Polyandrous females provide sons with more competitive sperm: Support for the sexy-sperm hypothesis in the rattlebox moth (*Utetheisa ornatrix*)

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Given the costs of multiple mating, why has female polyandry evolved? *Utetheisa ornatrix* moths are well suited for studying multiple mating in females because females are highly polyandrous over their life span, with each male mate transferring a substantial spermatophore with both genetic and nongenetic material. The accumulation of resources might explain the prevalence of polyandry in this species, but another, not mutually exclusive, possibility is that females mate multiply to increase the probability that their sons will inherit more-competitive sperm. This latter "sexy-sperm" hypothesis posits that female multiple mating and male sperm competitiveness coevolve via a Fisherian runaway process. We tested the sexy-sperm hypothesis by using competitive double matings to compare the sperm competition success of sons of polyandrous versus monandrous females. In accordance with sexy-sperm theory, we found that in 511 offspring across 17 families, the male whose polyandrous mother mated once with each of three different males sired significantly more of all total offspring (81%) than did the male whose monandrous mother was mated thrice to a single male. Interestingly, sons of polyandrous mothers had a significantly biased sex ratio of their brood toward sons, also in support of the hypothesis.

KEY WORDS: Lepidoptera, paternity, polyandry, sexual selection, sperm competition.

The standard paradigm in sexual selection theory is that males should maximize their reproductive success by mating as often as possible, whereas females should maximize their fitness by mating selectively with the highest quality male, at least when males do not transfer resources to females during mating (Bateman 1948; Trivers 1972; Arnold and Duvall 1994). Yet, the availability of molecular approaches in the last two decades has revealed that multiple mating by females across taxa is more prevalent in nature than previously thought (Andersson 1994; Eberhard 1996; Bogdanowicz et al. 1997; Hughes 1998; Simmons and Achmann 2000; Bretman et al. 2004; Behura 2006; Taylor et al. 2014). This phenomenon is challenging to explain in light of the fact that a single mating is often sufficient to provide a female with enough sperm to fertilize her entire complement of eggs (Ridley 1988;

Bezzerides 2004). Moreover, mating itself is costly. For example, males of many insect species transfer accessory substances to the female during mating that can negatively affect both her mating behavior and, consequently, her overall fitness (e.g., Fowler and Partridge 1989; Rowe et al. 1994; Wolfner 1997; Chapman et al. 2003). Additional matings also increase the risk of disease transmission as well as time and energetic costs incurred as a consequence of courtship and copulation (Thornhill and Alcock 1983; Watson et al. 1998; Thrall et al. 2000; Orsetti and Rutowski 2003). Given these significant costs incurred by polyandrous females, understanding how selection has maintained a high level of female promiscuity remains a major puzzle for evolutionary biologists (Slatyer et al. 2012; Parker and Birkhead 2013; Pizzari and Wedell 2013).

The fitness advantages that females derive from mating multiply may be broadly classified as either direct or indirect benefits. Direct (phenotypic) benefits are the result of nongenetic quantities that have a positive impact on the survivorship and offspring production of the female (Arnqvist and Rowe 2005). Such benefits can include enhanced paternal care (Davies 1992), the transmission of antipredator defensive chemicals (González et al. 1999), and the acquisition of nutrient rich nuptial gifts (Thornhill and Alcock 1983; Gwynne 1984). A review of over 100 experimental studies (Arnqvist and Nilsson 2000) shows that for species with nuptial gift-giving (i.e., nongenetic material is transferred at mating), the usual costs of multiple mating may be compensated for by the benefits of accumulating resources from multiple males. Thus, there appears to be substantial evidence that polyandry can be at least partially explained by the acquisition of material benefits. Indirect (genetic) benefits, on the other hand, are those that are reaped in the next generation in the form of increased survivorship and reproductive success of sons and daughters; as a result, by mating with multiple males, such females ensure that they increase their odds of finding a mate of superior genetic quality (Yasui 1998; Jennions and Petrie 2000; Sardell et al. 2012). In these cases where females mate multiply, sperm from the highest quality mate can be used preferentially; this postcopulatory selection leaves open the possibility that females accrue direct benefits from multiple males providing nuptial gifts while gaining the indirect benefits by having offspring of the highest genetic quality (Parker 1984; Birkhead and Møller 1995; Eberhard 1996).

