E&EB 240: Animal Behavior
Professor Suzanne Alonzo
Teaching Fellow: Andrea Hodgins-Davis

By submitting this essay, I attest that it is my own work, completed in accordance with University regulations. —Tse Yang LIM

Fly Sex: Adaptive manipulation of offspring sex ratio as a means of ameliorating sexual conflict in Drosophila melanogaster

by Tse Yang Lim

Introduction

Sexual selection was recognised as early as Darwin (1871) as an important component of evolution. It arises chiefly from competition between members of one sex, typically the males, for access to members of the other sex (Darwin 1871), or more specifically for access to fertile gametes of the other sex (Eberhard 1996). Traditionally, sex between mating partners has been considered an essentially harmonious and cooperative affair (Chapman et al. 2003, Arnqvist & Rowe 2005). This holds true in situations of true lifelong monogamy, but such situations are far more infrequent than Darwin had assumed (Arnqvist & Rowe 2005). More commonly, because mating partners are almost always genetically different and therefore have divergent genetic interests, sexual conflict can arise (Chapman et al. 2003, Parker 2006).

Sexual conflict is a specific type of evolutionary conflict of interests (Parker 2006). It occurs whenever the sexes differ over either the optimal value of or direction of selection on a trait controlled by the same locus in both sexes (intra-locus sexual conflict), or over the optimal outcome of an interaction such as parental investment or mating rate (inter-locus sexual conflict) (Arnqvist & Rowe 2005). An important precondition for sexual conflict is that the optima for each sex cannot be achieved simultaneously, such as by sex-limitation (Parker 2006). Human hip width is an example of intra-locus sexual conflict (Price & Hosken 2007) – the optimum width for females is wider to allow them to give birth, but narrower for males to allow more efficient

locomotion. If males and females share the genes for hip width, then they cannot both achieve their respective optimum hip widths simultaneously, unless some mechanism such as hormonal or epigenetic control evolves to allow different expression of hip-width genes in males and females.

Inter-locus sexual conflict arises when traits that increase the fitness of one sex decrease the fitness of the other sex (Parker 2006). In such a situation, the other sex is expected to evolve counter-adaptations to ameliorate these fitness costs (Rice 1996, Holland & Rice 1999, Cordero & Eberhard 2003, Arnqvist & Rowe 2005). Models of such sexually antagonistic coevolution include the sexual conflict model for costly female choice of Gavrilets et al. (2001) and the 'chase-away' sexual selection model of Holland & Rice (1998).

One interesting but largely ignored way in which such conflict mediation can occur is through parental control of offspring sex ratio (Cordero & Eberhard 2003). Trivers & Willard (1973) first proposed that parents should bias offspring sex ratio to maximise fitness (in the currency of grandchildren), based on the projected fitness of sons and daughters, leading to deviations from a 50/50 offspring sex ratio. Evidence for this hypothesis abounds, but most experiments focus on offspring sex ratio bias based on maternal condition or social rank (e.g. Nager et al. 1999) rather than as a form of conflict mediation. Only a handful of studies show sex ratio bias based on mate choice or condition, but their results are tantalising.

For instance, Calsbeek & Bonneaud (2008) showed that polyandrous female brown anoles, *Anolis sangrei*, differentially utilised sperm from different-sized males. They found that larger males sired more sons relative to daughters, and vice-versa for smaller males. Because these sex-ratio differences only occurred when females mated multiply, they concluded that this sex ratio bias was due to cryptic sperm choice by females. In addition, increased body size was

found to be positively correlated with fitness in males, but negatively correlated with fitness in females. Selectively sorting sperm by sire body size thus reduced the potential for intra-locus sexual conflict, resulting from different optimum body sizes for male and female offspring (Calsbeek & Bonneaud 2008). A similar result was found in the side-blotched lizard *Uta stansburiana*, in which fitness was once again differently correlated with body size for males and females (Calsbeek & Sinervo 2004). As expected, larger males sired relatively more sons and smaller males relatively more daughters, indicating that sperm sorting by females may be acting to mediate intra-locus conflict over body size (Calsbeek & Sinervo 2004).

Another example of adaptive offspring sex manipulation occurs in the fruit fly *Drosophila melanogaster*, in which older males produce more daughters and younger males produce more sons (Mange 1970, Long & Pischedda 2005). The fitness of sons from older sires is significantly lower than the fitness of sons sired by younger males, whereas fitness of daughters has a zero or positive correlation with sire age. The observed sex ratio bias in offspring is thus in accordance with the sex-allocation model of Trivers & Willard, and serves to partially resolve intra-locus conflict (Long & Pischedda 2005).

A fascinating twist on the Trivers & Willard hypothesis can occur in species in which inter-locus conflict occurs, particularly conflict over female reproductive output or male fertilisation rate. Such conflict often arises because the optimum female mating rate or reproductive output per mating is typically lower than that for males (Bateman 1948, Wigby & Chapman 2004), or because male adaptations for increased fertilisation success (e.g. advantages in sperm competition) can impose direct fitness costs on females (e.g. Rice 1996, Arnqvist & Rowe 2005). Females in such conflict scenarios may nonetheless benefit from having sons with

greater ability to manipulate females, despite any direct fitness costs (Cordero & Eberhard 2003, Eberhard 2005) – they may, as Eberhard (2005) puts it, 'gain by losing'.

In the case of sperm competition, females may practice polyandry despite direct costs of multiple mating if this results in sons which produce 'sexy sperm', and therefore have higher fertilisation success in turn (Keller & Reeve 1995, Jennions & Petrie 2000, Evans & Simmons 2008). Based on the Trivers & Willard sex-allocation model, female fitness should be further increased by producing more sons than daughters from 'sexy sperm'. In species where females exhibit such offspring sex manipulation ability, we would thus expect this differential sex allocation pattern to evolve.

