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SHORT REVIEW

Seminal fluid-mediated fitness traits in *Drosophila*

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The seminal fluid of male *Drosophila* contains a cocktail of proteins that have striking effects on male and female fitness. In *D. melanogaster*, seminal fluid proteins affect female receptivity, ovulation, oogenesis, sperm storage, sperm competition and mating plug formation. In addition, the seminal fluid contains antibacterial peptides and protease inhibitors. Some seminal fluid-encoding genes also show high rates of evolutionary change, exhibiting both significant between-species divergence and within-species polymorphism. Seminal fluid protein genes are expressed only in males, begging the question of how and why the reproductive processes of females are influenced by males. In this review I address these issues by bringing together evidence for the function, evolution, diversification, and maintenance of variation in, seminal fluid-mediated traits.

Keywords: accessory proteins, Acps, *Drosophila*, fitness, seminal fluid, variation.

Introduction

Male *Drosophila* transfer a cocktail of ejaculate proteins at mating that have striking effects on fitness. In *D. melanogaster*, seminal fluid proteins, specifically the accessory proteins (Acps) synthesized by the paired accessory glands, influence reproductive traits such as sperm transfer, sperm storage, female receptivity, ovulation and oogenesis (reviewed by Wolfner, 1997). Acps are diverse in nature, ranging from small peptides to large glycoproteins (Schafer, 1986; Chen *et al*., 1988; Wolfner, 1997; Wolfner *et al*., 1997; Swanson *et al*., 2001a). Some Acps also exhibit high rates of evolutionary change (e.g. Aguadé *et al*., 1992; Tsaur & Wu, 1997; Begun *et al*., 2000; Swanson *et al*., 2001a). There is to date little evidence to link sequence changes to phenotype, and the functional consequences of this rapid evolution currently remain unclear. Seminal fluid proteins are expressed only in males and seminal fluid-encoding genes are over-represented on the autosomes (Wolfner, 1997; Wolfner *et al*., 1997; Swanson *et al*., 2001a). I investigate these issues in this review, bringing together a large body of detailed data on the function, evolution and maintenance of variation in seminal fluid proteins and the genes that encode them. The rapidly expanding body of data in this field makes it possible to start to integrate theoretical and functional analysis from the molecular level upwards.

Seminal fluid-mediated traits

Seminal fluid comprises molecules from the accessory glands, ejaculatory duct and ejaculatory bulb. The 80 or so accessory gland proteins (Acps) (Swanson *et al*., 2001a) are the major seminal fluid components, but as yet, functions have been confirmed for only a few (Wolfner, 1997; see Table 1). Acps and sperm affect egg-laying and egg-production (Manning, 1962, 1967; Hihara, 1981; Chen *et al*., 1988; Kalb *et al*., 1993; Xue & Noll, 2000; see Table 1 for effects of specific Acps). Acps also affect female receptivity, egg fertility, sperm transfer, sperm storage and sperm competition (Table 1). Acps are components of the mating plug, a gelatinous structure formed in the distal part of the female's reproductive tract during mating (Table 1). Mating plugs may prevent premature sperm loss, or deter matings by other males. The seminal fluid contains protease inhibitors (Table 1), which could serve to protect sperm from degradation and/or promote sperm longevity. They could also protect other Acps from premature cleavage, as some Acps (e.g. Acp26Aa) are processed into active forms only after transfer to the female (Park & Wolfner, 1995). Protease inhibitors could also serve to prevent premature breakdown of the mating plug, or help to ensure the correct processing of the proteins that form it. In addition to the antimicrobial substances listed in Table 1, others may occur in the male reproductive tract and could be transferred in the ejaculate, although this is not confirmed. Drosomycin is an antifungal protein expressed in the female sperm-storage organs, but is sometimes detected in the male ejaculatory duct and bulb (Ferrandon *et al*., 1998). Drosocin is an additional antibacterial peptide induced in females by egg-laying or mating (Charlet *et al*., 1996). Anti-microbial peptides in the ejaculate may function to protect sperm, sanitize the female reproductive tract, protect against sexually transmitted pathogens and/or protect eggs from bacterial infection (e.g. Marchini *et al*., 1997).

