 1999; Saino et al., 1997; Westneat and Birkhead, 1998).

Vertebrates are exceptionally vulnerable to parasites because of their relatively long life span and highly regulated internal environment, and they have developed a complex set of immune reactions to deal with this threat (Wakelin, 1997). Several studies have shown that the ability to mount a strong immune response is dependent on an individuals' condition (Lochmiller and Deerenberg, 2000; Norris and Evans, 2000). This condition dependence may, for example, be manifested by a relation to ornamentation (Møller and Petrie, 2002), amount of reproductive effort (Moreno et al., 1999; Nordling et al., 1998), work load (Hasselquist et al., 2001), cold stress (Svensson et al., 1998), or amount of body fat (Demas et al., 2003). The condition dependence of the immune response may be most pronounced during the breeding season, when most individuals work hard and the immune system is suppressed (see Lozano and Lank, 2003; Nelson and Demas, 1996). However, individuals in prime condition may still be able to mount a strong immune response (Møller et al., 2003; Peters, 2000).

An indirect way of studying the immune response is to look at the amount of parasites in an individual, as high immune responses in theory would lead to lower amount of parasites. Many studies have found that females prefer less parasitized males in birds (Borgia and Collis, 1989; Buchholz, 1995; Hillgarth, 1990; Höglund et al., 1992a; Wiehn et al., 1997; Zuk et al., 1990), and fish (Houde and Torio, 1992; Milinski and Bakker, 1990). In general, these studies show a negative correlation between parasite load and male ornamentation. The latter is presumably used as the cue for female choice. Parasite prevalence may, however, be a crude measure of immunocompetence, because it is potentially affected by many things other than the immune response (Sheldon and

Verhulst, 1996). To avoid this problem, it is preferable to use more direct methods, such as an immune challenge test, to measure the strength of the immune response (Deerenberg et al., 1997; Hasselquist et al., 1999; Møller et al., 1999). Such studies have found positive relationships (Duffy and Ball, 2002; Garamszegi et al., 2003; Lindström and Lundström, 2000; Saino et al., 1997), negative relationships (Møller et al., 1996), or no relationships (Westneat et al., 2003) between the strength of immune response and mate choice or ornamentation.

The aim of the present study was to test if humoral immune response (level of antibody production to novel antigens) was related to mate choice in the great snipe. We measured humoral immune response by using an immune challenge test in the beginning of the mating season, when males perform extremely energetically costly display behavior (Höglund et al., 1992b). Because of the lek mating system of great snipe, in which the main benefits to females are most likely indirect (Sæther et al., 1999), this species is well suited for studying female mate preferences without having to take into consideration most of the usual direct benefits that might arise from mate choice. Furthermore, females are very choosy about whom they mate with, and male competition does not play a major role in the distribution of matings among territorial males (Sæther et al., 1999). In comparison to other lekking animals, the great snipe show very little sexual dimorphism, and males are in fact slightly smaller than are females (Höglund et al., 1990).

METHODS

Fieldwork

Fieldwork took place in Gåvålia near Kongsvoll (62°17′ N, 9°36′ E), Dovrefjell, in the Norwegian mountains during the early summer of 2001–2002 (data from 2003 on return rate of immunized and nonimmunized birds is also used). During nights, from mid May–early July, males gather at traditional lek sites to display and defend small territories that contain no obvious resources. The leks are situated in wet areas on mountain slopes just above the tree line (for a general description of the field site, see Løfaldli et al., 1992). Birds were caught in mist nets on the leks; blood samples were taken; and the birds were individually ringed with a metal ring and three plastic colored rings for identification. For a general introduction to the field methods, see Fiske and Kålås (1995). We recorded the behavior of the individually marked birds by observing the two largest leks in the study area from two or three elevated platforms at each lek when the birds were active. The male display in great snipes consists of a vocalization performed on the ground in an erect posture and of occasional jumps (up to 1 m high; Lemnell, 1978). In 2001, the display activity of males was monitored. The number of vocal displays and jumps during 2-min intervals was recorded for each male during the most intensive display period (23.45–02.15 h). The mean number of displays during two to 11 such intervals was used in further analyses. A female was considered to have chosen a partner if she was seen copulating with or soliciting to a male. If a female is seen soliciting to a particular male but not copulating, this means that she is very likely to mate with or has already mated with him on previous nights (Sæther et al., 2001). Male mating success was measured as the minimum number of individual females soliciting to a male (see Sæther et al., 2000). A neighbor to a chosen male was defined as a male with a territory that had a boundary to the territory of the chosen male on the night of copulation/solicitation.