Although many hypotheses regarding the genetic benefits of polyandry have been proposed, there have been relatively few empirical tests designed to identify the underlying mechanism by which female promiscuity is selected for and maintained in populations (Newcomer et al. 1999; Kokko et al. 2003; Slatyer et al. 2012; Parker and Birkhead 2013; Pizzari and Wedell 2013). Halliday and Arnold (1987) suggested that female promiscuity may be a by-product of selection for extreme male promiscuity through pleiotropic gene action, but this ignores the likelihood that subsequent selection on modifier genes should favor decoupling of male and female promiscuity (Reeve and Sherman 2001). We here test another, infrequently considered, hypothesis to explain the evolution of polyandry—the sexually selected, or "sexy-sperm" hypothesis—which posits that female multiple mating and male sperm competitiveness can coevolve via a runaway, or Fisherian, process (Keller and Reeve 1995). Specifically, females, by mating with multiple different males, create conditions favoring males with more competitive sperm, resulting in sons of promiscuous females possessing both the gene for the male trait (sperm competitiveness) and the gene for the female preference (for mating multiply). The direct selection on sperm competitiveness and genetic correlation between sperm competitiveness and female promiscuity create indirect selection on the female trait, subsequently promoting the spread of both the male and female traits in a positive-feedback process. Similar to other runaway sexual selection models, the sexy-sperm mechanism is especially likely to evolve in species with ZZ/ZW sex chromosome systems (e.g., lepidopterans and birds), because a Z-linked gene for female promiscuity will be transmitted to all rather than only half of sons with sperm competitiveness genes (Reeve 1993; Reeve and Shellman-Reeve 1997; Iyengar et al. 2002; Reeve and Pfennig 2003; Kirkpatrick and Hall 2004). Such a process should lead to mutual exaggeration of female promiscuity and male sperm competitiveness.

Here we test the "sexy-sperm" hypothesis in the rattlebox moth, *Utetheisa ornatrix* (Lepidoptera: Arctiidae), in which females are highly promiscuous and may mate with up to 22 mates over their three to four week life span (LaMunyon and Eisner 1993; Iyengar et al. 2001). In this species, we know that the degree of female polyandry is heritable, and that female promiscuity genes are Z-linked and inherited independently of those associated with male promiscuity (Iyengar and Reeve 2010). Although females exhibit mate choice based on a male courtship pheromone (Iyengar et al. 2001), females receive phenotypic benefits from each mating because each male delivers a substantial spermatophore containing nutrient and pyrrolizidine alkaloid (PA) transmitted in quantities proportional to body size (Dussourd et al. 1991; LaMunyon and Eisner 1994). The nutrient enhances female egg production (LaMunyon 1997), and the alkaloid provides chemical protection for herself (González et al. 1999) and her eggs (Dussourd et al. 1991). From the known direct benefits accrued by females via spermatophores, it might be hypothesized that such considerable male resources are sufficient to account fully for the evolution of female multiple mating in this species. However, this does not preclude that polyandry also benefits females through the genetic benefit of producing sons with more competitive sperm. For example, it is known that, in precopulatory female choice, females may obtain both direct and indirect benefits simultaneously because such benefits are not mutually exclusive and may both influence sexual selection (Iwasa et al. 1991; Kokko et al. 2003). Indeed, because body size is heritable in *U. ornatrix* (Iyengar and Eisner 1999a), females achieve genetic benefits from precopulatory choice for larger males because they produce both larger sons that are favored in courtship and larger daughters that are more fecund (Iyengar and Eisner 1999b). Most importantly, previous experiments have shown that multiply mated females are capable of selectively using sperm from the mate that transfers the largest spermatophore (LaMunyon and Eisner 1994) while benefiting from the nongenetic components of other male spermatophores (Bezzerides and Eisner 2002; Eisner and Meinwald 2003). Although the mechanism of postcopulatory female choice is not known, female *U. ornatrix*, like many lepidopterans, possess an elaborate series of ducts and chambers that

could be used to manipulate sperm after mating and be responsible for biased paternity by larger males (Engelmann 1970; LaMunyon and Eisner 1994; Rossini et al. 2001; Curril and LaMunyon 2006). The combination of female reproductive complexities, variation in male siring success, and Z-linked inheritance of polyandry in *U. ornatrix* set the stage for our study, as all of these conditions are required for sexy sperm to influence the evolution of polyandry.