Acps function to stimulate and regulate reproductive processes in females following mating (Table 1). Acp actions seem likely to help males to maximize their reproductive success, by stimulating egg production, reducing the

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Table 1 Seminal fluid proteins in *Drosophila* and their effects

Species	Seminal fluid protein	Site of synthesis†	Effect	References
D. melanogaster	Acp26Aa (ovulin)	AG	Increases egg-laying for 1 day after mating in virgin females, by increasing the rate of release of existing eggs (ovulation) from the ovaries.	Herndon & Wolfner, 1995; Heifetz *et al.* 2000, 2001.
			Can decrease early egg fertility.	Chapman *et al.* 2001.
	Acp70A (sex peptide)	AG	Increases egg-laying for 1–2 days after mating, acting at a different stage from Acp26Aa, namely oogenesis.	Chen *et al.* 1988; Aigaki *et al.* 1991; Soller *et al.* 1997, 1999.
			Decreases female receptivity for 1–2 days, the 'mating effect' (Manning, 1967).	Chen *et al.* 1988; Aigaki *et al.* 1991.
			Can bind to sperm, which could prolong Acp70A activity (sperm can be stored for several weeks).	S. Buesser, J. Peng & E. Kubli, pers. comm.
			Orthologues with amino acid similarity to Acp70A are present in all *melanogaster* species subgroup members, *D. suzukii* and probably *D. biarmipes*.	Chen & Balmer 1989; Chen 1996; Schmidt *et al.* 1993; Imamura *et al.* 1998.
	Acp36DE	AG	Males that lack Acp36DE transfer normal numbers of sperm, but only 15% of it is stored.	Neubaum & Wolfner, 1999.
			Necessary for success in sperm competition.	Chapman *et al.*, 2000.
			A likely component of the anterior mating plug.	Lung & Wolfner, 2001a.
	Acp62F	AG	Protease inhibitor.	Wolfner *et al.* 1997; Lung *et al.* unpubl.
	Acp76A	AG	Exhibits sequence similarity with protease inhibitors.	Coleman *et al.* 1995; Wolfner *et al.* 1997.
	Acp26Aa Acp29AB Acp36DE Acp53Ea	AG	Associations exist between alleles at these loci (as determined by single stand conformation polymorphism) and sperm defence (a male's ability to prevent removal of his sperm by a later mating male). The nature of allelic differences is unknown, and it is not yet confirmed that variation is attributable to these Acps.	Clark *et al.* 1995.
	Andropin	AG	Has antibacterial activity.	Samakovlis *et al.* 1991.
	Unnamed Acps	AG	Three proteins with antibacterial activity are transferred to the female at mating. One identified as Andropin (above).	Lung & Wolfner, 2001b.
	Unidentified Acps	AG	Contribute to sperm displacement and can temporarily decrease egg hatchability.	Harshman & Prout, 1994; Prout & Clark, 2000.
			Decrease female longevity and reproductive success.	Chapman *et al.* 1995.
			Necessary for sperm transfer.	Tram & Wolfner 1999.
	Unnamed Acps	AG	Eight putative protease inhibitors and nine putative proteases.	Swanson *et al.* 2001a.

	PEB-me	EB	A component of the posterior mating plug. PEB-me (Ludwig *et al*. 1991) with amino acid repeat motifs characteristic of spider silk proteins that form homopolymers. This suggests that PEB-me coagulates to form the mating plug.	Lung & Wolfner, 2001a.
	Dup99B	ED	Shares sequence similarity with Acp70A; may affect female receptivity and egg production.	Saudan, Hauck and Kubli, unpubl.
	Esterase-6	ED	Reported to influence progeny production, sperm use and mating frequency, although the results are equivocal.	Gilbert *et al*. 1981; Meikle *et al*. 1990; Gilbert 1981; Gilbert *et al*. 1981; Gilbert & Richmond 1982; Saad *et al*. 1994.
	Glucose dehydrogenase (GDH)	ED	Suggested to play a role in sperm storage. However, in a recent screen, no evidence was found for the involvement of GLD or esterase 6 (see above) in sperm competition (Clark *et al*. 1995).	Cavener & MacIntyre 1983.
			Across species, high GLD expression in adult males is associated with the presence of an expanded ejaculatory duct, a characteristic of the *melanogaster* species group.	Cavener 1985.
D. biarmipes	OSS	ED	Increases egg-laying. The OSS's of *D. biarmipes* and *D. suzukii* (below) share sequence similarity with the Dup99B peptide of *D. melanogaster*.	Sato *et al*. 1997; Imamura *et al*. 1998.
D. suzukii	OSS	ED	Increases egg-laying.	Ohashi *et al*. 1991.
D. funebris	PS-1	AG	Decreases female receptivity. It is not known if PS-1 and/or PS-2 (below) share sequence similarity with *D. melanogaster* Acps.	Baumann 1974a,b; Baumann *et al*. 1975.
	PS-2	AG	Increases egg-laying.	Baumann 1974a,b.
	Unnamed Acp	AG	Protease inhibitor.	Schmidt *et al*. 1989.

