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Ejaculate–female coevolution in *Drosophila mojavensis*

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Interspecific studies indicate that sperm morphology and other ejaculatory traits diverge more rapidly than other types of character in *Drosophila* and other taxa. This pattern has largely been attributed to postcopulatory sexual selection involving interaction between the sexes. Such divergence has been suggested to lead rapidly to reproductive isolation among populations and thus to be an 'engine of speciation.' Here, we test two critical predictions of this hypothesis: (i) there is significant variation in reproductive traits among incipient species; and (ii) divergence in interacting sex-specific traits exhibits a coevolutionary pattern among populations within a species, by examining geographical variation in *Drosophila mojavensis*, a species in the early stages of speciation. Significant among-population variation was identified in sperm length and female sperm-storage organ length, and a strong pattern of correlated evolution between these interacting traits was observed. In addition, crosses among populations revealed coevolution of male and female contributions to egg size. Support for these two important predictions confirms that coevolving internal characters that mediate successful reproduction may play an important part in speciation. The next step is to determine exactly what that role is.

Keywords: sperm; egg; sexual selection; coevolution; reproductive isolation; speciation

1. INTRODUCTION

Comparative studies of gene sequences among *Drosophila* species, and in other taxa, have revealed that reproductive traits, such as seminal fluid proteins (Acps), evolve more rapidly than other types of trait (Civetta & Singh 1998; Swanson & Vacquier 2002). Comparative investigations of genital morphology (Eberhard 1985) and of sperm and female sperm-storage organ length (e.g. Pitnick *et al.* 1995*a*, 1999) suggest a similar pattern for reproductive morphology. Postcopulatory sexual selection (including sexual conflict) involving evolutionary interaction between the sexes is believed to be the primary cause of this pattern (Eberhard 1985; Rice 1996; Parker & Partridge 1998; Gavrilets 2000).

For example, the cumulative evidence implicates postcopulatory sexual selection as the principal force driving sperm-length evolution in a diversity of animal groups (e.g. Gage 1998). Comparative studies have identified a positive relationship between sperm length and the risk of encountering sperm competition in birds (Briskie & Montgomerie 1992; Briskie *et al.* 1997), butterflies (Gage 1994) and nematodes (LaMunyon & Ward 1999), although not in mammals (Harcourt 1991; Hosken 1997; Gage & Freckelton 2003) or fishes (Stockley *et al.* 1997). In addition, there is a pattern of correlated evolution between sperm length and certain dimensions of the female reproductive tract in birds (Briskie & Montgomerie 1993; Briskie *et al.* 1997) and in a variety of insects (Dybas & Dybas 1981; Gage 1994; Pitnick *et al.* 1999; Presgraves *et al.* 1999; Morrow & Gage 2000). Finally, although the evolutionary causes are unknown, comparative studies of both fishes (Stockley *et al.* 1997) and echinoids (Raff *et al.* 1990) indicate that the sperm of internally fertilizing species are generally longer than those of externally fertilizing relatives. Collectively, these studies suggest that, in some animal taxa, the physical environment of the female reproductive tract selects for characteristics that enhance the competitive fertilization success of sperm (Keller & Reeve 1995; Eberhard 1996).

Among species within the genus *Drosophila*, sperm length is also highly variable (Pitnick *et al.* 1995*a*,*b*), and there is a strong positive relationship across species between the length of sperm and the length of the females' primary sperm-storage organ, the seminal receptacle (SR) (Pitnick *et al.* 1999). A recent investigation using experimental evolution in the laboratory revealed that differential male fertilization success is largely determined by an interaction between male sperm length and female SR length. In addition, evolutionary increases in SR length drove the correlated evolution of sperm length within experimental lines (Miller & Pitnick 2002, 2003). These data suggest that the correlated evolution between sperm and female reproductive tract morphology is the result of coevolution, with phenotypic variation in each sex-specific trait generating selection on the corresponding trait in the opposite sex.

