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Multiple paternity and sperm storage lead to increased genetic diversity in *Anolis* lizards

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ABSTRACT

Question: Are multiple mating and sperm storage important in the evolution of *Anolis* lizards?

Data description: Rates of multiple paternity in field-caught dams, timing of sperm storage from field-caught dams, sire order effects, genetic diversity of progeny, timing of sperm storage from controlled matings in the laboratory. Data are based on the use of eight microsatellite loci, natural and controlled breeding studies.

Search method: We used exclusionary paternity analysis to score the number of sires that fertilized each female's eggs. We scored the days since copulation that females continued to produce fertile eggs in the laboratory as a measure of the timing of sperm storage. We estimated differences in sire RS (field only) as a function of sire order. We compared proportions of shared microsatellite alleles between full-sibs and half-sibs relative to the population mean as an index of progeny genetic diversity.

Conclusion: *Anolis sagrei* is one of the most promiscuous amniote vertebrates studied. Most (80%) female *A. sagrei* mate with multiple males and can store sperm for more than 2 months. Mate order has little impact on sire reproductive success. The genetic diversity among progeny from females that mate with multiple males is higher, and closer to the total genetic diversity in the population, compared with progeny from monogamous females. We discuss potential adaptive explanations for multiple paternity, including the importance of sexual conflict in the mating system.

Keywords: island, lizard, multiple paternity, sexual selection, sperm.

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INTRODUCTION

Cryptic forms of sexual selection, whether sperm competition (Birkhead, 1995) or female choice (Eberhard, 1996; Kempenaers *et al.*, 2000), are increasingly recognized as important to the evolution of mating systems. Although mating with a single male usually provides sufficient sperm to fertilize all of a female's eggs (Lee and Hays, 2004), females of many taxa mate with multiple males and often produce progeny from multiple sires (Jennions and Petrie, 2000). In some cases, sperm stores are kept for extended periods [i.e. years (Pearse and Avise, 2001)] and males may continue to sire offspring even after they have died (Zamudio and Sinervo, 2000).

Multiple paternity within broods has been considered enigmatic, especially since the act of mating may itself be costly (Svensson *et al.*, 2005). However, recent studies have pointed out the potential benefits of mating with multiple males. For example, mating with multiple males sets the stage for sperm competition (Johnsen *et al.*, 1998), which may ensure that higher quality sperm fertilize the female's eggs (Tregenza and Wedell, 2002; Hosken *et al.*, 2003). Multiple paternity also allows females to produce a genetically diverse array of progeny, which may be a form of genetic bet-hedging, or a means to alleviate conflict in mating systems (Chippindale *et al.*, 2001). Whatever its adaptive significance, multiple paternity has proven to be a common pattern in diverse groups, including birds (Kempenaers *et al.*, 1992, 1999; Ratti *et al.*, 1995), reptiles (Hoggren and Tegelstrom, 1995; Olsson *et al.*, 1996; Laloi *et al.*, 2004), insects (Rubenstein, 1989; Price *et al.*, 1999; Simmons *et al.*, 1999), amphibians (Tennessen and Zamudio, 2003; Myers and Zamudio, 2004), fish (Evans and Magurran, 2001), and mammals (Solomon *et al.*, 2004).

Most studies of multiple paternity have focused on taxa that produce multiple offspring in any given reproductive effort. Less common are studies of multiple paternity in groups that produce only a single progeny per reproductive effort. This is an important shortcoming, since females that produce one versus many progeny might experience different selection pressures. For example, if the time between reproductive bouts depletes sperm stores (Jones *et al.*, 2002), or increases the uncertainty of environmental conditions experienced by progeny (Garant *et al.*, 2001), females may be under increased selection to mate with multiple males.

The brown anole, *Anolis sagrei*, is a small iguanid lizard with a broad tropical and subtropical range. Female *A. sagrei* typically lay one egg at a time (occasionally two) at approximately 10-day intervals (Jenssen and Nunez, 1998). Behavioural studies in the field and laboratory have demonstrated that female *A. sagrei* mate with multiple males (Tokarz, 1998), indicating the possibility of both sperm competition and multiple paternity. Moreover, anatomical studies confirm the existence of specialized sperm storage tubules in *A. sagrei* (Sever and Hamlett, 2002). However, multiple paternity has not been confirmed in anoles, nor has its potential adaptive significance been assessed. Here we use microsatellite-based estimates of paternity to quantify rates of multiple paternity in *A. sagrei*, and to show that multiple matings allow females to increase the genetic diversity among their progeny. We also estimate the timing of sperm storage by females and determine mate order effects. Finally, we discuss the possible adaptive significance of producing genetically diverse offspring from multiple sires.