More specifically, to be able to test this idea on a given species, it must meet the following criteria: 1) females must be naturally polyandrous; 2) multiple mating must impose a fitness cost (decreased survival or lifetime reproductive success) on the female involved; 3) variation in male fertilisation success must be heritable; and 4) females must be physiologically capable of biasing offspring sex ratios. If a species fulfils these four criteria, then adaptive sex ratio manipulation could occur. Conveniently, *Drosophila melanogaster* is just such a species, providing an excellent model system with which to test this hypothesis.

Polyandry in *D. melanogaster* is well established and forms the basis for hundreds of studies (e.g. Bateman 1948, Gromko & Pyle 1978, Rice 1996); we need not explore it further here. Polyandry is essential simply because sperm competition, and hence selection for 'sexy sperm', cannot exist without it (Evans & Simmons 2008).

Rice (1996) established that female mortality increases with mating rate, fulfilling our second criterion. Mating-induced mortality is an effect of male accessory gland products found in the seminal fluid (Chapman et al. 1995, Wolfner 1997). Besides being toxic to females, these

accessory gland products aid a male's sperm in sperm competition. They also increase female egg-laying rate and decrease her re-mating rate. The function of these accessory gland products in male manipulation of females is thus of clear benefit to males, while imposing a direct fitness cost on females (Chapman et al. 1995, Wolfner 1997). Females are in sexually antagonistic coevolution with males, continually increasing resistance to male manipulation and harm. Experimentally preventing this coevolution led to increased female mortality, as female resistance no longer kept pace with male accessory gland product toxicity (Rice 1996), further supporting the idea that this is a case of sexual conflict.

In a follow-up study, Holland & Rice (1999) demonstrated that enforced monogamy reduces both female resistance and male manipulative ability. For instance, monogamous females mated with control (promiscuous) males suffered increased mortality than did control females. In a monogamous situation, the fitness of each partner is identical to and dependent on that of the other partner, so sexual conflict is eliminated (Holland & Rice 1999). This result thus shows that male harm and female resistance are indeed the outcome of sexual conflict. In addition, both this result and that of Rice (1996) show that male manipulation ability is heritable to a substantial degree. A number of other studies have shown that male fertilisation success and ability to bias paternity in polyandrous situations is heritable (reviewed in Jennions & Petrie 2000), thus fulfilling our third criterion.

Finally, female *Drosophila* are known to be able to bias offspring sex ratios, and so meet our fourth requirement as well (Mange 1970, Long & Pischedda 2005). Fuller & Mousseau (2007) also found considerable deviations from a 50/50 sex ratio in the offspring of individual female *D. melanogaster*. Females allowed to choose their mates exhibited greater deviations in offspring sex ratio than control females with no mate choice permitted. These results indicate

that sex ratio manipulation is an adaptive response by females to some property or other of their mates (Fuller & Mousseau 2007). Considering that intra-locus sexual conflict is very common in *Drosophila* (Chippindale et al. 2001, Price & Hosken 2007), this result is unsurprising.

In addition to meeting the four criteria needed if we are to see adaptive sex ratio bias towards 'sexy sperm' producing sons, *D. melanogaster* has the added advantages of being one of the most thoroughly studied organisms known. After a century of work, we know its full genome (see Adams et al. 2000), and laboratory protocols for studying sexual conflict in *Drosophila* are well established (see Rice et al. 2006). This makes *D. melanogaster* an ideal model organism in which to test our hypothesis.

In summary, I postulate that in polyandrous *D. melanogaster*, females adaptively bias offspring sex ratios to gain indirect fitness benefits, as predicted in Cordero & Eberhard (2003) and elsewhere. This will be tested by examining how offspring sex ratios co-vary with paternity bias. The more competitive a male's sperm (and hence the more paternity is biased towards him), the more females should bias the sex ratios of his offspring towards males. I also predict that adaptive sex ratio manipulation is an evolved female response – a means of ameliorating sexual conflict – and thus should occur less in crosses with monogamous lines, which have decreased levels of sexual conflict. This would allow a further refinement and application of the Trivers & Willard sex-allocation model, and shed new light on the current debate over the benefits of polyandry and the 'sexy sperm' hypothesis (see Keller & Reeve 1995).

Methods

The key to testing this prediction lies in the reduction of sexual conflict through enforced monogamy and random mating, as established by Holland & Rice (1999). As explained above, this equalises the fitness of male and female mating partners. Selection will therefore favour

males which are *less* harmful, and after several generations (approximately 35-40), both male harm and female resistance will be significantly decreased compared to a population mating in a natural, promiscuous manner (Holland & Rice 1999). As both ability in sperm competition and harm inflicted on females are linked to the accessory gland products in the seminal fluid (Chapman et al. 1995, Rice 1996), males from monogamous lines should also produce sperm with reduced competitive ability.

Using both monogamous and promiscuous populations of *Drosophila* should thus provide us with males with greater differences in sperm competitiveness than males from a single promiscuous population, and females with or without the ability to resist or ameliorate male manipulation or harm.

Experimental Populations

A laboratory population of *D. melanogaster* will be maintained using the 'laboratory island' approach, detailed (along with its advantages) in Rice et al. (2006). This starting population will first be divided randomly into two sub-populations with equal sex ratios. Into one of these two sub-populations, a recessive autosomal marker (*bw*) which conveys a brown-eyed phenotype will be introgressed by repeated backcrossing (see Chippindale et al. 2001). The other sub-population will have the 'scraggly' or rd^s marker introgressed in the same way. Both these markers produce readily visible phenotypes in homozygotes, are largely benign, and have a minimal effect on fertility and reproduction compared to the wild type (Carver 1937, Long & Pischedda 2005).

From each sub-population, 500 males and 500 females will be sampled. The virgin offspring of these 1000 flies will be randomly assigned to 2 treatment groups, one monogamous (MN) and one promiscuous (PR), and maintained for 35 generations (see Holland & Rice 1999

for details). Note that for the remainder of the experiment, any comparisons or crosses of MN or PR *Drosophila* will be *between* sub-populations – with comparisons between MN-*bw* and PR-*rd*^{s} flies as one replicate of the experiment, and comparisons between MN-*rd*^{s} and PR-*bw* flies as a crossover replicate to control for any effects of the *bw* and *rd*^{s} markers. The treatments and data collection for each crossover replicate will be identical (except in the determination of paternity using the recessive markers), so the remainder of this section will detail only the methods for one replicate (MN-*bw* and PR-*rd*^{s}, which will be referred to here largely as just MN and PR).

