MOLECULAR ECOLOGY

Molecular Ecology (2009) **18**, 3340–3345 doi: 10.1111/j.1365-294X.2009.04301.x

FAST TRACK

Promiscuous females avoid inbreeding by controlling sperm storage

AMANDA BRETMAN, DEVI NEWCOMBE and TOM TREGENZA

Centre for Ecology and Conservation, University of Exeter, Cornwall Campus, Penryn TR10 9EZ, UK

Abstract

Recent studies in a variety of species have shown that polyandrous females are somehow able to bias paternity against their relatives postcopulation, although how they do so remains unknown. Field crickets readily mate with their siblings, but when also mated to an unrelated male, they produce disproportionately fewer inbred offspring. We use a new competitive microsatellite polymerase chain reaction technique to determine the contribution of males to stored sperm and subsequent paternity of offspring. Paternity is almost completely predicted by how much sperm from a particular male is stored, and unrelated males contribute more sperm to storage and have a corresponding higher paternity success.

Keywords: competitive microsatellite PCR, cryptic female choice, genetic incompatibility, inbreeding avoidance, polyandry, sperm competition

Received 21 May 2009; revision accepted 23 June 2009

Introduction

One of the many proposed explanations for the almost ubiquitous phenomenon of polyandry (females mating with more than one male) is as a mechanism to avoid reproducing with genetically incompatible mates (Zeh & Zeh 1996). Although genetic incompatibility may take many forms, one that is likely to be almost ubiquitous is inbreeding depression (Tregenza & Wedell 2000). Inbreeding generally results in reduced offspring fitness (Falconer & Mackay 1996; Charlesworth & Charlesworth 1999) and mechanisms that reduce matings between relatives are well documented (Pusey & Wolf 1996). A series of recent studies have additionally suggested that females that mate to both related and unrelated males are somehow able to bias paternity against their relatives (Olsson *et al.* 1996; Stockley 1999; Kraaijeveld-Smit *et al.* 2002; Mack *et al.* 2002; Bretman *et al.* 2004; Thuman & Griffith 2005; Simmons *et al.* 2006; Jehle *et al.* 2007), though the evidence is not universal (Stockley 1997; Jennions *et al.* 2004; Denk *et al.* 2005; Lane *et al.* 2007; Evans *et al.* 2008). In species where relatedness does affect paternity, there is little information about how females might bias fertilization after copulation.

Correspondence: Tom Tregenza, Fax: +44 1326 254205;
E-mail: t.tregenza@exeter.ac.uk

Field crickets are highly polyandrous in the laboratory and field (Bretman & Tregenza 2005) and will readily mate with their siblings, despite lower viability of inbred offspring (Tregenza & Wedell 2002). Polyandry allows females to overcome this cost, as females mated to a sibling and an unrelated male achieve the same hatching success as females mated to only unrelated males (Tregenza & Wedell 2002). This appears to be because the unrelated male receives higher paternity success (Bretman *et al.* 2004). Here, we use a recently developed competitive microsatellite polymerase chain reaction (CM-PCR) technique to study how females bias paternity. CM-PCR has been used to assess relative paternity of mixed samples of offspring (Wooninck *et al.* 2000), relative contribution to sperm in different storage organs (Bussiere *et al.* 2009), and relative biomass of fungi in mixed samples (Naef *et al.* 2006). Wooninck *et al.* (2000) and Bussiere *et al.* (2009) refer to this method as competitive PCR, however to avoid confusion with quantitative real-time PCR methods, we follow Naef *et al.* (2006) in using the term 'competitive microsatellite PCR'. CM-PCR allows us to assess the individual-specific alleles in mixed samples of DNA because the relative strength of signal (sequencer peak

area) for an allele is mainly determined by the initial amount of template DNA, such that a male contributing more sperm to a sample will produce a relatively larger peak. This method therefore enables us to assess the relative contribution of individuals to mixed DNA samples, such as the sperm stored from two mates. We mated females to a sibling and an unrelated male (in either order) and determined the contribution of each male to stored sperm and subsequent paternity in a sample of offspring.