METHODS

Our study populations are located in the Bahamas, around Georgetown Exuma (23°31′N, 75°49.5′W). During June 2004, we collected 50 females and 13 males from a small area

(< 500 m^2) adjacent to one of our long-term study populations (Calsbeek and Smith, 2003). Given that the mating season in this population begins in early spring (February/March) and extends through September, it is likely that all of the females we captured had already mated in the field. All lizards were transported back to the University of California at Los Angeles and housed in individual 10-gallon terraria. Each terrarium contained woodchips as a substrate, a potted plant for use as a perching and nesting site, full spectrum lighting, and a 40-W incandescent bulb as a heat source. Lizards were fed *ad libitum* with *Achaeta* crickets dusted with vitamins and calcium powder and supplied with fresh water daily. Plants were also watered daily to maintain the hydric conditions necessary for proper egg development.

Females were housed in individual terraria so that when their progeny hatched, maternity of offspring was known with certainty. Because we could not be certain that each female had mated in the field, a subset of 32 females (hereafter 'experimental females') was given additional access to two randomly determined males, for an average of 16 days each (range = 8–29 days). The remaining 18 females (hereafter 'control females') were not given further access to males. Females laid eggs in the plant pots, and eggs incubated and hatched from these pots. Thus, we cannot be certain of egg-laying dates and hereafter use hatching date as a proxy for laying date.

To better estimate the timing of sperm storage, we raised 20 virgin F1 females in tanks separate from males until they reached sexual maturity (gauged by palpation of maturing follicles). We then bred these 20 females randomly to F1 males (no matings within sibship) and allowed them to produce progeny as described above. We performed this second estimate because: (1) we did not know the exact timing of mating in the field, and (2) we wished to control for any confounding effects of having provided field-caught females with additional males once they reached the laboratory. Because of the controlled nature of breeding F1 progeny, we use these data only to estimate timing of sperm storage, and limit genetic analyses to progeny from field-caught females.

We obtained 226 F1 progeny in the laboratory between 11 June and 29 September 2004. Hatchlings were removed from terraria within one day of hatching and we removed a small (2 mm) piece of tail tissue for microsatellite analyses. Tissue sampling was non-destructive (Laloi *et al.*, 2004), as *A. sagrei* have natural tail autotomy, an adaptation for predator avoidance, and tails regenerate in a few weeks. Progeny were housed in terraria separately from adults, and fed *ad libitum* with curly winged *Drosophila* and baby crickets.

We extracted genomic DNA from tail tissue by overnight incubation at 55°C using standard DNA extraction kits (Qiagen) followed by centrifugation and 1 : 10 dilution of the extract. Eight microsatellite loci (Bardelbeden *et al.*, 2004) were amplified from the genomic template via multiplex polymerase chain reaction (PCR), and we assessed length polymorphism among individuals on an automated DNA sequencer (ABI 3700) using fluorescent labelling of one of the primers in each pair. We assigned paternity (95% confidence) using the software package CERVUS (Marshall *et al.*, 1998). We calculated the average number of shared microsatellite alleles at each locus for pairs of individuals. We calculated band-sharing values for three classes of individuals: full-sibs ($n = 33$ comparisons), half-sibs ($n = 85$ comparisons), and the total population of offspring ($n = 3619$ comparisons for progeny sired in both the field and laboratory).

Statistical analyses

The distributions of count data for numbers of progeny and numbers of sires were non-normal, even after attempts at transformation, so we used non-parametric Spearman correlations in our analyses involving counts. We calculated the difference in number of progeny sired by first and second males in the laboratory experiment using a paired signed rank test, and compared hatch dates of progeny from different sires using analysis of variance (ANOVA). We report two-tailed probabilities for all of these comparisons.

Randomization tests

We predicted that pairs of full-sibs would be, on average, less diverse than the population as a whole (i.e. would share more microsatellite alleles than the population mean), while diversity of maternal half-sibs would not differ statistically from the population mean (and thus would be more diverse than full-sibs). We tested these predictions by randomizing microsatellite band-sharing values across sibships. Our randomization test entailed random draws with replacement from the distribution of all pair-wise comparisons of band-sharing values measured in the laboratory. The number of draws for each sibship class was identical to the actual number of pair-wise comparisons made in the laboratory (33 draws for full-sibs, 85 draws for half-sibs). We calculated the probability that the average pair-wise band-sharing value would be as great as that observed in each sibship class by chance (10,000 randomizations). We predicted *a priori* that band-sharing values would be higher for siblings compared with the population mean, so we report the one-tailed probabilities for all randomization tests.

