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NOTE

The Coevolution of Ova Defensiveness with Sperm Competitiveness in House Mice

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ABSTRACT: Theoretical models have suggested that sperm competition can lead to increased ova resistance to fertilization. While there is some comparative evidence that this might be true, there is no experimental evidence to show that ova defensiveness evolves in response to sperm competition. We performed a series of in vitro fertilization assays to gauge the fertilizability of ova produced by female house mice from experimental populations that evolved either with or without sperm competition. Our analysis revealed that after 24 generations of experimental evolution, females that evolved under a polygamous regime produced more defensive ova than females that evolved under a monogamous regime. We therefore provide the first direct line of evidence that sperm competition can generate sexual conflict at the gametic level and lead to asymmetries in fertilization rates among populations. Our results show that females respond to sperm competition via fertilization barriers that have the potential to mediate sperm entry.

Keywords: sexual conflict, polyspermy, house mice, experimental evolution, reproductive barriers, zona pellucida.

Introduction

Sexual conflict over the postmating interests of males and females is ubiquitous in polygamous mating systems and arises when the sperm of rival males compete for fertilizations (Arnqvist and Rowe 2005). For example, males evolving under sperm competition are selected to produce greater numbers of sperm and sperm of greater motility and fertilization capacity, allowing them to outcompete rival males and achieve high rates of fertilization (Hosken and Ward 2001; Gomendio et al. 2006; Simmons and García-González 2008; Fitzpatrick et al. 2009; Firman and Simmons 2011). However, increased sperm aggressiveness will generate an elevation in the risk that more than one sperm will enter the ovum during fertilization (poly-

spermy; Levitan et al. 2007). Simultaneous fertilization by multiple sperm is a lethal condition that results in zygote mortality and thus impedes female fertility (Gilbert 1997). Consequently, females might be expected to evolve strong defenses against polyspermy (Jaffe and Gould 1985; Snook et al. 2011). Indeed, theoretical modeling predicts that an increase in male sperm competitiveness is expected to provoke the counteradaptation in females of increased resistance to fertilization (ova defensiveness; Frank 2000; Gavrillets 2000). Sexual conflict theory thus views females as walking an evolutionary tightrope; reduced defenses increase the risk of polyspermy, while overly efficient defenses may prevent fertilization altogether (Arnqvist and Rowe 2005). Such a delicate balance in the fertilizability of ova may explain why polyspermy persists, albeit at low rates, in natural populations (Austin and Braden 1953; Braden 1957; Franke et al. 2003).

Empirical support for the sperm competitiveness–ova defensiveness sexual conflict paradigm is limited to just two comparative studies. Both within and between free-spawning marine invertebrate species, ova resistance to fertilization has been shown to covary with the number of sperm at the site of fertilization (Levitan et al. 2007), and among *Mus* species, female ovum defenses appear to covary with male sperm competitiveness (Martin-Coello et al. 2009). While informative, these interspecific investigations provide only correlational support. Covariation between ovum defensiveness and sperm competitiveness could be explained by the mutual covariation of these traits with some other unrelated variable(s). Ova defensiveness has not been demonstrated to respond to selection from sperm competition as theory predicts.

Here, we combined the powerful techniques of experimental evolution (Firman and Simmons 2010a) and in vitro fertilization (IVF; Martin-Coello et al. 2009) to determine whether postcopulatory sexual selection drives the coevolutionary divergence of ovum defenses with sperm-fertilizing potential among laboratory populations of

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house mice (*Mus domesticus*). It is well known that there is a positive link between fertilization rates and polyspermy in house mice, and it has been shown that several factors can lead to polyspermic fertilizations (Fraser and Drury 1975; Fraser and Maudlin 1978, 1979; Wortzman and Evans 2004). In natural monogamous matings, polyspermy has been shown to occur at a rate of 2%–4% (Fraser and Maudlin 1979; Ueno and Niimura 2008), and higher fertilization rates are accompanied by higher rates of polyspermy (Fraser and Maudlin 1978). By isolating the gametes and utilizing IVF we were able to eliminate confounding factors, such as variation in sperm concentration and/or other factors dictated by the female reproductive tract, and assess fertilization rates under standardized conditions. This allowed us to specifically test the hypothesis that postcopulatory sexual selection elicits ovum defenses to polyspermy, as opposed to female defenses in general.

