

## Sex-linked Correlated Responses in Female Reproductive Traits to Selection on Male Eye Span in Stalk-eyed Flies<sup>1</sup>

GERALD S. WILKINSON,<sup>2</sup> EMILY G. AMITIN, AND PHILIP M. JOHNS

*Department of Biology, University of Maryland, College Park, Maryland 20742*

**SYNOPSIS.** Coevolution between male and female traits can result from correlated responses to selection or correlated selection on genetically independent traits. This study examines the possibility that traits involved in precopulatory sexual selection may influence the evolution of traits involved in postcopulatory sexual selection due to the existence of correlated selection or correlated responses to selection. Artificial selection on male eye span in *Cyrtodiopsis dalmanni*, a sexually dimorphic stalk-eyed fly, is used to test for correlated changes in reproductive traits of male and female flies. Flies from replicate lines that had been under selection for 57 generations were matched for age and genotyped at four X-linked microsatellite loci. Egg number and testis size increased with age, but did not differ among lines. Spermathecal areas and duct lengths differed among replicates, but not among selection treatments. Female relative eye span, size of the ventral receptacle and egg size exhibited significant correlated responses to selection on male relative eye span. The absence of any change in sperm length or testis size between lines indicates that changes in female traits are unlikely due to correlated selection mediated by sperm competition. Significant effects of X-linked microsatellite genotypes indicate instead that the correlated responses to selection were due, in part, to X-linked genes in linkage disequilibrium or that exhibit pleiotropy. The presence of nonadditive allelic effects on genetically correlated female traits combined with additive allelic effects on a male ornament provides a previously unrecognized mechanism by which genetic variation could be maintained despite strong sexual selection.

### INTRODUCTION

Comparative studies in insects (Dybas and Dybas, 1981; Morrow and Gage, 2000; Pitnick *et al.*, 1999; Pitnick *et al.*, 2003; Presgraves *et al.*, 1999) and birds (Briskie and Montgomerie, 1993; Briskie *et al.*, 1997) have found evidence for correlated evolution between male and female reproductive traits while recent experimental studies on *Drosophila melanogaster* have shown that selection for longer sperm leads to changes in female sperm storage organ size (Miller and Pitnick, 2002). These examples of male-female coevolution could result from either correlated responses to selection, correlated selection or both. Correlated responses in female traits are expected under several models of sexual selection. For example, both runaway (Kirkpatrick, 1982; Lande, 1981) and good genes (Iwasa *et al.*, 1991; Pomiankowski, 1987) models predict that linkage disequilibrium will accumulate as a result of female choice and cause coevolutionary change in male ornaments and female preferences. Evidence in support of a genetic correlation between precopulatory ornaments and preferences has been obtained from artificial selection (Houde, 1994; Wilkinson and Reillo, 1994) and breeding studies (Bakker, 1993; Gray and Cade, 2000).

Alternatively, correlated selection can lead to coevolution between genetically independent traits if change in one trait influences how selection operates on the other trait. For example, antagonistic coevolu-

tion due to sexual conflict (Gavrilets *et al.*, 2001; Parker, 1979; Parker and Partridge, 1998) occurs when a trait that enhances male competitive ability leads to correlated selection on females to resist mating. Experimental evidence in support of antagonistic coevolution comes from studies on *Drosophila melanogaster* which show that when males are permitted to evolve greater offensive and defensive mating ability, females exhibit higher mortality (Rice, 1996). Selection on the reproductive tract of females could also produce correlated selection on sperm size or number to the extent that females create the arena in which sperm interact prior to fertilization (Eberhard, 1996).

Connecting the pattern of variation in reproductive traits observed among taxa to the process responsible for producing that pattern requires an understanding of both the pattern of selection and the genetic architecture. Disentangling effects of correlated selection from those due to correlated responses to selection is complicated by pleiotropy and genetic linkage because these factors can cause genetic correlations between traits involved in different biological functions. Given that most animals have thousands of genes, linkage might seem unlikely; however, the opportunity for linkage increases if selection favors gene clustering in the genome. For example, several scenarios could cause genes that influence reproductive traits to occur preferentially on the sex chromosomes. If a gene affects a trait that increases male competitive ability but is costly to females, then it will spread more rapidly when recessive and X-linked (Charlesworth *et al.*, 1987; Rice, 1984). Alternatively, if selection fluctuates on males, then X-linked ornamental traits are expected to persist longer than autosomal traits (Reinhold,

<sup>1</sup> From the Symposium *Selection Experiments as a Tool in Evolutionary and Comparative Physiology: Insights into Complex Traits* presented at the Annual Meeting of the Society for Integrative and Comparative Biology, 5–9 January 2004, at New Orleans, Louisiana.

<sup>2</sup> E-mail: wilkinso@umd.edu

1999). Finally, in male heterogametic species, X chromosomes occur twice as often in females than males assuming an equal sex ratio. Consequently, X-linked traits experience stronger selection in females and weaker selection in males each generation. Under a good genes model of sexual selection X-linked inheritance of female preferences and male traits changes female preferences more than autosomal inheritance of preferences and traits (Kirkpatrick and Hall, 2004).

Evidence in support of X-linked inheritance of sexually selected traits is mixed. Reviews of the inheritance of sexually selected traits based on line crosses have found a bias towards X-linkage (Lindholm and Breden, 2002; Reinhold, 1998). Similarly, genes expressed in the testes tend to be X-linked in mice (Wang *et al.*, 2001), as are genes associated with reproduction in humans (Saifi and Chandra, 1999). In contrast, in *Drosophila melanogaster* a survey of gene annotations found no evidence for an X-linked bias of genes involved in mating behavior, although such genes tend to have pleiotropic effects (Fitzpatrick, 2004), and genes with male-biased expression were significantly less likely to be found on the X chromosome (Parisi *et al.*, 2003; Ranz *et al.*, 2003). However, the X chromosome contained the majority of genes with sexually antagonistic effects on fitness (Gibson *et al.*, 2002).

In this study we examine the possibility that traits involved in precopulatory sexual selection may influence the evolution of traits involved in postcopulatory sexual selection due to the existence of correlated selection or correlated responses to selection. We also evaluate the possibility that these traits exhibit linkage to regions of the X chromosome. After exerting selection on a trait known to influence mating success in males we measure male and female traits involved in determining fertilization success after copulation and test for evidence of correlated change. We use the stalk-eyed fly, *Cyrtodiopsis dalmanni*, because this species experiences intense precopulatory and postcopulatory sexual selection. Male relative eye span influences the outcome of male-male competition (Panhuis and Wilkinson, 1999) and female mate choice (Wilkinson *et al.*, 1998a; Wilkinson and Reillo, 1994). Selection imposed by sperm competition is expected to be high due to extreme promiscuity. In *C. dalmanni* males compete for access to groups of females at dusk and then mate with multiple females at dawn (Wilkinson and Reillo, 1994). Females routinely mate multiple times per day (Wilkinson *et al.*, 1998a), do not reduce mating activity after copulation (Grant *et al.*, 2002) and live for many months (Wilkinson and Reillo, 1994). Multiple mating does not appear to have deleterious effects on females (Reguera *et al.*, 2004). Instead, multiply mated females have higher fecundity than single mated females (Baker *et al.*, 2001) probably because they receive more sperm. Males transfer seminal material in a spermatophore (Kotrba, 1990) but pass few sperm. On average, only 65 sperm are found in the spermathecae of a *C. dalmanni* female after a single copulation (C.L. Fry, personal commu-

nication). In the closely related species, *C. whitei*, sperm survival can also be reduced by seminal fluid from competitors (Fry and Wilkinson, 2004). Sperm are stored in three spermathecae, but must move to individual chambers in the seminal receptacle before entering an egg and effecting fertilization.

Comparative studies have revealed that sexually dimorphic eye span has evolved rapidly and recurrently among species of stalk-eyed flies (Baker and Wilkinson, 2001). Furthermore, male and female reproductive traits exhibit evidence of correlated evolution. A comparative analysis utilizing 17 species of diopsids revealed dramatic differences in sperm length, presence or absence of sperm heteroplasmy, and correlated changes in the size, shape, complexity and location of sperm storage organs in females (Presgraves *et al.*, 1999).