Based on these general patterns and the demonstrated coevolutionary process, we and others have suggested that divergence among populations in traits such as these may result in compromised ejaculate–female compatibility when members of these populations interbreed, thus limiting gene exchange. This phenomenon might be a common mechanism leading to reproductive isolation and speciation (Markow 1997; Civetta & Singh 1998; Parker & Partridge 1998; Pitnick *et al.* 1999; Brown & Eady 2001; Eady 2001; Miller & Pitnick 2002). The broadly observed pattern of conspecific sperm and pollen precedence supports this contention (reviewed by Howard 1999; see also

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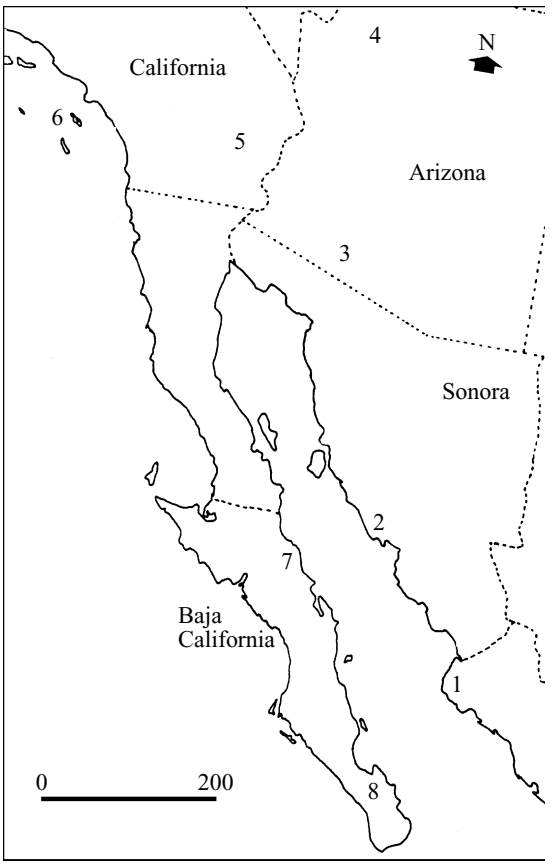

Figure 1. Map of southwestern United States and northwestern Mexico. Numbers indicate collection localities for eight populations of *Drosophila mojavensis* examined (month/year): (1) Topolobampo, Sinaloa (11/1996); (2) San Carlos, Sonora (2/1997); (3) Organ Pipe National Monument, AZ (4/1998); (4) Whitmore Canyon of Grand Canyon, AZ (9/1996); (5) Anza Borrego, CA (3/1996); (6) Catalina Island, CA (3/1997); (7) South Mulege, Baja (4/1997); (8) Cape region of La Paz, Baja (11/1996). Scale bar, 200 miles.

Price *et al.* 2000, 2001). The possibility that divergence in the reproductive traits in question did not occur until after speciation, however, has never been ruled out. If such a pattern were found, then any causal role for such divergence in the speciation process would be excluded.

We test two critical predictions of the hypothesis that coevolution of sex-specific reproductive traits contributes to speciation: (i) there is significant variation in reproductive traits among incipient species; and (ii) divergence in interacting sex-specific traits exhibits a coevolutionary pattern among populations within a species. To test the first prediction, we quantified among-population variation in sperm and SR length across the range of *Drosophila mojavensis* (figure 1). Because this species appears to be in the early stages of speciating (Markow & Hocutt 1998), the level of heritable geographical variation in this species can be used to determine the extent to which traits have diverged relative to the speciation process. To test the second prediction, we examined the correlated evolution of sperm and SR length among the geographical populations. Additionally, because ejaculates of *D. mojavensis* include a 'nutritive donation' that females use to make eggs (Markow & Ankney 1984; Pitnick *et al.* 1997), we also tested for male–female coevolution underlying this phenomenon.

2. MATERIAL AND METHODS

Flies were collected from eight locations across their range between 1996 and 1998 as indicated in figure 1. All experimental flies were reared under standardized conditions. For each population, 50 first-instar larvae were transferred to each of several 36 mm shell vials containing 8 ml of medium with live yeast. On the day of eclosion, virgin flies were sorted according to sex following anaesthetization with CO_2 and maintained in vials with medium and live yeast until reproductively mature (4–6 days for females; 6–8 days for males). All geographical populations were thus reared contemporaneously and all traits were measured on an equal number of flies from each population or cross on each day of an experiment. As an index of total body mass, the thorax length of flies was measured (Robertson & Reeve 1952) using an ocular reticule under a stereomicroscope.