†AG, Accessory Gland; ED, Ejaculatory Duct; EB, Ejaculatory Bulb.

probability that their mates will mate again, and ensuring that their sperm are stored efficiently. Receipt of high levels of Acps can be costly to females, causing a decrease in female longevity and reproductive success (Chapman *et al*., 1995). This cost is thought to be a deleterious side-effect of Acp functions that serve to increase male per-mating fitness (Chapman *et al*., 1995). If true, one or more Acp-mediated fitness traits should be directly linked to reductions in female longevity. Recent support for this type of sexually antagonistic interaction comes from the negative relationship observed between male sperm defence (success of a first mating male after subsequent matings) and early female mortality (Civetta & Clark, 2000a; but see Sawby & Hughes, 2001). This type of sexual conflict (Trivers, 1972; Parker, 1979; West-Eberhard, 1984; Rice & Holland, 1997), involving actions that have the potential to benefit males and harm females, has received considerable empirical support (e.g. Rice, 1992; Arnqvist & Rowe, 1995; Rice, 1996; Holland & Rice, 1998; Civetta & Clark, 2000a; Chippindale *et al*., 2001).

Sites of Acp action

None of the receptors to Acps has yet been characterized, but the sites of Acp action may offer clues to the identity of target tissues. Acp70A binding sites appear to be accessible via the haemolymph, as injections of synthetic Acp70A into the abdomen of virgin females causes an increase in egg production and decrease in receptivity (e.g. Chen *et al*., 1988). Binding sites for Acp70A were investigated by ectopic expression in transgenic females (Nakayama *et al*., 1997). Membrane-bound Acp70A decreased female receptivity and increased egg production, but cytoplasmic Acp70A did not. Responses were highest when Acp70A was expressed in the head. Acp70A therefore appears to exert its effects outside cells, with the head as a potentially important site of action. Potential binding sites of Acp70A and Dup99B (Table 1) were also investigated by incubating thin sections of females with iodinated peptides (Ottiger *et al*., 2000). Binding of both peptides was indicated in peripheral nerves, suboesophageal and thoracic ganglia, and the genital tract. Sites in the afferent nervous system suggested a role in the modulation of sensory inputs. The range of potential binding sites was wider than that suggested by Nakayama *et al*. (1997). This may be because the existence of binding sites as determined by the labelling of thin sections does not necessarily prove that peptides delivered via the normal route actually access these sites.

Antibodies raised to Acps such as Acp36DE and Acp26Aa show localization consistent with known functions of these peptides. After mating, Acp36DE is found tightly bound to sperm and anterior to the openings of the female sperm storage organs (Bertram *et al*., 1996). It is also probably a component of the mating plug (Lung & Wolfner, 2001a). This is consistent with its role in corralling the sperm into storage (Bertram *et al*., 1996; Neubaum & Wolfner, 1999). Acp26Aa is found primarily at the base of the ovary after mating, consistent with its role in ovulation (Monsma *et al*., 1990; Heifetz *et al*., 2000). Some Acps such as Acp36DE are restricted to the female reproductive tract after mating (Lung & Wolfner, 1999). Others such as Acp26Aa, Acp26Ab and Acp62F and esterase 6 pass from the reproductive tract into the haemolymph (Meikle *et al*., 1990; Monsma *et al*., 1990; Lung & Wolfner, 1999). Acp26Aa and Acp62F can be detected in the female's reproductive tract from 3 min into mating (Lung & Wolfner, 1999). Acp62F and Acp26Aa haemolymph levels decline towards the end of mating even though levels in the reproductive tract continue to increase. In addition, portions of the Acp26Aa protein that are known to be cleaved 10 min after the start of mating are not found in the female haemolymph (Monsma & Wolfner, 1988; Lung & Wolfner, 1999). This suggests that the route into the circulatory system starts to close after 10 min, or that Acps are degraded at an increased rate as mating proceeds.