Several lines of evidence indicate that the X chromosome influences sexually selected traits in this species. Analysis of line cross data indicates that genes on the X chromosome explain 30% of the variation in relative eye span (Wolfenbarger and Wilkinson, 2001). Furthermore, all populations tested from different islands in southeast Asia contain an X-linked factor that when present in males causes them to produce predominantly female progeny (Presgraves *et al.*, 1997; Wilkinson *et al.*, 2003) due to abnormal development of Y-bearing sperm (Wilkinson and Sanchez, 2001). Artificial selection on male eye span altered brood sex ratios suggesting that factors on the X chromosome jointly affect a male ornament and sperm development (Lande and Wilkinson, 1999; Wilkinson *et al.*, 1998b). In many species of *Drosophila*, cases of X chromosome meiotic drive or *sex ratio* are associated with chromosomal inversions (Jaenike, 2001). Paracentric inversions restrict recombination between heterokaryotypes (Navarro *et al.*, 1997) and recombination map length negatively correlates with the size of successful inversions in flies (Caceres *et al.*, 1999). Consequently, regions of linkage disequilibrium may be greater for X-linked genes found in inversions or under strong selection, such as is likely to occur with meiotic drive (Derome *et al.*, 2004; Palopoli and Wu, 1996).

## MATERIALS AND METHODS

### *Selection regime*

Artificial sexual selection was exerted on the ratio of eye span to body length in male *Cyrtodiopsis dalmanni* descended from animals captured in peninsular Malaysia in 1989. These flies had been reared in the lab in mass culture for approximately seven generations prior to the onset of selection (Wilkinson, 1993). Beginning in March 1991, two lines each were selected for high and low ratios by taking 10 of 50 males and housing them with 25 females chosen at random from within each selected line in 40 × 40 × 40 cm cages. Two unselected control lines were maintained at the same time by randomly selecting 10 males and 25 females from each line. To produce offspring for

the next generation, we collected eggs from each of the six cages twice each week in four cups containing 50 ml of food after allowing selected flies to mate for at least two weeks. Thus, larvae typically developed at low density and produced large-bodied adult flies. Every generation after eclosion we separated flies from each replicate line by sex and maintained them in single-sex cages as virgins until selection was imposed after at least four weeks of age. This selection regime was continued for 30 generations. At generation 31, selection was relaxed to reduce inbreeding such that 25 of 50 measured males were chosen for breeding each generation. Note that this change in selection intensity also altered the sex ratio of adult flies in each cage from 1:2.5 to 1:1 male:female, which could have potentially increased the intensity of sperm competition within each line. In this study we measured reproductive traits from male and female flies in each of the six lines after 57 generations of selection.

All cages are lined with moist cotton and blotting paper and kept at  $25 \pm 1^\circ\text{C}$  with at least 70% relative humidity on a 12 hr light-dark cycle. Twice each week pureed corn is provided in disposable dishes as food. Adding 5 ml of a 10% solution of methylparaben in 90% ethanol to each liter of corn pulp after autoclaving for 30 min inhibits mold growth. To rear flies food cups are placed inside larger 500 ml containers lined with damp cotton and plugged with foam stoppers to permit the larvae to climb out of the cups and pupate in the cotton. Any pupae remaining in cups are transferred to the cotton before eclosion.

Flies are measured under  $\text{CO}_2$  anesthesia while resting on their orbital and thoracic spines. Using the program Image 1.59 we measure eye span from the outer edges of the ommatidia and body length from face to wing tip on each animal at  $11\times$  (resolution of 50 pixels/mm). The ratio of eye span to body length is referred to as relative eye span throughout this paper.

#### Reproductive trait measurements

Because these flies take two to three weeks to reach reproductive maturity, it is possible that some reproductive traits could change with age. Consequently, we dissected two or three males and females from each line every other day between 14 and 32 days of age. We supplemented these measurements of young flies with measurements of older females that were 40–48 days of age and older males that were 58–86 days of age. All flies were kept in single-sex cages prior to dissection.

To isolate reproductive structures, abdomens of anesthetized flies were removed with small scissors and placed in a single drop of phosphate buffered saline (PBS) on a clean glass slide. The remainder of the body was frozen and later measured for eye span, body length and thorax width as described above. For males, testes were removed into the PBS and the area of both testes, whenever possible, was measured at  $100\times$  (575 pixels/mm). The contents of the testes were then released into the PBS and the sperm tails of five fully

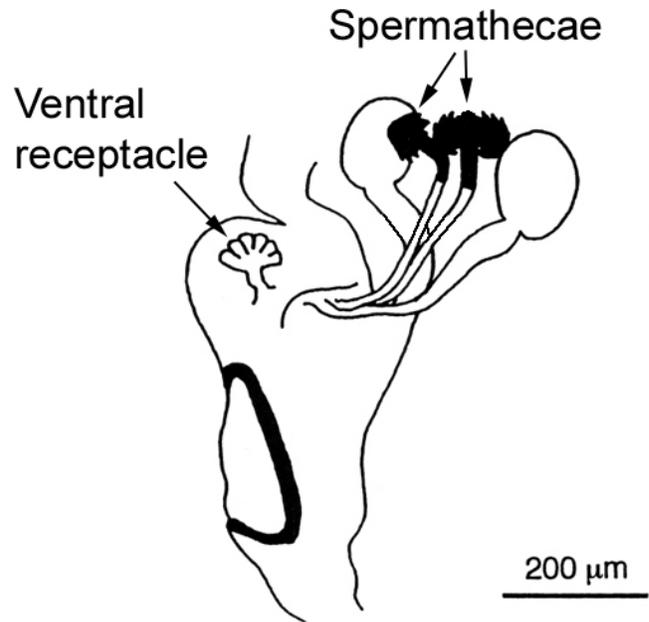


FIG. 1. Schematic drawing of a female *Cyrtodiopsis dalmanni* reproductive tract.

elongated sperm bundles were measured at  $400\times$  (2.3 pixels/ $\mu\text{m}$ ). Sperm heads were not included because of the possibility that sperm head length is modified in males that carry sex chromosome meiotic drive (Wilkinson and Sanchez, 2001).

For females, the reproductive tract (see Fig. 1) including ovaries was removed from the abdomen into a drop of PBS on a clean glass slide, and the rest of the abdomen was discarded. The number of mature eggs, defined as those at least 0.7 mm in length, were counted, if present, and moved to the side of the slide. The tract was oriented with the ventral sclerite (Kotrba, 1995) facing up and a cover slip was gently placed over the reproductive tract. The areas of all three spermathecae, the lengths of the two spermathecal ducts, the width of the ventral receptacle, and for a subset of the sample ( $n = 80$ ), the number of chambers in the ventral receptacle were measured at  $400\times$  (2.3 pixels/ $\mu\text{m}$ ). Egg size was measured for three mature eggs at  $100\times$  (575 pixels/mm).

#### Genotyping

We extracted DNA from all flies and then used the polymerase chain reaction (PCR) to amplify microsatellite alleles using four pairs of X-linked primers (Wright *et al.*, 2004). These primer pairs were previously found to produce products that differ in size between high and low line flies and their relative positions on the X chromosome have been determined by linkage mapping using F2 progeny resulting from a cross between two selected lines (P.M.J. and G.S.W., unpublished data). The order of the loci and their recombination map intervals are: ms125–14.2 cM–ms244–8.2 cM–ms54–2.5 cM–ms 395. Unique alleles at three of the four loci have also been found to occur

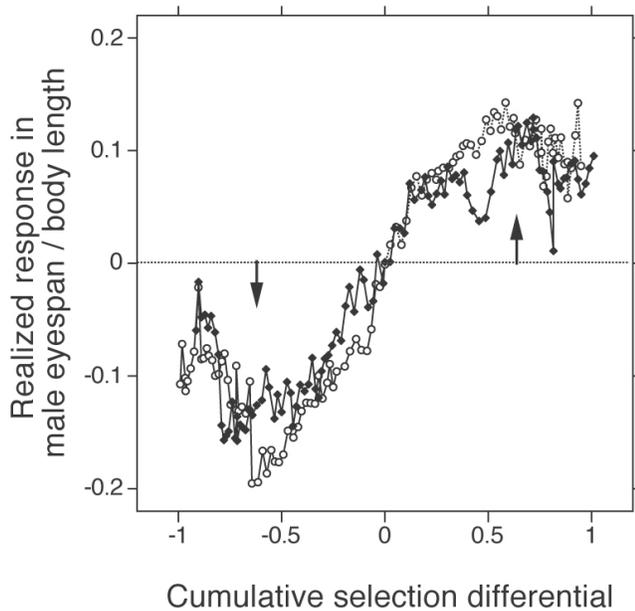


FIG. 2. The realized response in male relative eye span, *i.e.*, the difference between each selected line and the average of the two control lines at each generation, plotted against the cumulative selection differential, *i.e.*, the cumulative difference between the males selected for breeding and the average of all measured males for each replicate line each generation divided by two because only one sex is under selection. The arrows indicate when selection was relaxed from 20% to 50% of males chosen for breeding each generation.

in males that carry X chromosome meiotic drive and produce predominantly female offspring (P.M.J. and G.S.W., unpublished data).