Immunization

In the beginning of each mating season, males ($n = 59$ in 2001 and $n = 41$ in 2002) were caught and injected with 100 µl of diphtheria and tetanus vaccine (2001, SBL Vaccin Sweden; 2002, Statens serum institut, Denmark) in the pectoral muscle. We also collected a preinjection blood sample from each male. There is no indication that injection with this vaccine causes a sickness response such as a fever in birds (Hasselquist D, unpublished data). However, in some passerine birds injection with these antigens causes a limited reduction in feeding rates (Ilmonen et al., 2000; Råberg et al., 2000). After 11 to 15 nights, i.e., around the time of the peak of the primary antibody response (see Hasselquist et al., 1999), 51 of the immunized birds were recaught and a new blood sample was taken. All but six of these males were recaught after 12 nights; excluding these six males from the analyses did not affect the conclusions but lowered the statistical significances of the results. They were therefore kept in the analyses. The collected blood was mixed with a small drop of heparin to avoid clotting and was kept cool until later the same night when it was centrifuged at 3000 rpm for 7min. The clear plasma on the top was collected and kept at −20°C until further analysis.

Humoral immune response

We assessed humoral immune responses by measuring specific antibody production against diphtheria and tetanus toxoid by using an enzyme-linked immunosorbent assay, ELISA, according to the methods described by Hasselquist et al. (1999; 2001). Ninety-six–well ELISA plates coated with diphtheria or tetanus antigen were incubated overnight. Plates were then blocked by using a dilution of 3% milk powder in 0.01 M phosphate buffered saline (PBS, pH 7.2). A diluent of 1% milk powder in 0.01 M PBS was used to produce 1:400 and 1:1200 dilutions of each serum sample. After incubation and washing (in PBS and Tween 20), we added a secondary rabbit anti–red-winged blackbird immunoglobulin antiserum diluted 1:1000 in 1% milk powder in 0.01 M PBS. After a second incubation and wash, a commercial peroxidase-labeled goat anti-rabbit antiserum (Kirkegaard and Perry) diluted 1:2000 in 1% milk powder in 0.01 M PBS was added. After incubation and washing, the peroxidase substrate (2,2-azino-bis-3-ethylbenzthiazoline-6-sulfonic acid, ABTS) and peroxide were added, and the plates were immediately transferred to a Vmax (Molecular Dynamics) kinetics ELISA reader. Plates were read at 30-s intervals for 14 min by using a 405-nm wavelength filter. All antibody concentrations are given as the slope of the substrate conversion over time, measured in the unit $10^{-3} \times$ optical density per minute (mOD/min; analyzed using KineticCalc software), with a higher slope indicating a higher concentration of antidiphtheria or antitetanus antibodies in the sample. For each dilution and individual, serum samples were added to the ELISA plates in duplicate, and the average of these was our measure of antibody titer for each individual.

Statistical methods

Antibody response was defined as antibody titer in the primary response sample minus antibody titer in the preinjection sample. The levels of antibodies in the control samples differed between the 2 years, so the responses in 2002 were corrected for these differences by multiplying the values with the ratio of the 2001 and 2002 mean control values. Because the distribution of antibody titer values differed from a normal distribution, nonparametric statistics were used for
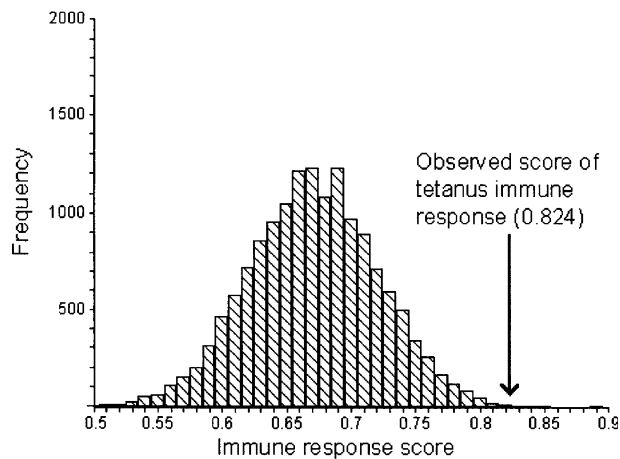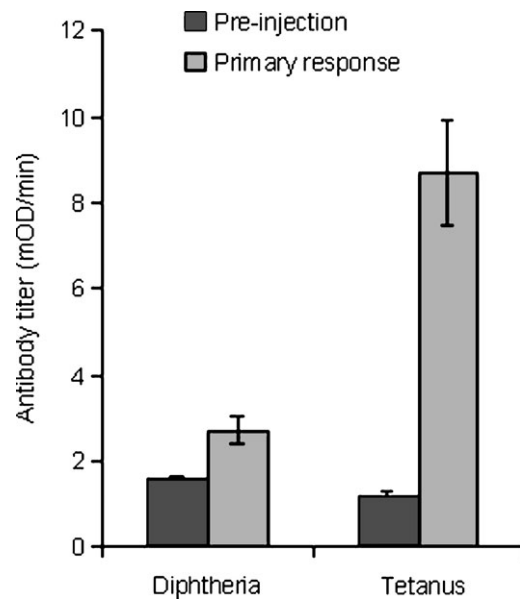
Figure 1
The frequency distribution of female choice of male humoral immune response scores from 15,000 simulations of the null model. Higher scores indicate that the chosen male of an individual female had higher humoral immune responses than did nonchosen neighbors. The arrow shows the mean of observed female choice scores for male tetanus response.

