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Biol. Lett. 2009 **5**, 232–234 first published online 25 February 2009
doi: 10.1098/rsbl.2008.0796

References

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Consistent male–male paternity differences across female genotypes

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In a recent paper, we demonstrated that male–female genetic relatedness determines male probability of paternity in experimental sperm competition in the Peron's tree frog (*Litoria peronii*), with a more closely related male out-competing his rival. Here, we test the hypothesis that a male–male difference in siring success with one female significantly predicts the corresponding difference in siring success with another female. With male sperm concentration held constant, and the proportion of viable sperm controlled statistically, the male–male difference in siring success with one female strongly predicted the corresponding difference in siring success with another female, and alone explained more than 62 per cent of the variance in male–male siring differences. This study demonstrates that male siring success is primarily dictated by among-male differences in innate siring success with less influence of male–female relatedness.

Keywords: genetic compatibility; good genes; paternity; relatedness; sperm traits; amphibian

1. INTRODUCTION

Recent work on probability of paternity in situations of sperm competition and cryptic female (or egg) choice has primarily focused on whether less related males have a paternity advantage (or not) compared with a male more closely related with the female (and/or rival male) across a range of taxa from insects (e.g. Wilson *et al.* 1997; Tregenza & Wedell 2000), spiders (Bilde *et al.* 2007), to fishes (Evans *et al.* 2003), frogs (Sherman *et al.* 2008a), marsupials (Fisher *et al.* 2006) and mice (Firman & Simmons 2008). However, early in the history of sperm competition biology, Martin & Dziuk (1977) demonstrated that differences in probability of paternity between breeds of roosters were consistent across females, suggesting that male–male trait differences dictated probability of paternity rather than compatibility effects between the male and female genomes. These two processes could, however, operate simultaneously if, for example, some components of fertilization success are dictated by male–male interactions (e.g. differences in sperm longevity;

Hunter & Birkhead 2002; Simmons *et al.* 2007), whereas others are more influenced by male–female interaction (e.g. genetic compatibility at the major histocompatibility complex; Olsson *et al.* 2003; Roberts & Gosling 2003).

In a previous study (Sherman *et al.* 2008a), we demonstrated in artificial fertilization experiments that sperm from a more closely related male to the female had a siring advantage when in competition with a male more distantly related to the female in Peron's tree frog. Thus, male–female compatibility was a significant component in determining the probability of fertilization. However, logistic constraints (sperm number in ejaculates) made it difficult to assess fertilization success for combinations of males with more than one female (a male's ejaculate can only be divided into a few replicates without affecting fertility). Thus, those experiments did not allow us to assess consistency of male–male differences in siring success among females. We therefore designed a new set of mating trials to determine siring success of males across different females, while controlling for sperm concentration and the proportion of live/dead sperm in the ejaculates.

2. MATERIAL AND METHODS

(a) *In vitro* fertilizations and sperm competition trials

We collected adult male and female frogs (*Litoria peronii*) for *in vitro* sperm competition trials from a pond at Darkes Forest, NSW, Australia. We followed the protocol for artificial fertilization outlined by Sherman *et al.* (2008a,b). For each of 16 trials, ejaculates from two randomly chosen males were obtained for sperm competition trials with two randomly chosen females (total of 32 males and 32 females). We induced males to release sperm after a subcutaneous injection of luteinizing hormone releasing hormone (LHRH Sigma-Aldrich). Males were injected within 2 min of each other to ensure that longevity of sperm did not confound our experiments. One hundred microlitres of each male's sperm was taken to determine the proportion of viable sperm within each male's ejaculate using Live/Dead sperm viability kits (Molecular Probes). Sperm concentration for each male was determined using a Hawksley haemocytometer (four separate counts) and the sperm samples diluted to equal concentrations. An intraclass correlation coefficient, Rho (ρ), showed a high correlation between sperm counts ($\rho = 0.994$, $F_{31,93} = 163.96$, $p < 0.0001$). Two millilitres of the males' sperm solutions were mixed together and divided between two Petri dishes. The eggs from two randomly chosen females were harvested by squeezing their abdomen directly into the Petri dishes. Thus, the mixed sperm from each pair of males in a sperm competition trial were used to fertilize eggs from two different females. After 2 min, the egg/sperm mixture was flooded with 100 ml of water. After 3 h, all eggs were transferred to a 750 ml container and held at a constant temperature of 23°C until hatching.