We maintained selection lines of mice evolving with (polygamous) and without (monogamous) postcopulatory sexual selection for 24 generations. Previously, it was established that males from our polygamous lines produced more competitive ejaculates (Firman and Simmons 2011), which were characterized by greater numbers of sperm and greater sperm motility (Firman and Simmons 2010a), compared to males from our monogamous lines. We thus performed IVF assays (Martin-Coello et al. 2009) between sperm and ova retrieved from individuals from replicate polygamous and monogamous selection lines. In so doing, we provide evidence for the coevolution of ovum resistance to fertilization with sperm competitiveness in house mice.

Methods

Source Population

House mice are actively polygamous in nature, with both males and females copulating with multiple mates (Dean et al. 2006; Firman and Simmons 2008). However, laboratory colonies of house mice are typically maintained under enforced monogamy. A wild-derived colony of house mice was established at the Animal Resources Centre (ARC; Murdoch, Western Australia) with 90 breeding pairs sourced from six wild Australian *Mus domesticus* populations. The colony was maintained as an outbred population under the Poiley outbreeding system, after which the outbreeding regime changed to a dedicated program based on the coefficient of inbreeding (Firman and Simmons 2010a). After approximately 30 generations of enforced monogamy, sexually mature mice ($n = 120$) were transported to and held at the Centre for Evolutionary Biology (University of Western Australia). Male/female pairs ($n = 60$) reproduced to establish a base population from which our selection lines were derived. In so doing,

we referred to the ARC colony pedigree to ensure that close relatives did not breed (Firman and Simmons 2010a).

Experimental Evolution

We established four monogamous and four polygamous selection lines by recruiting animals at random from the 60 litters generated by the wild-derived colony mice. The mating design of the lines has been described in detail previously (Firman and Simmons 2010a) and therefore is reported in brief here. Postcopulatory sexual selection remained absent in the monogamous lines with females mating with only a single male. In contrast, postcopulatory sexual selection was reinstated in the polygamous lines with females mating with three males in quick succession. Each polygamous line male had the opportunity to contribute to the next generation by being positioned as the first, second, and third male to mate. Precopulatory sexual selection (male contest competition and female mate choice) did not operate in either the monogamous or polygamous selection lines (Firman and Simmons 2010a). Litters were weaned and sexed at 3 weeks of age and separated at 4 weeks. After 12 generations of selection, microsatellite data revealed that individuals from the two selection treatments did not differ in the level of heterozygosity or average inbreeding coefficient (Firman and Simmons 2011). Animals from the twenty-fourth generation were used in this experiment.

In Vitro Fertilization

The IVF assays were performed as described in Martín-Coello et al. (2009). Adult females were induced to superovulate by intraperitoneal injections of 5IU PMSG and 5IU hCG that were administered 48 h apart (Martín-Coello et al. 2008). Fourteen hours after the administration of hCG, the female reproductive tracts were removed and placed under mineral oil. Cumulus-oocyte complexes (COCs) were released from the ampullae using a pair of needles and transferred to a 200- μ L drop of M2 medium. The COCs were washed in three 100- μ L drops of M2 medium before being used in the IVF.