The Crosses

Note: Unless otherwise stated, protocols for conducting the crosses and the collection and counting of progeny from crosses will follow those of Long & Pischedda (2005). In addition, both males and females in crosses will be controlled for age, size, external morphology (except for the *bw* and *rd*^{s} phenotypes), and health (e.g. minimal parasite load), since these are known to affect offspring sex ratios (Mange 1970, Long & Pischedda 2005, Fuller & Mousseau 2007). Also, because female mate choice and mating behaviour is affected by learning and exposure to courting males (Dukas 2005), females used in crosses will be isolated from the moment of hatching and therefore be naïve at mating. As any given experimental female is mated only once or twice, with the second mating immediately following the first (and thus before learning has time to occur), the role of learned mate choice will be minimal. Finally, in any cross involving two males – one MN-*bw* and one PR-*rd*^{s} – mating with a female of either type, paternity of the offspring will be easily determinable based on the expression of the *bw* and *rd*^{s} phenotypes. In a cross with a MN-*bw* female, for instance, offspring sired by the MN-*bw* male will express the brown-eyed *bw* phenotype but those sired by the PR-*rd*^{s} male will not; conversely, in a cross with a PR-*rd*^{s} female, offspring sired by the PR-*rd*^{s} male will show the 'scraggly' phenotype and

those sired by the MN-*bw* male will not. This allows for easy determination of paternity in all such crosses.

The experiment will involve three main sets of crosses, along with various control crosses. The first set of crosses will be a pilot, aimed at confirming that PR males perform better in sperm competition than MN males, as predicted above. The next set of crosses will test whether PR females are able to bias offspring sex ratios based on paternity, and the final set will test whether MN females have the same ability. We shall now examine each of these types of crosses in detail.

1) Sperm Competition Assessment

To assess the performance of MN and PR males in sperm competition, 50 pairs of males, each with one MN and one PR male, will be sampled from the relevant groups. Each pair of males will mate with 10 MN females and 10 PR females. As second-male sperm precedence is common in *D. melanogaster* (Gromko et al. 1984), a reciprocal design is necessary, with the MN male of each pair mating first in 5 of the matings with MN females and 5 with PR females, and the PR males mating first in the rest. To minimise order effects such as sperm depletion, the order of matings will be randomised. Eggs laid by each female within the first 24 hours will be isolated and incubated until the offspring can be assessed for paternity and sexed (see above). Second-male paternity (P_2) values (i.e. the percentage of offspring sired by the second male to mate) will be recorded for each cross.

One-tailed t-tests* will be performed for each male pair to test for the repeatability of P_2 values for the females inseminated by a given male pair, and thereby determine how much of the variance in P_2 values is attributable to differences between males of a pair, i.e. sperm competitive

* Since we predict, based on strong evidence from previous studies, that PR males will have *higher* (not just different) rates of paternity than MN males, a one-tailed test may be used here.

ability and other male effects (see Birkhead 1998). An ANOVA will also be performed on the entire data set to determine how much of the variance in P_2 values is attributable to overall differences between MN and PR males.

2) Sex Ratio Manipulation – PR Females

For the second set of crosses, 100 MN-PR pairs of males will be sampled, and each pair will be mated with 10 PR females in a reciprocal pattern, again in randomised order. Eggs laid by each female within the first 24 hours of each cross, and each subsequent 24 hours thereafter, will be isolated and incubated until the offspring can be assessed for paternity and sexed (see above, also Long & Pischedda 2005). P_2 values for each female, as well as the number of male and female offspring sired by each male with each female, will be recorded for every cross. These values will be recorded both for eggs laid within the first 24 hours and for total eggs laid by each female.

As a control, each male in the 100 pairs will be mated individually with 5 PR females, and the sex ratios of offspring produced (both within the first 24 hours and overall) will be recorded.

Deviations from an offspring sex ratio of 0.50 will be standardised using a binomial distribution computed from the number of male and female offspring from each pairing (see Fuller & Mousseau 2007). I will use ANCOVA to examine whether sex ratio deviation co-varies with P_2. These computations will be performed on both the first-24-hr data and the total offspring data.

3) Sex Ratio Manipulation – MN Females

The third set of crosses will be similar to the second set, except that a fresh sample of 100 MN-PR male pairs will be taken, and each pair mated with 10 MN females instead. Once again,

P_2 values for each female, as well as the number of male and female offspring sired by each male with each female, will be recorded for every cross. These values will be recorded both for eggs laid within the first 24 hours and for total eggs laid by each female. The control will be performed using 5 MN females per male, and offspring sex ratios will be recorded for the first 24 hours and in total. The data will be standardised and analysed just as for the second set.

Expected Results

1) Sperm Competition Assessment

There is considerable evidence that performance in sperm competition and degree of harm or manipulation inflicted on females are correlated (see especially Holland & Rice 1999; also Chapman et al. 1995, Wigby & Chapman 2004, Friberg 2005). I thus firmly expect that in the pilot crosses, P_2 values will be significantly higher ($p<0.05$) for PR males than for MN males, for crosses with both MN and PR females (see Box 1). In addition, the data will be monitored for order effects, especially sperm limitation, which can be a major confounding factor in sperm competition studies (García-González 2004). Sperm or ejaculate depletion after multiple successive matings occurs in many *Drosophila* species such as *D. pachea* (Pitnick & Markow 1994). In *D. melanogaster*, ejaculate depletion rates may vary based on operational sex ratio (Linklater et al. 2007). Since OSR is different for MN and PR populations, ejaculate depletion may cause considerable variation in paternity between earlier and later matings. Sperm limitation can be detected statistically by monitoring sex ratio (see Fuller & Mousseau 2007). Should order effects like ejaculate depletion consistently observed, it may be necessary to correct for them in the later crosses, such as by decreasing the number of crosses per male and instead increasing the number of males or male pairs used.