Interestingly, there are no sequences common to Acps that might mediate their entry into the haemolymph via a specific transport mechanism (Monsma & Wolfner, 1988; DiBenedetto *et al*., 1990; Monsma *et al*., 1990; Lung & Wolfner, 1999). Instead, Acps pass into the female circulatory system through a permeable area in the female reproductive tract, the vaginal intima, which is potentially open to all except the largest Acps (Lung & Wolfner, 1999). The closing of this area 10 min into mating may be facilitated by the formation of the mating plug (Lung & Wolfner, 2001a). The non-specific mode of transport provides the opportunity for non-adaptive leakage of Acps into areas where they have no specific targets. This may provide a mechanism by which Acps have deleterious side-effects on females at high doses (Wolfner *et al*., 1997; Wolfner, 1997).

Sex limitation in seminal fluid protein encoding genes

Seminal fluid proteins presumably evolved to initiate and effectively co-ordinate post-mating reproductive processes. However, seminal fluid-mediated traits could subsequently have become influenced by sexual selection through male–male competition (sperm competition), male–female competition (sexual conflict) or cryptic female choice (Eberhard, 1997). This subject is excellently reviewed in several articles (see Cordero, 1995, 1996; 1998; Eberhard & Cordero, 1995).

Genes encoding seminal fluid proteins are expressed only in males. It is of considerable interest to consider why these genes are not switched on in females, and therefore why the reproductive processes of females are under the influence of seminal fluid proteins transferred by mating males. Ejaculate transfer may act to kick-start reproduction once mating has occurred. Females would start laying eggs and refrain from further matings (at least temporarily) only upon receipt of sperm. This could provide a mechanism for the expression of cryptic female choice against matings by inadequate males (Eberhard & Cordero, 1995). Assuming that a male's Acp complement correlates with important aspects of ejaculate (e.g. quality or quantity), insufficient ejaculate transfer could result in females remating sooner than would otherwise be the case. The fitness consequences of female choice based on differences in ejaculate quality have not yet been tested experimentally, but could result in differential egg production, fertility, mating frequency, or reproductive costs.

A second hypothesis for the maintenance of sex limitation proposes that seminal fluid proteins represent a nuptial gift (Wickler, 1985). Females could thus synchronize their reproductive processes with receipt of a gift from males. In *D. melanogaster*, there is no evidence that remating confers nutritional benefits for females (Chapman *et al*., 1994). However, nuptial feeding may have been important in the evolutionary past of this species, an idea that would be interesting to address in a phylogenetic context. Acps stimulate reproductive processes in females, but can be deleterious at high doses (Chapman *et al*., 1995). This stimulation of reproductive processes by Acps to a costly level should select for damage limitation in females. This would be expected to lead to silencing of Acp genes in females if they expressed them and selection to neutralize the deleterious effects of Acps. Damage limitation therefore represents a third factor that could promote sex limitation in seminal fluid-encoding genes. For example, consider a situation in which males could induce females to commit a level of resources to the current batch of offspring that compromised future female reproductive potential. Sex limitation would be promoted, and females would be expected to evolve resistance to counter the deleterious effects of receiving high levels of seminal fluid proteins (see Holland & Rice, 1998). Selection for damage limitation in females would also be promoted if the potential toxicity of seminal fluid was exploited by males to cause deliberate harm to their mates (Johnstone & Keller, 2000). It is increasingly essential to know the mechanism by which Acps reduce female fitness. This would show whether selection was acting directly on toxicity, as envisaged by Johnstone & Keller (2000) or whether the selection is on Acp function with toxicity as a side-effect (Chapman *et al*., 1995).

