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ORIGINAL ARTICLE

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MHC, health, color, and reproductive success in sand lizards

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Abstract "Good genes" are genetic elements that contribute to lifetime reproductive success, regardless of an individual's additional genotype. Their existence is debated, and most work has targeted their viability benefits to the offspring of choosy females. In the present study, we analyze a case of potential good genes effects in adult male sand lizards (*Lacerta agilis*). We show that males with a particular RFLP (Restriction Fragment Length Polymorphism) MHC genotype (O-males), as opposed to those that lack this genetic element (NO-males), have less ectoparasites under increasing physiological stress (indexed by baseline corticosterone level), and are not constrained by parasites at production of status coloration. Furthermore, O-males are more successful at mate acquisition and guard their partners longer. Ultimately, they have a higher genetic reproductive success as assigned by microsatellites.

Keywords MHC · "Good genes" · Reproductive success · *Lacerta agilis*

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Introduction

In the last decade, the Major Histocompatibility Complex (MHC) genotype has become increasingly appreciated as an important determinant of a number of behavioral categories in vertebrate evolutionary ecology, such as aggregation patterns, social behavior, and mate choice (Penn and Potts 1998; Landry et al. 2001). One underlying reason for this is likely to be that selection favors the ability of individuals to detect genes that strongly influence the immunocompetence and risk of autoimmune disease in their offspring. Recent elegant work in the laboratory demonstrates such phenomena in sticklebacks (*Gasterosteus aculeatus*). Females adjust their mate preference in relation to their own genotypes, preferring more MHC polymorphic males when they are less polymorphic themselves and vice versa (Reusch et al. 2001). Complementary work demonstrates that down-regulating the number of MHC alleles via mate choice may lead to superior parasite defense compared to more polymorphic genotypes, probably due to the reduction of possible combinations of self-antigens and MHC proteins (Kurtz et al. 2004).

In natural populations, evolution of immune function has primarily been studied from a perspective of natural selection, sensu strictu, such as evolution of Major Histocompatibility Complex genes for HIV resistance in humans (Rowland-Jones et al. 1998). Lately, however, sexual selection resulting from female genetic mate choice has also received attention (Johnsen et al. 2000). Reports on such genetic effects on male health, vigor, and mate acquisition are, however, virtually non-existent. Here we present evidence of such effects in a natural Swedish population of the sand lizard (*Lacerta agilis*), a small (mass to 20 g), ground-dwelling, sexually dichromatic species.

Two to three weeks subsequent to emergence from hibernation (i.e., early May), males develop conspicuously bright-green color on their body sides (henceforth the "badge"), whereas females are grayish-brown. Manipulation experiments have revealed that less colorful males are more likely to be attacked first in staged contests, that more

similarly colored males take longer to resolve a conflict, and that more colorful males are more likely to win contests in a natural population and are more successful at mate acquisition (Olsson et al. 2000; Anderholm et al. 2004). Male sand lizards typically vary in the degree to which they engage in contests for females, with small "sneaky" males having delayed expression of nuptial colors, which makes them less conspicuous to overtly aggressive males. However, among-male differences in coloration and fighting tactics (e.g., aggression) could depend on several factors, e.g., status, fighting ability, health, and the degree to which an individual tolerates elevated levels of corticosterone, a hormone released in response to stress. In birds, corticosterone also modulates integumental processes such as skin shedding and molting (Bentley 1998). Hence, physiological stress level is potentially linked to the degree to which integumental colors are expressed and contribute to making status signals honest. To the best of our knowledge, there are as yet no published reports of similar phenomena in lizards.