Overall, the sexy-sperm hypothesis has remained incompletely tested for the evolution of female promiscuity in *U. ornatrix* and is infrequently tested in general. However, there is growing evidence that sons of polyandrous versus monandrous females may have siring advantages due to increased sperm number and viability in organisms including red flour beetles, field crickets and bank voles (Bernasconi and Keller 2001; Pai and Yan 2002; Klemme et al. 2014; McNamara et al. 2014). In *U. ornatrix*, it is known that female promiscuity genes are primarily Z-linked (Iyengar and Reeve 2010), as would be expected if sperm competitiveness genes and female promiscuity genes coevolved via a Fisherian runaway process. Our current study focuses on whether sons of polyandrous females (i.e., females mated thrice, once each to three different males) indeed have greater sperm competitiveness than sons of monandrous females (i.e., females mated thrice to the same male). This experimental design standardizes the number of matings between the monandrous and polyandrous treatments and controls for phenotypic benefits received by females (McNamara et al. 2014; reviewed in Slatyer et al. 2012). Additionally, we examined whether sons of polyandrous females exhibit offspring sex ratios unusually biased toward males, as might be expected if such males indeed have especially high sperm competitiveness compared to sons of monandrous females. This sex ratio bias is predicted because females mated to males of especially high sperm competitiveness should favor making sons over daughters given that their sons would also tend to excel at sperm competition and thus be especially valuable relative to daughters (the relative value of sons to daughters would be less to females who mated with sons of monandrous mothers). This is a "sexy-sperm" extension of Trivers and Willard's (1973) hypothesis that females mated to especially attractive males should bias their offspring sex ratio toward males (the "Silver Spoon Hypothesis"; see also Charnov 1982; Hardy 2002).

Methods
OFFSPRING PRODUCTION FOR PATERNITY ANALYSES
All *U. ornatrix* used in this experiment were from a laboratory colony established from wild stock collected at the Archbold Biological Station in Lake Placid, FL. The experimental individuals, reared in separated, genetically independent family lines, were only one to two generations removed from the field to maintain a genetic diversity representative of natural populations. Larvae were reared on a pinto bean-based diet supplemented with the seeds of *Crotalaria spectabilis* (its primary food plant; Conner et al. 1981), and adults were maintained on sponges moistened with honey water. Throughout our experiments, we controlled for adult body size due to its important fitness consequences for both sexes, and therefore define "size-matched" moths to be same-aged individuals within 5% body mass (Iyengar and Eisner 1999a). For all matings, males and females of known body mass were paired individually for 24 h in small, paper cylindrical containers (0.35 l), and pairs were monitored at 6-h intervals to confirm mating success (copulation lasts 10–12 h; LaMunyon and Eisner 1994).

For our experiments, we created unique family lines by isolating pairs of size-matched full sisters to randomly selected, two-day-old virgin males from the colony; one sister was assigned to a monogamous treatment in which she mated thrice to the same male (at two-day intervals on days 2, 4, and 6), whereas the other sister was assigned to a polyandrous treatment in which she was mated three times to three different males (the males in this treatment were the same ages, and had the same mating status and mating intervals as males in the monogamous treatment to control for spermatophore size; Fig. 1). Overall, we had 17 families in which we reared sons of monandrous mothers (M males) and sons of polyandrous mothers (P males). From these matings, four size-matched males from each family line (two M males and two P males) were selected to mate with two size-matched sisters (unrelated to any other family used and from the colony). To control for mating order, we mated one sister to an M male on day 2 (posteclosion) and then a P male on day 4, and we mated the other sister to an P male on day 2 and an M male on day 4. Size matching among males was important to control for differences in spermatophore size based on size and mating history, and the two-day mating interval for females provided a sufficient period to empty the bursa that receives the spermatophore (LaMunyon and Eisner 1994). We confirmed that such size-matched pairs also transferred same-sized spermatophores by conducting a paired *t*-test on spermatophore mass, which was measured indirectly by taking the average between the weight loss of the male and the weight gain of the female for each mating pair (LaMunyon and Eisner 1994). Because the M males in these sets of matings were size-matched brothers and the P males were also size-matched brothers, this protocol allowed us to account for potential order effects when we examined the offspring from these two sisters (who were size matched to control for biases based on female phenotype and genotype).