The Acps which influence the reproductive process in females have been isolated for the most part by virtue of their sex-specific expression in males. However females, of course, also have a significant influence on Acp-mediated traits. For instance, virgin females can lay nonviable eggs in the absence of cues from males. The number of eggs laid by virgins is low compared to mated females, although it does increase in older virgins (Boulétreau-Merle, 1978; Partridge *et al*., 1986). Old virgins are also reported to be refractory to mating (Neckameyer *et al*., 2000). High egg production and low receptivity are characteristics of the actions of Acp70A (see Table 1), which may suggest some baseline, 'leaky' level of Acp70A synthesis in old virgin females, although this has not been tested. It is clear that high levels of expression of Acps such as Acp70A in females would be deleterious; this would cause low receptivity and females would rarely, if ever, mate. We would therefore predict strong selection against Acp70A expression in females, as appears to have occurred in females of a transgenic stock in which Acp70A was constitutively expressed (Aigaki *et al*., 1991; E. Kubli, pers. comm.). Future work could employ transgenic stocks in which Acps are expressed in females, to examine the effects of non sex-limited Acp expression.

Chromosomal location of Acp encoding genes

All 75 of the currently mapped Acps reside on the autosomes (Wolfner *et al*., 1997; Wolfner, 1997; Swanson *et al*., 2001a). For genes located on any chromosome apart from the Y, which is only present in males, mechanisms to switch genes on or off in either sex are required for sex-specific expression. In this respect, there is no obvious advantage for genes expressed only in males to be restricted to autosomes. Any Acp-encoding genes that arise on the sex chromosomes must therefore presumably be rapidly eliminated or translocated to autosomes. As yet it is not known whether there are pseudogene relics of Acps on the sex chromosomes that could be indicative of past duplication/translocation events. The lack of Acps on the sex chromosomes could be due to an incompatibility with the machinery of dosage compensation (Wolfner, 1997; Wolfner *et al*., 1997). However, it is difficult to see why this class of sex-limited genes and not others should be affected. The actions of Acps sometimes indicate sexual conflict, since they can benefit males, but harm females (Chapman *et al*., 1995). Therefore, whilst Acps are not sexually antagonistic as defined by (Rice, 1992, i.e. genes expressed in both sexes with different alleles favoured in each), they can exhibit sexually antagonistic features. The lack of Acp genes on the sex chromosomes is therefore perhaps even more surprising, given that the X chromosome is expected to be a hot spot for sexual antagonism (Rice, 1984). Other genes expressed specifically in males, such as those involved in spermatogenesis, are not restricted to the autosomes, although the majority of them are found there (Andrews *et al*., 2000).

The prediction that the X chromosome is a hot spot for sexually antagonistic alleles holds for fully and partially recessive alleles whose actions favour males, and dominant alleles that favour females (Rice, 1984). Therefore, dominant alleles whose actions benefit males might be expected to be under-represented on the X chromosome. Therefore, if new Acp genes arise through dominant gain of function mutations, and if the benefit they provide to males is greater than the fitness cost to females, they should be under-represented on the X. It is possible therefore, that the lack of Acps on the X could be explained by dominance relationships.

Some genes that encode seminal fluid proteins show high rates of evolutionary change

Male reproductive tract proteins in general exhibit high levels of polymorphism (Coulhart & Singh, 1988) and interspecific divergence (Thomas & Singh, 1992). This suggests that reproductive tract protein evolution is driven by selection, or that constraints on sequence changes are low. Male reproductive tract genes are estimated to evolve at twice the rate of non-reproductive tract genes (Civetta & Singh, 1995). This is in agreement with the latest estimates of the speed of Acp evolutionary change, and a number of novel accessory gland protein genes are putative targets of positive Darwinian selection (Swanson *et al*., 2001a). An analysis of 12 Acp sequences (Aguadé *et al*., 1992; Cirera & Aguadé, 1997; Aguadé, 1998, 1999; Begun *et al*., 2000), found evidence for the operation of directional selection (Begun *et al*., 2000). Exclusion of the two most heterogeneous loci (Acp26Aa and Acp36DE) removed the evidence for directional selection operating on the remaining pool. The following section

reviews the currently available data for divergence and polymorphism in specific genes (for Acp functions, refer to Table 1).

