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THE ROLE OF HALDANE'S RULE IN SEX ALLOCATION

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Abstract.—Sex allocation theory predicts that parents should bias their reproductive investments toward the offspring sex generating the greatest fitness return. When females are the heterogametic sex (e.g., ZW in butterflies, some lizards, and birds), production of daughters is associated with an increased risk of offspring inviability due to the expression of paternal, detrimental recessives on the Z chromosome. Thus, daughters should primarily be produced when mating with partners of high genetic quality. When female sand lizards (*Lacerta agilis*) mate with genetically superior males, exhibiting high MHC Class I polymorphism, offspring sex ratios are biased towards daughters, possibly due to recruitment of more Z-carrying oocytes when females have assessed the genetic quality of their partners. If our study has general applicability across taxa, it predicts taxon-specific sex allocation effects depending on which sex is the heterogametic one.

Key words.—Heterogamety, MHC polymorphism, sex ratio adjustment, sex-specific mortality (daughters).

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Early this century, J.B.S. Haldane made the famous empirical observation ''when in the offspring of two different animal races one sex is absent, rare, or sterile, that sex is the heterozygous sex'' (Haldane 1922). Although Haldane seems to have identified this phenomenon at both a between- and within-species level (referring to ''races'' in his original statement), the rule that carries his name has mostly been applied in contexts of hybridization. The underlying reason for this is likely to be because genes that are not detrimental (or less so) in within-population matings may disrupt normal ontogenetic development when expressed on a hybrid genetic background with, not surprisingly, stronger effects of genetic incompatibility at outbreeding. Subsequent work has identified several genetic mechanisms that may wholly or partly explain such sterility effects, for example, faster evolution of sex chromosome genes in males (due to sexual selection), faster evolution of X-linked genes, and meiotic drive (Coyne and Orr 2004). However, only one hypothesis has the capacity to explain differences in both offspring viability and sterility in the heterogametic sex, regardless of whether this is the male or the female, and regardless of chromosome morphology (e.g., Z, W, or X, Y systems): this is the dominance theory (Muller 1940, 1942). Assume that a female is heterogametic (e.g., ZW), and depict her genotype $A_1A_1B_1B_1$, and that of her partner $A_2A_2B_2B_2$. Assume further that allele A_1 is incompatible with B_2 (with the effect of incompatibility varying in severeness along an inbreeding-outbreeding continuum with some intermediate optimum). Then, when both loci are autosomal, the resulting offspring will be $A_1A_2B_1B_2$, and inviable only if both factors are relatively dominant (with dominance referring to effects on fitness on this particular genetic background). However, when locus A is Z-linked and locus B is autosomal, females will be $A_1B_1B_2$, and males $A_1A_2B_1B_2$, Thus, females will be inviable regardless of the dominance of A, whereas males will only be dominant if both A_1 and B_2 are both relatively dominant (reinterpreted from Coyne and Orr's hybridization example (2004) into a female ZW system, with assumed allo- and conspecific incompatibility set by inbreeding-oubreeding effects). Thus, when there is no genetic counterpart on the homologous chromosome, the risk of expressing detrimental alleles is 100%, regardless of whether these have direct pathological effects (e.g., sex-linked genetic disease), or act disruptively via interaction with other parts of the genome (epistasis) (Haldane 1922; Orr 1997). For example, in humans, the X chromosome may contain some 4000 genes coding for a host of diseases such as color blindness and muscular dystrophy (Strachan and Read 2001). Thus, Haldane's empirical observation of reduced fitness in the heterogametic sex can be applied along an inbreeding-outbreeding continuum, with different genetic mechanisms explaining sterility and inviability along its range (Haldane 1922; Orr 1997; Coyne and Orr 2004).

With costs of heterogametic gene expression incorporated into sex allocation theory, applied to a model system with heterogametic females, female fitness returns from investments into daughters are likely to parallel (or surpass) those from sons only when females mate with males with little genetic load, that is, when the risk of expressing paternal, detrimental recessives are particularly low in the heterogametic sex (Charnov 1982; Frank 1990; Hardy 2002)). We test this prediction using sand lizards (*Lacerta agilis*), in which females are heteromorphic (ZW), with daughters hence obtaining the Z chromosome from the siring male, and the W from the female, whereas sons are homomorphic (ZZ) (Rykena 1991; Odierna et al. 1993). In a recent publication, we demonstrate that daughters from population-crossings of the same subspecies have more than 300% higher risk of expressing malformations than sons, and that hatchability is reduced by more than 10% in female-biased clutches (Olsson et al. 2004a). Thus, there is reason to believe that genetic costs of offspring production can be sex specific and that females may have evolved facultative sex ratio adjustment in relation to partner genetic quality.