Starting 24 h after the second mating (to allow sperm to reach the spermatheca; LaMunyon and Eisner 1993), eggs were collected daily, and larvae were reared to pupation to determine sex. Upon eclosion, we placed all moths in individually labeled

A Rearing sons from monandrous (M) and polyandrous (P) mothers
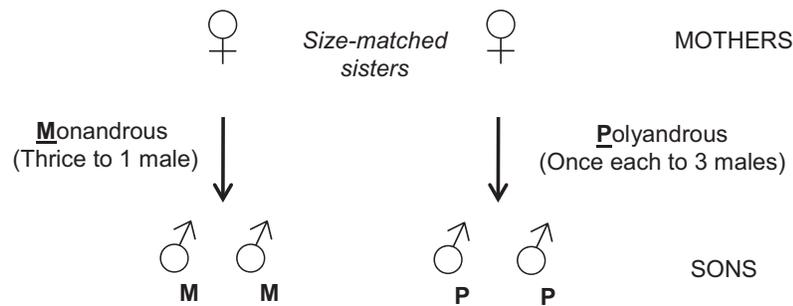
B Competitive double matings of M vs. P sons that controls for mating order
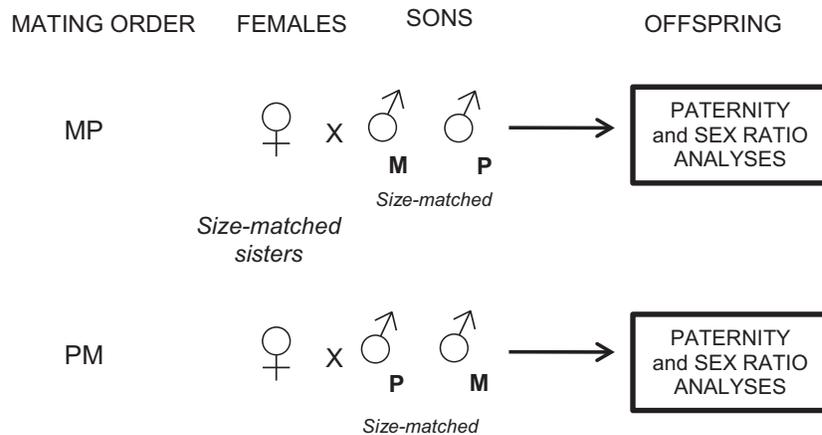
Figure 1. Experimental design for testing whether sons of polyandrous females (P sons) have more competitive sperm than sons of monandrous females (M sons). In (B), the two sets of matings are comparable because, for each pair of size-matched females, M sons and P sons were similar both genotypically (mothers were sisters) and phenotypically (body size).

microcentrifuge tubes and stored them in −80°C in preparation for paternity analyses.

PATERNITY ANALYSES USING MICROSATELLITES

We determined paternity using three polymorphic microsatellite loci (*Utor*7, *Utor*28, and *Utor*TAC1) previously developed specifically for *U. ornatrix* (Bezzerides et al. 2004). Genomic DNA was extracted from the moths' abdomen by use of a DNEasy™ Tissue Kit (QIAGEN) for each individual (mother, potential male sires, and all offspring) for each family. For polymerase chain reaction (PCR) amplification, we did not multiplex because of the similar size of these locus fragments. Loci *Utor*7 and *Utor*28 were modified on the forward primer by addition of a fluorescent label, whereas locus *Utor*TAC1 was modified by a fluorescent label on the reverse primer. The sequence GTTT was added to the 5′ end of all unlabeled primers to promote adenylation. PCR reactions (10 μl) contained 10–100 ng of genomic DNA, 2.5 U/μl Jumpstart *Taq* polymerase, 3.25 mM MgCl₂, 10 mM dNTPs, and 10 μM each of forward and reverse primer.

The PCR cycling profile consisted of one cycle at 95°C for 2 min, 35 cycles of 50 sec at 95°C, 1 min at 50°C, and 1 min at 72°C, followed by 5 min at 72°C, and a final extension cycle of 30 min at 10°C. We carried out PCR by using a Dyad thermocycler (Bio-Rad Laboratories), and we electrophoresed a subset of PCR products through polyacrylamide gels to ensure DNA amplification. PCR products were genotyped at Cornell University using an ABI Prism 3100 Genetic Analyzer (Applied Biosystems), and allele sizes were estimated with GeneMapper version 3.7 (Applied Biosystems; Chaterji and Pachter 2006).