Sperm length of each anaesthetized male ($n = 3$ sperm per male, 15 males per population) was measured by dissecting the seminal vesicles into phosphate-buffered saline (PBS) on a subbed slide. After releasing a few hundred sperm into the saline, preparations were dried in a 60 °C oven, fixed in methanol : acetic acid (3 : 1), and then mounted with glycerol : PBS (9 : 1) under a glass coverslip. Digital images of sperm using darkfield optics at a magnification of ×200 were obtained using a Dage CCD72 camera (Dage-MTI Inc., Michigan City, IN, USA) mounted on an Olympus BX60 microscope (Olympus America Inc., Melville, NY, USA). Sperm were measured to the nearest 10 μm using NIH Image public domain software (http://rsb.info.nih.gov/nih-image).

For each female ($n = 40$ per population), following anaesthetization with ether, the reproductive tract was dissected into PBS on a microscope slide. A glass coverslip was placed on top with clay at the corners that allowed flattening of the SR to two dimensions without stretching the organ. The preparation was then viewed at magnification ×200 using differential interference contrast microscopy. A digitized image of the SR was obtained and organ length determined by tracing its lumen using NIH Image.

To examine male, female and male × female interaction effects on egg size, we conducted crosses between flies from two populations: Organ Pipe National Monument, AZ (OP) and the Grand Canyon, AZ (GC). There were two control (OP × OP and GC × GC) and two reciprocal (OP × GC and GC × OP) crosses. For each cross, virgin flies were randomly assigned to cross treatment and paired en masse on oviposition plates for 48 h ($n = 50$ females and 50 males per cross). Plates from the first 24 h contained many eggs, which we discarded. Eggs measured ($n = 50$ per cross for each of two experimental replicates; $n = 400$ total) were from the latter 24 h and thus presumed to have been manufactured following insemination. Egg volume was determined by aligning each egg with a similar orientation on the surface of the medium using a fine probe. A digitized image of each egg was obtained at a magnification of ×130 through an Olympus SZX12 stereomicroscope. The length and width of each egg was measured using NIH Image. Egg volume was then calculated as the volume of a prolate spheroid using the formula $(\text{length} \times \text{width}^2 \times \pi)/6$. Experimental replicates were conducted several generations apart.

Table 1. Results of nested analysis of variance to partition variation among sources for sperm length ($n = 3$ sperm per male; 15 males per population; eight populations) and seminal receptacle (SR) length ($n = 40$ females per population; eight populations).

variance source	d.f.	mean square	F	p	% of total
sperm length					
total	359	0.0071			100.00
populations	7	0.2338	34.14	< 0.0001	64.92
males within populations	112	0.0068	10.32	< 0.0001	26.54
sperm within males	240	0.0007			8.54
SR length					
total	319	0.2202			100.00
populations	7	1.4236	7.38	< 0.0001	13.91
females within populations	312	0.1928			86.09

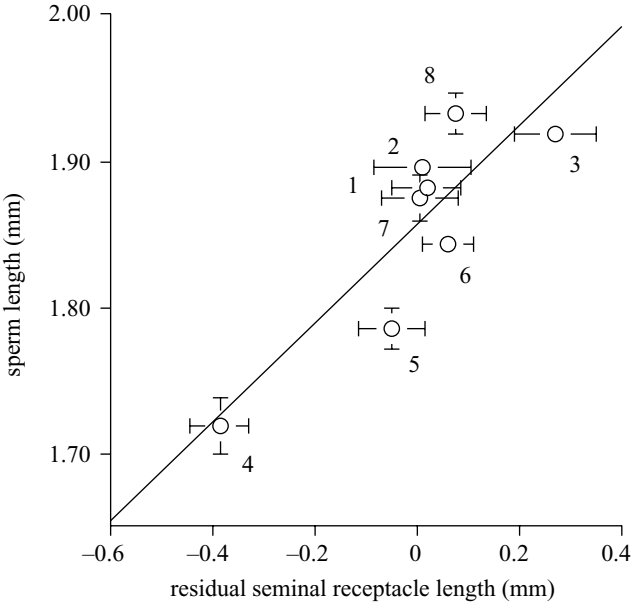

Figure 2. Relationship between mean population sperm length and residual mean population SR length, after removing effects of body size on SR length. Numbers indicate populations as in figure 1. Bars indicate 1 s.e.