Materials and methods

Crickets originated from the vicinity of Valencia, Spain and had been kept in a large, panmictic laboratory population for 20 generations. In the laboratory, crickets were maintained under a 14L:10D photoperiod at 28 °C, and given free access to food (rodent diet) and water. Crickets were isolated as late instar nymphs to ensure virginity, and mated at 7 days postadult eclosion. To obtain full-sib families, 14 separate crosses were set up and the offspring of these crosses used. Pairs that did not mate within an hour were discarded. Males produce spermatophores before introduction to the female, so could not later alter the content of the spermatophore depending on their relatedness to the female (Hall *et al.* 2000). After mating, pairs were kept together for an hour to standardize spermatophore attachment time, since males prevented spermatophore removal by females, and this was sufficient time to ensure complete sperm transfer (Simmons 1986a). Hence 1 h after the first mating, the first male was removed and the second male introduced. After mating, males were frozen at −20 °C until DNA extraction. Females were allowed to lay eggs for 24 h after their second mating and were then immediately preserved in 100% ethanol. Offspring hatched 7–8 days after laying and a random collection of up to 30 newly emerged nymphs (mean ± SE = 27.6 ± 1.1) were frozen until DNA extraction. In all 28 matings were performed, 14 with the sibling as the first mate and 14 with the unrelated male first, such that each treatment contained one sister from each full-sib family.

DNA was extracted from adult legs and whole nymph mixtures (i.e. all offspring collected from one female combined in one extraction) using a salting out protocol (see Bretman & Tregenza 2005). Females were rehydrated for 16 h before spermathecae were dissected out and the sperm DNA immediately extracted using a chelex protocol (see Bretman & Tregenza 2005). Adult and nymph DNA was standardized to 30 ng/μL using a Nanovue (GE Healthcare).

Adults were screened with eight microsatellite loci [*Gbim*15 (Dawson *et al.* 2003); *Gbim*26, 29, 35, 48, 49, 66 and 72 (Bretman *et al.* 2008)] on an ABI 3130XL sequencer (Applied Biosystems). The CM-PCR technique requires one locus to be identified at which both males have one unique allele (i.e. not shared by the other male or the mother). Of the 28 triads mated, we could only identify unique alleles for 19 triads (nine sibling first mate) of which only 17 (eight sibling first) females laid eggs in the first 24 h. Therefore, the sperm and offspring of these triads were only genotyped at the locus at which both males had unique alleles, but this was not the same locus for all the triads.

To estimate the contribution to paternity and sperm in storage from the unrelated male, we compared relative peak areas (peak area is a standard variable calculated from the raw sequencer data by the fragment analysis software GENEMAPPER v 4.0, Applied Biosystems) of the unique alleles in these samples to standard curves derived from mixtures of each pair of males. Standard curves were made by mixing male DNA such that the unrelated male accounted for 6.25%, 12.5%, 25%, 50%, 75%, 87.5% and 93.75% of the mix. As females necessarily contributed to the offspring DNA, and potentially contaminate the sperm sample, we repeated this with the addition of a standard amount of female DNA, representing half the total DNA mixture (i.e. 1 μL female DNA at 30 ng/μL plus 1 μL mixed male DNA for the seven different male mixtures). The relative peak area of the unrelated male's allele (unrelated allele peak area/unrelated + sibling allele peak area) was used in a regression with the initial proportion. No significant differences ($P > 0.05$) were found in comparisons of the gradient and elevation of the regressions with and without female DNA (Zar 1984). The initial proportion of DNA almost entirely predicted the relative peak area (R^2 range 0.86–0.99). As we used the actual competing pair of males or full mated triads for the standards, rather than a subsample of randomly paired males representing the distribution of alleles in the population (Bussiere *et al.* 2009), no other covariates (such as relative allele size in base pairs) were added to the model. Sperm and offspring samples were genotyped with the relevant locus and solved for the pertinent regression equation.

Results

All data and residuals were normally distributed (Kolmogorov–Smirnov tests, $P > 0.05$), and variances were homogeneous (Levene's test, $P > 0.05$). Mating order had no effect on male representation in the spermatheca (ANOVA $F_{1,19} = 0.408$, $P = 0.531$) or on paternity ($F_{1,17} = 0.377$, $P = 0.548$), hence this was removed from further analysis. Unrelated males contributed significantly more than half the sample of both sperm and