RESULTS

We obtained 226 F1 progeny from our laboratory rearing experiment. Simulations in CERVUS indicated a total paternity exclusionary power of 0.993. Of these 226 progeny, we could assign paternity with at least 95% confidence to 107 offspring from 11 sires, 32 experimental females, and 12 control females. We assume that unknown males from the field sired the remaining 119 progeny before we had capture dams. Within clutches produced by these females, 58.3% were co-sired by multiple males in the wild. This estimate, however, is biased downwards by ten females from whom we obtained only a single progeny. When we exclude these females and consider only the females that produced multiple progeny, rates of multiple paternity in the wild were 80.7%.

When we considered only the data from experimental females that mated with each of two males in the laboratory, first males sired significantly more progeny than second males (mean progeny = 2.82 ± 0.55 vs. 1.00 ± 0.36 for first and second laboratory sires respectively; signed rank test: $Z = -2.37$, $P < 0.01$). This result did not arise from attenuated egg production, or from differences in the timing of fertilization in the laboratory, since hatching dates of progeny did not differ for progeny sired by first and second laboratory sires respectively ($P = 0.21$). The total number of sires that could be assigned to a female's progeny increased as a function of the number of progeny produced by the female (Spearman's rank correlation: $Z = 4.85$, $P < 0.0001$, $n = 35$) (Fig. 1).

Hatching dates of progeny from control females (i.e. those that did not mate in the laboratory) did not differ from hatching dates of progeny from unknown sires produced by

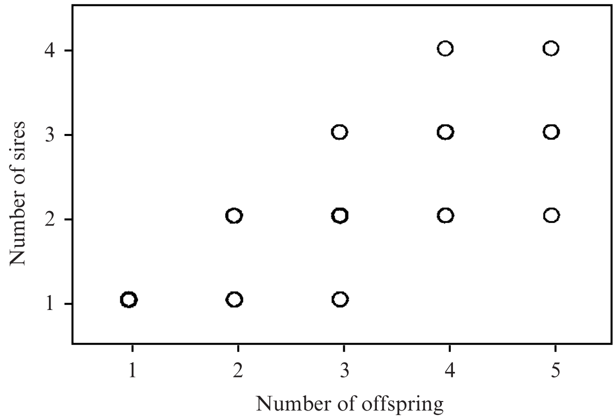

Fig. 1. The total number of males that co-sired a female's offspring increased with increasing female reproductive success, indicating that females produce genetically diverse offspring with the sperm from multiple males. Total samples sizes are 107 progeny from 35 dams and 11 sires.

experimental females (ANOVA: $F_{1,19} = 0.009$, $P = 0.93$), presumably because progeny from both groups were sired by males in the field rather than the laboratory. However, hatching dates of progeny from experimental females differed significantly depending on whether they were sired by males in the field or by males in the laboratory (ANOVA: $F_{2,38} = 9.03$, $P < 0.0005$). Following Bonferroni correction for multiple comparisons, the difference between progeny sired in the field and progeny sired by the first male in the laboratory remained significant, but the comparison between field sires and second laboratory sires did not ($P = 0.06$). The mean difference in hatching dates of progeny sired in the field versus those sired in the laboratory is a rough estimate of the timing of sperm storage from these field-caught females, because switching to the production of progeny by laboratory sires indicates either that stored sperm from field sires became depleted, or that females began preferentially using the sperm from laboratory sires. The mean hatching date of progeny sired in the laboratory was 19 (± 4) days later than progeny sired in the field, suggesting that field-mated females store sperm for about 3 weeks before using the sperm from additional sires. F1 females continued producing progeny ($n = 90$ total progeny) for on average 55 days (range = 17–107) following mating, providing a much longer upper estimate for the ultimate timing of sperm storage in this group.

Mean microsatellite band-sharing values are given in Table 1. Pair-wise band-sharing values among full-sibs were significantly higher than the average pair-wise band-sharing

Table 1. Mean numbers of shared microsatellite alleles at the eight loci scored in this study

Class	No. of comparisons	Mean band-sharing value	Standard error
Full-sibs	85	0.73	0.22
Maternal half-sibs	33	0.61	0.20
Total population	3917	0.46	0.21

Note: Values were calculated based on all possible pair-wise comparisons among full-sibs, maternal half-sibs, and the entire population.

value in the population (randomization test, one-tailed P-value = 0.045); however, the pair-wise band-sharing values of maternal half-sibs (i.e. progeny from multiply sired clutches) did not differ significantly from the population mean (randomization test, one-tailed P-value = 0.09). Thus, producing maternal half-sibs results in a genetically diverse array of progeny that represent more of the allelic diversity of the total population compared with full-sibs.