We extracted DNA by grinding thorax and leg tissues before incubating at 65°C for at least 2 hr in 400  $\mu$ l of 6.25% (w/v) Chelex 100 solution with 2 U proteinase K (Fisher Scientific). We then incubated samples at 95°C for 20 min and then centrifuged them for 3 min at 2,000 rpm on a microcentrifuge. PCR was performed in 10  $\mu$ l reactions containing 1  $\mu$ l extraction supernatant as template DNA, 0.125 U Taq DNA polymerase (Invitrogen, Carlsbad, CA), 1 $\times$  PCR Buffer (Invitrogen), 0.2 mM of each dNTP, 2.5 mM MgCl<sub>2</sub> and 0.5  $\mu$ M of each primer. Forward primers were labeled with NED (Applied Biosystems), HEX, or FAM fluorescent dyes. We used a touchdown cycle of 17 cycles with initial annealing at 63°C and decreases of 1°C per cycle followed by 20 cycles with annealing at 47°C. PCR products were separated on a 3100 DNA Analyzer (Applied Biosystems) using Pop-4 polymer and evaluated with Genescan 3.1.2 software (Applied Biosystems). Alleles were sized and scored using Genotyper 2.5 software (Applied Biosystems).

#### Statistical analysis

We used mixed model nested analyses of covariance (ANCOVA) to determine if age, body size, replicate or selection treatment influenced male and female traits. We nested replicate within selection treatment and assumed that replicate was a random effect. If se-

TABLE 1. *F*-ratios from nested analyses of covariance for male reproductive traits on selected line flies.

Source (df)	Eye span	Testis	Sperm length
Log age (1,142)	0.1	72.1***	0.1
Body length (1,142)	253.6***	2.3	1.0
Selected line (2,3)	24.3*	3.7	0.6
Replicate (3,142)	9.5***	1.3	1.3
Model (7,142)	107.5***	11.7***	1.1

\*  $P < 0.05$ ; \*\*  $P < 0.01$ ; \*\*\*  $P < 0.0001$ .

lection on male relative eye span causes change in other characters due to pleiotropy or linkage, the magnitude of change in the other traits should correlate with the magnitude of change in male relative eye span. Therefore, we quantified the correlated response by fitting regressions to the least squares means of female traits that differed between selection treatments to male relative eye span for each replicate within each treatment. We tested for evidence of X chromosome linkage by conducting an ANCOVA on those traits that differed between selection treatment using microsatellite genotype as a main effect and body length as covariate. Age was not included in these analyses because it had not explained a significant fraction of the variation in these traits in the previous ANCOVAs. Statistical analyses were conducted using JMP v5.0.1.2 (SAS-Institute, 2003).

#### RESULTS

Artificial sexual selection resulted in rapid bidirectional response in male relative eye span (Fig. 2). Response to selection was consistent for 15 generations in the replicate high lines and for 23 generations in the replicate low lines. At generation 16 one of the high lines underwent a temporary reversal in response to selection, while at generation 24 one of the low lines reached a plateau. When selection was reduced in intensity at generation 30, all four lines stopped responding. Subsequently, each of the low lines and one of the high lines exhibited reversals at independent generations. Nevertheless, at generation 57 significant differences in relative eye span existed between selected lines and between replicates for both males (Table 1) and females (Table 2). Each replicate high line differed in relative eye span from each low line by at least 3.3 SD and by as much as 5.0 SD.

Differences in relative eye span between selection treatments are caused predominantly by changes in absolute eye span rather than by changes in body length. Selection produced consistent responses in eye span between low and high lines, as compared to control lines, for both males and females (Fig. 3). For male body length, no effect of selection treatment could be detected ( $F_{2,3} = 0.1$ ,  $P = 0.9$ ) but significant differences among replicates occurred ( $F_{1,141} = 6.8$ ,  $P = 0.0003$ ). In contrast, for female body length significant effects of line ( $F_{2,3} = 27.9$ ,  $P = 0.012$ ), but not of replicate ( $F_{1,172} = 0.6$ ,  $P = 0.6$ ), were found. However,

TABLE 2. *F*-ratios from nested analyses of covariance for female reproductive traits on selected line flies.

Source (df)	Eye span	Egg size	Egg number	Ventral receptacle	Singlet spermatheca		Doublet spermatheca	
					Duct length	Area	Duct length	Area
Log age (1,172)	0.0	0.0	12.3**	0.1	0.1	1.7	0.2	4.3
Body length (1,172)	213.6***	0.1	0.3	0.8	0.8	2.9	3.1	9.5**
Selected line (2,3)	17.6*	4.3	0.1	9.8*	0.3	0.2	0.8	0.0
Replicate (3,172)	14.7***	1.8	1.3	2.8*	47.5***	5.1**	38.6***	5.5**
Model (7,172)	129.2***	3.1**	2.4*	10.3***	25.3***	3.4**	27.5***	1.5

\*  $P < 0.05$ ; \*\*  $P < 0.01$ ; \*\*\*  $P < 0.0001$ .

the line effect in females is due to control line flies being smaller than flies from either selected line, which do not differ from each other (Fig. 3).

Two traits, average testis size in males and the number of mature eggs carried by females, showed significant effects of age but no effects of body length, selection line or replicate (Tables 1 and 2). Testis area exhibited a biphasic growth pattern (Fig. 4) with rapid growth occurring during the first three weeks of age followed by slow growth that appears to continue for five months or longer. The number of mature eggs in females also increased with age (Fig. 4), although egg number appears to plateau at four weeks of age and does not show subsequent change. The remaining trait in males, sperm length, was not influenced by body length, age, selection treatment or replicate (Table 1).

In females, nested ANCOVA explained significant variation in every reproductive trait (Table 2). Significant effects of replicate, but not selection treatment, were detected for the singlet and doublet spermathecal areas and duct lengths (Table 2, Fig. 5). All of these traits tended to increase with body length, but only for

the average area of the doublet spermathecae was this effect significant (Table 2). The width of the ventral receptacle was independent of age and body length, but exhibited significant differences between selection treatments and replicates within treatments (Table 2). Selection line explained 29% of the variance in ventral receptacle width while replicate explained only 4%. Ventral receptacle width correlated with the number of chambers counted in the ventral receptacle ( $r^2 = 0.55$ ,  $P < 0.0001$ ). Because no significant effect of replicate could be detected for egg size, the ANCOVA was re-run after pooling replicates within selected lines. A significant effect of selection treatment on egg size ( $F_{2,3} = 6.9$ ,  $P = 0.0015$ ) was then detected and explained 16% of the variation in average egg size.

Differences between selection treatments are consistent with correlated responses to selection. The regression of female relative eye span on male relative eye span (Fig. 6a) was highly significant ( $t = 10.2$ ,  $P = 0.0005$ ) and explained 96% of the variation in female relative eye span. In addition, the regression between the number of chambers in the ventral receptacle and male relative eye span (Fig. 6b) was significant ( $r^2 = 0.88$ ,  $P = 0.006$ ). The regression between average egg size and male relative eye span approached significance ( $r^2 = 0.61$ ,  $P = 0.066$ ).