Positive selection appears to have favoured amino acid evolution in Acp26Aa (ovulin) but not Acp26Ab (Aguadé *et al*., 1992; Aguadé, 1997, 1999; Tsaur & Wu, 1997; Tsaur *et al*., 1998). Acp26Aa and Acp26Ab (whose function is currently unknown) exist in tandem 20 nucleotides apart, but exhibit no sequence similarity to one another (Monsma & Wolfner, 1988). Thus rates of evolutionary change can vary significantly over very short chromosomal distances. Amino acid substitutions caused by directional selection can be associated with reduced variation in the surrounding region resulting from 'selective sweeps' (e.g. Maynard Smith & Haigh, 1974). Such directional selection is not expected to contribute strongly to within-species genetic variation because polymorphisms are transient. However, if sweeps are numerous or recent, the imprint of directional selection may still be evident. There was no evidence for selective sweeps in the Acp26Aa locus, indicating significant nucleotide diversity and thus high within-species polymorphism, in addition to divergence (Tsaur *et al*., 1998). Focusing on particular regions of Acp26Aa has shown that the amino (N) terminus exhibits the highest number of amino acid replacements in *D. melanogaster*/*D. mauritiana* comparisons (Tsaur *et al*., 2001). Tsaur *et al*. (2001) suggest a role for the N terminus of Acp26Aa in sperm competition, but extensive functional analysis of Acp26Aa provides no evidence for this (Herndon & Wolfner, 1995; Heifetz *et al*., 2000, 2001; Chapman *et al*., 2001).

Sequence analysis of the Acp36DE protein shows an excess of amino acid substitutions in *D. melanogaster*/*D. simulans* comparisons (Begun *et al*., 2000). Acp62F in *D. simulans* exhibits high intraspecific polymorphism, which may be maintained by balancing selection, although neutrality could not be rejected (Begun *et al*., 2000). Analysis of Acp98AB, which has as yet no known function, shows a fixed length difference and eight amino acid differences between *D. melanogaster* and *D. simulans* sequences, suggesting adaptive evolution (Begun *et al*., 2000). The Acp29AB locus also shows a high level of amino acid substitutions in *D. melanogaster*/*D. simulans* comparisons (Aguadé, 1999; Begun *et al*., 2000), a pattern consistent with positive selection during speciation, followed by balancing selection (Aguadé, 1999). Acp53Ea exhibits a single amino acid polymorphism in non-African samples (Begun *et al*., 2000).

Sequence analysis of Acp70A (the sex peptide) sequences in *D. melanogaster*, *D. sechellia*, *D. simulans* and *D. mauritiana* revealed a polymorphism in the signal peptide of *D. melanogaster* (Cirera & Aguadé, 1997). Low variation in the most derived allele is consistent with its recent origin and subsequent increase in frequency due to selection. However, there was no significant evidence of selection in Acp70A divergence (Cirera & Aguadé, 1997). The Acp70A gene is also duplicated in *D. subobscura* (Cirera & Aguadé, 1998), the significance of which is not clear. Acp32CD, whose function is currently unknown, shows few silent or replacement site changes and little polymorphism (Begun *et al*., 2000). Turning to the data on other seminal fluid proteins, there is no evidence of excess non-synonymous fixed changes in Esterase-6 sequences (Karotam *et al*., 1993). The antimicrobial protein Andropin does not exhibit exceptional levels of polymorphism, but differs significantly from neutrality (Clark & Wang, 1997).

In conclusion, there is mounting evidence for high rates of evolutionary change in seminal fluid proteins within and between species: those exhibiting evidence of amino acid change driven by positive selection are Acp26Aa, Acp29AB and Acp36DE, and those exhibiting significant within-species polymorphism are Acp26Aa, Acp62F, Acp70A and Andropin. However, statistical power is low in many of the tests for positive selection and nucleotide diversity, because of the small size of many Acp genes (Begun *et al*., 2000). Nonetheless, high rates of evolutionary change in loci encoding seminal fluid proteins appear to be common.