If males using more aggressive strategies suffer from elevated physiological stress, associated costs may be high because immune function may be compromised by corticosterone. This could result in increased susceptibility to parasites (Folstad and Karter 1992; Penn and Potts 1999; Goldsby et al. 2002). We expect variation in such parasite immunocompetence among males if some genes for this trait are not fixed in the population. Support for this scenario comes from a closely related species, *L. vivipara*, for which we demonstrated strong family effects on health and survival under exposure to a pathogen (Uller et al. 2003). This finding supports the existence of a genetic link between tick-related pathology and male fitness. Thus, there is reason to suspect that male MHC genotype may influence tick resistance, in particular during the physiologically stressful period of intense male-male aggression, and hence may influence concomitant effects on a male's badge size. Since badge size is known to be a strong determinant of mate acquisition in male sand lizards (Olsson and Madsen 2001), we would also expect that males with an MHC genotype that renders them successful at combating parasites should also have higher success in mate acquisition, be better at mate defense, and have higher genetic reproductive success. We specifically test all these predictions in the current report.

Methods

Field and husbandry techniques

Males and females were captured upon emergence from hibernation (late April) at the Asketunnan field site, 50 km south of Gothenburg on the Swedish west coast. A 100-µl blood sample was collected with a capillary tube within 30 s of capture from the *sinus angularis* (in the corner of the mouth) and stored on ice until the end of the field day. Upon return to the laboratory that night, the blood samples were centrifuged at 5,000 rpm for 5 min, the plasma stored at −20°C until radioimmunoassay was conducted (see below), and the pellet dispersed in EDTA buffer for MHC RFLP (Restriction Fragment Length Polymorphism) and microsatellite analyses (see below). Subsequent to blood sampling, each lizard was weighed (to the nearest 0.001 g), measured snout to vent (to the nearest mm), and toe-clipped with a unique individual code for permanent identification. Ectoparasite ticks (*Ixodes ricinus*) were counted (all ticks attached to the lizard), square root transformed and standardized by genotype (setting mean to zero, and standard deviation to unity), which successfully normalized the data (Shapiro-Wilks' statistics, W, normal=0.98, P=0.66). Tick counts were then regressed on corticosterone to assess the difference in immunocompetence under stress in MHC categories of males. We then used the residuals from this regression to assess the degree to which stress-related immunocompetence influenced a male's ability to develop status coloration. A lateral photograph was taken in a standardized way using Ektachrome 200 ASA. Once the film was developed, the size of the lateral area with green pigmentation ("the badge"; Olsson and Madsen 2001) was estimated by taking the ratio between the green area and the total area of the side of the trunk. This ratio was left untransformed in the statistical analyses, since the trait conformed to normal distribution (Shapiro-Wilks' statistics, W, normal=1.0, P=0.4).

Temporary scars accumulate in males during the mating season as a result of male-male aggressive interactions. These are blue in color for ca. 2–3 days, then become black and disappear 3–4 weeks later (i.e., at the very end of the mating season) and can, hence, be temporally separated and counted. We used these as a proxy of male involvement in aggressive interactions.

After the morphology and parasite data had been collected, the lizards were released at the place of capture and monitored for associations with partners (facilitated by the prolonged mate guarding, Olsson and Madsen 2001) every day of the mating season when the weather permitted lizard activity (late April–mid-June). To assess the degree to which males with larger badges also suffered consequences in terms of more aggressive interactions, we looked for a correlation between male badge size and number of accumulated scars.

Once females showed egg contours on their body sides, they were brought to facilities at the Department of Zoology, University of Gothenburg. The females were housed separately in 50×50×60 cm cages with sand as bottom substrate, a 40-W spotlight at one end of the cage to allow thermoregulation to their preferred body temperature (ca. 36°C), and a moist patch under a flat rock to direct egg-laying and avoid desiccation of oviposited eggs. The cages were checked at least twice daily for eggs, which were immediately transferred to vermiculite mixed with water in a 7:1 volume relationship and incubated at 25°C until hatching (ca. 40 days).

Offspring (n=154) produced by 23 females from within the home ranges of MHC screened males were analyzed with respect to paternity using microsatellites (see protocol below). This represents 6.7 young per female, which is

on average 76% of the number of young produced per clutch [8.8±0.1 (SE)]. Thus, we scored both male success in mate acquisition (i.e., number of partners in relation to number of sightings) by observing pairs in the wild, and post-copulatory (genetic) reproductive success in our MHC screened males. Since larger males have larger testes and transfer more sperm, we controlled for this by using residuals from a regression between number of young sired and body mass.