To determine if linkage between X-linked factors could be partly responsible for the correlated responses to selection, we compared trait values across genotypes at four X-linked microsatellite loci. Those traits that differed between selected line treatments also exhibited significant differences between genotypes at the X-linked loci (Table 3). For male eye span, all four X-linked loci exhibited significant genotype effects, although the effect of ms125 genotype was much weaker than for the other three loci. The effect of each of the six alleles at locus ms244 was nearly additive while for locus 244 only one of the four alleles appeared to influence male eye span (Fig. 7). All four X-linked loci also exhibited significant genotypic effects on female eye span (Table 3). However, because females are diploid at X-linked loci, rather than haploid, homozygous and heterozygous genotypes are present. Examination of mean female eye span for each genotype revealed strong dominance effects for some pairs of alleles at every locus. For example, at locus ms125 three different heterozygotes exhibited underdominance, while at locus ms244, ms54 and ms395, one heterozygous

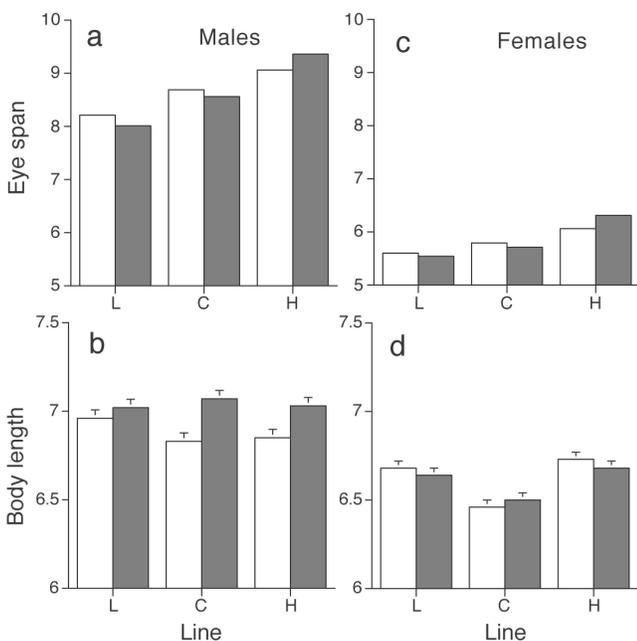


FIG. 3. Least squares means for a) male eye span, b) male body length, c) female eye span, and d) female body length from the six replicate selected lines: L = low, C = control, H = high after 56 generations of selection. Error bars represent one standard error.

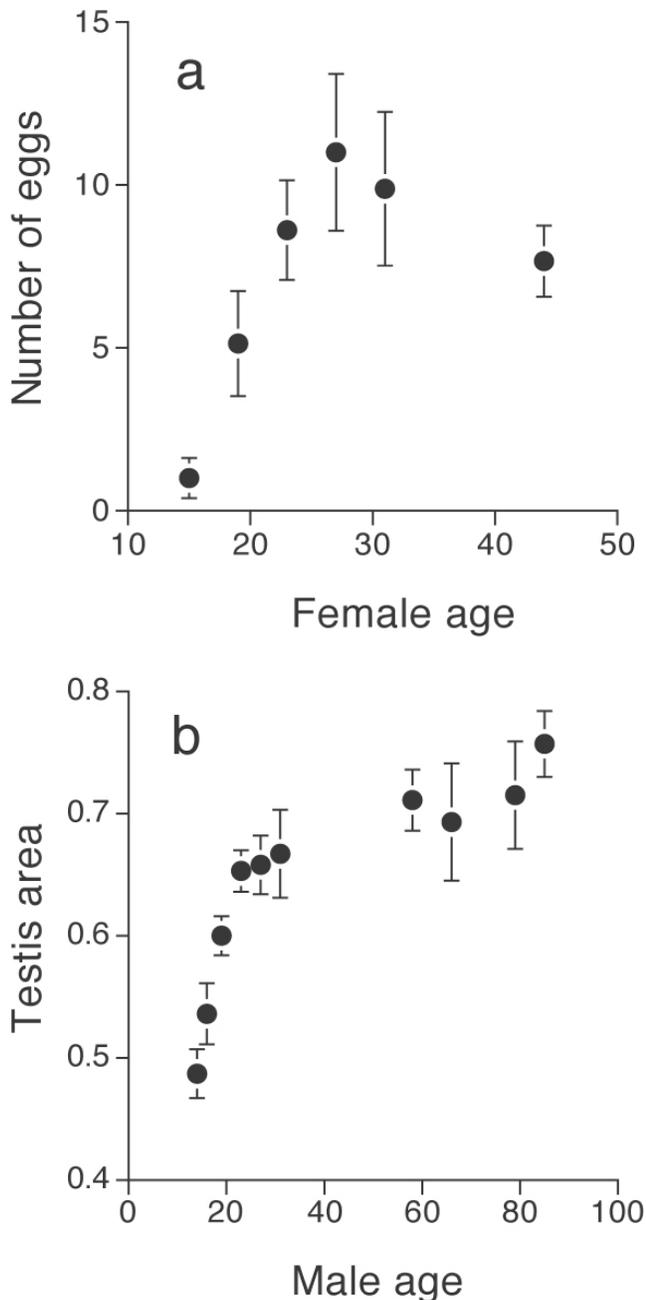


FIG. 4. Least squares mean a) number of mature eggs and b) average testis area (mm<sup>2</sup>) as a function of fly age in days. Measurements are pooled among replicates and lines. Error bars represent one standard error.

genotype was overdominant (Fig. 8). For ventral receptacle width significant effects of genotype were detected at the same three loci that exhibited highly significant genotypic effects on male eye span (Table 3). Mean ventral receptacle width at each ms395 genotype showed the same pattern of overdominance as for female eye span while the homozygous genotypes at ms244 exhibited additivity (Fig. 9). The genotypic effects of ms54 on ventral receptacle width were signif-

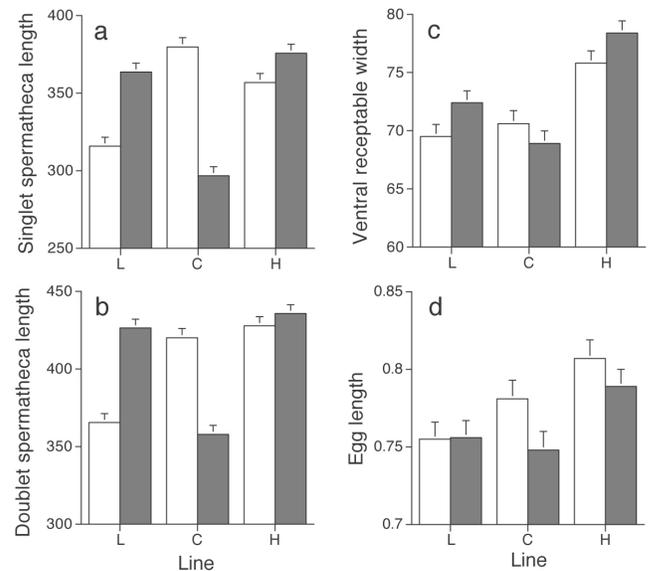


FIG. 5. Least squares means for female reproductive traits: a) duct length for the singlet spermatheca, b) duct length for the doublet spermathecae, c) width of the ventral receptacle, and d) average length of a mature egg for the six selected lines. Error bars represent one standard error and if not visible are less than the width of the symbol.

icant, but exhibited less similarity to the pattern observed for female eye span.

Unique alleles at three of the four loci define a male haplotype that is always associated with highly female-biased sex ratios. We did not find any of these drive alleles in any male or female that was genotyped from the selected lines.

#### DISCUSSION

Long-term artificial sexual selection resulted in bidirectional change in male relative eye span in replicate selected lines as long as selection was intense. The strong correlated response in female relative eye span, which has been noted previously (Wilkinson, 1993; Wolfenbarger and Wilkinson, 2001), suggests that the genes that influence male eye span also influence female eye span. Direct support for this inference has recently been obtained from a QTL mapping study (P.M.J. and G.S.W., unpublished data), which has found four QTL in the same locations in both males and females and only one QTL unique to males. The correlated responses found for ventral receptacle size and egg size, both of which exhibit differences between selection treatments after adjusting for body size, have several possible explanations.