All three microsatellite loci were highly polymorphic with the number of alleles ranging from nine to 12 (see Table S1). After initial paternity assignments using GeneMapper, we viewed each allele and manually corrected scoring errors that can occur when using such automated programs (Bonin et al. 2004). This manual inspection of all individuals (mother, two potential fathers, and offspring) also allowed us to determine which calls from the offspring were real alleles (as they were found consistently in at least one of the parents) and which calls were false or contained

a mutation (allele was not present in any parent). In cases where the sample appeared to have more than two alleles, we evaluated which peaks were consistent with those found in the parents. All samples that originally failed at multiple loci or were called as homozygous were reanalyzed to obtain additional data and to check for errors (i.e., null alleles). We then used CERVUS 3.0 to verify our results, by setting the amount of offspring for the simulation of paternity test to 10,000 and the potential typing error to 0.01%. At a strict confidence level of 95%, the analysis showed an assignment rate of 51% given the father alone and a 99% assignment rate at a relaxed confidence level of 80%. Assignment rates given the known mother were 100% at both the strict and relaxed confidence intervals.

We excluded all offspring that showed any conflicting paternity across loci (offspring with at least one locus showing a different father than the other two loci) or whose loci were not variable enough to make a distinction between fathers. Overall, we were able to unambiguously assign approximately 70% of all offspring, and these individuals were used in our subsequent analyses.

STATISTICAL ANALYSES OF PATERNITY AND SEX RATIO

All statistical analyses were conducted using R version 3.1.2 (R Core Team 2014). We analyzed offspring sired by sons of polyandrous females and offspring sex ratio for both male sires with a generalized linear mixed model (GLMM) using the glmer function from the "lme4" R package (Bates et al. 2014). We used a binomial probability distribution and a logit link function. To account for the sibling design in the experiment, family was included as a random factor. Mating order was included as a fixed effect in our initial model, but it was not significant ($P = 0.26$) and was subsequently omitted from our final analyses. This allowed us to combine the offspring from both sisters within each family. We compared models using Akaike Information Criterion (AIC) and only the best fitting model is reported here. Additionally, we used chi-square goodness-of-fit tests to examine paternity biases within each family.

Results

SIZE ANALYSES AND CHARACTERIZATION OF LOCI

Across all 17 families, we confirmed that each set of sisters not only mated with males of similar body mass (paired $t = 1.27$; df $= 33$; $P = 0.214$) but also with males transferring spermatophore of similar mass (paired $t = 1.60$; df $= 33$; $P = 0.118$). Furthermore, spermatophore masses transferred to each pair of sisters was within 3% for all cases, with the exception of families 10, 12, 15, and 17, where the spermatophores of sons
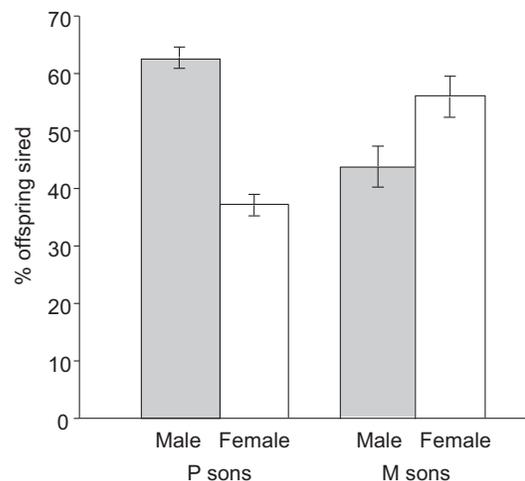
Figure 2. The sex ratio of offspring sired by sons of polyandrous females (P sons) versus sons of monandrous females (M sons) across all families (mean ± SE). Overall, the offspring of P sons were male biased, whereas there was no offspring sex bias for the offspring of M sons.

of monandrous females were between 4% and 5% larger than those of sons of polyandrous females.

Based on the CERVUS analysis, the mean number of alleles across the three loci was 11 (see Table S1). The mean proportion of individuals typed was 0.9869, mean expected heterozygosity was 0.7239, and the mean polymorphic information content (PIC) was 0.6856. The ranges of allele sizes (in base pairs) were as follows: *Utor*7, 244–271 bp; *Utor*28, 242–286 bp; *Utor*TAC1, 201–227 bp.

PATERNITY ANALYSES

Sons of polyandrous females sired significantly more offspring than did sons of monandrous females (binomial GLMM: $n = 34$, $z = 4.393$, $P = 1.12 \times 10^{-5}$). The resulting probability of offspring sired by male P is 0.81, 95% CI [0.69, 0.89]. We also determined the percentage of offspring sired by male P within each family, and we found biased paternity towards male P in 12 of 17 families (Table 1). There was no bias in paternity among the other five families (10, 11, 12, 15, and 17).