Males had 11.6±3.3 SE, RFLP-fragments on average in their genotype. Exploratory data analysis revealed that one category of MHC genotype, presence or absence of RFLP fragment "O" (named in alphabetic order of occurrence on the autoradiogram), differed in several aspects, e.g., reproductive success. We therefore singled out this genotype for explicit tests of our predictions. Our comparison of O-males and NO-males (i.e., males lacking the O-band) in terms of within-season reproductive success should well represent their differences in life-time reproductive success, since we found no difference between genotypes in snout-vent length, which is highly correlated with age in this species (Olsson and Shine 1996). The lack of a size difference between MHC categories suggests that there is no difference between the two genotypes in age distribution or longevity that could bias our estimates of life-time reproductive success.

Restriction Fragment Length Polymorphism (RFLP)

RFLP of sand lizard MHC class I genes was analyzed using an MHC class I species-specific probe (Madsen et al. 2000). The probe was a cloned and sequenced PCR fragment (21.207) spanning 261 base pairs of the hypervariable exon 3 of a class I gene. Initially, three sand lizards were tested in a Southern blot analysis using five different restriction enzymes; Hind III, Pst I, Sac I, Taq I and Pvu II. All enzymes revealed polymorphism in combination with the MHC class I probe. However, Pvu II revealed the highest degree of polymorphism and was subsequently used in the RFLP analysis. Ten milligrams of genomic DNA was digested with 24 units of restriction enzyme for 3 h, and then run in a 0.8% agarose gel so that all fragments longer than 500 bp remained in the gel. Lambda DNA digested with Hind III was used as size markers. The DNA in the gel was transferred to a nylon membrane (Micron Separations, Westborough, Mass.) using a vacuum blotter model 785 (Bio-Rad, Hercules, Calif.), and the membranes were cross-linked in an XL-1500 UV cross-linker (Spectronics, Westbury, N.Y.). The membranes were prehybridized in a prehybridization solution (0.5 M Na_2HPO_4, 1% SDS) for 45 min at 62°C and then hybridized overnight at 62°C in a solution containing 0.2 M Na_2HPO_4, 1.0% SDS, 1.0% BSA, 6% PEG 6000 and the probe labelled with (α-^{32}P) dCTP (Amersham). The membranes were washed at 62°C for 15 min in preheated 2×SSPE, 20 min in 1×SSPE, and finally for 20 min in 0.5×SSPE. The washed membranes were exposed to X-ray film (Eastman Kodak, Rochester, N.Y.) in intensifying screens for 1–5 days at −80°C.

Microsatellite analysis

The sand lizards were genotyped using three polymorphic microsatellite DNA loci (La-3, Lv4-72, Lv4-x) described by Gullberg et al. (1997) and Boudejamadi et al. (1999). The PCR reactions were run in a 25-µl reaction mixture containing 25 ng total genomic DNA, 1U of AmpliTaq polymerase (Applied Biosystems), 1.0 mM $MgCl_2$, 0.125 mM of each nucleotide, 5 µl of Perkin-Elmer GeneAmp ×10 PCR buffer (100 mM Tris-HCl, 500 mM KCl and 0.01% gelatine) and 0.4 µM of each primer (forward primers were labelled with fluorescein, MWG Biotech). The reaction mixture was heated to 94°C for 3 min and then amplification was performed through 30 cycles at 94°C for 30 s, 54°C for 30 s and 72°C for 1 min. Following the 30 cycles, there was a final 10-min extension at 72°C. DNA from females and their offspring were run alongside, and in order to avoid contamination, negative controls were run with each set of reactions, using 1 µl of sterile Milli-Q water in place of the template (all other reagent concentrations remained the same). The gels were run for an hour, and scanned in a fluorimager (Molecular Dynamics, Sunnyvale, Calif.).