First, the expression of each of these traits, eye span, ventral receptacle size and egg size, may be under the influence of juvenile hormone (JH) and either JH titer, receptor distribution, or esterase concentration could have been changed by selection. Juvenile hormone has been implicated in the development of the imaginal discs that produce horns in beetles (Emlen and Nijhout, 1999, 2001). Juvenile hormone is also associ-

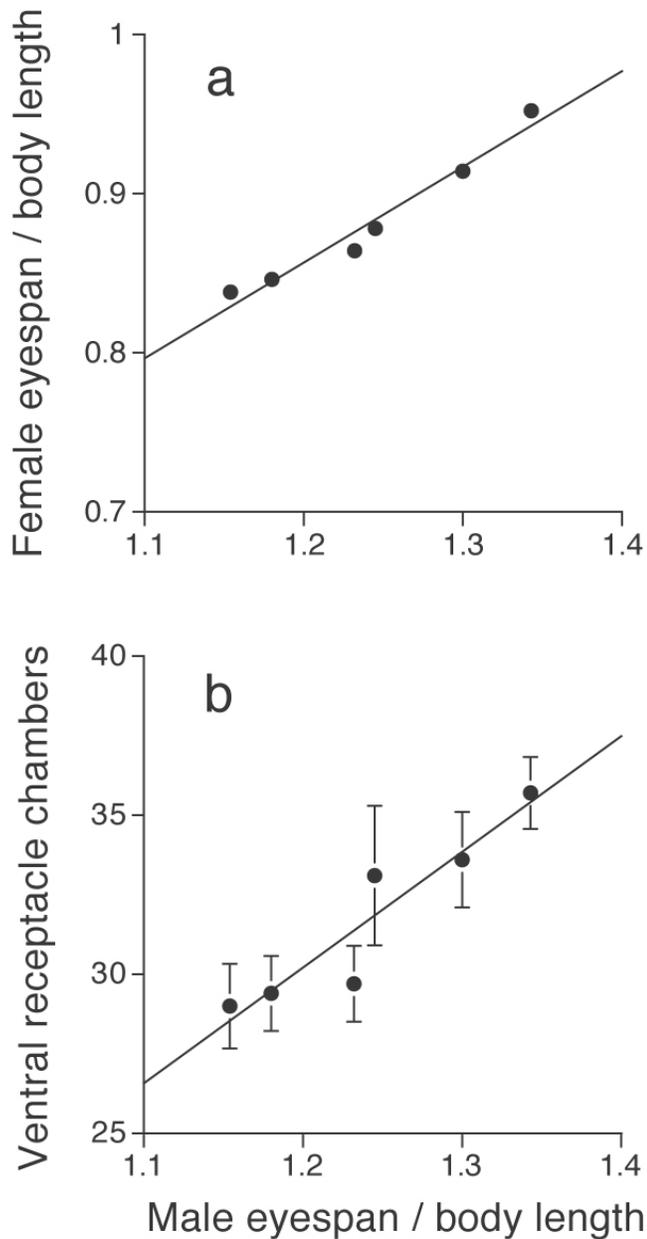


FIG. 6. Correlated response in a) female relative eye span (eye span/body length) and b) the number of chambers in the ventral receptacle plotted as a function of average male relative eye span. Error bars represent one standard error and if not visible are less than the width of the symbol.

ated with reproductive development in insects and can influence ovulation and mating behavior in flies (Dubrovsky *et al.*, 2002; Soller *et al.*, 1999). However, a difficulty with any hormonal explanation is that the stage at which these traits develop is different, so hormone production would have to be altered for extended periods. Eyestalks form from eye-antennal discs in third instar larvae (Buschbeck *et al.*, 2001) while the ventral receptacle forms from genital discs that differentiate late in pupal development in stalk-eyed flies. Recent work indicates that exogenous application of

TABLE 3. *F*-ratios from analyses of covariance for those traits that differed between selection lines using microsatellite genotype as main effect and body length as a covariate.†

Source	ms125	ms244	ms54	ms395
<i>Male eye span</i>				
Body length	59.7***	62.1***	101.4***	88.6***
Genotype	3.3*	27.9***	20.4***	47.4***
<i>Female eye span</i>				
Body length	96.9***	120.4***	100.2***	110.0***
Genotype	6.4***	39.3***	17.0***	22.4***
<i>Ventral receptacle</i>				
Body length	0.2	2.1	0.8	6.2*
Genotype	3.2	10.4***	5.6***	3.4*

\*  $P < 0.05$ ; \*\*  $P < 0.01$ ; \*\*\*  $P < 0.0001$ .

† Microsatellites are ordered as they occur on a recombination map of the X chromosome (see text for intervals).

synthetic juvenile hormone on stalk-eyed fly third instar larvae increases relative eye span of small-bodied males and reduces testes growth (C.L. Fry, personal communication). However, no effect on female eye span, egg size or egg number has been observed when synthetic juvenile hormone is applied to larvae. Thus, the available evidence does not strongly support JH-mediation of the correlated responses observed in this study.

A second possibility is that the genes that influence eye span could have pleiotropic effects on the development of female reproductive traits. A recent survey of genes putatively under sexual selection in *Drosophila melanogaster* found that 73% of them exhibited pleiotropy (Fitzpatrick, 2004). Furthermore, one gene, *icebox*, influences both female courtship behavior and seminal receptacle development while another gene, *ovarian tumor*, influences both female courtship behavior and ovary development. While no genes have yet been found to influence a male ornament and a female reproductive trait, these examples show that differential expression of a single gene could alter traits involved in precopulatory and postcopulatory sexual selection. However, given that only 2 of 100 genes examined were found to influence both courtship behavior and female reproductive traits in *Drosophila* (Fitzpatrick, 2004), it seems unlikely that three out of four X-linked microsatellites, which were arbitrarily selected from a genomic library and do not occur in the coding regions of any known genes (Wright *et al.*, 2004), would show pleiotropic effects on both male eyespan and ventral receptacle size.

A third possibility is that genes that influence eye span may be in linkage disequilibrium with genes that influence egg and ventral receptacle size. For this to occur, genes that influence all three traits would need to lie on the same chromosome. In *C. dalmanni*, the X chromosome explained 30% of the variation in relative eye span between lines after 30 generations of selection (Wolfenbarger and Wilkinson, 2001) and a major QTL that affects male and female relative eye span has been identified on the X chromosome be-

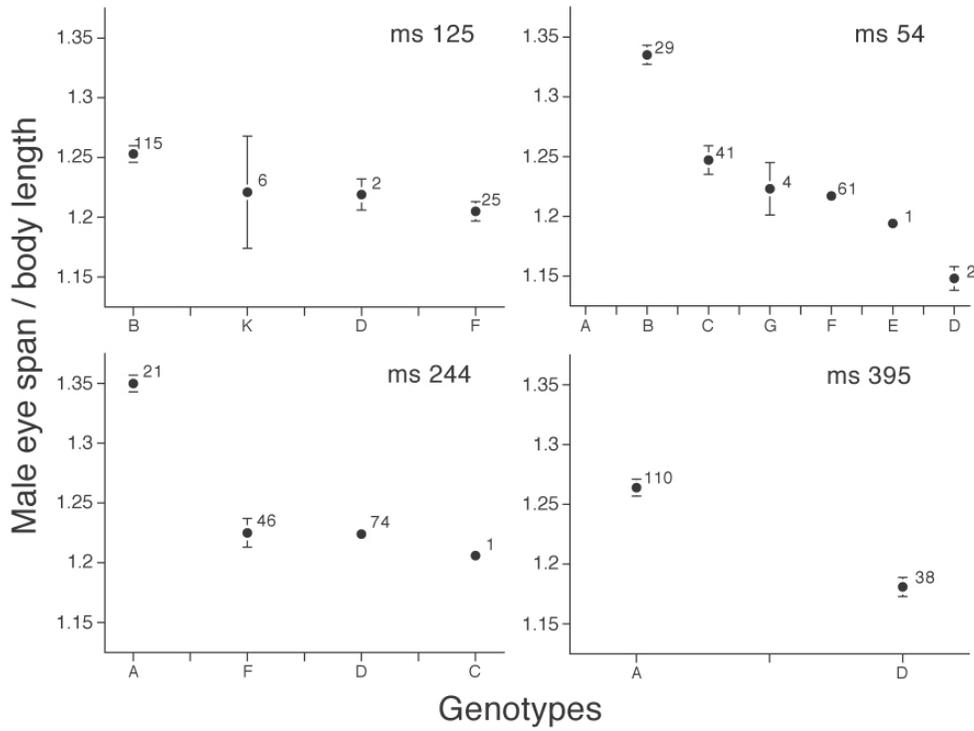


FIG. 7. Mean male relative eye span ordered from largest to smallest for each genotype at four X-linked microsatellite loci. Error bars represent one standard error and sample size is indicated next to each point. The length of each microsatellite allele is as follows. MS125: B–152 bp, F–156 bp, D–158 bp, K–174 bp; MS244: C–217 bp, D–220 bp, A–226 bp, F–230 bp; MS54: A–160 bp, B–162 bp, C–164 bp, G–166 bp; D–168 bp; E–170 bp, F–177 bp; MS395: D–195 bp, A–201 bp.