Box 1: Sperm Competition Assessment

Note: All statistical analyses presented throughout this paper were carried out using Minitab® 15.1.1.0.

The hypothetical results presented here illustrate the prediction that PR males are more successful in sperm competition than MN males, and hence have higher P_2 values – comparing both within a male pair and between 5 male pairs (a subset of the 50 to be tested).

a) 1-tailed t-test for P_2 within a male pair

The mean P_2 for the MN male in the pair is significantly lower than mean P_2 for the PR male ($T = -8.88$, $p = 0.000$)

Two-sample T for MN1 vs PR1

	N	Mean	StDev	SE Mean
MN1	10	0.6700	0.0576	0.018
PR1	10	0.8733	0.0439	0.014

T-Test of difference = 0 (vs <): **T-Value = -8.88 P-Value = 0.000** DF = 18
Both use Pooled StDev = 0.0512

b) ANOVA for P_2

Nested analysis of variance conducted on 5 male pairs shows that over 80% of the variance in P_2 is attributable to the difference between MN and PR males ($p = 0.000$).

Analysis of Variance for P2

Source	DF	SS	MS	F	P
2nd Male	1	1.0609	1.0609	429.128	0.000
Pair	8	0.0198	0.0025	0.481	0.866
Error	90	0.4621	0.0051		
Total	99	1.5428			

Variance Components

Source	Var Comp.	**% of Total**	StDev
2nd Male	0.021	**80.48**	0.145
Pair	-0.000	**0.00**	0.000
Error	0.005	**19.52**	0.072
Total	0.026		0.162

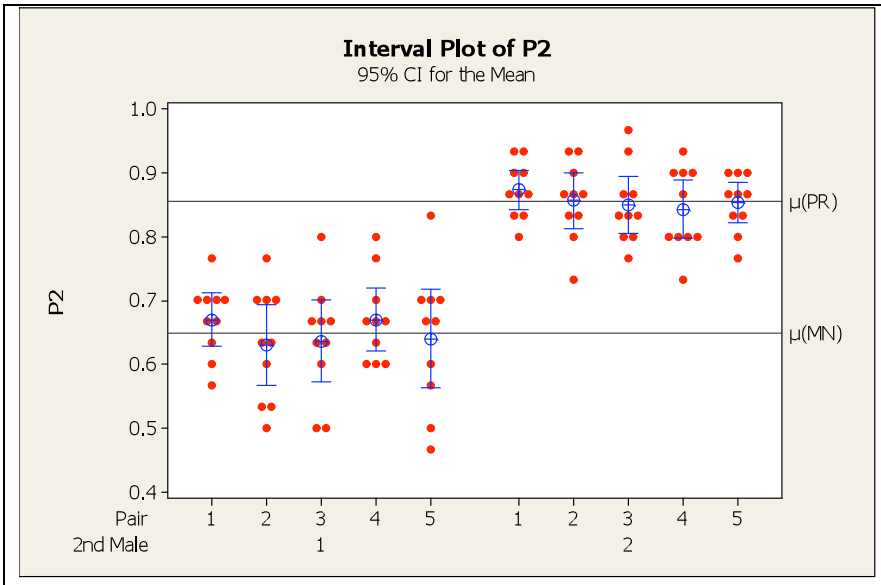

Fig. 1: Mean values of P_2 for five male pairs. Error bars represent a 95% CI for the mean of each male's P_2; horizontal lines are the mean P_2 for PR males (upper line) and MN males (lower line). 2nd Male: 1 = MN, 2 = PR.

2) Sex Ratio Manipulation – PR Females

Positive Results

I expect that for the second set of crosses, the majority of PR females will show significant deviations ($p < 0.05$) from a 0.50 sex ratio in the offspring sired by each male (though not necessarily in overall offspring sex ratio) (see Fig. 2 & 3). The proportion of individual females producing broods deviating from a 0.50 sex ratio should be distinctly lower for the control crosses. Recall also that data collected on offspring sex ratios and paternity was separated into data for the first 24 hours after mating and overall cumulative data for that mating, and that a separate set of calculations will be performed for each data set. Previous studies found that the data for the first 24 hours indicate which sperm females preferentially use (Mange 1970, Long & Pischedda 2005). This first data set is thus where the most significant results are expected; note that all predictions given below apply to this data set. I expect that the cumulative data will show no significant sex ratio deviation, as found by Long & Pischedda (2005).

