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Sons are made from old stores: sperm storage effects on sex ratio in a lizard

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Sperm storage is a widespread phenomenon across taxa and mating systems but its consequences for central fitness parameters, such as sex ratios, has rarely been investigated. In Australian painted dragon lizards (*Ctenophorus pictus*), we describe elsewhere that male reproductive success via sperm competition is largely an effect of sperm storage. That is, sperm being stored in the female reproductive tract outcompete more recently inseminated sperm in subsequent ovarian cycles. Here we look at the consequences of such sperm storage for sex allocation in the same species, which has genetic sex determination. We show that stored sperm have a 23% higher probability of producing sons than daughters. Thus, shifts in sex ratio, for example over the reproductive season, can partly be explained by different survival of son-producing sperm or some unidentified female mechanism taking effect during prolonged storage.

Keywords: sperm storage; sex ratios; lizard

1. INTRODUCTION

Sperm storage occurs in a wide range of taxa and mating systems, from insects, molluscs, amphibians to reptiles, birds and mammals; from monogamous to polygynandrous populations; and with a huge variation in duration from hours to years (Birkhead & Møller 1998). Often, this is argued to be the result of selection arising from rarity of mating opportunities (e.g. sexual segregation of males and females in time and space; Ruckstuhl & Neuhaus 2005), or as an insurance mechanism against male sperm limitation (Preston *et al.* 2003). A number of proximate mechanisms have been sought and linked to the duration of sperm storage, such as sperm length (Miller & Pitnick 2002), female sperm storage crypt characteristics (Miller & Pitnick 2002) and oviposition interval (Olsson & Madsen 1998). However, evolutionary corollaries of sperm storing, outside of gamete and primary sex trait anatomy and physiology, remain largely uninvestigated.

In humans, older men have a tendency to overproduce sons (Lazarus 2002). Thus, if widespread, characteristics of the spermatozoa in relation to the age of males, or the way sperm is produced or may change during storage in either sex, may have adaptive, non-adaptive or even maladaptive consequences for their production of offspring, depending on optimal sex allocation tactics (Fisher 1930; Williams 1966; Hardy 2002). For example, if the haploid genotype of the sperm is genetically compromised through ageing (e.g. due to higher free radical attacks on their mitochondria; Harman 1956), their genes may be more suited for the production of a given sex offspring (typically the homogametic, which may suffer the least from genetic pathology; Olsson *et al.* 2004, 2005*a*,*b*). Thus, if sperm characteristics change with sire age or prolonged storage, this warrants investigation of sperm storage effects on life-history traits in species where pronounced sperm storage forges a link between copulatory tactics and life-history evolution.

2. MATERIAL AND METHODS

The Australian painted dragon (*Ctenophorus pictus*) is a small, (adult snout–vent length 65–95 mm and mass 8–16 g) diurnal lizard of typically sandy habitats and low vegetation, with a range covering central and western New South Wales to Western Australia. It is short-lived (only approx. 10% live to a second year) and strongly territorial. We show elsewhere using molecular genetic analysis that sperm storage in this species is pronounced, with male reproductive success in staged sperm competition experiments following largely from their success at fertilizing eggs in ovarian cycles subsequent to—not within—the cycle in which their sperm is currently competing with those of rivals. Owing to this, we designed an experiment in which we allowed females to first mate in the wild subsequent to their emergence from hibernation (i.e. in the 'natural' way). We then brought them, and additional wild-caught males, to facilities at Wollongong University where we staged matings repeatedly with females and different males in consecutive ovarian cycles to allow the sperm from males to compete under natural storage circumstances in the female reproductive tract. We then assessed the paternity of the offspring as they hatched out with species-specific microsatellites (so that we knew exactly from which copulation the sperm came). We then analysed the sex ratio of the offspring from stored versus 'fresh' sperm.

(a) *Field and laboratory procedures*

Males and females were captured by noose at Yathong Nature Reserve NSW 145° 35′ E; 32° 35′ S, NSW, along a two-dimensional transect (a fence line) and brought to holding facilities at Wollongong University where they were held separately in $600\times600\times500$ mm cages with a 40 W spot light at one end to allow thermoregulation to their preferred body temperature (approx. 36–37°C as per cloacal temperatures of free-ranging territorial, displaying males, M. Olsson & E. Wapstra 2000, unpublished data). Lizards were fed crickets and meal worms dusted with calcium and multivitamins ad libitum every second day and sprayed with a mist of water twice daily. Over the following four months (September–December), i.e. during the time of the year corresponding to the natural mating season in the wild, matings were staged in the laboratory. Female follicles are palpable and reach a size of approximately 4–6 mm during the receptive part of the ovarian cycle. At this stage, males were introduced to the female. Availability of males made it impossible to mate each female with a unique male in each ovarian cycle, but all females were mated with males in rotation, making sure that a given female was only mated once with a given male except in one case. On average, females produced 2.05 clutches during the mating season (±0.12, $n=80$). Female cages were checked for recently laid eggs at least twice daily, which were immediately removed and placed in moist vermiculite (mixed with water in a 1 : 7 ratio) and incubated at approximately 30°C until hatching. Offspring were sexed by hemipenis eversion (Uller *et al.* 2006).