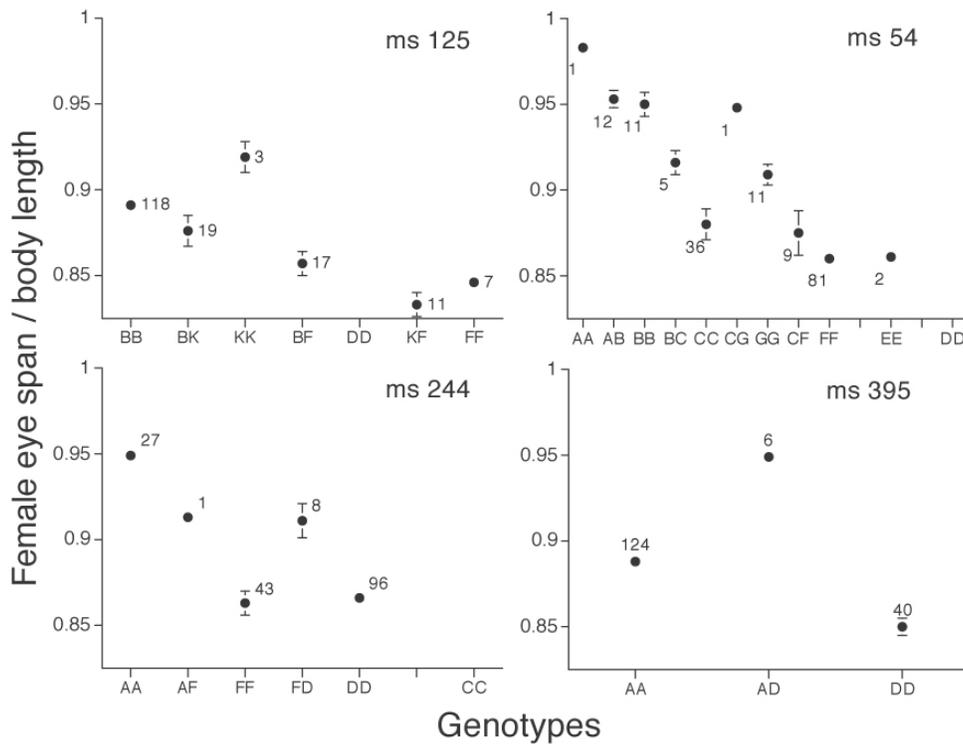


FIG. 8. Mean female relative eye span for each genotype at four X-linked microsatellite loci. Error bars represent one standard error and sample size is indicated next to each point. Genotype order and allele sizes as in Figure 7.

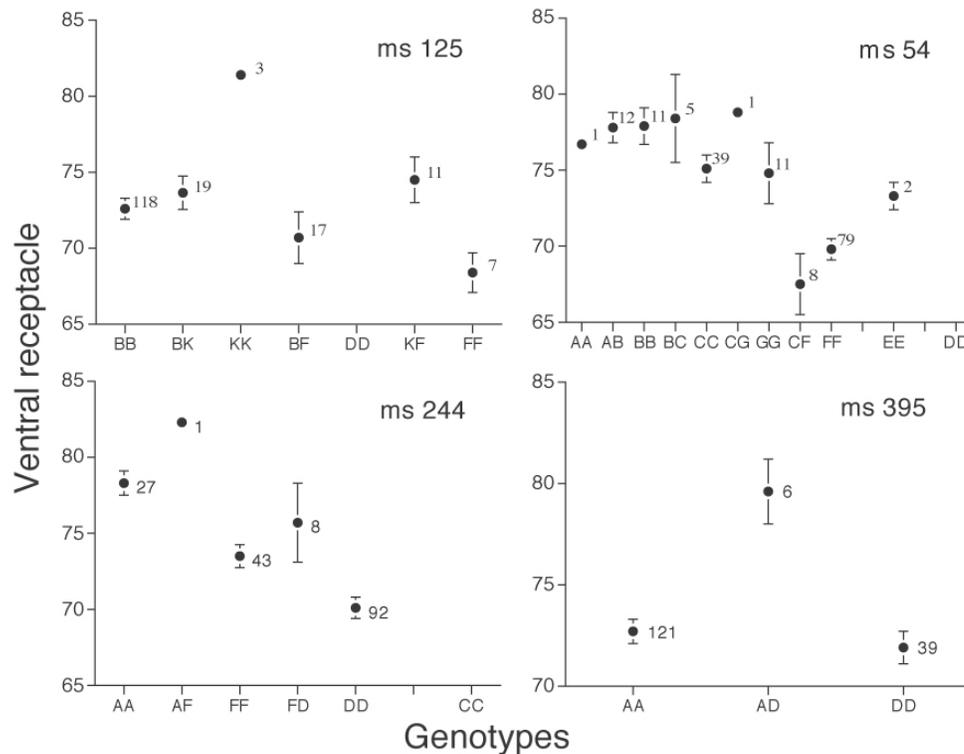


FIG. 9. Mean female ventral receptacle width for each genotype at four X-linked microsatellite loci. Error bars represent one standard error and sample size is indicated next to each point. Genotype order and allele sizes as in Figure 7.

tween microsatellite markers ms244 and ms54 (P.M.J. and G.S.W., unpublished data). If linkage disequilibrium was responsible for the correlated responses, we would expect different trait means for flies that differ in X genotype, with this difference being greatest near the X-linked QTL for male relative eye span. Examination of Table 3 reveals that the genotypic effect on all three traits was greatest for the two loci, ms244 and ms54, that flank the eye span QTL and that significant genotypic effects on ventral receptacle size occur at three loci which span 10.7 cM of the X chromosome (P.M.J. and G.S.W., unpublished data). This evidence strongly supports linkage among genes on the X chromosome as the cause of the correlated changes in ventral receptacle and egg size.

Because strong selection, such as that imposed by long term artificial selection, can generate linkage disequilibrium, the most generalizable result of this study may be that genes which influence ventral receptacle and egg size are X-linked. Nevertheless, there is reason to consider the possibility that linkage may have influenced the correlated evolution of reproductive traits among species of diopsid stalk-eyed flies (Presgraves *et al.*, 1999). Every population of *Cyrtodiopsis dalmanni* and *C. whitei* that has been examined to date contains appreciable frequencies of X chromosomes that exhibit meiotic drive (Presgraves *et al.*, 1997; Wilkinson *et al.*, 2003). Meiotic drive can increase the size of regions that persist in linkage disequilibrium because it prevents random assortment of alleles and

reduces effective population size. Furthermore, drive chromosomes are often associated with paracentric inversions that reduce recombination (Jaenike, 2001; Jiggins *et al.*, 1999). We have found that recombination is extremely reduced between drive and nondrive X chromosomes in *C. dalmanni* (P.M.J. and G.S.W., unpublished data). Recent studies on *sex ratio* X chromosomes in *Drosophila simulans* have found evidence of selective sweeps that reduce genetic variation near the meiotic drive factor (Derome *et al.*, 2004). These observations suggest that a population genetic study designed to assess the degree of linkage disequilibrium across the X chromosome is warranted to determine the degree to which X-linked traits segregate independently in natural populations of stalk-eyed flies.