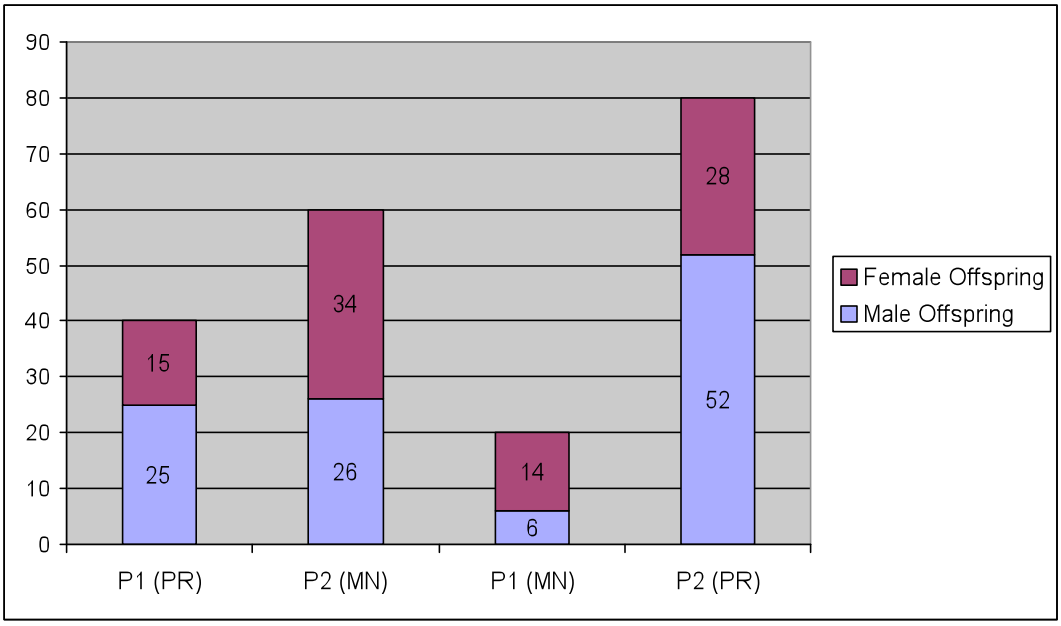

Fig 2: Offspring paternity and sex from two separate crosses of male pairs with PR females, assuming a total clutch size of 100 offspring. P1 = paternity (and type) of the first male to mate; P2 = second-male paternity (and type). Note that offspring sex ratio of MN males is female-biased, and vice-versa for PR males, regardless of overall paternity. For instance, due to differences in paternity, the PR male in the second cross (4th bar) sires more female offspring than the MN male (3rd bar), despite having a male-biased offspring sex ratio. Overall sex ratio from each cross is slightly, though not significantly ($p = 0.764, 0.089$ respectively), male-biased (see Discussion).

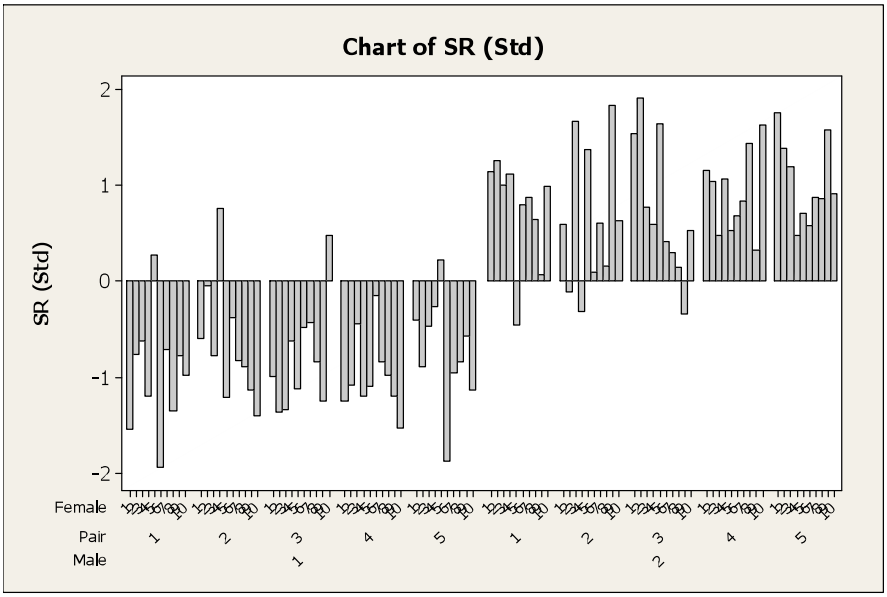

Fig. 3: Deviations from a 0.50 sex ratio for 5 pairs of males crossed with 10 females each, standardised using a binomial distribution [SR (Std)]. Male: 1 = MN, 2 = PR. These results assume the sex ratio from the control crosses is 0.50; if not, sex ratio will be standardised against the control crosses. The chart clearly shows that MN males tend to have female-biased offspring sex ratios and vice-versa.

Assuming that PR males do indeed have a fertilisation advantage over MN males, as I hope to establish in the pilot crosses, then I predict that sex ratios will be consistently male-biased for the offspring of PR males, and female-biased for the offspring of MN males. A series of t-tests for sex ratios of the offspring of each male within pairs should show this (see Box 2). Ideally, an ANCOVA should show that sex ratio bias for each male's offspring co-varies positively with P_2 value (see Box 2) – i.e. the greater the paternity bias in favour of a given male, the more male-biased sex ratio of his offspring. This would imply that selection for 'sexy sperm' is, in fact, the predominant factor behind adaptive sex-ratio bias in the females.

If the co-variance between P_2 and offspring sex ratio is low, but the direction of sex ratio bias is nonetheless consistently correlated with P_2, this would indicate that sperm competitive ability is not the only or predominant factor involved. This would be unsurprising given that intra-locus sexual conflict is widespread in *Drosophila* (Chippindale 2001, Pischedda & Chippindale 2006, Price & Hosken 2007), and that females seem to adaptively manipulate offspring sex allocation for a number of reasons related to avoiding such conflict (Long & Pischedda 2005, Fuller & Mousseau 2007).

Box 2: Sex Ratio Manipulation (Positive Results)
These results illustrate the prediction that PR males will have male-biased offspring sex ratios (as computed from SR (Std), binomial-standardised sex ratios), and vice-versa for MN males. SR (Std) is also predicted to co-vary to some extent with Pat (Std), standardised as deviations in paternity (first- or second-male).

a) 2-tailed t-test for SR (Std) within a male pair
The mean standardised sex ratio for the MN male in the pair is significantly lower (more female-biased) than mean SR (Std) for the PR male (T = -6.65, p = 0.000)

Two-sample T for SR (Std)

Male	N	Mean	StDev	SE Mean
1	10	-0.960	0.604	0.19

2 10 0.745 0.540 0.17

T-Test of difference = 0 (vs not =): **T-Value = -6.65 P-Value = 0.000** DF = 18
Both use Pooled StDev = 0.5732

b) ANCOVA of SR (Std)

Analysis of covariance between SR (Std) and Pat (Std) for five pairs of males. Both deviation in paternity and male type significantly affect sex ratio deviation (p = 0.000), and sex ratio co-varies strongly with paternity (see Fig. 5). Note that the predicted co-variance is likely to be a lot less than this (see Discussion).