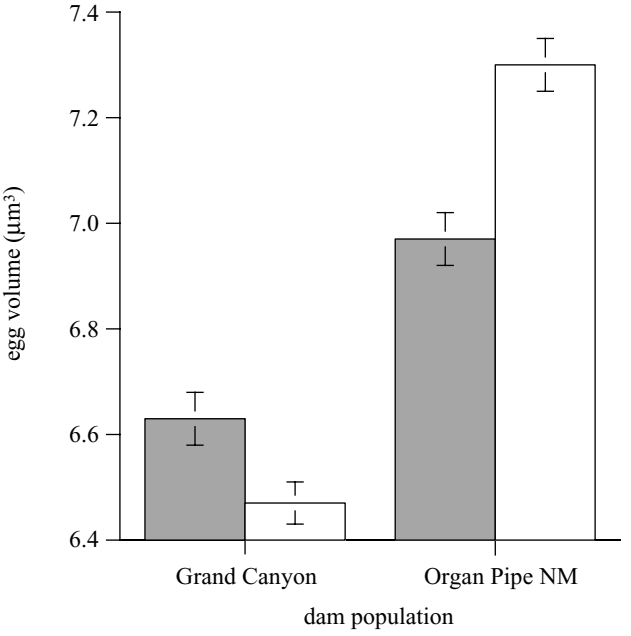

Figure 3. Mean ± s.e. egg volume for crosses with sires from Organ Pipe National Monument (open bars; figure 1, no. 3) and Grand Canyon (filled bars; figure 1, no. 4), AZ populations.

3. RESULTS

We found evidence of significant evolutionary divergence among geographical populations of *D. mojavensis* for all three reproductive traits examined. Variation in sperm length was partitioned among sources (i.e. among sperm within males, among males within populations, and among populations) by nested analysis of variance using SAS (SAS 1989). Variation in SR length was similarly partitioned into components (i.e. among females within populations and among populations). Both traits exhibited highly significant divergence among populations (table 1; figure 2). For sperm length, 65% of the variation was attributable to differences among populations. Consistent with studies of other taxa, significant variation (27%) was attributable to differences among males within populations (Ward 1998) and there was relatively little variation among sperm within males (Morrow & Gage 2001). For SR length, the majority of variation (86%) was attributable to differences between females within populations. Analysis of variance comparing egg volume between the two within-population crosses (GC × GC and OP × OP), with replicate also entered as a factor, revealed a highly significant difference between these populations for this trait ($F_{1,196} = 105.35$, $p < 0.0001$), with OP females making significantly larger eggs than GC females (figure 3). This difference is consistent with differences in body size, OP flies being larger (analysis of thorax length from SR length experiment: OP mean ± s.e. = 1.115 ± 0.004, GC mean ± s.e. = 1.086 ± 0.004; $F_{1,78} = 28.02$, $p < 0.0001$). Unfortunately, owing to the mass culturing to obtain eggs, thorax length was not recorded in the egg volume experiment and thus cannot be included in the analysis as a covariate.