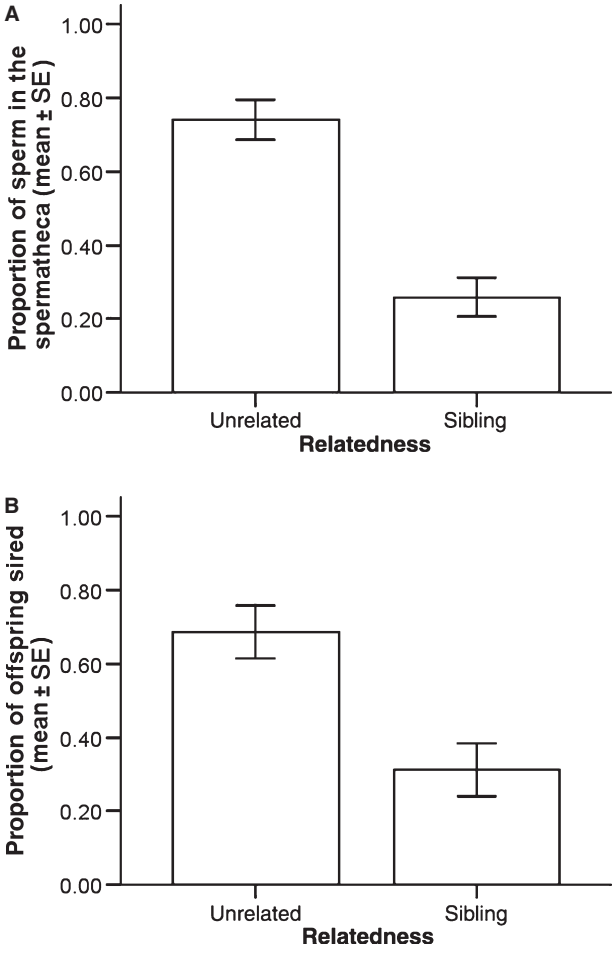

Fig. 1 Relative contribution to stored sperm and offspring paternity. Females were mated to a sibling and an unrelated male in either order. The related male contributes significantly more in (A) proportion of sperm in the spermatheca (mean ± SE) and (B) proportion of offspring sired (mean ± SE).

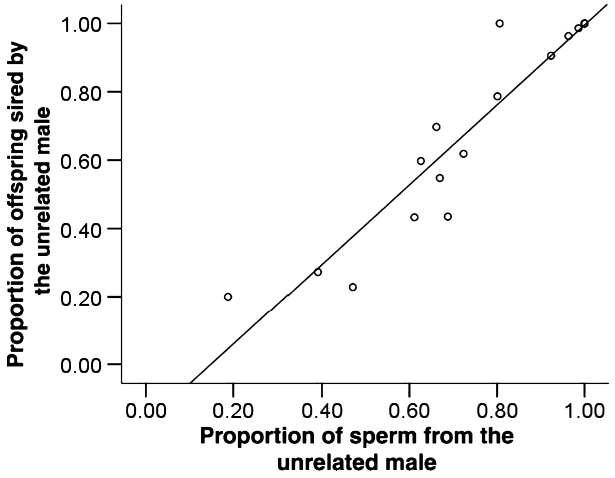

Fig. 2 Correlation between the proportion of sperm in the spermatheca and the proportion of offspring sired by the unrelated male; sperm storage almost completely predicts paternity.

offspring (mean [±standard error (SE)]; sperm, mean = 0.74 [0.06]; offspring, mean = 0.69 [0.07]. One sample *t*-tests compared to 0.5: sperm, $t_{18} = 4.46$, $P < 0.0001$: offspring, $t_{16} = 2.60$, $P = 0.019$. Fig. 1). Males with more sperm in storage had correspondingly higher paternity (Pearson $r = 0.938$, $n = 17$, $P < 0.0001$; Fig. 2).

Discussion

Our data provide evidence for a simple mechanism by which polyandry enables females to avoid the cost of inbreeding. By assessing the contribution of unrelated and related males to sperm extracted from the spermatheca, we find that sperm from unrelated males is stored in preference to that from siblings. This corresponds to paternity bias in favour of unrelated males, assessed by using the same method to genotype a mixed sample of offspring. Although crickets were kept in a large, panmictic laboratory population, some inbreeding may have occurred, however if the 'unrelated' males were more related to the females than in a fully outbred population, this would make it more remarkable that females distinguish between these males and full-sib males. In contrast to our previous findings (Bretman *et al.* 2004) in which unrelated males only achieved higher paternity when they mated first, there was no effect of mating order; the unrelated male achieved more sperm storage and higher paternity whether the first or second to mate. Further work is needed to understand this difference; crickets were from a different population to the one studied previously (from Botswana), so there may be population level variation in the extent of sperm precedence due to mating order.