Source	DF	Seq SS	Adj SS	Adj MS	F	**P**
Pat (Std)	1	85.4892	11.7693	11.7693	124.38	**0.000**
Male	1	3.7406	3.2063	3.2063	33.89	**0.000**
Pair(Male)	8	1.3487	1.3487	0.1686	1.78	**0.091**
Error	89	8.4215	8.4215	0.0946		
Total	99	99.0000				

S = 0.307609 **R-Sq = 91.49%** **R-Sq(adj) = 90.54%**

Variance Components, using Adjusted SS

Source	Estimated Value
Error	0.09462

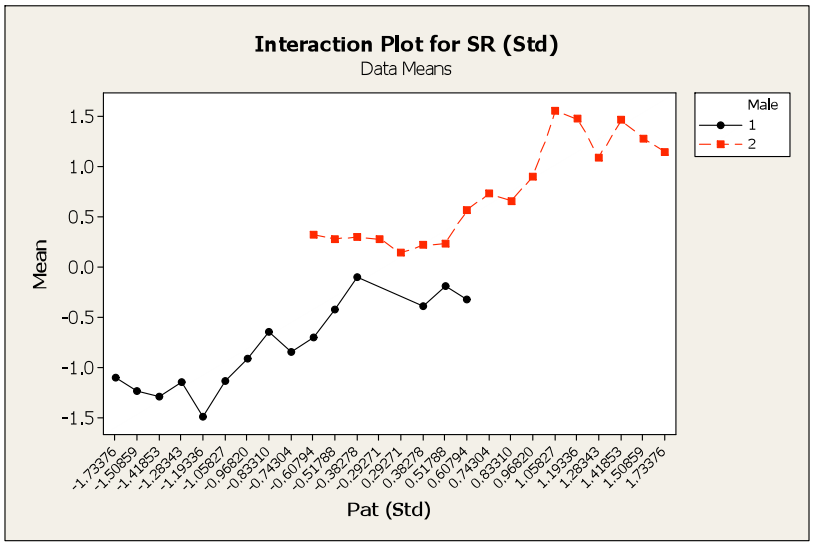

Fig. 4: Interaction plot for SR (Std) with Pat (Std). Note that sex ratio bias and paternity bias are generally higher for PR males (=2, squares) than for MN males (=1, circles), with some overlap. Within male types, SR is also positively correlated with paternity.

Box 2 cont'd

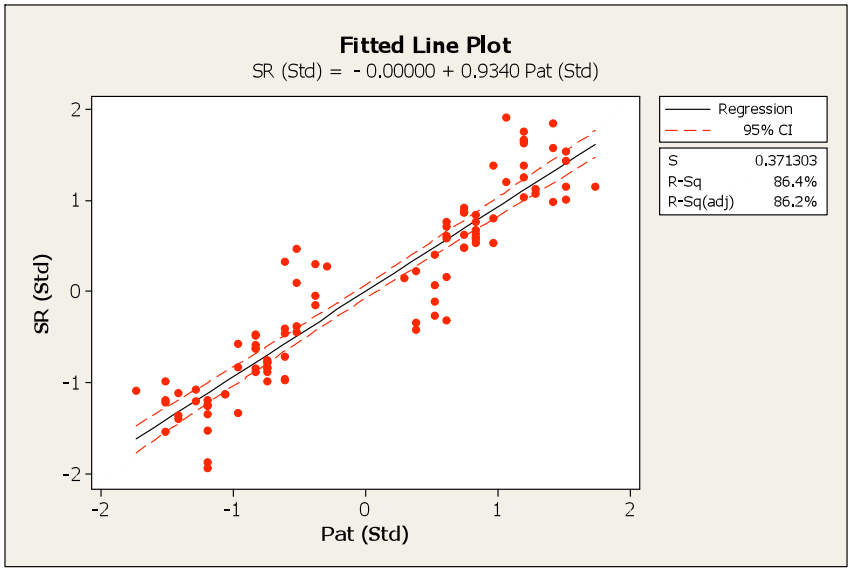

Fig. 5: Linear regression line for SR (Std) against Pat (Std). With this hypothetical data, a fairly high proportion of the variance in SR (Std) is explained by paternity (R^2[adj] = 86.2%). In reality, the R^2 value is likely to be considerably lower.

Negative Results

If, on the other hand, offspring sex ratio bias is frequent but the direction of such bias is inconsistent (i.e. whether a male is an MN or PR male, and a male's P_2 value, do not predict whether his offspring will be male- or female-biased (see Box 3), this would indicate that sex allocation is being manipulated without regard for whether a male's sperm are 'sexy', effectively ruling out the hypothesis that females bias sex ratios to obtain sons with 'sexy sperm'. In this case, we should expect the pattern of sex ratio deviation for each male in the experimental crosses to be similar to that observed for the same males in the control crosses, indicating that some other, uncontrolled factor is driving the sex ratio bias. Note that if the control crosses show significant deviations from a

0.50 sex ratio, it may be necessary to standardise the sex ratios in the experimental crosses against the control sex ratios.

Box 3: Sex Ratio Manipulation (Negative Results)

This is an example of negative results that do not support the predictions about offspring sex ratios. In this case, deviations in offspring sex ratios are observed, but with no consistent pattern. This would indicate that females bias offspring sex ratios without regard to paternity or sperm competition.

a) ANCOVA of SR (Std)

Analysis of covariance between SR (Std) and Pat (Std) for five pairs of males. Here the pattern of sex ratio deviation is apparently random, not significantly affected by paternity or male type (p = 0.931, 0.996 respectively) (see Discussion for explanations).