Corticosterone Radioimmunoassay (RIA)

Plasma corticosterone levels were measured with a radioimmunoassay developed by Silverin (1997), Silverin et al. (1999), and Cockrem and Silverin (2002). Plasma samples were extracted with dichlormethane and then assayed according to Wingfield et al. (1992). After equilibration with tritiated corticosterone (for recovery determination), plasma samples (10–20 µl) were diluted to 400 µl with distilled water. Samples were extracted overnight at 4°C with 4 ml redistilled dichlormethane. The following day, the dichlormethane phase was evaporated under nitrogen, and samples were reconstituted in 500 µl PBSG buffer. Samples were assayed in duplicate by a direct radioimmunoassay (Wingfield et al. 1992). Recoveries after extraction were 88–95%. All samples were sampled in single assay. In the assay, three blanks and four samples from a mallard (*Anas platyrhynchos*) plasma pool were included. All blanks showed nondetectable levels of corticosterone.

To ensure that our statistical analyses linking corticosterone levels to male coloration and reproductive tactics were not correlated effects of testosterone, a separate RIA was run to assay plasma levels of testosterone. This confirmed that there was no covariation between corticosterone and testosterone and, hence, that testosterone is not a confounding factor in our analysis [mean plasma level of testosterone, 10.45 ng ml^{-1}±0.75 (SE), Pearson's correlation coefficient, r=−0.07, P=0.73, n=28].

Results

Body mass was positively correlated with both the number of scars accumulated through male-male interactions

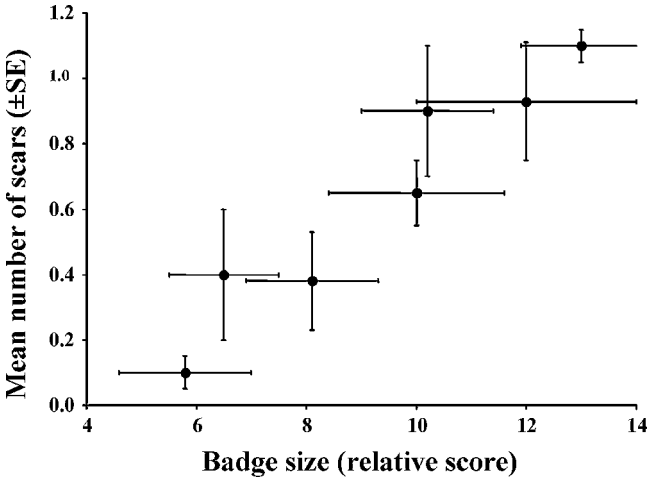

Fig. 1 The number of scars from male-male contests in samples of males with different badge size in size classes of mature males. For clarity of presentation, the sample is plotted in 2-mm SVL increments from 64 mm (approximate maturation).

(Fig. 1; r_s=0.16, P=0.0001, n=409), and with plasma levels of corticosterone, both at the beginning and end of the mating season (r=0.42, P=0.026, and r=0.47, P=0.012; n=26). Furthermore, baseline corticosterone level was significantly higher at the beginning of the mating season, i.e., during the time when badges develop, than at the end (pairwise t-test, t=−2.34, n=26, P=0.027).

MHC-dependent ability to withstand pathogen exposure at increasing physiological stress levels was evident from differing responses by O- and NO-males. In NO-males, tick load increased with corticosterone at the time of badge development (Fig. 2a). O-males, unexpectedly, showed a significant negative relationship between tick load and corticosterone level (Fig. 2b). The difference in slope coefficients depicting the tick load-corticosterone relationship was statistically significant between MHC categories (homogeneity of slopes test, genotype×corticosterone interaction, $F_{2,\ 25}$=4.08, P=0.029, R^2=0.25, β_{NO}=0.48±0.26, t=1.88, P=0.07, β_O=−0.51±0.24, t=−2.16, P=0.04). Consequently, O-males had a significantly lower corticosterone-dependent parasite load than NO-males (Fig. 2; homogeneity of slopes test, $F_{2,\ 25}$=4.08, P=0.029), with an average of 19.8 (±4.9, SE) accumulated ticks across males on average.