The difference in the number of offspring sired by related and unrelated males could also have been driven by differential mortality of offspring. Tregenza & Wedell (2002) found that hatching success of inbred offspring was half that of outbred offspring, hence in Bretman *et al.* (2004) a correction was made for this in the calculation of offspring sired by the unrelated male. We did not make such a correction here as we have evidence that the manifestation of inbreeding depression can differ dramatically between populations of the same species. R. Rodríguez-Muñoz and T. Tregenza (unpublished) found no effect of inbreeding on hatching success in a population of *Gryllus bimaculatus* from Seville, Spain, in contrast to that found by Tregenza & Wedell (2002) in a population from Botswana. Population level differences in the costs of inbreeding have been demonstrated in other taxa (Frankel & Soule 1981; Thornhill 1993). The population in this study came from Valencia, Spain. Given the very strong correlation between sperm stored and offspring sired in this study, it is unlikely

that differential mortality had a large effect on our results. In addition, inbreeding could result in a difference in size between inbred and outbred offspring which would bias the calculation of relative contribution (as larger nymphs would contribute more DNA to the mixed extraction), however, we minimized this possibility by sampling newly emerged nymphs.

Studies similar to this one have been criticized because they cannot control pre- and postcopulatory behavioural mechanisms (Evans *et al.* 2008). However, crickets have a particular advantage for this type of study in that we can allow them to mate naturally and still rule out bias being due to males varying ejaculate size. As males had already produced the spermatophore before introduction to the female, they could not alter investment to transfer fewer sperm to their sisters (Hall *et al.* 2000). Spermatophore attachment time has been previously suggested as a way for females to exert choice (Simmons 1986b), however, as we standardized attachment time this could not have influenced sperm storage. Interestingly, the two existing studies that explicitly control for prefertilization behaviours through artificial insemination found no paternity bias (Denk *et al.* 2005; Evans *et al.* 2008), which perhaps indicates that contact with males is generally necessary for the females to assess male relatedness, or that behaviour such as differential sperm storage is a more parsimonious mechanism than cell–cell interactions.

Existing evidence for how polyandry might allow females to reduce the costs of inbreeding comes from only two studies. Pseudoscorpions with brood care can abort entire inbred broods (Zeh & Zeh 2006), and polyandry rescues inbred offspring because spontaneous abortion is suppressed in broods containing a mixture of inbred and outbred offspring. However, within mixed broods, paternity is actually biased in favour of related fathers, therefore, this mechanism increases reproductive success by reducing abortion and not by biasing paternity in favour of unrelated males. In single matings, female jungle fowl eject sperm from related males (Pizzari *et al.* 2004), however, it is unknown whether this leads to a paternity bias when siblings and unrelated males are in competition. Indeed, males actually increase the amount of sperm transferred when mating with sisters (Pizzari *et al.* 2004). Apart from relatedness, various male characteristics have been shown to influence sperm storage. For example, in chickens females store more sperm from dominant males (Pizzari & Birkhead 2000) and in *Drosophila simulans* females store more sperm from males lacking meiotic drivers (Angelard *et al.* 2008). Sperm storage or rejection could therefore provide a general mechanism for female choice where rejection of mating is costly, or where precopulatory choosiness is more costly than postcopulatory choice or where close contact with males is required to assess the relevant traits.

Our experiment also provides direct evidence that sperm numbers determine paternity success. The idea of the raffle is central to sperm competition theory (Parker *et al.* 1997; Parker 1998). There is abundant evidence across many taxa that males adjust sperm number when faced with varying competition (Wedell *et al.* 2002). For example, a greater number (or more viable) sperm are ejaculated when mating with nonvirgin females (Cook & Wedell 1996; Thomas & Simmons 2007), when males experience rivals before or during copulation (Gage 1991; Candolin & Reynolds 2002; Pilastro *et al.* 2002; Pizzari *et al.* 2003; del Barco-Trillo & Ferkin 2004; del Barco-Trillo & Ferkin 2007; Pound & Gage 2004) or when males employ alternative mating tactics (Vladić & Järvi 2001). In yellow dung-flies, under enforced monogamy or polyandry, males in polyandrous lines evolved larger testis and therefore increased potential sperm production (Hosken *et al.* 2001). The link between paternity and sperm number has been demonstrated through artificial insemination (Martin *et al.* 1974), and indirectly using experimentally manipulated lines in which males produce few, large or many, small sperm (Gage & Morrow 2003). We have shown a remarkably tight correlation between sperm number and paternity, which to our knowledge this is the first time that this relationship been directly demonstrated in natural matings.