Maintenance of variation in seminal fluid-mediated traits

Positive selection acting upon Acps suggests a role in species divergence. Polymorphism in seminal fluid-mediated traits could be transient and associated with recent selective sweeps, or actively maintained by antagonistic pleiotropy, frequency dependence, overdominance or other, unknown forces (e.g. Haldane, 1962; Prout & Clark, 1996; Hughes, 1997). There has been little attempt as yet to link Acp sequence variation to differences in phenotype. Therefore the functional significance of the high rates of evolutionary change remains to be investigated. However, the existence of significant polymorphism in Acps, and in the traits that they influence, requires explanation. In assessing the forces involved, we are confronted by the problem of whether to attribute variation to male–male competition, male–female competition, or cryptic female choice (see Birkhead, 1998, 2000; Eberhard, 2000; Kempenaers *et al*., 2000; Pitnick & Brown, 2000). The potential complexity can be illustrated as follows. There is evidence that male Acps interfere with the sperm of previous males in store (Harshman & Prout, 1994; Prout & Clark, 2000). Males also differ in their sperm competitive ability relative to one another (e.g. Prout & Bundgaard, 1977; Clark *et al*., 2000). These findings could be attributed to male–male competition. We also know that a male's ability in sperm competition may be negatively correlated with his effect on female early mortality (Civetta & Clark, 2000a), which supports the idea of a conflict between the sexes. On the other hand, a male's mating success is partly determined by Acps. Therefore the Acp genotype at the relevant loci will reflect male quality. For instance, males that are null for Acp36DE are deficient in sperm storage and thus represent low quality mates for females. The possibility that females could discriminate between males on the basis of Acps thus provides a potential mechanism for female choice (Eberhard, 1996). Further work is required to distinguish between these alternative views.

Evidence of significant variation in the seminal fluid-mediated traits themselves comes predominantly from studies of sperm competition. In the first rigorous study of the population genetics of sperm displacement Prout & Bundgaard (1977) investigated the performance of three stocks in double

and triple matings. The results showed average differences between male genotypes in the degree of sperm displacement and a linear order of displacement ability. More recently, the sperm precedence of six chromosome-extracted lines was tested against a panel of marked strains, and indicated complex, non-transitive relationships (Clark *et al*., 2000). Flies artificially selected for early and late age reproduction also showed correlated responses in sperm defence ability (Service & Fales, 1993). Evidence for male, female (Clark & Begun, 1998), male × female (Clark *et al*., 1999) and chromosomal (Civetta & Clark, 2000b) effects on sperm competition, suggests that interactions with females are important for maintaining variation. Antagonistic effects of sperm competition on females could also be an important factor (Chapman *et al*., 1995; Prout & Clark, 1996; Civetta & Clark, 2000a).

The first evidence of a relationship between phenotypic variation in sperm competition and underlying Acp sequences is provided by the study of Clark *et al*. (1995) mentioned earlier. Significant associations were found between sperm defence and variation at four Acp loci. There was no evidence from Clark *et al*. (1995) of significant differences in sperm displacement ability of strains carrying different esterase-6 alleles as reported by Gilbert & Richmond (1981). Hughes (1997) analysed the quantitative genetics of first and second male sperm precedence and demonstrated significant standing genetic variation for sperm precedence, with a small number of genes contributing effects. The genetic architecture of sperm precedence was found to be unusual, comprising lots of non-additive and little additive genetic variation (Hughes, 1997). Fitness traits are more typically characterized by the opposite pattern. Non-additive variation could be caused by balancing selection (Haldane, 1949), recessive alleles at low frequency, or mutation–selection balance with weak selection (Hughes, 1997). However, it should be noted that the Hughes (1997) study used lines that were homozygous for approximately 40% of their genomes. The genetic variation observed may therefore have been caused in part by inbreeding depression, rather than additive fitness variation.

The potential forces maintaining variation in sperm competition have also been subject to theoretical investigation. Prout & Bundgaard (1977) showed that stable polymorphisms in genes influencing sperm competition could be maintained given overdominance or non-transitivity in the relationships between males. Prout & Clark (1996) showed stable polymorphisms were maintained if sperm competition alleles had pleiotropic effects on fecundity and mating ability. In a further model Clark *et al*. (2000), showed that variation was maintained when six lines competed against three marker strains, but disappeared when smaller numbers of marker strains were used. The inclusion of a locus for mating behaviour in the model of Curtsinger (1991) provided no evidence that polymorphism in sperm displacement could drive the evolution of multiple mating in females. However, a further model in which female choice was incorporated, provided evidence that non-transitivity in sperm types could drive female choice and multiple mating (Keller & Reeve, 1995). The result was not stable polymorphism, but oscillating evolution with 'rock, scissors, paper' dynamics. The role for non-transitive dynamics is intriguing given the empirical evidence (Clark *et al*., 2000).