Correlated selection mediated by sperm competition seems to be the least likely explanation for the correlated responses we observed in this study, although it may explain the patterns of correlated evolution found between sperm and female reproductive traits among species of diopsids (Presgraves *et al.*, 1999) and *Drosophila* (Pitnick *et al.*, 1999; Pitnick *et al.*, 2003). Even though our selection regime altered the breeding sex ratio and probably increased sperm competition midway through the experiment, all lines experienced the same regime and, therefore, sperm competition should not have differed between lines. The absence of any significant treatment effects on testis size supports this interpretation. However, in *Drosophila melanogaster* selection on seminal receptacle length resulted in

changes in sperm length, presumably due to correlated selection (Miller and Pitnick, 2002). If sperm length is heritable in diopsids, as it is in dung flies (Ward, 2000), dung beetles (Simmons and Kotiaho, 2002), crickets (Morrow and Gage, 2001), and drosophila (Joly *et al.*, 1995; Pitnick and Miller, 2000), then sperm length should have changed due to selection exerted by the change in ventral receptacle size observed between treatments. The absence of any detectable differences in sperm length may be due to weak correlated selection, low heritability, or low experimental power. Comparison of sperm lengths and female reproductive tracts among recently isolated populations of *Cyrtodiopsis* is underway to determine the rate at which these traits evolve and coevolve.

The existence of genetic correlations between genes that influence a male ornament and female traits involved in fertilization success has potential consequences for the maintenance of genetic variation for sexually selected traits. How genetic variation can be maintained for traits under strong sexual selection is commonly known as the lek paradox (Borgia, 1979; Taylor and Williams, 1982). Possible solutions to the lek paradox include nonlinear selection with an accumulation of modifiers that increase phenotypic variation (Pomiankowski and Møller, 1995), genic capture of condition dependence (Rowe and Houle, 1996), and antagonistic pleiotropy between genes that influence both male and female reproductive success (Rice and Chippindale, 2001). Our results provide another alternative: male ornament genes may be linked or exhibit pleiotropy with genes having nonadditive effects on female traits under selection. This possibility is suggested by the presence of nonadditive X-linked effects for female reproductive traits while the same chromosomal regions exhibit additive effects on male eye span. In effect, under or over-dominance in females at loci that exhibit additive allelic effects in males is a form of sexual antagonism because males and females have different genetic optima. Thus, sexual antagonism is possible even though we observed a positive, rather than a negative (Chippindale *et al.*, 2001), correlation between the expression of a male ornament and a female reproductive trait. Whether such sex-biased nonadditive genetic effects occur in other sexually selected species merits additional study.

#### ACKNOWLEDGMENTS

We are especially grateful to M. Muscedere and E. Kelleher for assistance in the lab and to two anonymous reviewers for useful comments. This work was supported by a graduate fellowship to EGA from the program in Behavior, Ecology, Evolution and Systematics, by a postdoctoral fellowship to PMJ from an NIH training grant in Neuroethology, and by grants DEB-0065427 and DEB-0343617 to GSW from the National Science Foundation.

#### REFERENCES

- Baker, R. H., R. I. S. Ashwell, T. A. Richards, K. Fowler, T. Chapman, and A. Pomiankowski. 2001. Effects of multiple mating

- and male eye span on female reproductive output in the stalk-eyed fly, *Cyrtodiopsis dalmani*. *Behav. Ecol.* 12:732–739.
- Baker, R. H. and G. S. Wilkinson. 2001. Phylogenetic analysis of eye stalk allometry and sexual dimorphism in stalk-eyed flies (Diopsidae). *Evolution* 55:1373–1385.
- Bakker, T. C. M. 1993. Positive genetic correlation between female preference and preferred male ornament in sticklebacks. *Nature* 363:255–257.
- Borgia, G. 1979. Sexual selection and the evolution of mating systems. In M. S. Blum, and N. A. Blum, (eds.), *Sexual selection and reproductive competition in insects*, pp. 19–80. Academic Press, New York.
- Briskie, J. V. and R. Montgomerie. 1993. Patterns of sperm storage in relation to sperm competition in passerine birds. *Condor* 95: 442–454.
- Briskie, J. V., R. Montgomerie, and T. R. Birkhead. 1997. The evolution of sperm size in birds. *Evolution* 51:937–945.
- Buschbeck, E. K., J. L. Roosevelt, and R. R. Hoy. 2001. Eye stalks or no eye stalks: A structural comparison of pupal development in the stalk-eyed fly *Cyrtodiopsis* and in *Drosophila*. *J. Comp. Neurol.* 433:486–498.
- Caceres, M., A. Barbadilla, and A. Ruiz. 1999. Recombination rate predicts inversion size in diptera. *Genetics* 153:251–259.
- Charlesworth, B., J. A. Coyne, and N. H. Barton. 1987. The relative rates of evolution of sex chromosomes and autosomes. *Am. Nat.* 130:113–146.
- Chippindale, A. K., J. R. Gibson, and W. R. Rice. 2001. Negative genetic correlation for adult fitness between sexes reveals ontogenetic conflict in *Drosophila*. *Proc. Natl. Acad. Sci. U.S.A.* 98:1671–1675.
- Derome, N., K. Metayer, C. Montchamp-Moreau, and M. Veuille. 2004. Signature of selective sweep associated with evolution of *sex-ratio* drive in *Drosophila simulans*. *Genetics* 166:1357–1366.
- Dubrovsky, E. B., V. A. Dubrovskaya, and E. M. Berger. 2002. Juvenile hormone signaling during oogenesis in *Drosophila melanogaster*. *Insect Biochem. Mol. Biol.* 32:1555–1565.
- Dybas, L. K. and H. S. Dybas. 1981. Coadaptation and taxonomic differentiation of sperm and spermathecae in featherwing beetles. *Evolution* 35:168–174.
- Eberhard, W. G. 1996. *Female control: sexual selection by cryptic female choice*. Princeton University Press, Princeton.
- Emlen, D. J. and H. F. Nijhout. 1999. Hormonal control of male horn length dimorphism in the dung beetle *Onthophagus taurus* (Coleoptera: Scarabaeidae). *J. Insect Physiol.* 45:45–53.
- Emlen, D. J. and H. F. Nijhout. 2001. Hormonal control of male horn length dimorphism in *Onthophagus taurus* (Coleoptera: Scarabaeidae): A second critical period of sensitivity to juvenile hormone. *J. Insect Physiol.* 47:1045–1054.
- Fitzpatrick, M. J. 2004. Pleiotropy and the genomic location of sexually selected genes. *Am. Nat.* 163:800–808.
- Fry, C. L. and G. S. Wilkinson. 2004. Sperm survival in female stalk-eyed flies depends on seminal fluid and meiotic drive. *Evolution* 58:1622–1626.
- Gavrilets, S., G. Arnqvist, and U. Friberg. 2001. The evolution of female mate choice by sexual conflict. *Proc. Roy. Soc. London B* 268:531–539.
- Gibson, J. R., A. K. Chippindale, and W. R. Rice. 2002. The X chromosome is a hot spot for sexually antagonistic fitness variation. *Proc. Roy. Soc. London B* 269:499–505.
- Grant, C. A., K. Fowler, and T. Chapman. 2002. No reduction of female sexual receptivity following mating in a stalk-eyed fly, *Cyrtodiopsis dalmani* (Diptera: Diopsidae). *J. Evol. Biol.* 15: 210–215.
- Gray, D. A. and W. H. Cade. 2000. Sexual selection and speciation in field crickets. *Proc. Natl. Acad. Sci. U.S.A.* 97:14449–14454.
- Houde, A. E. 1994. Effect of artificial selection on male colour patterns on mating preference of female guppies. *Proc. Roy. Soc. London B* 256:125–130.
- Iwasa, Y., A. Pomiankowski, and S. Nee. 1991. The evolution of costly mate preferences. II. The “handicap” principle. *Evolution* 45:1431–1442.