In order to test this scenario we use data from: (1) a long series of mating experiments in a laboratory population originating from southern Sweden, and (2) a long-term intensive

study of free-ranging sand lizards in the same population. A Z-chromosome-specific, multipathology, molecular marker is not available for any species to our knowledge. Therefore, we screened a subsample of lizards at the major histocompatibility class (MHC) I loci, using a probe for species-specific restriction fragment length polymorphism (RFLP) (Madsen et al. 2000), and assigned paternity in the wild using microsatellites. Previous work has shown that MHC polymorphism is strongly indicative of genome-wide polymorphism in this species (Madsen et al. 2000) and is correlated with several fitness components in addition to immunocapacity (Madsen et al. 2000; Olsson et al. 2003). Furthermore, odor-based female choice at the MHC in this species (Olsson et al. 2003, 2004b) may constitute a coevolutionary axis between perception of partner genetic quality and female sex allocation adjustment.

Materials and Methods

Two assessments of offspring viability were made at hatching: (1) hatchability, which underestimates daughter inviability if females are capable of adjusting the sex ratio towards sons, and (2) malformations in hatchlings and dead embryos in late stages of development, which is a conservative estimate of daughter inviability, because some may have died of malformation-related effects during development. Hatchlings were screened for kinked vertebrate columns, and asymmetries in toe numbers and jaw shape (Olsson et al. 1996a). Mean malformation frequency (1 = deformed, 0 = nondeformed), hatchability (1 = hatched, 0 = dead in incubation; egg mortality can be distinguished from infertility in this species (Olsson and Shine 1997)), and sex ratio (1 = son, 0 = daughter), were calculated per clutch (mean number of young per female = 8.05 ± 0.67, SE). Because of the inherent problem with sex-specific embryonic mortality in analysis of female sex ratio adjustment, we used clutches with 100 percent hatchability from MHC-screened parents in our laboratory and field populations. Thus, embryonic mortality cannot explain facultative sex-ratio adjustment in our study (and there is no resorption of oocytes in the oviduct in this species (Olsson and Shine 1997)).

Laboratory Husbandry

Sand lizards (*Lacerta agilis*) were captured in southern Sweden (~N57°22′ E11°58′) and brought back to facilities at the University of Gothenburg, Sweden (Madsen et al 2000; Olsson et al. 2004b). After an artificial hibernation (Olsson et al. 1996a; Olsson and Madsen 2001), matings were allowed and the time spent in copula monitored (seconds). Subsequent to oviposition (Olsson et al. 1996a; Olsson and Madsen 2001), the eggs were incubated at 25°C, which is the ideal incubation temperature at which incidence of offspring malformations are minimized and hatching success maximized (Zakharov 1989; Olsson and Madsen 2001). At hatching, male hemipenes can be gently everted and this was used to sex all offspring (Harlow 1996), and reconfirmed to have near 100 percent repeatability (Olsson and Shine 2001). In the mating trials in which sex-ratio effects of male RFLP genotype were assessed, a female was mated twice with two different males selected at random, and their mean number of RFLP fragments used as a male polymorphism index. This approach was adopted in order to make laboratory results comparable to those collected in the field where females are known to be promiscuous (Olsson et al. 1996b; Olsson and Shine 1997).

Field Techniques

The population at our main study site (Asketunnan) has been studied for nearly two decades and detailed descriptions of field techniques can be found elsewhere (Olsson and Madsen 2001). In short, sand lizards are strongly sexually dimorphic, small (to 20 g), ground-dwelling lizards. Males develop a bright green nuptial coloration, the ''badge,'' and its size is known to influence male reproductive success (Olsson et al. 2000; Anderholm et al. 2004). We therefore monitored individually marked lizards throughout the mating season with respect to presence of partners, and scored paternity of males in clutches from collected females that oviposited in our laboratory (> 90% of all females available to a male). Female body condition was indexed using residuals from a body mass, snout-vent length regression, and used to assess condition effects on mean clutch sex ratios. Throughout the study, nonparametric statistics were resorted to when traits failed to conform to normality even after attempts at transformation.