The evidence for significant variation in other seminal fluid-mediated traits is distinctly lacking. Differences in the refractory period of females mated to different genotypes of males can be demonstrated (e.g. Sgro *et al*., 1998). However, Hughes (1997) showed no significant variation in either first or second male genotype and female remating probability. Genetic variation in the effect of the first mating male on female egg-laying and remating behaviour has also been demonstrated (Service & Vossbrink, 1996). There also appears to be genetic variation in female *D. biarmipes* responses to a seminal fluid protein that stimulates egg-production (Imamura *et al*., 1998). It is not yet known, however, whether any variation in male effects on female refractory period or egg-laying is associated with Acp genotype.

Implicit so far is the assumption that selection will act on Acp proteins that have quantitatively different effects and that are encoded for by different Acp alleles. However selection could also create variation in seminal fluid-mediated traits through regulatory changes such as increased expression levels, response thresholds, receptor sensitivity or through redundancy (multiple peptides with the same function). This would be interesting to address in future studies.

Future directions

Although much progress has been made, there is clearly much work to be done to clarify the roles of Acps, patterns of evolutionary change and the selective forces that maintain variation in traits mediated by seminal fluid proteins. I highlight here some promising areas for future investigation.

More functional studies are required of the effects of individual Acps with unknown functions. In addition, there is a lack of studies in which the consequences of DNA and protein sequence changes upon phenotypes have been addressed. There is also a need to identify the receptors to which seminal fluid proteins bind. This is a difficult task because receptors may not be restricted to one tissue. However, the isolation of receptors will pay dividends in elucidating mechanisms of Acp action and in providing evidence for the selective forces important in maintaining variation. For example, under sexual conflict and other scenarios, there is an assumption that the high rates of evolutionary change in Acp will be matched by variation in the receptors to which those proteins bind. This is supported by the recent analysis of three mammalian fertilization proteins (Swanson *et al*., 2001b).

It is also interesting to consider whether males or females have the most control over the reproductive processes influenced by seminal fluid proteins. Clearly, there are circumstances when the transfer of Acps to females is costly, and males appear to 'win' (Chapman *et al*., 1995; Rice, 1996). However this would be expected to be context-dependent and particularly apparent under non-equilibrium conditions. The extent to which fitness costs are incurred will depend upon the life history stage at which they are manifested and upon the evolutionary history of the population in question. Considerable standing genetic variation for interactions between male genotype and female survival

within populations has been identified (Sawby & Hughes, 2001), along with negative relationships between adult male and female fitness (e.g. Chippindale *et al*., 2001). Future work could focus on investigating the costs and benefits of sexual interactions and the contexts in which they are expressed.

Signatures of past evolutionary interactions between the sexes could be evident as lineage-specific effects between Acps and their receptors, within or between species. Such changes could reflect different evolutionary histories in the level of multiple mating and therefore the strength of selection due to male–male competition, male–female competition or female choice. There is evidence of lineage-specific effects in between-species comparisons of Acp sequences (Begun *et al*., 2000). An example comes from the higher rate of replacement substitutions in *D. simulans* than in *D. melanogaster* (Begun, 1996). Lineage-specific effects have also been identified in the pattern of variation in the N terminus of the Acp26Aa gene (Tsaur *et al*., 2001), which appears to be evolving faster in *D. mauritiana* than in *D. sechellia*, *D. simulans* or *D. melanogaster*. Evidence for general lineage-specific effects within species has been provided by selection experiments in *D. melanogaster* (e.g. Rice, 1992; Rice, 1996; Holland & Rice, 1999; Pitnick *et al*., 2001). The possibility that lineage-specific effects between or within species indicate the degree of sexual selection or sexual conflict is enticing. However, differences due to drift, effective population size, and the degree of inbreeding could all produce lineage-specific effects, and should be controlled for (see Snook, 2001). A challenge in future experimental work will be to vary lineage-specific effects caused by sexual interactions whilst keeping all else equal, and then to examine the consequences in terms of Acps variants and expression levels.

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