Analysis of Variance for SR (Std), using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	**P**
P2	1	0.050	0.008	0.008	0.01	**0.931**
Male	1	0.000	0.000	0.000	0.00	**0.996**
Pair(Male)	8	5.705	5.705	0.713	0.68	**0.707**
Error	89	93.245	93.245	1.048		
Total	99	99.000				

S = 1.02357 R-Sq = 5.81% R-Sq(adj) = 0.00%

Variance Components, using Adjusted SS

Source	Estimated Value
Error	1.048

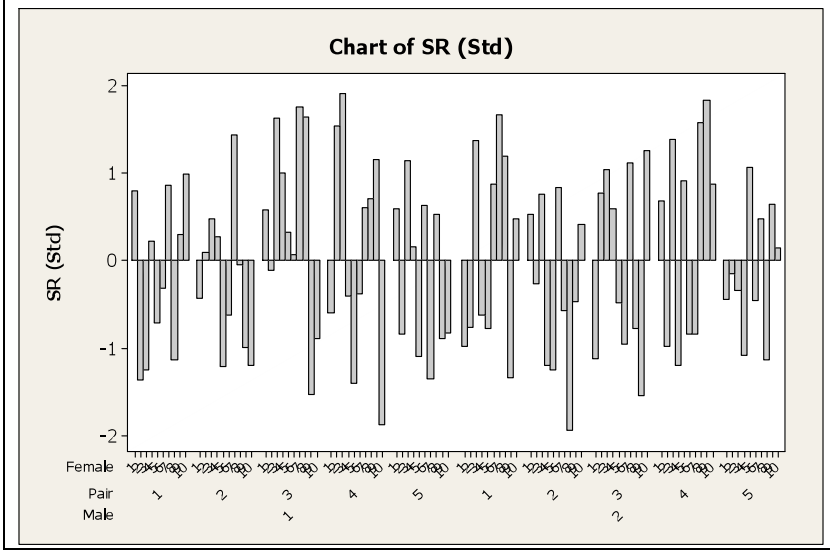

Fig. 6: Deviations from a 0.50 sex ratio for 5 pairs of males crossed with 10 females each, standardised using a binomial distribution [SR (Std)]. Male: 1 = MN, 2 = PR. There is no clear pattern behind whether males have male- or female-biased offspring sex ratios (contrast with Fig. 3).

3) Sex Ratio Manipulation – MN Females

The data from the third set of crosses is of especial interest when contrasted with that from the second set. I predict that manipulation of offspring sex ratios is an adaptive response by females to maximise the indirect benefits of mating with males with 'sexy sperm' (but harmful seminal fluid)

Box 3 cont'd

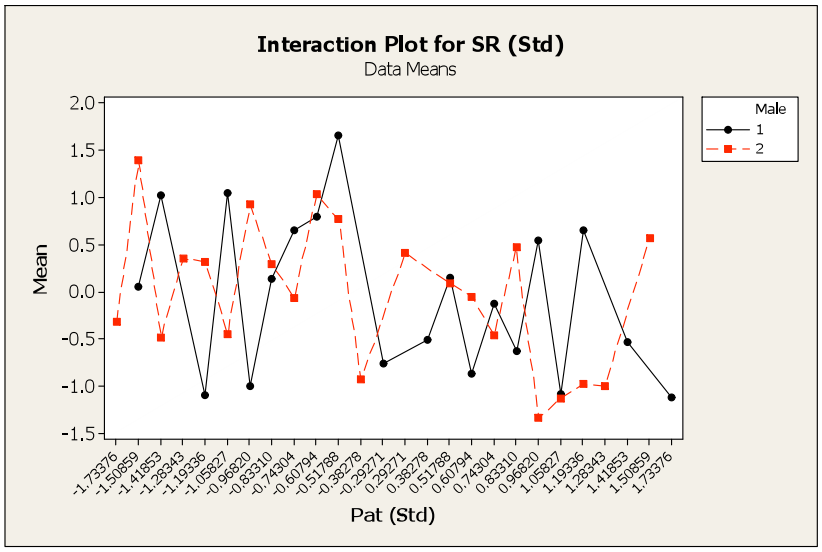

Fig. 7: Interaction plot for SR (Std) with Pat (Std). Once again, there are no clear interaction patterns between SR (Std), Pat (Std), and male type (contrast with Fig. 4).

– an evolved means of alleviating the costs of inter-locus sexual conflict. If this is the case, then MN females should have considerably reduced sex-ratio manipulation capabilities due to their reduced exposure to sexual conflict. Deviations from a 0.50 sex ratio should therefore be much smaller in both the control and experimental MN female crosses. In the experimental crosses

especially, there should be only very low, if any, co-variance between P_2 (or whether a male is MN or PR) and deviations in offspring sex ratio.

3) Sex Ratio Manipulation – MN Females

The data from the third set of crosses is of especial interest when contrasted with that from the second set. I predict that manipulation of offspring sex ratios is an adaptive response by females to maximise the indirect benefits of mating with males with 'sexy sperm' (but harmful seminal fluid) – an evolved means of alleviating the costs of inter-locus sexual conflict. If this is the case, then MN females should have considerably reduced sex-ratio manipulation capabilities due to their reduced exposure to sexual conflict. Deviations from a 0.50 sex ratio should therefore be much smaller in both the control and experimental MN female crosses. In the experimental crosses especially, there should be only very low, if any, co-variance between P_2 (or whether a male is MN or PR) and deviations in offspring sex ratio.

The MN females may show a similar degree, or at least a similar direction, of co-variance between P_2 and sex ratio bias. In this case, the observed deviations in offspring sex ratios are likely due at least in part to some male effect, as opposed to differential female utilisation of sperm or some other form of female cryptic choice (see Eberhard 1996).

Discussion

Interpretation of Results

Thus far, we have delineated three categories of possible results for the second set of crosses. First, there could be no significant bias in offspring sex ratio. This result would be unexpected and, based on existing data described earlier, unlikely (see Chippindale 2001, Long & Pischedda 2005, Pischedda & Chippindale 2006, Fuller & Mousseau 2007, Price & Hosken 2007).

Second, there could be significant deviations from a 0.50 offspring sex ratio which are not, however, correlated with sire paternity rates. This result, while negative with regard to our predictions, does still lend support to the Trivers & Willard model of sex allocation. As explained above, it is fully expected that adaptive offspring sex ratio manipulation will evolve, as it increases fitness. The lack of any correlation with male manipulative ability and performance in sperm competition would be puzzling, though, given the considerable negative impact of this sexual conflict on reproductive success (Holland & Rice 1999). It is possible over several generations of separate breeding, the informational cues linked to sperm competitive ability within each population (MN or PR) may differ. This would effectively "blind" PR females to differences in sperm competitive ability between MN and PR males. Females would thus exercise their sex ratio manipulation abilities based not on sperm competition but other factors (Fuller & Mousseau 2007). However, as sperm competition and male harm are mediated by closely linked physiological mechanisms (Wolfner 1997), and increased resistance to male harm in PR females applies to both PR and MN males (Holland & Rice 1999), it is unlikely that PR females will not also be able to recognise differences in sperm competitive ability across populations.