Epididymides were removed from males and placed in 1-mL drops of human tubal fluid (HTF; Quinn et al. 1985; Martín-Coello et al. 2008) under oil and incubated at 37°C under 5% CO₂/air for 10 min to allow sperm to disperse into the medium (Martín-Coello et al. 2009). The epididymal tissue was removed, and the suspensions were incubated for a further 50 min to allow the sperm to capacitate. Sperm number and motility was quantified using a CEROS computer-assisted sperm analysis system (ver. 10, Hamilton and Thorne Research) under standard mouse parameters (Nayernia et al. 2003) following the

initial 10-min incubation and then every hour for the duration of the IVF. The decline in the percentage of motile sperm was both weak and similar for males from both the monogamous and polygamous lines (table A1). Divergence in sperm number and quality has been observed at different generations across the lines' evolutionary histories (Firman and Simmons 2010a, 2010b; Firman et al. 2011). Here, on average, the polygamous line males had higher sperm concentrations than the monogamous line males, although average sperm motility traits did not differ between males with different selection histories in this sample (tables A1, A2).

The IVF assays were performed in 500- μ L drops of HTF under mineral oil. The COCs from three females of different replicate selection lines were pooled in a single assay. Previous IVF experiments both within and between *Mus* species revealed (on average) higher fertilization rates of conspecific ova-sperm combinations compared with heterospecific combinations (Martin-Coello et al. 2009), and trial assays performed on the selection line mice at sperm concentrations of 2.0×10^6 motile sperm/mL provided evidence of a ceiling effect such that the monogamous $\times$ monogamous and polygamous $\times$ polygamous sperm-ova combinations reached fertilization rates $\sim 100\%$. Thus, to ensure that higher fertilization rates generated from between-line crosses would be detectable, the IVF assays were mixed with a final concentration of 1.5×10^6 motile sperm/mL. The gametes were coincubated for 4 h at 37°C under 5% CO₂/air, which allowed the sperm to penetrate the ova, fuse with the oolemma, decondense, and form a pronucleus (Martin-Coello et al. 2009). After the incubation period the ova were washed in 100- μ L drops of HTF to remove remaining cumulus cells and/or attached sperm. The oocytes were then stained with a DNA fluorochrome (Hoechst 33342), and viewed under a Zeiss AxioImager A1 fluorescent microscope. Fertilization rate was determined as the number of ova with stained pronuclei over the total number of mature ova.

Experimental Design

The IVF assay combinations were generated via a full factorial design: monogamous line ova $\times$ monogamous line sperm, polygamous line ova $\times$ polygamous line sperm, monogamous line ova $\times$ polygamous line sperm, and polygamous line ova $\times$ monogamous line sperm (table A3). Each assay combination was repeated four times, so a total of 16 assays were performed. Three females and one male donated gametes in each replicate assay. Individuals that had coevolved within the same replicate selection line were never used in the same replicate assay. Thus, the three ova donors for each assay were always taken from different replicate lines. Additionally, for the within selection history

assays (i.e., monogamous $\times$ monogamous, polygamous $\times$ polygamous), sperm and ova donors were always taken from different replicate lines. The total number of ova scored and proportion of ova fertilized for each assay are presented in appendix table A3. Our data were deposited in the Dryad Digital Repository, <http://dx.doi.org/10.5061/dryad.7287d> (Firman et al. 2014).

Results

We analyzed the IVF rates among ova and sperm from our selection lines using a generalized linear mixed model (GLMM) in the R statistical analysis package (R Development Core Team 2011; Firman and Simmons 2012). GLMMs combine the properties of two statistical frameworks, linear mixed models (which incorporate random effects) and generalized linear models (which handle non-normal data by using link functions and exponential family distributions, such as binomial distributions; Bolker et al. 2008). Thus, IVF rate was modeled with a GLMM fit by the Laplace approximation using the "lme4" library (Pinheiro and Bates 2000). The number of fertilized ova was modeled as the response variable, and the total number of ova scored as the binomial n . Fixed effects in the GLMM were the sperm donor selection history, ova donors selection history, and their interaction, while replicate assay identification was modeled as a random effect (table 1).