Figure 2
Antibody titer (mOD/min) measured before antigen injection (control) and 12 days after injection ($n = 51$). Bars represent standard errors.

all analyses, that included these variables. Statistical analyses were performed by using SPSS 11.5, ResamplingStats 4.0.7 and GenStat 4.2. All significance levels are two-tailed, and the critical p value for significance was 0.05.

We made two kinds of analyses relating immune response to mating success. First, we applied a sexual selection approach to look for a relationship between immune response and mating success. We analyzed variation in mating success by building a generalized linear model assuming a poisson error distribution and a logarithmic link function, fitting diphtheria response and tetanus response (as covariates), and fitting lek and year (as factors). Second, we used an individual female mate choice approach, calculating an immune response score for each female's choice of partner as follows: the chosen male and all his neighbors for which we had immune response data were ranked in ascending order according to their immune response. The rank for the chosen male was then divided by the total number of ranked males for the focal female to obtain an immune response score between 0 and 1. We have used this rank-based transformation because of the nonnormal properties of the antibody response data. To use the neighbors of the chosen male as a control group may have at least two advantages. First, these males may be a representative sample of nonchosen males in the population. Second, females are likely to have seen and compared these males with the chosen male. To calculate the underlying distribution and confidence intervals for the mean immune response score for all the choosing females, we used a null model obtained by randomization. Here we distributed the same females randomly between the same males and then calculated the mean immune response score in the same way as described above. We ran the simulation 15,000 times and compared the distribution of the mean immune response scores to the observed value (Figure 1). Because the choice of each female is used as an independent data point, some males appear more than once in the analysis. More specifically, out of 20 chosen males, four were chosen by two different females, one by three females, and one by four females. Also, some males appear in the analyses both as chosen male and as a neighbor to another male chosen by a different female. Each female appears only once in the analysis. We argue that this is a correct way of analyzing the data because it has been

shown that each female makes her own choice rather than copying the choice of other females (Fiske et al., 1996). There remains a possible problem with the independence of the different data points if some males are preferred by many females because of something that is not related to the immune response. However, the mating success is not heavily skewed in the present study, and a majority of the males were only chosen by a single female.

To see if the immunization had any adverse effects on the birds, we compared the display behavior between vaccinated and nonvaccinated males. We also compared return rates for the next breeding season between vaccinated males and males that were not vaccinated but were displaying on the leks at the time of vaccination.

RESULTS

Immune response

Birds responded to immunization by producing specific antibodies to both diphtheria (Wilcoxon signed-rank test comparing pre- and postinjection titers, $Z = -4.3$, $n = 51$, $p < .001$) (Figure 2) and tetanus (Wilcoxon signed-rank test comparing pre- and postinjection titers, $Z = -6.2$, $n = 51$, $p < .001$) (Figure 2). There was a significant correlation between the responses to the two antigens (Spearman's $\rho = 0.40$, $p = .003$), but the response was much stronger for tetanus than for diphtheria (Wilcoxon signed-rank test, $Z = -5.3$, $n = 51$, $p < .001$) (Figure 2). There was no difference in response between the 2 years (Mann-Whitney test, tetanus: $U = 291$, $n = 51$, $p = .91$; diphtheria: $U = 232$, $n = 51$, $p = .20$).