SEX RATIO ANALYSES

Sons of polyandrous females sired significantly more male offspring than did sons of monandrous females (binomial GLMM: $n = 31$, $z = 3.654$, $P = 0.000259$; Fig. 2). Across all families, sons of polyandrous females had offspring with a male-biased sex ratio, as the probability of offspring being male for those sired by P sons was 0.64, 95% CI [0.59, 0.69]. Sons of monandrous females, on the other hand, had offspring with an even sex ratio, as the probability of offspring being male for those sired by the M sons was 0.45, 95% CI [0.37, 0.54].

Table 1. The percentage of offspring sired by P sons (males with a polyandrous mother) based on the total number of offspring sired by sisters within a family line. P sons sired more offspring than M sons in 12 of 17 families.

Family	N	Percentage of offspring sired by P son	P son favored sire over M son?
1	32	100.00	Yes ($\chi^2 = 32.00$; df $= 1$; $P < 0.001$)
2	31	67.74	Yes ($\chi^2 = 3.90$; df $= 1$; $P = 0.048$)
3	32	75.00	Yes ($\chi^2 = 8.00$; df $= 1$; $P = 0.005$)
4	38	86.84	Yes ($\chi^2 = 20.63$; df $= 1$; $P < 0.001$)
5	35	77.14	Yes ($\chi^2 = 10.31$; df $= 1$; $P = 0.001$)
6	19	100.00	Yes ($\chi^2 = 19.00$; df $= 1$; $P < 0.001$)
7	25	84.00	Yes ($\chi^2 = 11.56$; df $= 1$; $P = 0.001$)
8	25	72.00	Yes ($\chi^2 = 4.84$; df $= 1$; $P = 0.028$)
9	34	94.12	Yes ($\chi^2 = 26.47$; df $= 1$; $P < 0.001$)
10	30	56.67	No ($\chi^2 = 0.53$; df $= 1$; $P = 0.465$)
11	23	65.22	No ($\chi^2 = 2.13$; df $= 1$; $P = 0.144$)
12	43	55.81	No ($\chi^2 = 0.58$; df $= 1$; $P = 0.446$)
13	25	88.00	Yes ($\chi^2 = 14.44$; df $= 1$; $P < 0.001$)
14	30	76.67	Yes ($\chi^2 = 8.53$; df $= 1$; $P = 0.004$)
15	22	50.00	No ($\chi^2 = 0.00$; df $= 1$; $P = 1.00$)
16	35	100.00	Yes ($\chi^2 = 35.00$; df $= 1$; $P < 0.001$)
17	32	34.38	No ($\chi^2 = 3.13$; df $= 1$; $P = 0.077$)

Discussion

In this study, we used the moth *U. ornatrix* to test the sexy-sperm hypothesis, an evolutionary mechanism by which female polyandry is maintained at high levels in populations due to a genetic correlation of the male trait for sperm competitiveness and female preference for multiple mating (Keller and Reeve 1995). Because this runaway selection operates via a Fisherian process, it is more likely to occur in ZZ/ZW systems where female promiscuity alleles found on the Z chromosome are transmitted to *all* sons (as opposed to a subset of sons, as is the case for XX/XY systems). This Z-linkage leads to a genetic correlation of the sperm competitiveness and female promiscuity, further fueling their mutual coevolution (Reeve 1993; Reeve and Shellman-Reeve 1997; Iyengar et al. 2002; Reeve and Pfennig 2003; Iyengar and Reeve 2010). Indeed, it was recently discovered that female promiscuity genes appear to localize to the Z chromosome in *U. ornatrix* (Iyengar and Reeve 2010), strengthening the possibility that such coevolution has occurred in this polyandrous moth. A critical, previously untested, prediction of the sexy-sperm hypothesis is that sons of polyandrous mothers should have a higher siring success than sons of monandrous mothers.

We used microsatellite paternity analyses to examine siring success among males based on their mother's mating history in *U. ornatrix*. We found that sons of polyandrous mothers sired over three times more offspring than sons of monandrous mothers, thus supporting the sexy-sperm hypothesis. This biased siring success is likely due to more-competitive sperm inherited from their father because we controlled for genetic effects from the mother (within

each family, P and M males had mothers that were size-matched sisters) and phenotypic effects of the spermatophore on paternity by using size-matched P and M sons. Furthermore, because both females mated the same number of time with spermatophores of similar sizes, maternal effects such as increased investment by females mating multiply or receiving larger spermatophore is less likely to be occurring (Simmons 2005; Slatyer et al. 2012).