- Jaenike, J. 2001. Sex chromosome meiotic drive. *Annu. Rev. Ecol. Syst.* 32:25–49.
- Jiggins, F. M., G. D. D. Hurst, and M. E. N. Majerus. 1999. How common are meiotically driven sex chromosomes? *Am. Nat.* 154:481–483.
- Joly, D., C. Bazin, L.-W. Zeng, and R. S. Singh. 1995. Genetic basis of sperm and testis length differences and epistatic effect on hybrid inviability and sperm motility between *Drosophila simulans* and *D. sechellia*. *Heredity* 78:354–362.
- Kirkpatrick, M. 1982. Sexual selection and the evolution of female choice. *Evolution* 36:1–12.
- Kirkpatrick, M. and D. W. Hall. 2004. Sexual selection and sex linkage. *Evolution* 58:683–691.
- Kotrba, M. 1990. Sperm transfer by spermatophore in an acalyptrate fly (Diptera: Diopsidae). *Entomol. Gener.* 15:181–183.
- Kotrba, M. 1995. The internal female genital organs of *Chaetodiopsis* and *Diasemopsis* (Diptera: Diopsidae) and their systematic relevance. *Ann. Natal Mus.* 36:147–159.
- Lande, R. 1981. Models of speciation by sexual selection on polygenic traits. *Proc. Natl. Acad. Sci. U.S.A.* 78:3721–3725.
- Lande, R. and G. S. Wilkinson. 1999. Models of sex-ratio meiotic drive and sexual selection in stalk-eyed flies. *Genet. Res.* 74:245–253.
- Lindholm, A. and F. Breden. 2002. Sex chromosomes and sexual selection in poeciliid fishes. *Am. Nat.* 160:S214–S224.
- Miller, G. T. and S. Pitnick. 2002. Sperm-female coevolution in *Drosophila*. *Science* 298:1230–1233.
- Morrow, E. H. and M. J. G. Gage. 2000. The evolution of sperm length in moths. *Proc. Roy. Soc. London B* 267:307–313.
- Morrow, E. H. and M. J. G. Gage. 2001. Artificial selection and heritability of sperm length in *Gryllus bimaculatus*. *Heredity* 87:1–7.
- Navarro, A., E. Betran, A. Barbadilla, and A. Ruiz. 1997. Recombination and gene flux caused by gene conversion and crossing over in inversion heterokaryotypes. *Genetics* 146:695–709.
- Palopoli, M. F. and C.-I. Wu. 1996. Rapid evolution of a coadapted gene complex: Evidence from the *Segregation Distorter* (SD) system of meiotic drive in *Drosophila melanogaster*. *Genetics* 143:1675–1688.
- Panhuis, T. M. and G. S. Wilkinson. 1999. Exaggerated male eye span influences contest outcome in stalk-eyed flies. *Behav. Ecol. Sociobiol.* 46:221–227.
- Parisi, M., R. Nuttall, D. Naiman, G. Bouffard, J. Malley, J. Andrews, S. Eastman, and B. Oliver. 2003. Paucity of genes on the *Drosophila* X chromosome showing male-biased expression. *Science* 299:697–700.
- Parker, G. A. 1979. Sexual selection and sexual conflict. In M. S. Blum and N. B. Blum (eds.), *Sexual selection and reproductive competition in insects*, pp. 123–166. Academic Press, New York.
- Parker, G. A. and L. Partridge. 1998. Sexual conflict and speciation. *Philos. Trans. R. Soc. London B* 353:261–274.
- Pitnick, S., T. Markow, and G. S. Spicer. 1999. Evolution of multiple kinds of female sperm-storage organs in *Drosophila*. *Evolution* 53:1804–1822.
- Pitnick, S. and G. T. Miller. 2000. Correlated response in reproductive and life history traits to selection on testis length in *Drosophila hydei*. *Heredity* 84:416–426.
- Pitnick, S., G. T. Miller, K. Schneider, and T. A. Markow. 2003. Ejaculate-female coevolution in *Drosophila mojavensis*. *Proc. Roy. Soc. London B* 270:1507–1512.
- Pomiankowski, A. 1987. Sexual selection: The handicap principle does work-sometimes. *Proc. Roy. Soc. London B* 231:123–145.
- Pomiankowski, A. and A. P. Møller. 1995. A resolution of the lek paradox. *Proc. Roy. Soc. London B* 260:21–29.
- Presgraves, D. C., R. H. Baker, and G. S. Wilkinson. 1999. Coevolution of sperm and female reproductive tract morphology in stalk-eyed flies. *Proc. Roy. Soc. London B* 266:1041–1047.
- Presgraves, D. C., E. Severence, and G. S. Wilkinson. 1997. Sex chromosome meiotic drive in stalk-eyed flies. *Genetics* 147:1169–1180.
- Ranz, J. M., C. I. Castillo-Davis, C. D. Meiklejohn, and D. L. Hartl. 2003. Sex-dependent gene expression and evolution of the *Drosophila* transcriptome. *Science* 300:1742–1745.
- Reguera, P., A. Pomiankowski, K. Fowler, and T. Chapman. 2004. Low cost of reproduction in female stalk-eyed flies, *Cyrtodiopsis dalmanni*. *J. Ins. Physiol.* 50:103–108.
- Reinhold, K. 1998. Sex linkage among genes controlling sexually selected traits. *Behav. Ecol. Sociobiol.* 44:1–7.
- Reinhold, K. 1999. Evolutionary genetics of sex-limited traits under fluctuating selection. *J. Evol. Biol.* 12:897–902.
- Rice, W. R. 1984. Sex chromosomes and the evolution of sexual dimorphism. *Evolution* 38:735–742.
- Rice, W. R. 1996. Sexually antagonistic male adaptation triggered by experimental arrest of female evolution. *Nature* 381:232–235.
- Rice, W. R. and A. K. Chippindale. 2001. Intersexual ontogenetic conflict. *J. Evol. Biol.* 14:685–693.
- Rowe, L. and D. Houle. 1996. The lek paradox and the capture of genetic variance by condition-dependent traits. *Proc. Roy. Soc. London B* 263:1415–1421.
- Saifi, G. M. and H. S. Chandra. 1999. An apparent excess of sex- and reproduction-related genes on the human X chromosome. *Proc. Roy. Soc. London B* 266:203–209.
- SAS\_Institute. 2003. JMP. SAS Institute, Inc., Cary, North Carolina.
- Simmons, L. W. and J. S. Kotiaho. 2002. Evolution of ejaculates: Patterns of phenotypic and genotypic variation and condition dependence in sperm competition traits. *Evolution* 56:1622–1631.
- Soller, M., M. Bownes, and E. Kubli. 1999. Control of oocyte maturation in sexually mature *Drosophila* females. *Dev. Biol.* 208:337–351.
- Taylor, P. D. and G. C. Williams. 1982. The lek paradox is not resolved. *Theor. Popul. Biol.* 22:392–409.
- Wang, P. J., J. R. McCarrey, F. Yang, and D. C. Page. 2001. An abundance of X-linked genes expressed in spermatogonia. *Nat. Genet.* 27:422–6.
- Ward, P. I. 2000. Sperm length is heritable and sex-linked in yellow dung fly (*Scatophaga stercoraria*). *J. Zool.* 251:349–353.
- Wilkinson, G. S. 1993. Artificial sexual selection alters allometry in the stalk-eyed fly *Cyrtodiopsis dalmanni* (Diptera: Diopsidae). *Genet. Res.* 62:213–222.
- Wilkinson, G. S., H. Kahler, and R. H. Baker. 1998a. Evolution of female mating preferences in stalk-eyed flies. *Behav. Ecol.* 9:525–533.
- Wilkinson, G. S., D. C. Presgraves, and L. Crymes. 1998b. Male eye span in stalk-eyed flies indicates genetic quality by meiotic drive suppression. *Nature* 391:276–278.
- Wilkinson, G. S. and P. R. Reillo. 1994. Female preference response to artificial selection on an exaggerated male trait in a stalk-eyed fly. *Proc. Roy. Soc. London B* 255:1–6.
- Wilkinson, G. S. and M. I. Sanchez. 2001. Sperm development, age and sex chromosome meiotic drive in the stalk-eyed fly, *Cyrtodiopsis whitei*. *Heredity* 87:17–24.
- Wilkinson, G. S., J. G. Swallow, S. J. Christianson, and K. Madden. 2003. Phylogeography of *sex ratio* and multiple mating in stalk-eyed flies from southeast Asia. *Genetica* 117:37–46.
- Wolfenbarger, L. L., and G. S. Wilkinson. 2001. Sex-linked expression of a sexually selected trait in the stalk-eyed fly, *Cyrtodiopsis dalmanni*. *Evolution* 55:103–110.
- Wright, T. F., P. M. Johns, J. P. Walters, A. P. Lerner, J. G. Swallow, and G. S. Wilkinson. 2004. Microsatellite variation among divergent populations of stalk-eyed flies, genus *Cyrtodiopsis*. *Genet. Res.* 84:27–40.