The offspring genotyped in this study were from the first 24 h of egg laying. In our previous study (Bretman *et al.* 2004), females were allowed to lay for 3 days, and so this could contribute to the difference between our current and previous findings. Female *G. bimaculatus* can lay for up to 3 weeks, and hence this will represent a fraction of the full progeny. However, most females laid substantially more eggs than the sample of offspring taken (up to 200). There is a trade-off between having a representative sample of offspring and being able to genotype the stored sperm. Leaving females for longer than 24 h could have compromised the ability to amplify the sperm (already a forensically small sample) and would also raise doubts over whether the sperm contents remained properly representative of what was stored initially. Nevertheless, further investigation of the change in sperm usage patterns over time would be far easier using this CM-PCR method.

The CM-PCR technique that we employ is clearly a powerful tool that could be applied to many situations, allowing samples that necessarily contain mixed DNA (such as sperm stored from two males) to be analysed, and saving the researcher considerable time and money. It is remarkable that this technique, first proposed by Wooninck *et al.* (2000) has remained unused (or at least

uncited) until being updated by Bussiere *et al.* (2009). We advocate its use and anticipate that it will be very fruitful for future research. One important caveat is that neither we nor previous studies (Wooninck *et al.* 2000; Bussiere *et al.* 2009) have investigated whether CM-PCR can be used in cases where more than two males are in competition. However, Naef *et al.* (2006) were able to identify and quantify four different strains of fungi in a mixed sample. Another limitation is that it is necessary to be able to genotype all individuals involved, therefore that is not useful for mixed sperm from wild caught females (for example as in Bretman & Tregenza 2005). It is important to note that this method does not actually quantify DNA, like quantitative PCR, but can employ existing markers to assess relative contributions (microsatellites are now very widely available; nearly 240 000 loci have been submitted to the NCBI database).

In conclusion, our data show that females mated to a sibling and an unrelated male can preferentially store sperm from the unrelated male, and that the relative amount of sperm in storage accurately predicts paternity. This bias must be a female effect as males produced their spermatophores in advance of introduction to the female. This is the first demonstration that females can manipulate sperm storage to avoid inbreeding, revealing a simple process of cryptic female choice that may apply across taxa.

Acknowledgements

We thank Dave Hosken, Nick Royle, Thor Veen and Clarissa House, Robert Wayne and two anonymous referees for comments on the manuscript, and Luc Bussiere for allowing us early access to the competitive PCR technique. A.B. was funded by the NERC and T.T. by a Royal Society Fellowship.

References

Angelard C, Montchamp-Moreau C, Joly D (2008) Female-driven mechanisms, ejaculate size and quality contribute to the lower fertility of sex-ratio distorter males in *Drosophila simulans*. *BMC Evolutionary Biology*, **8**, 326–337.

Bretman AJ, Tregenza T (2005) Measuring polyandry in wild populations: a case study using promiscuous crickets. *Molecular Ecology*, **14**, 2169–2179.

Bretman A, Wedell N, Tregenza T (2004) Molecular evidence of post-copulatory inbreeding avoidance in the field cricket *Gryllus bimaculatus*. *Proceedings of the Royal Society of London. Series B: Biological Sciences*, **271**, 159–164.

Bretman A, Dawson DA, Horsburgh GJ, Tregenza T (2008) New microsatellite loci isolated from the field cricket *Gryllus bimaculatus* characterized in two cricket species, *Gryllus bimaculatus* and *Gryllus campestris*. *Molecular Ecology Resources*, **8**, 1015–1019.

Bussiere LF, Demont D, Pemberton AJ, Hall MD, Ward PI (2009) The assessment of insemination success in yellow dung flies using competitive PCR. *Molecular Ecology Resources* (in press).

Candolin U, Reynolds JD (2002) Adjustments of ejaculation rates in response to risk of sperm competition in a fish, the bitterling (*Rhodeus sericeus*). *Proceedings of the Royal Society of London. Series B: Biological Sciences*, **269**, 1549–1553.

Charlesworth B, Charlesworth D (1999) The genetic basis of inbreeding depression. *Genetical Research*, **74**, 329–340.