NO-males also showed a positive relationship between our estimate of immunocompetence and coloration (slope coefficient, β=0.77±0.24 SE, P=0.005), whereas no such effect could be identified for O-males (β=−0.12±0.22 SE, P=0.60), and with slopes being significantly heterogeneous ($F_{2,\ 20}$=7.23, P=0.014, our estimate of immunocompetence used as a covariate was also not significant, F=3.90, P=0.062).

O-males also differed from NO-males in behavior. Males with the O-fragment had significantly greater mate acquisition (observations with partners in relation to total number of observations, Kruskal Wallis test, with chi-square approximation; X^2=4.32, df=1, P=0.038). This estimate varied from an average of 0.08 (±0.11 SD, n=16) in NO-males to an average of 0.21 (±0.22 SE, n=17) in O-males (Fig. 3). O-males showed longer mate-guarding behaviors, lasting for 1.7 days, as opposed to 1.0 day on average for NO-

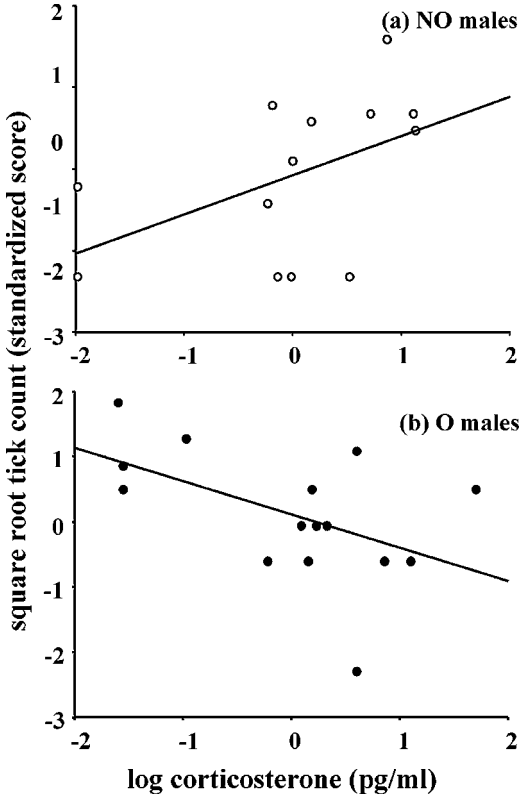

Fig. 2 Tick load at the time of badge development for **a** NO-males, and **b** O-males in relation to level of corticosterone in the plasma. Tick counts were square root transformed and standardized by genotype (setting mean to zero, and standard deviation to unity), which successfully normalized the data.

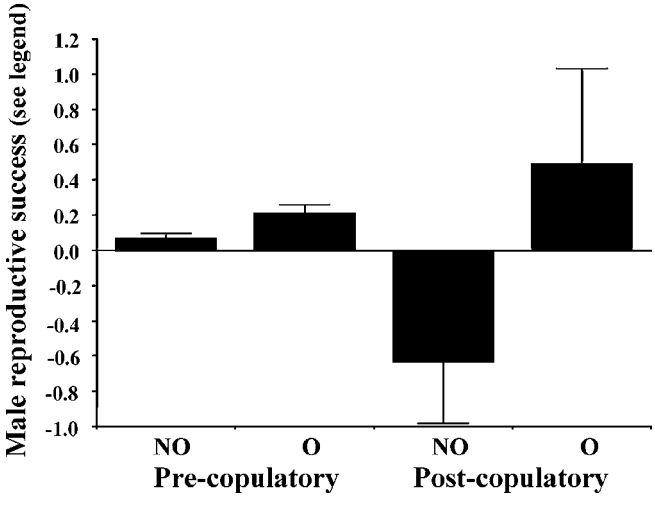

Fig. 3 Mate acquisition and genetic reproductive success were significantly higher in O-males than in NO-males. Mate acquisition was proxied by a male's number of pairings with a receptive female divided by his total number of observations. Male genetic reproductive success, assigned by microsatellites, was assessed in relation to male body size (since larger males are likely to transfer more spermatozoa).

males (Pitman's permutation test, $P=0.05$, $n_{NO-males}=5$, $n_{O-males}=8$). This predicts that O-males should also exhibit higher genetic reproductive success, while accounting for post-copulatory events such as cryptic female choice and sperm competition. This was also the case. More young were produced by O- than NO-males as revealed by microsatellite paternity analysis (Fig. 3; Kruskal Wallis test, with chi-square approximation, $X^2=3.88$, $df=1$, $P=0.049$). On average, NO-males sired 1.07 young ($\pm$1.2 SD, $n=15$), whereas O-males sired 1.71 ($\pm$2.1 SD, $n=17$), before body mass was taken into account in the analysis.