Microsatellite techniques

The sand lizards were genotyped using three polymorphic microsatellite DNA loci (La-3, Lv4-72, Lv4-x) (Gullberg et al. 1998; Boudejamadi et al. 1999; Madsen et al. 2000). The PCR reactions were run in a 25 μl reaction mixture containing 25 ng total genomic DNA, 1 U of AmpliTaq polymerase (Applied Biosystems, Foster City, CA), 1.0 mM $MgCl_2$, 0.125mM of each nucleotide, 5 μl of Perkin-Elmer (Wellesley, MA) GeneAmp 10× PCR buffer (100 mM Tris-HCl, 500mM KCl and 0.01% gelatine) and 0.4 μM of each primer (forward primers were labelled with fluorescein, MWG Biotech AG, Ebersberg, Germany). The reaction mixture was heated to 94°C for 3 min and then amplification was performed through 30 cycles at 94°C for 30 sec, 54°C for 30 sec, 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 to avoid contamination, negative controls were run with each set of reactions, using 1 μl of sterile Milli-Q water (Millipore, Billerica, MA) 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, CA).

MHC Class I Screening

Restriction fragment length polymorphisms of sand lizard MHC Class I genes were analyzed using a 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,

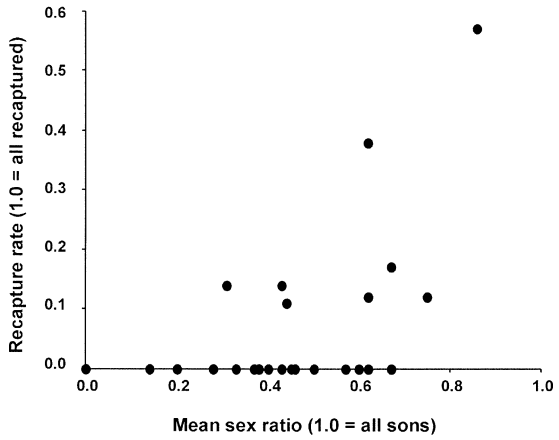

FIG. 1. Biased production of offspring towards sons increases a female's probability of recruiting young into their second year of life. This result was further reinforced by controlling for offspring mass at release in a Spearman's nonparametric partial correlation analysis ($r_s = 0.43$, $P = 0.028$, $n = 27$).

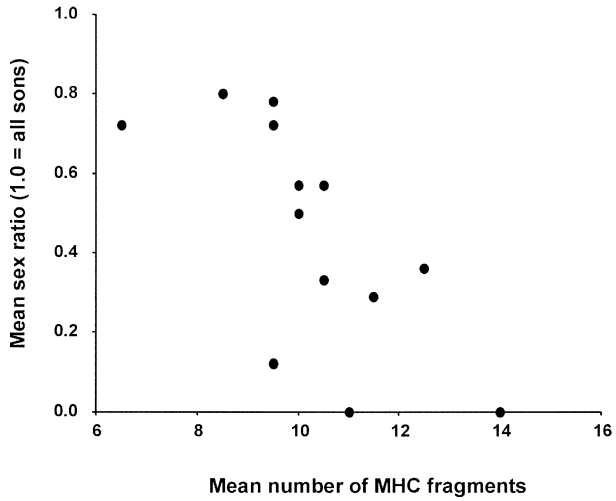

FIG. 2. Relationship between mean male MHC polymorphism (number of RFLP-MHC Class I fragments) and mean sex ratio in staged experiments. When copula duration was held constant, the correlation between MHC polymorphism and sex ratio became even stronger ($r_s = -0.78$, $P = 0.0027$, $n = 13$).

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 were 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 Inc, Westborough, MA) using a vacuum blotter model 785 (Bio-Rad, Hercules, CA) and the membranes were crosslinked in an XL-1500 UV crosslinker (Spectronics Corporation, Westbury, NY). 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 over night 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 labeled with ($\propto$-^{32}P) dCTP (Amersham Biosciences, Piscataway, NJ). 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, NY) in intensifying screens for 1–5 days at −80°C.

RESULTS AND DISCUSSION

In the laboratory, clutches with more males showed less deformities ($r = -0.50$, $P = 0.0001$, $n = 62$; range 0–1.00, mean = 0.22 ± 0.038 (SE)), and higher hatchability ($r = 0.53$, $P = 0.001$, $n = 69$, range 0–1.00, mean = 0.76 ± 0.039 (SE); sex ratios were normally distributed and left untransformed, Shapiro-Wilkes statistics, W:Normal = 0.92, $Pr < W = 0.26$). The relationship between deformation frequency and hatchability was strongly negative ($r = -0.81$, $P = 0.0001$, $n = 69$).