Our results run counter to some previous tests of the sexy-sperm hypothesis in the bulb mite *Rhizoglyphus robini* (Konior et al. 2009) and the field cricket, *Teleogryllus oceanicus* (Simmons 2003), in which no difference in sperm competitiveness was found between sons of monandrous and polyandrous females. However, in these studies it is possible that any potential effects were obscured by uncontrolled maternal effects and other nongenetic effects, such as spermatophore nutrient content, which greatly influence overall siring ability and fitness of offspring (Simmons 2005). Because it was known that *U. ornatrix* males transferring larger spermatophores have an advantage in paternity (LaMunyon and Eisner 1994), we carefully controlled for such nongenetic effects to ensure they were not masking the effects of sperm competitiveness. For each family, the body mass of males were size matched within 5%, and, in all but four cases, the subsequent spermatophores transferred were within 3% in mass. Notably, these four cases where the spermatophore mass of M male was between 4% and 5% larger than male P (with one additional case) were the only instances where paternity did not favor the P male. In each of these five families, there was no siring bias toward either male, which indicates that spermatophore

size trumps all other influences of paternity and remains the primary criterion for siring success, in support of previous work (LaMunyon and Eisner 1994). However, our results show that, when spermatophores are size matched (within 3% in most cases), males with polyandrous mothers will have an advantage in paternity over males with monandrous mothers and that male sperm may play an important role in postcopulatory selection.

Mating order affects paternity in many insects, and the most common fertilization pattern in members of Lepidoptera and other insects is last-male precedence (Walker 1980; Watt et al.1985; de Jong et al. 1998; Wedell and Cook 1998; Platt and Allen 2001; Kehl et al. 2013). In this study, we found no first- or last-mate advantages with respect to paternity, which confirms previous studies in this species demonstrating that order does not affect paternity (LaMunyon and Eisner 1993, 1994). Interestingly, among the only four families in which order played a role in the proportion of offspring sired, there were three in which the male whose mother was monogamous transferred a spermatophore 4–5% larger than the male whose mother was polyandrous (whereas the rest of the families consisted of males with spermatophores within 3% of each other). This further demonstrates the primary importance of spermatophore size in siring success, as the superior competitiveness of sperm from sons of polyandrous mothers is only realized when spermatophores are close in size. We found no overall mating order effects, although our study involved females mated to only two males when *U. ornatrix* females in the field mate with an average of 11 in the field (Iyengar et al. 2001). Thus, it would be important to examine potential order effects on paternity when females are mated to more than three males.

Interestingly, we also found that sons of polyandrous females had male-biased offspring whereas sons of monandrous females did not. Male-biased sex ratios have been observed in other instances where females mate with high-quality males (Trivers and Willard 1973; Calsbeek and Sinervo 2004; Neff and Pitcher 2005; Cox and Calsbeek 2010) as it is plausible that the genetic value of sons of such matings may exceed that of daughters by virtue of the sons' especially attractive male traits (Charnov 1982; West et al. 2000). Because we do not know whether females or males are responsible for these offspring sex ratio biases, this intriguing result raises many questions about the sperm competition and fertilization processes in *U. ornatrix*. Regardless, to our knowledge, this is the first study demonstrating increased siring success due to sperm competition concomitant with sex ratio biases.

These results show that, in *U. ornatrix*, key elements are in place for the sexy-sperm phenomenon to occur via a Fisherian runaway process, a mechanism that explains the correlation between exaggerated male traits and female preferences for these traits in a postcopulatory context. The association between polyandry and increased sperm quality genes indicates runaway selection is enhancing both female polyandry and male sperm competitiveness in a positive-feedback coevolutionary loop (Keller and Reeve 1995). Further morphological evidence in this species corroborates this idea. *Utetheisa ornatrix* females, like many lepidopterans, have an elaborate reproductive tract with numerous ducts and chambers within which she could sort sperm via cryptic female choice or create an environment in which sperm must compete for fertilization (Engelmann 1970; Eberhard 1996; Rossini et al. 2001; Curril and LaMunyon 2006; Yan et al. 2013). In species where females mate multiply, males may influence postcopulatory processes (siring success) via behavioral tactics, as observed in the traumatic insemination employed by male bed bugs (Lange et al. 2013) or via chemical warfare, as seen in spermicides and toxins in the spermatophore of fruit flies (Wolfner 1997, 2002). Although previous work showing that *U. ornatrix* females are capable of sorting sperm suggests that females are in control of postcopulatory selection, males may also influence siring success. Lepidopteran males produce both nucleated, fertilizing (eupyrene) and anucleated, nonfertilizing (apyrene) sperm (Silberglied et al. 1984), and the exact function of apyrene sperm remains elusive (Wedell and Cook 1999; Curril and LaMunyon 2006) .