(b) *Assignment of paternity*

After hatching, approximately 60 tadpoles per sperm competition trial (mean per trial \pm s.e., 58 ± 3.08) were collected for the assignment of paternity. A toe clip from each adult was used for DNA extraction and the assignment of paternity. Genomic DNA was isolated from whole tadpoles using Qiagen DNAeasy Tissue Kit as per the manufacturer's instructions. Nine microsatellite loci (LP01, LP03, LP04, LP05, LP07, LP13, LP19, LP22 and LP23) were used to assign paternity (Sherman & Olsson 2007). Paternity was unambiguously assigned to all offspring (1856 tadpoles) according to allele sharing between putative sires, dam and offspring.

(c) *Statistical analysis*

Using a general linear model, we investigated the relationship between the difference in siring success between male 1 and male 2 (male 1–male 2) with female 1 and the differences in (i) siring success with female 2, (ii) condition index of the two males, (iii) the number of viable sperm between males and (iv) genetic similarity between each female and each of the males. We calculated a condition index for each male based on the residuals from the regression of mass against snout–vent length ($F_{1,30} = 14.9$, $p < 0.001$). Genetic similarity

Table 1. General linear model analysis with the difference in male probability of paternity between two randomly selected males in a sperm competition trial with a second female and the difference between these two males in a number of tabulated traits. β , regression coefficient; s.e., standard error; t , t -statistic; p , probability value. Model $R^2=0.71$, $F_{1,11}=6.8$, $p=0.005$.

trait difference	β	s.e.	t	p -value
siring success with female 1	1.27	0.27	4.67	0.0007
male condition	−6.53	6.78	−0.96	0.36
live sperm	−0.24	0.16	−1.45	0.18
male–female relatedness	31.8	47.7	0.67	0.52

among individuals was determined as the proportion of shared alleles and calculated using GENALEX (V6; Peakall & Smouse 2006). All variables met the assumptions of normality and homogeneity of variances.

3. RESULTS

We first confirmed the relationship between genetic similarity of competing males in sperm competition with a female and siring success by pooling our data from this experiment ($n=16$) with our previous analysis ($n=30$; Sherman *et al.* 2008a). The means and variances of all traits analysed in these datasets were approximately equal. A linear regression analysis confirmed that the difference in genetic similarity with the female was positively related to a male's siring success with the more closely related male siring most of the offspring ($F_{1,44}=5.97$, $p=0.019$, $R^2=0.12$, $\beta=1.52\pm0.62$, s.e.).

We then performed a multiple regression analysis based on the current experiment for which we had data on male relative reproductive success with a second female. This analysis showed that the only significant predictor of male–male difference in siring success in a second sperm competition trial with a different female drawn at random was the corresponding difference in siring success between the males in a first sperm competition trial ($p=0.0007$; see table 1 for full model). After backward elimination at $p>0.25$ of traits from the model, the proportion of live sperm ($p=0.14$) and the male–male difference in siring success with the first female ($p<0.0001$) were the only traits remaining in the model, with male–male difference in siring success explaining more than 62 per cent of the variation (figure 1). To explore whether the relationship was driven by two outliers (figure 1), we reran the final model with these data points removed. This relationship remained significant after the removal of the two outliers ($p=0.039$, $R^2=0.35$) confirming the robustness of our original analysis. We also tested whether male–male relatedness (within trials) and female–female relatedness (between trials) influenced this outcome, but none of the variables were significant in these models ($p>0.70$). The proportion of viable sperm within ejaculates was high (mean \pm s.e., $84\%\pm2\%$) while relatedness estimates (mean allele sharing \pm s.e., 0.19 ± 0.1) were similar to that previously reported for this species (Sherman *et al.* 2008a).