DISCUSSION

Multiple paternity as a form of cryptic sexual selection is increasingly recognized as important in many taxa (Newcomer *et al.*, 1999; Wedell and Tregenza, 1999; Jennions and Petrie, 2000) and is nearly ubiquitous in reptiles (Madsen *et al.*, 1992; Olsson and Madsen, 1998). Here we demonstrate a form of multiple paternity in lizards in which female *A. sagrei* store sperm from multiple males and produce a genetically diverse array of progeny. More than 80% of field-caught *A. sagrei* females that produced two or more progeny had mated with multiple males. This makes *A. sagrei* one of the most promiscuous amniote vertebrates studied to date [e.g. some estimates of multiple paternity include: turtles, 30% (Pearse *et al.*, 2002); voles, 56% (Solomon *et al.*, 2004); birds, 4–67% (Garamszegi and Moller, 2004); most other lizards, 50–60% (Gullberg *et al.*, 1997; Laloi *et al.*, 2004); but up to 80% in one other lizard (Zamudio and Sinervo, 2000)].

Our data suggest that although females produce progeny from multiple sires, earlier males have a statistical advantage in the production of offspring (Evans and Magurran, 2001). Data from field-caught females suggested that sperm are used for a relatively brief period of approximately 3 weeks, at which point the female might choose to re-mate [i.e. 'topping-off' (Jones *et al.*, 2002)] and then continue to fertilize eggs. In this case, the tendency for females to use sperm from early sires first would not necessarily result in a net early-sire fitness advantage. Data from mating virgin females in the laboratory indicated that females are physiologically capable of storing sperm for much longer (mean = 55 days) and that the change in sperm use observed in the laboratory could be behavioural (e.g. choice, see below), rather than the result of exhausted sperm stores.

We detected increased numbers of sires used to fertilize a female's offspring as a function of increasing egg number. We emphasize that this is not the trivial result of additional offspring providing new opportunities to detect multiple sires, since an alternative possibility would have been for females to sire all of their offspring with the sperm from just one or two males. Rather, we suggest that females engage in a sort of genetic bet-hedging (Yasui, 2001), in which mating with multiple males allows the female to diversify her progeny genotypes and increase their likelihood of survival in unpredictable environments. If progeny encounter novel pathogens (Palmer and Oldroyd, 2003; Spielman *et al.*, 2004; Pearman and Garner, 2005), or other unpredictable environmental conditions, then diversifying progeny genotypes may ensure that at least some offspring survive to adulthood. This hypothesis has been supported by previous studies (Garant *et al.*, 2001) and is currently being tested with *Anolis* at our field site.

Another element of the *Anolis* mating system that could explain the extreme rates of multiple paternity in this group is the emerging picture of sexual conflict in the *Anolis* mating system. Our recent work suggests that large and small males carry genes for creating high-quality sons and daughters respectively (R. Calsbeek *et al.*, submitted). Intra-locus sexual conflict (Parker, 2006) results from large sires carrying male benefit/female detriment genes, and small sires carrying female benefit/male detriment genes. Females that mate with a single sire would thus be faced with an adaptive dilemma, whether to make high-quality sons or daughters. However, others (Chippindale *et al.*, 2001) have suggested that mating with multiple males may allow females to choose the best sire genes for their progeny and thereby alleviate the costs of sexual conflict (Bonduriansky and Rowe, 2005). Results from our laboratory suggest that intra-locus sexual conflict may be an important factor in the evolution of multiple paternity (R. Calsbeek *et al.*, submitted), and we suggest that females that mate with multiple males may do so as a means of engaging in adaptive mate choice, rather than simply avoiding the use of sperm from small males.

Finally, creating a genetically diverse array of offspring may also facilitate colonization of new islands. *Anolis sagrei* is an extremely efficient disperser (Schoener and Schoener, 1984) and is found on islands throughout the Greater Antilles (Roughgarden, 1993). Recent studies have shown that island populations devastated by hurricanes are quickly re-colonized by over-water dispersal of *A. sagrei* from adjacent islands (Spiller *et al.*, 1998; Schoener *et al.*, 2000), and gene flow among islands plays an important role in the evolution of these lizards (Calsbeek and Smith, 2003). Given the high rates of multiple paternity in *A. sagrei*, over-water dispersal by a single gravid female could establish a new island population while mitigating the potentially deleterious effects of a genetic bottleneck. Such dispersal events are rare, thus multiple paternity is unlikely to have evolved solely as an adaptation for island colonization. However, maternal half-sibs could mate with each other at reduced risk of inbreeding depression (Tregenza and Wedell, 2002) and this would likely be an ancillary adaptive benefit of genetic bet-hedging. Further tests of the adaptive significance of multiple paternity in *A. sagrei* are clearly warranted.

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