Effects of immunization

We observed no differences in display behavior between immunized individuals ($n = 20$) and nonimmunized individuals ($n = 25$). This was true both for the number of vocal displays ($t_{43} = 0.029$, $p = .98$) (Figure 3a) and the number of jumps ($t_{43} = 0.86$, $p = .39$) (Figure 3b). Return rates of birds to the breeding area the year after immunization (a proxy for
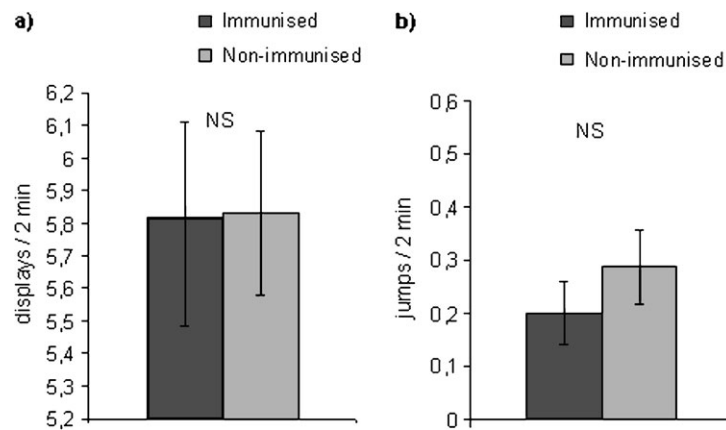
Figure 3
The effect of immunization on display frequency, measured as the mean number of displays (a) or jumps (b) during 2-min periods. Number of immunized males is 20; number of nonimmunized males is 25. Bars represent standard errors.

survival) did not differ between immunized (41.0 %, $n = 100$) and nonimmunized (44.8 %, $n = 143$) males ($G_1 = 0.34$, $p = .56$). Furthermore, the antibody response did not differ between returning and nonreturning males (Mann-Whitney test, tetanus: $U = 283$, $n = 51$, $p = .43$; diphtheria: $U = 319$, $n = 51$, $p = .92$).

Immune response and mating success

We could not detect any covariance between mating success and either diphtheria or tetanus responses (Table 1). However, when we analyzed each female's choice, we found that the chosen males had a stronger tetanus response than did their neighbors. Twenty-nine females (17 in 2001 and 12 in 2002) were observed copulating with, or soliciting to, males for which humoral immune response data from that year were available ($n = 20$ males). A total number of 35 males (chosen males and their neighbors) were included in this analysis. The mean observed tetanus immune response score (0.82) of males chosen by females (see Methods) was higher than expected by chance ($p = .001$, average of 15000 simulations = 0.67, 95% confidence interval: 0.57–0.77) (Figure 1). No such effect was found when analyzing the diphtheria response ($p = .39$, observed score = 0.63). All the above results were based on data combined for the two study years. Analyzing each year separately showed that the results for tetanus were similar in both years (2001, $p = .054$; 2002, $p = .012$).

DISCUSSION

The present study indicates that male humoral immune responses may have significance for female mate choice in the great snipe. In this lekking species, we found that males chosen as mates have a higher humoral immune response against a foreign antigen (tetanus) than do their neighbors. The results of this analysis should, however, be interpreted with care because of the possible problem with pseudo-replication (see Methods). For diphtheria, there was no significant relationship with mate choice. The antibody response of diphtheria and tetanus were correlated, but the response against diphtheria was of a much smaller magnitude than was the response against tetanus. Similar response patterns have been found previously by using these antigens (Westneat et al., 2003). Because the response was so small and the measurement error at low response values high, random effects had a considerably greater influence on the diphtheria response and may have masked possible relationships for this antigen. However, antibody responses to these two antigens have been found to differ also in other bird species. In blue tits (*Parus caeruleus*), primary antibody responses were found to be under stabilizing selection for diphtheria and no clear selection pattern for tetanus, whereas secondary antibody responses were under positive directional selection for tetanus and no clear selection pattern for diphtheria (Råberg and Stjernman, 2003). Thus, we are not surprised to find that patterns of (sexual) selection differed between these two antigens also in the great snipe.

We did not, however, find that males with higher mating success have a higher immune response. Such an effect may have been asked by females using more than one single trait when choosing mates in this species, or by females sampling a limited number of males in their mate choice (Benton and Evans, 1998; Fiske and Kålås, 1995; Sæther et al., 2000). In addition, given the rather low sample size of this analysis, we may have a low chance of detecting small or even medium effects.