(b) *Paternity assignment and statistical analyses*

DNA was isolated from adult and juvenile toe clips with the Qiagen DNAeasy 96-well Tissue Kits (Qiagen). Samples were genotyped at four microsatellite loci (CP01, CP02, CP10, CP17). Loci were amplified via fluorescent PCR according to Schwartz *et al.* (in press) and were electrophoresed on an ABI 3900 Genetic Analyzer (Applied Biosystems). Genotypes were scored with the assistance of GENEMAPPER (Applied Biosystems).

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Paternity analyses were conducted in Cervus (Marshall *et al.* 1998). Genotypes from the adults were used to calculate allele frequencies, observed and expected heterozygosites, frequency of null alleles and the polymorphic information content of the loci (Botstein *et al.* 1980; Hearne *et al.* 1992). Mother–offspring genotypes were compared for the presence of null alleles. Paternity was assigned using two simulation analyses: zero genotyping error rate (complete exclusion) and a 0.01 genotyping error rate. Results were compared and assigned fathers were assessed by eye across mother–offspring pairs and the clutch mates.

We used a generalized linear mixed model (Proc Glimmix, SAS; binomial response variable, logit link function) with the number of sons entered over the total number of offspring as our response variable, sperm storage category (stored versus non-stored sperm) as a fixed factor and female identity as a random factor (Littell *et al.* 1996). The degrees of freedom were estimated using Satterthwaite's approximation.

3. RESULTS

From 54 clutches of 34 females and 26 males, 180 offspring were split into either of two sperm storage categories, depending on whether they had been sired from stored sperm ('stored') or a recent laboratory mating ('fresh'). Each ovarian cycle in this species takes approximately three weeks resulting in an approximate sperm storage difference of two months between stored and fresh sperm at fertilization (early September versus early November on average). A descriptive analysis showed a 23% higher sex ratio from stored sperm than fresh sperm (0.55 ± 0.06 s.e., $n=34$, and 0.32 ± 0.09 s.e., $n=20$, respectively), which was statistically significant ($F_{1,50}=6.58$, $p=0.013$).

4. DISCUSSION

Previous work has shown that sex ratios may vary with a number of temporal factors across taxa, e.g. the mating season in reptiles (Shine & Olsson 2003) and birds (Daan *et al.* 1996), sexual rest in Norway rats (*Rattus norvegicus*; Hornig & McClintock 1996), and with paternal age and coital frequency in humans (reviewed in Lazarus 2002). The underlying mechanism(s) to this is unknown but is likely to involve multiple factors since these taxa comprise different systems of sex determination (e.g. genetic versus environmental; Shine 1999) and sex-specific heterogamety (e.g. XY in human males and ZW in female birds). Thus, for example, age-dependent changes in X–Y bearing sperm ratio due to senility, pathology or other factors as in humans (Kidd *et al.* 2001; Paulson *et al.* 2001) cannot explain sex ratio bias in species with female heterogamety (such as birds and many reptiles; Hardy 2002). In addition, the capacity for sperm storage varies hugely within and across higher taxa (Birkhead & Møller 1998). Thus, the current literature gives us little support in identifying a potential proximate mechanism for the increased production of sons from stored sperm in painted dragons.

In a previous study, prior to molecular paternity analyses of this dataset, we found that females showed consistency in their sex ratios among clutches produced over the reproductive season, with an overall bias towards daughters (Uller *et al.* 2006). Furthermore, in 1 out of 2 years, there was also a slight, but statistically significant, increase in the sex ratio over the season. Our present data may explain this trend and also suggest that variation among years in seasonal shifts in sex ratio to some extent may be driven by annual variation in sperm storage. However, the proximate mechanism behind the higher proportion of sons from stored sperm remains elusive.

This study was approved by and conducted in compliance with Wollongong University animal ethics protocols; AE 04/03, AE 04 and AE 05. A scientific licence was also issued under the National Parks and Wildlife Act 1974 by the National Parks and Wildlife Service, NSW, Australia.

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