Third, we could see a significant positive systematic co-variance between paternity and sex ratio bias. The degree of co-variance could range from relatively weak to fairly strong (e.g. >0.8 S.D.), indicating the relative importance of sperm competition for indirect fitness benefits through sons. The degree of co-variance is predicted to be intermediate in value. This may be due partly to sperm limitation, which reduces the ability of females to bias offspring sex ratio (Fuller & Mousseau 2007). The main reason, though, is that females sired by manipulative PR fathers suffer no obvious reduction in fitness. In intra-locus conflict scenarios in *Drosophila* and

other species, 'good male' genotypes are often actually detrimental in females (Calsbeek & Sinervo 2004, Price & Hosken 2007). This results in strong selection pressure on females to manipulate offspring sex allocation. As there is no apparent cost to having female offspring from PR sperm, there is no selection pressure to *reduce* the number of daughters sired by PR males in favour of daughters from MN males (see Fig. 2); there is only a selective advantage to having more sons by PR males and fewer by MN males. The effects of differences in male manipulative ability on offspring sex allocation are thus likely to be fairly moderate compared to the effects of intra-locus conflict. In addition, populations in which the predicted sex ratio manipulation occurs are expected, as a whole, to have somewhat male-biased sex ratios. Based on Fisherian sex allocation principles, too great a deviation from a 0.50 sex ratio will counteract the benefits of having more sons with 'sexy sperm'. This too will act to moderate the degree of sex ratio bias by females.

Even a moderate positive co-variance would strongly support the Trivers & Willard model, unless the results of our third set of crosses show that offspring sex ratio manipulation is primarily due to male rather than female effects. This is highly unlikely, though, as previous studies have shown that although the precise physiological mechanism is not known, differential fertilisation success of X- and Y-bearing sperm is apparently under female control (Mange 1970, Long & Pischedda 2005).

Budget & Feasibility

The proposed study should be fairly straightforward to carry out, as *Drosophila*-handling protocols are very well developed. *Drosophila* are easy and inexpensive to maintain in a laboratory environment. With their short generation times, the experiment should take no longer than 2-3 years, including breeding the MN and PR populations. Due to the large number of

replicates, a fair amount of space – several standard-sized teaching laboratory rooms – will be required. The crosses will also be labour-intensive, requiring a large number of man-hours. The protocols are not complex, however, and thus can easily be performed by undergraduate research assistants with a minimum of training. In addition, since paternity analyses are purely visual (based on the *bw* and rd^s phenotypes), expensive molecular methods can be dispensed with. While not exceptionally fast or easy, therefore, the proposed study is by no means unfeasible, comparable perhaps with a standard dissertation.

Based on the grants used to fund a number of similar studies (e.g. Holland & Rice 1999, Long & Pischedda 2005), I estimate that this study will require on the order of $250,000 in funding over two or three years. Most of this will go towards hiring research assistants to help with the crosses. Maintaining the *Drosophila* populations, carrying out the crosses, and collecting the data will directly require only a small amount of funding. The amount of funding required is well within the average NSF funding award for the biological sciences – an average amount of approx. $115,000 per year for 2.8 years in 2006 and 2007 (NSF).

Relevance

Few existing studies have examined the role of the Trivers & Willard sex allocation model in sexual conflict, and most of these deal with intra-locus conflict. In addition to supporting the model, our predicted results will give it a fascinating new dimension – the interaction between inter-locus sexual conflict and sex allocation.

When we consider that female *Drosophila* actually prefer mating with males which are more detrimental to their survival (Friberg & Arnqvist 2003), these results would seem to support the 'sexy sperm' hypothesis of Keller & Reeve (1995) and Evans & Simmons (2008). However, under the Trivers & Willard model, females in a polyandrous situation are expected to

bias offspring sex ratios according to paternity *regardless of* the overall fitness effects of polyandry, because in either case differential sperm use confers indirect benefits. Whether these indirect benefits are enough to offset the cost of multiple mating and thereby allow females to 'gain by losing' (Cordero & Eberhard 2003) is not shown by the results of these experiments. The question of whether indirect fitness benefits through 'sexy sons' or sons with 'sexy sperm' are enough to actually increase female fitness is much debated. There is evidence both in favour of (see Arnqvist & Nilsson 2000) and against (see Brown et al. 2004) the 'gain by losing' idea. Neither side of the argument contradicts our predictions, and adaptive offspring sex ratio bias is expected to evolve in either case. To decide whether 'gain by losing' truly occurs would require further experiments with the F_1 sons of our experimental females, to assay indirect fitness benefits gained in the currency of grandchildren.

Until such experiments are performed, therefore, we cannot rule in favour of or against the 'gain by losing' scenario. Nevertheless, given the results of Friberg & Arnqvist (2003), our predicted results would lend support to the 'sexy sperm' hypothesis by providing females with a means of mitigating the costs of sexual conflict. As this hypothesis has been proposed as an explanation for the origin and maintenance of polyandry (Keller & Reeve 1995, Jennions & Petrie 2000, Evans & Simmons 2008), our results could help shed further light on this issue, and on the connection between polyandry and sexual conflict.

Even more interestingly, if our results (and those of follow-up fitness assays) support the 'gain by losing' scenario, they would imply that by evolving to adaptively manipulate offspring sex ratios, females may not only alleviate the costs of sexual conflict, but even resolve a conflict situation into a cooperative one in which the fitness of both partners increases. This would add a whole new level to our understanding of sexual conflict. The phenomenon of sexual conflict lies

at the conjunction of the two most important and fundamental processes in biology, natural and sexual selection, and offers insight into them both. A question which deepens our understanding of it is thus well worth exploring.

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