Cook PA, Wedell N (1996) Ejaculate dynamics in butterflies: a strategy for maximizing fertilization success? *Proceedings of the Royal Society of London. Series B: Biological Sciences*, **263**, 1047–1051.

Dawson DA, Bretman AJ, Tregenza T (2003) Microsatellite loci for the field cricket *Gryllus bimaculatus* and their cross-utility in other species of Orthoptera. *Molecular Ecology Notes*, **3**, 191–195.

del Barco-Trillo J, Ferkin MH (2004) Male mammals respond to a risk of sperm competition conveyed by odours of conspecific males. *Nature*, **431**, 446–449.

del Barco-Trillo J, Ferkin MH (2007) Increased sperm numbers in the vas deferens of meadow voles, *Microtus pennsylvanicus*, in response to odors of conspecific males. *Behavioral Ecology and Sociobiology*, **61**, 1759–1764.

Denk AG, Holzmann A, Peters A, Vermeirssen ELM, Kempenaers B (2005) Paternity in mallards: effects of sperm quality and female sperm selection for inbreeding avoidance. *Behavioral Ecology*, **16**, 825–833.

Evans JP, Brooks RC, Zajitschek SRK, Griffith SC (2008) Does genetic relatedness of mates influence fertilization success in guppies? *Evolution*, **62**, 2929–2935.

Falconer DS, Mackay TFC (1996) *Introduction to Quantitative Genetics*, 4th edn. Longman, New York.

Frankel OH, Soule ME (1981) *Conservation and Evolution*. Cambridge University Press, Cambridge.

Gage MJG (1991) Risk of sperm competition directly affects ejaculate size in the Mediterranean fruit-fly. *Animal Behaviour*, **42**, 1036–1037.

Gage MJG, Morrow EH (2003) Experimental evidence for the evolution of numerous, tiny sperm via sperm competition. *Current Biology*, **13**, 754–757.

Hall MD, Beck R, Greenwood M (2000) Detailed developmental morphology of the spermatophore of the Mediterranean field cricket, *Gryllus bimaculatus* (De Geer) (Orthoptera : Gryllidae). *Arthropod Structure & Development*, **29**, 23–32.

Hosken DJ, Garner TWJ, Ward PI (2001) Sexual conflict selects for male and female reproductive characters. *Current Biology*, **11**, 489–493.

Jehle R, Sztatecsny M, Wolf JBW, Whitlock A, Burke T (2007) Genetic dissimilarity predicts paternity in the smooth newt (*Lissotriton vulgaris*). *Biology Letters*, **3**, 526–528.

Jennions MD, Hunt J, Graham R, Brooks R (2004) No evidence for inbreeding avoidance through postcopulatory mechanisms in the black field cricket, *Teleogryllus commodus*. *Evolution*, **58**, 2472–2477.

Kraaijeveld-Smit FJL, Ward SJ, Temple-Smith PD, Paetkau D (2002) Factors influencing paternity success in *Antechinus agilis*: last-male sperm precedence, timing of mating and genetic compatibility. *Journal of Evolutionary Biology*, **15**, 100–107.

Lane JE, Boutin S, Gunn MR, Slate J, Coltman DW (2007) Genetic relatedness of mates does not predict patterns of