The strong correlation between diphtheria and tetanus responses within a male indicates that the responses measured here may represent some general ability to produce antibodies against a novel antigen in these birds (Svensson et al., 1998). That said, we have still only measured a small part of the complex set of immune reactions that determine how well an individual is able to protect itself against parasites and diseases. Recent studies have investigated the relation between the level of response to an immune challenge and fitness components such as survival, and there is generally a rather strong positive correlation between these traits (for review, see Møller and Saino, 2004). It has also been pointed out that there may be serious costs involved in mounting a strong immune response, so the highest level of immune

Table 1
Generalized linear model of great snipe male mating success on two leks in relation to immune response

Term	Estimate (SE)	p
Constant	−0.425 (0.349)	.22
Tetanus response	−0.119 (0.026)	.65
Diphtheria response	0.026 (0.056)	.65
Lek	0.793 (0.380)	.04

Year (2001 or 2002) did not have any significant effect and was removed from the final model. Deviance ratio for model fit = 1.74, approximate $p = .16$ with df = 3,35.

response may not always be optimal (Råberg et al., 1998; Westneat and Birkhead, 1998; Zuk and Stoehr, 2002).

There may be both direct and indirect benefits for a female in choosing a male with high humoral immune response. Because we have studied a lekking species with no paternal care, most of the direct benefits, such as access to food or good nesting territories, can be ruled out. Instead, we suggest that the main benefits to female great snipe are indirect (Sæther et al., 1999). By choosing a male with an efficient humoral immune system, the offspring may inherit this capacity and thereby gain increased survival and reproductive success, which ultimately enhances female fitness. Råberg et al. (2003) showed that there is a strong heritable component of antibody response to tetanus in vaccinated blue tits. Note, however, that there is one direct benefit, decreased disease transmission during copulation (Sheldon, 1993), which cannot be ruled out in this case.

Females are not likely to be able to assess male immune response directly, and future studies need to address what indirect cues females potentially use to find high response males. Immunocompetence often seems to be affected by the general condition of the individual, something that in turn may be reflected in the size, ornamentation, or territory quality of the male (Blount et al., 2003; Lochmiller and Deerenberg, 2000; Møller and Petrie, 2002; Norris and Evans, 2000, but see Ohlsson et al., 2002; 2003). We suggest that female great snipe may use some general condition or health character when making their choice of mate. It is difficult to study the exact nature of such cues, but behavioral traits such as level of activity may be good candidates (Fiske et al., 1994; Hoglund and Lundberg, 1987). The antibody response is also to some extent linked to genetically determined parts of the immune system, for example, the major histocompatibility complex (MHC). MHC genes are highly polymorphic (Hedrick, 1994), also in the great snipe (Ekblom et al., 2003), and have been shown to influence mate choice and odor preference in mammals (for review, see Penn and Potts, 1999), lizards (Olsson et al., 2003), and birds (Freeman-Gallant et al., 2003). von Schantz et al. (1996) found that pheasant males with certain MHC genotypes have longer spurs (a sexually selected character; von Schantz et al., 1989) and higher survival and reproductive success. Recent studies of pheasants have found positive correlations between spur length and both cell-mediated and humoral immunity (Ohlsson et al., 2002; 2003).

Another possibility is that females assess male immunocompetence in a more direct manner, for example, on the basis of parasite prevalence. It has been shown that females sometimes avoid the smell (Kavaliers and Colwell, 1993) and appearance of parasitized males (Milinski and Bakker, 1990). There are no published accounts on parasites in great snipe, but we know that ectoparasites are rare. In future studies it would be interesting to investigate the functional link between mate choice and immune response by means of manipulative experiments.

We found no negative effects of vaccination in terms of individuals' display rate (an energetically costly signal; Höglund et al., 1992b) or survival. This is in agreement with a study on replacement egg laying in starlings (Williams et al., 1999) but in contrast to several other studies on birds that have found negative effects of nonpathogenic antigen injections on parental effort, reproductive success or survival (Andersson, 2001; Hanssen et al., 2004; Ilmonen et al., 2000; Råberg et al., 2000). This indicates that effects of a nonpathogenic immune system activation differs depending on which traits and species that are investigated. The reasons for these differences still remain obscure, and the costs of immune system activation definitely deserve further attention.

We conclude that the strength of the male humoral immune response may affect female mate choice in lekking great snipe and suggest that females thus may be able to enhance the immunocompetence of their offspring. Still, however, experimental studies are needed to draw conclusions about the exact nature of this relationship.

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