Our analysis revealed variation in fertilization rates that were explained by the selection history of both the sperm donor and ova donors. As expected, fertilization rates within selection histories were comparable, and sperm from polygamous lines had greater fertilizing capacity than sperm from monogamous lines (table 1). More importantly, we found that ova selection history was highly significant ($P < .001$); ova from the polygamous lines had greater resistance to fertilization than did ova from the

Table 1: Analysis of fertilization rates of house mice from selection lines

	Variance	SD		
Random effects:				
Assay ID	<.001	<.001		
	Estimate	SE	<i>z</i>	<i>P</i>
Fixed effects:				
Intercept	.546	.176	3.100	<.001
Sperm selection history	1.806	.375	4.818	<.001
Ova selection history	-1.963	.288	-6.823	<.001
Interaction term	.499	.482	1.034	.301

Note: A generalized linear mixed model fit by the Laplace approximation using "lme4" library from the R statistical analysis package to test fertilization rates among reciprocal crosses between sperm and ova derived from monogamous and polygamous selection lines.

monogamous lines (table 1). The robustness of these effects is clearly apparent in figure 1.

Our analysis failed to detect a statistically significant interaction between sperm and ova selection history (table 1). The sample size required to detect an interaction is fourfold that to detect a main effect of the same magnitude (Leon and Heo 2009). Thus, we calculated partial effect size estimations (Pearson's r) and their 95% confidence intervals (CIs) to gauge the magnitude of the observed effect of the sperm selection history $\times$ ova selection history ($r = 0.776$ [0.156, 0.957]) on fertilization rate (Nakagawa and Cuthill 2007). The CIs did not include zero, which suggests caution in rejecting an interaction between sperm and ova selection history (fig. 1).

Discussion

We performed IVF assays on experimental populations of house mice evolving under enforced monogamy or polygamy. Higher fertilization rates among the sperm and ova of individuals from genetically diverged populations might have been expected simply because the gametes were not coevolving. However, the patterns of fertilization re-

vealed that postcopulatory sexual selection drove an evolutionary response in males for increased fertilization efficiency and a response in females for increased ovum defenses. Thus, our investigation provides the first empirical demonstration that the fertilizability of the female gamete evolves in response to postcopulatory sexual selection and can lead to asymmetries in fertilization rates among populations.

Interestingly, different mouse strains have been found to differ in their rates of ova fertilization when similar IVF conditions are used (Niwa et al. 1980). Variation in fertilization rates in laboratory mouse strains may be due to founder effects, mutations, and/or genetic drift. In our study, the only difference between our evolving selection lines was the absence or presence of postcopulatory sexual selection. Although mice mate with multiple partners in nature, the population from which the lines were sourced had experienced many generations of enforced monogamy (Firman and Simmons 2010a). Thus, we reinstated sperm competition and sexual conflict in the polygamous lines by ensuring that all individuals mated with three partners.

We found that under controlled IVF conditions, sperm from donors taken from populations with a history of se-

Figure 1: The proportion of fertilized ova in in vitro fertilization assays using gametes derived from monogamous and polygamous selection lines of house mice (mean $\pm$ SE).

lection from sperm competition were more likely to effect fertilization than sperm taken from donors with an evolutionary history of enforced monogamy. Experimental evolution has been widely adopted in studies of insect postcopulatory sexual selection and has confirmed that sperm competition selects for increased sperm production (Holland and Rice 1999; Hosken and Ward 2001; Pitnick et al. 2001; Simmons and García-González 2008) and enhanced sperm competitive ability (Hosken and Ward 2001; Simmons and García-González 2008). The *in vitro* patterns of fertilization that we report here are consistent with previous investigations of the experimentally evolving mouse lines. After eight generations of experimental evolution, males from lines evolving with sperm competition had evolved ejaculates containing more sperm and sperm with greater motility than males from lines evolving without sperm competition (Firman and Simmons 2010a). Moreover, after 12 generations of experimental evolution, when males from these evolving lines were allowed to compete for fertilizations *in vivo*, males from lines evolving with sperm competition achieved a higher competitive fertilization success than males from lines evolving without sperm competition (Firman and Simmons 2011). Thus, sperm competition has led to the divergence in sperm fertilization efficacy among these experimental populations.