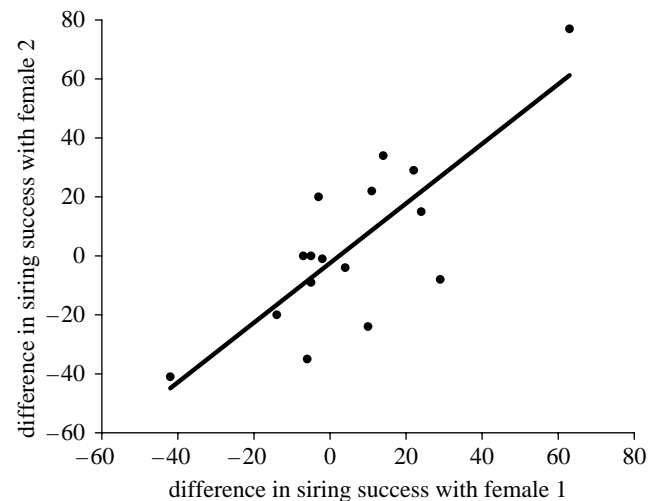


Figure 1. Relationship between the relative siring success of two males in sperm competition with females 1 and 2 ($F_{1,14}=23.3$, $p<0.0001$).

4. DISCUSSION

Our revisit of sperm competition in *L. peronii* revealed that regardless of the female genotype, male–male differences in siring success was consistent when males were mated across different females. What may explain this result? First, we can rule out the effect of the number of viable sperm within a males' ejaculate. We found that the proportion of viable sperm within a males' ejaculate was not a significant predictor of siring success in sperm competition, which is consistent with our previous study (Sherman *et al.* 2008a). Thus, although other sperm traits such as sperm motility and morphology may well contribute to the probability of paternity in many taxa (reviewed in Snook 2005), it appears highly unlikely that those traits would override the effects of, and not be captured in, sperm viability (Damiens *et al.* 2002; Hunter & Birkhead 2002; Garcia-Gonzalez & Simmons 2005). A number of studies have shown that sperm traits may be condition dependent (e.g. Urbach *et al.* 2007); however, our analysis showed that potential male condition effects on sperm performance could not explain the observed variation in siring success.

The rationale for our study was to determine factors that may influence male–male differences in paternity, in particular genetic relatedness with the female and intrinsic male quality (including good-genes effects). In our previous study, we showed strong effects of male–female relatedness on the probability of paternity. In the present study, this result was not significant by itself. However, when the data from the two studies were pooled, more closely related males still had a siring advantage. That said, the relative effect of male siring success with a first female was a much stronger predictor of siring success with a second female ($R^2>0.62$), compared with the effect of relatedness ($R^2=0.12$; combined data). Needless to say, these analyses are not immediately comparable owing to the differences in sample size, but the more than 50 per cent higher resolution of variance of the male–male difference in siring

success with a second female indicates that intrinsic male quality (potential ‘good-genes/good-sperm’ effects) plays a significant role in determining siring success in this species. Our results supports a growing number of studies showing that, at least in some species, traits contributing to fertilization success can be explained by intrinsic differences among males (e.g. Dziuk 1996; Konior *et al.* 2006). However, both additive and non-additive genetic benefits to polyandry may not necessarily be mutually exclusive, and accumulating evidence from a wide range of studies suggest that both are likely to play a role in the evolution and maintenance of polyandry (reviewed in Zeh & Zeh 2001, 2008).

All work was carried out in accordance with NPWS permit S11186 and the University of Wollongong ethics permit AE04/03-05.

This work was supported by ARC grants to M.O. and E.W. We would like to thank two anonymous reviews for their comments that helped to improve the manuscript.

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