One intriguing possibility is that postcopulatory processes and apyrene sperm are correlated. Perhaps sons of multiply mated females produce more effective or proportionally greater amounts of apyrene sperm. If so, what role does apyrene sperm play in sperm competition and do sons of multiply mated mothers benefit as a result? Another possible mechanism for a male's influence in sperm competition are the characteristics of the sperm themselves. Perhaps sons of multiply mated females produce sperm with longer tails and greater swimming ability (Katz and Drobnis 1990; Gomendio and Roldan 1991; Firman et al. 2013) or more especially potent spermicides that influence paternity as is found in *Drosophila melangoster* (Imhof et al. 1998)? Future studies that test the sexy-sperm hypothesis should more closely examine the precise mechanisms by which the two types of sperm interact to lead to a bias in fertilization success and how sons of polyandrous and monandrous mothers differ in their efficacy of these interactions.

This study adds to a small, yet growing body of evidence supporting the sexy-sperm hypothesis. Klemme et al. (2014) found that sons of multiply mated females had higher siring success in bank voles, but this pattern was primarily due to increased mating duration and greater numbers of transferred sperm rather than more competitive sperm per se. Although sperm competitiveness was not directly tested, McNamara et al. (2014) found that sons of polyandrous (vs. monandrous) females had higher sperm viability in field crickets. Pai and Yan (2002) found that

in the red flour beetle *Tribolium castaneum,* male offspring from mothers with multiple mates had higher fitness (the proportion of offspring in a test population) than those from mothers with single mates. Further work in this beetle species has revealed that sons of polyandrous males had a paternity advantage over sons of monandrous males when they were the female's second mate, but they are at a siring disadvantage as the female's first mate (Bernasconi and Keller 2001). Although this highlights the potential importance of mating order in siring success, we found no such order effects in this study.

Overall, our results support the sexy-sperm hypothesis in *U. ornatrix*; the sons of polyandrous mothers not only sired a higher proportion of offspring than did sons of monandrous mothers, but they also produced a more male-biased offspring sex ratio. It has been suggested that the sexy-sperm hypothesis may not be a common mechanism driving the evolution of polyandry because it cannot overcome direct selection against polyandry, nor can it work when there is a lottery system of sperm selection (Bocedi and Reid 2013). In *U. ornatrix*, we know that both of these are not the case; with each mating, females receive defensive alkaloids and nutrients to boost fecundity to use at her discretion (LaMunyon 1997; Bezzerides and Eisner 2002) while paternity remains biased toward the partner transferring the largest spermatophore (LaMunyon and Eisner 1994). Furthermore, the ZZ/ZW genetic architecture of this moth increases the likelihood of a Fisherian process like sexy-sperm selection to operate (Reeve and Pfennig 2003; Kirkpatrick and Hall 2004), and, indeed, female promiscuity genes have been shown to have *Z*-linked heritability in *U. ornatrix* (Iyengar and Reeve 2010). We propose that the sexy-sperm mechanism may occur more often than previously thought; while opposing direct selection could supersede indirect benefits (Iwasa et al. 1991; Kirkpatrick and Ryan 1991), the widespread occurrence of polyandry (Pizzari and Wedell 2013; Taylor et al. 2014) suggests that direct selection against female multiple mating is not pervasive. Knowledge about sperm competition and elaborate female reproductive tracts across taxa and an increasing number of documented examples of biased paternity (Parker and Pizzari 2010; Slatyer et al. 2012) also suggest the prevalence of conditions that could promote the evolution of polyandry via the sexy-sperm mechanism, particularly in ZZ/ZW systems including lepidopterans and birds.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Table S1. Characterization of individual loci obtained through analyses in CERVUS 3.0.