Theoretical models predict that the female response to increased fertilization efficacy should be an increase in ova defensiveness to avoid the costs of polyspermy (Frank 2000; Gavrillets 2000). Here, our IVF assays produced fertilization rates that are consistent with such a response in females. Under controlled environmental conditions and controlled sperm density, ova from donors evolving with sperm competition were less likely to be fertilized than ova from donors evolving without sperm competition. It is interesting to note that the lowest fertilization rates were between sperm from donors evolving without sperm competition and ova from donors evolving with sperm competition, while the highest rates of fertilization were between sperm from donors evolving with sperm competition and ova from donors evolving without sperm competition. While the interaction effect was not statistically significant with our sample size, the effect size analysis suggests caution in accepting the null hypothesis of no interaction. Certainly, the observed effect is what we would expect to see under the sexual conflict model. The population used to establish the lines had a long history of enforced monogamy, which was likely to have contributed to low ova defensiveness in the monogamous lines. For example, increased ova fertilizability might be favored in response to sperm limitation in monogamous pairings. However, it is clear from our data that the reinstatement of postcopulatory sexual selection generated a rapid evolutionary increase in ova resistance to fertilization.

Proteins involved in fertilization are known to have higher rates of evolutionary divergence than other proteins and have been shown to evolve in response to sexual conflict (Gavrillets 2000; Wyckoff 2000; Swanson and Vacquier 2002a, 2002b; Swanson et al. 2003). When sperm densities are high and polyspermic conditions prevail, selection may favor ovum surface proteins that reduce the speed of sperm entry, ultimately generating a counteradaptation in sperm proteins to become more efficient at entering the ova due to selection for sperm competitive ability (Palumbi 2009). Studies on sperm-ova interactions among sea urchins have suggested that the molecular coevolution of gamete proteins could be attributable to conflict over variation in the fertilization interests of males and females (Palumbi 1999; Clark et al. 2009). Thus, although we cannot discard other processes related to sperm-ovum interactions, a divergence in reproductive proteins that affect fertilization offers one potential mechanism for the divergence in ova defensiveness we report among our experimental populations of house mice.

Theoretical models suggest that sexual conflict and coevolutionary “chases” have the potential to generate genetic divergence in reproductive characters (Gavrillets and Hayashi 2005; Hayashi et al. 2007). Reproductive isolation driven via postcopulatory sexual selection may become evident when individuals from allopatric populations attempt to reproduce but fail due to a high degree of genetic incompatibility (Rice 1998). Isolating mechanisms that involve the gametes are physiological, subtle, and therefore difficult to investigate. To circumvent this problem, recent studies of species within the genus *Mus* have utilized IVF techniques to show that conspecific fertilization rate is faster than heterospecific fertilization rate (Dean and Nachman 2009) and that ova resistance to fertilization correlates with relative testes size, a widely used proxy for the strength of selection from sperm competition (Martin-Coello et al. 2009). Thus, current evidence from interspecific studies of *Mus* align with the notion that postcopulatory sexual selection has the potential to contribute to the evolution of reproductive barriers during the early stages of speciation (Snook et al. 2011). Our data show that ova fertilizability has the capacity to respond to postcopulatory sexual selection, which is necessary for the evolution of postcopulatory prezygotic barriers to reproduction.