Discussion

NO-males showed less immunocompetence under elevated corticosterone level in the wild than did O-males. Their development of status coloration was affected by immunocapacity (as indicated by tick load in relation to physiological stress). In contrast, development of status coloration by O-males seemed to be unconstrained by immunocompetence. Furthermore, O-males: (i) showed better mate acquisition, (ii) longer mate guarding, and (iii) higher genetic reproductive success than did NO-males. Thus, our results paint a coherent picture with the presence of the O-band in a male's genotype being linked to male fitness-enhancing traits at multiple levels, from reduction in parasite load under increasing plasma corticosterone level, better performance in mate acquisition and mate defense, to higher genetic reproductive success.

Although we are aware that males may differ in corticosterone level for other reasons than stress in relation to male-male competition, e.g., starvation (Wikelski and Thom 2000), from a perspective of immunocompetence under elevated steroid level, it is irrelevant what caused these levels to vary and we therefore let baseline corticosterone index overall, multifactor-induced physiological stress.

How robust are our results with respect to genotype-dependent immune function? Analysis of Restriction Fragment Length Polymorphism is, simply put, a molecular technique in which parts of the genome, coded as well as non-coded, are excised with restriction enzymes and visually exposed on a gel. Using a species-specific MHC class I probe guarantees that the fragments observed are situated in this part of the genome, but not that the fragments contain functional genes. We do not know the sequence(s) and functions(s) of the gene(s) that link the O-fragment to, e.g., higher genetic reproductive success. However, it is highly probable that this genotype is directly or epistatically part of a genetic-phenotypic interaction that makes O-males better at combating ectoparasites, produce status coloration, acquiring and defending partners, and producing more young. This provides evidence strongly suggesting a genetic basis for parasite resistance and other factors affecting male fitness and, hence, "good genes" effects of this fragment in its classical sense (a genetic element with fitness-enhancing effects population-wide). This calls for an answer to the obvious question, "If all individuals in the population would benefit from having the O-fragment, why has this gene (or gene cluster) not gone to fixation"?

One potential explanation to this question is that multiple genes may have similar function and that the fitness loss from lacking the O-fragment is compensated by the presence of some other gene or gene combination. Possibly, such gene combinations are kept in control by selection against autoimmunity (as suggested by Råberg et al. 1998). Furthermore, our data strongly suggest that O-males show a more effective, or perhaps even qualitatively different, parasite resistance than do NO-males. This increasing level of corticosterone may perhaps even depress parasite load in O-males, which is not predicted from theory, and for which we currently have no explanation.

A second potential explanation to the lack of O-bands in some male genotypes may be that cost of mounting an immune response is sufficiently high to prevent males from evolving an MHC genotype that identifies a certain pathogen for destruction. To the best of our knowledge, no study has assessed such costs of immunity, although recent work clearly demonstrates survival penalties for mounting a full immune response in bumblebees (Moret and Schmid-Haempel 2000) and eider ducks (Hanssen et al. 2004).

A third potential explanation is that sand lizard pathogen resistance linked to the O-band is currently evolving, but is still lagging behind ongoing selection. Two decades ago, when the studies of sand lizards started in this population (1984), tick prevalence was much lower than today (less than one tick per lizard, M. Olsson, unpublished thesis). Today, the average gain in ticks over a few weeks early in the mating season is close to 30 (Olsson et al. 2000), and lizards with more than 100 ticks have been recorded. Thus, it appears that tick abundance has increased considerably over the course of the study period and, consequently, so has selection pressure for tick resistance.

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