Next, the correlated evolution of sperm and SR length was examined. We first examined relationships between these traits and body size by regressing (least squares) mean sperm length or SR length on thorax length for all flies in the study. Consistent with other experiments examining condition-dependence of sperm phenotype in *Drosophila* (S. Pitnick, unpublished data), the relationship between sperm length and body size was not significant ($R^2 = 0.007$, $F_{1,119} = 0.86$, $p = 0.357$). There was, however, a significant positive relationship between SR length

Table 2. Results of analysis of variance of egg volume in crosses between OP (figure 1, #3) and GC (figure 1, #4) populations.

variance source	d.f.	mean square	F	p
dam population	1	3.38×10^{-5}	156.27	< 0.0001
sire population	1	6.69×10^{-7}	3.09	0.0795
replicate	1	6.69×10^{-7}	3.09	0.0794
dam × sire	1	6.06×10^{-6}	27.99	< 0.0001
dam × replicate	1	7.01×10^{-6}	32.40	< 0.0001
sire × replicate	1	1.72×10^{-6}	7.95	0.0051
dam × sire × replicate	1	2.56×10^{-6}	11.80	0.0007
residual	392	2.16×10^{-7}		

and female size ($R^2 = 0.037$, $F_{1,315} = 12.19$, $p < 0.001$). We thus examined the correlated evolution of sperm and SR length by regressing mean population sperm length on mean population residual SR length, after removing the effects of body size on SR length. As predicted, there was a significant pattern of correlated evolution between the sex-specific traits (figure 2; $R^2 = 0.736$, $F_{1,6} = 16.76$, $p < 0.01$).

Analysis of egg volume variation similarly suggests coevolution of male and female contributions to this trait. We performed an ANOVA of egg volume with dam population, sire population and replicate as the main factors with full interaction (table 2). Most notably, females of both populations made larger eggs when inseminated by males from their own population (figure 3; one-tailed *t*-tests with male source and replicate as factors: OP: $t_{198} = 4.391$, $p < 0.0001$; GC: $t_{198} = 2.536$, $p < 0.01$). This pattern contributed to the highly significant dam by sire interaction effect on egg volume (table 2). The significant interaction effects involving replicate may be attributable to between-replicate differences in the quality of medium or quantity of yeast in vials, as such factors are known to influence egg production (e.g. Robertson & Sang 1944) and may, perhaps, also affect ejaculatory donation quality and/or quantity.

4. DISCUSSION

Comparative studies reveal correlated macroevolution of sperm and SR length in the genus *Drosophila* and suggest that these traits are evolving rapidly (Pitnick *et al.* 1995*a*, 1999). Experimental evolution studies in the laboratory suggest that the interspecific pattern is attributable to coevolution driven by postcopulatory sexual selection mediated by female sperm choice (Miller & Pitnick 2002), a type of cryptic female choice (Birkhead 1998; Pitnick & Brown 2000; Simmons 2001). These studies have led to speculation that coevolution of sperm and female reproductive tract morphology may contribute to reproductive isolation between populations. Because they are believed to be incipient species (Markow & Hocutt 1998), geographical populations of *D. mojavensis* were used to test whether: (i) they differed in their sperm and SR lengths; and (ii) any divergence reflects a pattern of coevolution between the sexes as indicated by laboratory experiments (Miller & Pitnick 2002). Significant differences among populations in sperm and SR length clearly indicate that these reproductive traits can diverge extremely rapidly, and at a rate relevant to the speciation process. Measuring these traits under standardized conditions in the laboratory strongly implicates a genetic basis to such variation. This conclusion is supported by the results of quantitative genetic analyses using crosses between the Grand Canyon, AZ and Organ Pipe National Monument, AZ populations of *D. mojavensis* to resolve the genetic architecture underlying population differences in sperm and SR length (Miller *et al.* 2003).

The pattern of correlated evolution between sperm and SR length observed among populations of *D. mojavensis* is striking, with 73.6% of the variation in one trait explained by a variation in the corresponding trait of the opposite sex (figure 2). This result suggests that the process of female sperm choice identified to underlie coevolution of sperm and SR length in the laboratory (Miller & Pitnick 2002) has been important in nature. The far greater extent of among-population variation in sperm length over SR length is probably attributable to differences in the condition-dependence of these traits. The rearing environment has virtually no effect on sperm length but strongly influences SR length (E. Amitin and S. Pitnick, unpublished data). Studies to discern the role of divergence of these traits on reproductive isolation would benefit from the inclusion of an examination of gene by environment interaction on SR length and of the phenotypic distribution of SR lengths of wild females from different populations.