It is possible that cryptic female choice at the gametic level may also have contributed to the divergence in ova defensiveness we have observed in our experimental populations (Eberhard 1996). Indeed, cryptic female choice is a key component of sexual conflict, and these processes are unlikely to be mutually exclusive (Arnqvist and Rowe 2005). Theory predicts that females mate multiply to incite sperm competition and to ensure that males of superior genetic quality achieve high fertilization rates and produce

high-quality offspring (Yasui 1997). While it has long been known that selective fertilization occurs in *Mus* (Edwards 1955; Bateman 1960; Levine 1967) and fertilization biases can “favor” specific sperm types (Wedekind et al. 1996), we suggest that females may have greater control over the outcome of fertilization than has been appreciated. When the sperm of more than one male reaches the site of fertilization, ovum defenses or barriers may act as mechanism to mediate sperm entry and bias fertilization toward a specific sperm type that will provide a fitness advantage. Among our experimentally evolving mouse populations, monogamously mated females may have experienced greater costs associated with cryptic female choice at the gametic level because they risk compete reproductive failure, leading to an evolutionary relaxation of ova defenses. In contrast, the greater ovum defenses of polygamous line females may act as a selective mechanism to ensure that sperm which would return the highest fitness fertilized their ova. Studies of these populations have shown that while there was no effect of female selection history on embryo viability, males from the polygamous lines do sire embryos of higher viability compared with males from the monogamous lines, suggesting that postcopulatory mechanisms have selected for improved male quality (Firman and Simmons 2012).

In conclusion, we utilized the powerful technique of experimental evolution to experimentally test the sperm competitiveness–ova defensiveness sexual conflict paradigm. By performing IVF assays we were able to focus directly on the interaction between the gametes. We found that females respond to postcopulatory sexual selection by increasing their ovum resistance to fertilization. Thus, we provide empirical evidence that postcopulatory sexual selection can create evolutionary responses in ova defensiveness within populations and generate asymmetries in fertilization rates among populations.

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APPENDIX

Supplementary Tables

Table A1: Mean ($\pm$ SE) sperm number and sperm motility traits of monogamous line and polygamous line mice used in the in vitro fertilization assays

Trait	Monogamous	Polygamous
Sperm number ($\times 10^6$)	9.4 $\pm$ 2.4	22.3 $\pm$ 1.9
% motile	72.3 $\pm$ 7.8	65.1 $\pm$ 8.1
% progressive	26.3 $\pm$ 4.3	16.7 $\pm$ 5.6
% rapid	59.6 $\pm$ 10.1	39.4 $\pm$ 10.6
Velocity ($\mu\text{m}/\text{sec}$)	91.7 $\pm$ 10.0	73.4 $\pm$ 10.2
Longevity	−1.8 $\pm$.7	−1.0 $\pm$.6

Note: Longevity is the slope of motility (%) against time since activation.

Table A2: ANOVA comparing sperm trait values between monogamous line ($n = 8$) and polygamous line ($n = 8$) mice used in the in vitro fertilization assays

Trait	SS	df	F	P
Sperm number	663.06	1, 14	18.45	<.001
% motile	206.64	1, 14	.41	.535
% progressive	367.68	1, 14	2.37	.146
% rapid	1,636.20	1, 14	1.91	.189
Velocity	1,337.73	1, 14	1.64	.221
Longevity	2.72	1, 14	.89	.362

Table A3: The total number of ova scored and the proportion of ova fertilized in each replicate in vitro fertilization assay

	Total no. of ova scored				Proportion of ova fertilized			
	M $\times$ M	P $\times$ P	P (sp) $\times$ M (ova)	P (ova) $\times$ M (sp)	M $\times$ M	P $\times$ P	P (sp) $\times$ M (ova)	P (ova) $\times$ M (sp)
1	48	46	40	40	.66	.71	.88	.25
2	26	25	30	15	.65	.72	.93	.07
3	35	16	20	43	.63	.69	.85	.19
4	30	33	25	25	.60	.70	1.00	.20
Sum/mean	139	120	115	123	.64	.71	.92	.18

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Single house mouse (*Mus domesticus*) sperm. Image prepared and taken by Renée Firman.