parentage in North American red squirrels. *Animal Behaviour*, **74**, 611–619.
Mack PD, Hammock BA, Promislow DEL (2002) Sperm competitive ability and genetic relatedness in *Drosophila melanogaster*: similarity breeds contempt. *Evolution*, **56**, 1789–1795.
Martin PA, Reimers TJ, Lodge JR, Dzuik PJ (1974) The effect of ratios and numbers of spermatozoa mixed frim two males on proportions of offspring. *Journal of Reproduction and Fertility*, **39**, 251–258.
Naef A, Senatore M, Defago G (2006) A microsatellite based method for quantification of fungi in decomposing plant material elucidates the role of *Fusarium graminearum* DON production in the saprophytic competition with *Trichoderma atroviride* in maize tissue microcosms. *FEMS Microbiology Ecology*, **55**, 211–220.
Olsson M, Shine R, Madsen T, Gullberg A, Tegelström H (1996) Sperm selection by females. *Nature*, **383**, 585.
Parker GA (1998) Sperm competition and the evolution of ejaculates: towards a theory base. In: *Sperm Competition and Sexual Selection* (eds Birkhead TR, Møller AP), pp. 3–54. Academic Press, London.
Parker GA, Ball MA, Stockley P, Gage MJG (1997) Sperm competition games: a prospective analysis of risk assessment. *Proceedings of the Royal Society of London. Series B: Biological Sciences*, **264**, 1793–1802.
Pilastro A, Scaggiante M, Rasotto MB (2002) Individual adjustment of sperm expenditure accords with sperm competition theory. *Proceedings of the National Academy of Sciences of the United States of America*, **99**, 9913–9915.
Pizzari T, Birkhead TR (2000) Female feral fowl eject sperm of subdominant males. *Nature*, **405**, 787–789.
Pizzari T, Cornwallis CK, Løvlie H, Jakobsson S, Birkhead TR (2003) Sophisticated sperm allocation in male fowl. *Nature*, **426**, 70–74.
Pizzari T, Lovlie H, Cornwallis CK (2004) Sex-specific, counteracting responses to inbreeding in a bird. *Proceedings of the Royal Society of London. Series B: Biological Sciences*, **271**, 2115–2121.
Pound N, Gage MJG (2004) Prudent sperm allocation in Norway rats, *Rattus norvegicus*: a mammalian model of adaptive ejaculate adjustment. *Animal Behaviour*, **68**, 819–823.
Pusey A, Wolf M (1996) Inbreeding avoidance in animals. *Trends in Ecology and Evolution*, **11**, 201–206.
Simmons LW (1986a) Female choice in the field cricket *Gryllus bimaculatus* (de Geer). *Animal Behaviour*, **34**, 1463–1470.
Simmons LW (1986b) Intermale competition and mating success in the field cricket, *Gryllus bimaculatus* (de Geer). *Animal Behaviour*, **34**, 567–579.
Simmons LW, Wedell N, Beveridge M, Tregenza T (2006) Post-copulatory inbreeding avoidance by female crickets only revealed by molecular markers. *Molecular Ecology*, **15**, 3817–3824.
Stockley P (1997) No evidence of sperm selection by female common shrews. *Proceedings of the Royal Society of London. Series B: Biological Sciences*, **264**, 1497–1500.
Stockley P (1999) Sperm selection and genetic incompatibility: does relatedness of mates affect male success in sperm competition? *Proceedings of the Royal Society of London. Series B: Biological Sciences*, **266**, 1663–1669.
Thomas ML, Simmons LW (2007) Male crickets adjust the viability of their sperm in response to female mating status. *The American Naturalist*, **170**, 190–195.
Thornhill NW (1993) *The Natural History of Inbreeding and Outbreeding: Theoretical and Empirical Perspectives*. University of Chicago Press, Chicago.
Thuman KA, Griffith SC (2005) Genetic similarity and the nonrandom distribution of paternity in a genetically highly polyandrous shorebird. *Animal Behaviour*, **69**, 765–770.
Tregenza T, Wedell N (2000) Genetic compatibility, mate choice and patterns of parentage. *Molecular Ecology*, **9**, 1013–1027.
Tregenza T, Wedell N (2002) Polyandrous females avoid costs of inbreeding. *Nature*, **415**, 71–73.
Vladić TV, Järvi T (2001) Sperm quality in the alternative reproductive tactics of Atlantic salmon: the importance of the loaded raffle mechanism. *Proceedings of the Royal Society of London. Series B: Biological Sciences*, **268**, 2375–2381.
Wedell N, Gage MJG, Parker GA (2002) Sperm competition, male prudence and sperm limited females. *Trends in Ecology and Evolution*, **17**, 313–320.
Wooninck LM, Warner RR, Fleischer RC (2000) Relative fitness measures with competitive PCR. *Molecular Ecology*, **9**, 1409–1414.
Zar JH (1984) *Biostatistical Analysis*. Prentice-Hall, New Jersey.
Zeh JA, Zeh DW (1996) The evolution of polyandry I: intragenomic conflict and genetic incompatibility. *Proceedings of the Royal Society of London. Series B: Biological Sciences*, **263**, 1711–1717.
Zeh JA, Zeh DW (2006) Outbred embryos rescue inbred half-siblings in mixed-paternity broods of live-bearing females. *Nature*, **439**, 201–203.

A.B. uses molecular techniques to answer behavioural questions related to sexual selection and polyandry in insects. D.N. was an undergraduate student on this project and is about to start a PhD on maternal effects in milkweed bugs. T.T. uses insects and other model systems in the hope of improving our understanding of speciation and the evolutionary consequences of sexual reproduction.