A role for pleiotropy in generating the correlated divergence in sperm and SR length is not supported by the collective evidence. First, the pattern of correlated change in sperm length, occurring in laboratory populations of *D. melanogaster* artificially selected for longer but not shorter SR length, provides compelling support for selection rather than pleiotropy as the causal mechanism (Miller & Pitnick 2002). Second, while not ruling out the possibility of pleiotropy, very different quantitative genetic models are supported for these two traits in *D. mojavensis*. SR length is largely an autosomal additive trait, whereas additive effects, dominance and epistasis all contributed to variation in sperm length (Miller *et al.* 2001, 2003). Moreover, pleiotropy is hardly expected between the length of female-specific somatic organ (consisting of a thin layer of visceral muscle, a basement membrane, cells making up the lumen wall and a cuticle-lined lumen; Blaney (1970)) and the length of male-specific sex cells (consisting primarily of an axial filament, the electron-dense and paracrystalline derivatives of the mitochondria and a plasma membrane; Lindsley & Tokuyasu (1980), Fuller (1993)).

Similarly, a pattern of population divergence and the coevolution of male and female reproductive traits were observed in the volumes of eggs produced by crosses

between flies from different populations (figure 3). Males of some species transfer proteins in their semen that females incorporate into developing oocytes (reviewed by Eberhard 1996; Markow 1996). Such 'ejaculatory donations' are larger in *D. mojavensis* and their close relatives than in any other of the numerous *Drosophila* species examined (Markow & Ankney 1984; Pitnick *et al.* 1997). Further, in some *Drosophila* species male-derived phosphorus is transferred in semen and incorporated into nucleic acids of the females' ovaries, and subsequently into mature oocytes (Markow *et al.* 2001). The significant sire by dam effect on egg volume suggests coevolution between quantity and/or quality of what males transfer and the physiological processes by which females use this material to make eggs.

To understand how new species come into existence, we need to understand why traits diverge and how they create barriers to interbreeding between incipient species (Rice & Hostert 1993). The selective environment for sperm length is the female reproductive tract (Pitnick *et al.* 1999; Miller & Pitnick 2002). Sperm-length divergence is thus relatively unconstrained by the physical environment. Divergence between populations may thus occur despite environmental homogeneity, requiring nothing more than restricted gene flow. The adaptive significance of SR length is unknown. Its rapid evolution supports a model of sexually antagonistic coevolution with sperm length, driven by intergenomic conflict (Rice & Holland 1997; Holland & Rice 1998), but other non-mutually exclusive models of sexual selection (e.g. Fisherian runaway selection, good genes) may also be relevant (Miller & Pitnick 2002). By contrast, coevolutionary divergence in genes underlying the male and female contributions (and their interaction) to egg volume, are more probably related to geographical variation in host adaptation. Some of the populations examined here (including OP and GC) use different host cacti (see fig. 17.2 in Markow & Hocutt 1998) that differ substantially in their chemical ecology and the community of micro-organisms that they support (Starmer 1982; Fogleman & Heed 1989; Fogelman & Abril 1990). Irrespective of whether male ejaculatory donations represent 'mating effort' or 'parental effort' (Gwynne 1986; Simmons & Parker 1989; Wedell 1993), ejaculate composition and female sequestration of male-derived molecules into oocytes may adapt to geographically varying nutritional limitations to female reproduction.

A recent study using among-population crosses of *D. mojavensis* also demonstrated divergence in sex-specific contributions to the duration of the insemination reaction (Knowles & Markow 2001), a large opaque vaginal mass that forms after mating and delays oviposition (Alonso-Pimentel *et al.* 1994). We can thus conclude that ejaculate–female coevolution in *D. mojavensis*, and presumably other taxa, is characterized by extremely rapid divergence and can simultaneously involve multiple reproductive processes including morphological, physiological and biochemical traits. Coevolving internal characters that mediate successful reproduction, therefore, can have a far greater role in speciation than previously assumed and may arise through cryptic female choice as argued by Eady (2001). It is now necessary to examine the correlation between quantifiable divergence in specific reproductive traits and the extent of postcopulatory, prezygotic reproductive isolation among populations for each species of interest.

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