We then examined whether mean sex ratio also affected offspring viability in the natural population. Offspring from 27 females captured in the field were hatched out in the laboratory, released back into the wild and then recaptured prior to oviposition. Female body condition was unrelated to offspring sex ratio ($r = -0.10$, $P = 0.62$, $n = 27$). However, in agreement with our observations in the laboratory, the clutches showed a negative relationship between sex ratio and malformation frequency (Spearman's rank-order correlation coefficient, $r_s = -0.40$, $P = 0.038$), a similar negative trend between hatchability and malformations ($r_s = -0.33$, $P = 0.091$, $n = 27$), and an enhanced mean recapture rate with more sons per clutch ($r_s = 0.41$, $P = 0.036$, $n = 27$; Fig. 1). Sex-specific migration cannot explain sex-specific recapture, because a 300 m wide corridor was searched for migrants around the study site, representing > 10 times the shift in home range by any sand lizard neonate (Olsson and Madsen 2001).

Analysis of our MHC screened partners revealed that more polymorphic males copulated for longer ($r_s = 0.58$, $P = 0.029$, $n = 14$). This is not a trivial observation since females are the larger sex and have the capacity to reject males and interrupt copulations. Thus, females may accept to copulate for longer with more polymorphic males, which increases probability of paternity through transfer of more spermatozoa during the continuous ejaculation typical of squamate reptiles (Olsson and Madsen 1998). Thus, promiscuous females may prolong copulations with more polymorphic males in order to increase their probability of paternity in a situation of sperm competition and cryptic female choice. However, a female's sex ratio adjustment should be conducted in direct response to a male's genotype and only be indirectly linked to copula duration. This turned out to be the case. With number of male RFLP fragments held constant at its mean in a partial correlation analysis, the relationship between copula duration and sex ratio vanished ($r = 0.42$, $P = 0.17$, $n = 13$), but in the corresponding partial correlation with copula duration held constant, the relationship between male MHC polymorphism and offspring sex ratio was even reinforced ($r_s = -0.69$, $P = 0.009$, $n = 13$; Fig. 2). No significant effect

of parental band sharing (relatedness) (Wetton et al. 1987) could be identified on sex ratio or copula duration ($P > 0.18$), which suggests a maternal decision on offspring sex based on the paternal genotype alone.

In free-ranging MHC-screened males, there was a strong trend for more polymorphic males to have more breeding coloration (larger ''badges''; $r_s = 0.34$, $P = 0.079$, $n = 27$), a trait that we have shown in previous work to be strongly related to male reproductive success (Olsson et al 2000; Anderholm et al. 2004). We therefore held badge size constant in a partial correlation analysis, which revealed independent effects of male MHC polymorphism on male reproductive success (number of sired young assigned by microsatellites; $r_s = 0.59$, $P = 0.054$, $n = 12$; range sired young, 0–7, mean $= 1.41 \pm 0.31$ (SE), median $= 0.5$). The sex ratios of the young sired by these males strongly agreed with our laboratory results with more daughters being produced from fertilizations by more MHC polymorphic males ($r_s = -0.56$, $P = 0.028$, $n = 14$).

How, then, do females make the sex ratio adjustment per se? In reptiles, once secondary follicles have been recruited from first ones, clutch size cannot be increased (Byskov 1978; Saint Girons 1985). However, females could fine-tune their allocation decisions by follicle resorption (atresia) (Byskov 1978; Saint Girons 1985). If follicles with W-oocytes and Z-oocytes are produced or resorbed at different rates through the ovarian cycle, this provides a potential mechanism by which females can adjust offspring sex ratio. In sand lizards, ovulation is sometimes interrupted after a first few eggs have been laid and hatching therefore takes place over several days. Males siring such clutches were never MHC-screened, which rules out tests of ovulation patterns in response to male genotype. Nevertheless, these clutches provide an opportunity to test whether W-oocyte ovulation is less common late in the cycle, that is, when females have had ample opportunity to assess partner genetic polymorphism. This turned out to be the case ($n = 15$; mean sex ratios ($\pm$ SE), 0.48 ± 0.08, vs. 0.69 ± 0.09, in first vs. last date hatchlings, Wilcoxon test, $P = 0.039$, one-tailed probability).

In conclusion, our results unanimously show that production of daughters entails genetic recruitment costs and provide support for adaptive female sex-ratio adjustment. Females offered genetically more polymorphic males responded by producing more daughters, that is, the sex most susceptible to inviability from detrimental Z-chromosome alleles. This is in close agreement with predictions from one of the most universally consistent paradigms in evolutionary genetics (Haldane's rule), here applied from a ''cost'' perspective in sex allocation theory. Furthermore, our results predict contrasting sex allocation patterns among taxa in response to parental genotype, depending on which sex is the heterogametic one.

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