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Reviews

The accessory gland proteins in male *Drosophila*: structural, reproductive, and evolutionary aspects

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Abstract. Recent results from biochemical and molecular genetic studies of the accessory gland proteins in male *Drosophila* are reviewed. The most prominent feature is the species-specific variability. However, the analysis of the sex peptide in *D. melanogaster* shows that there is a strong homology in the molecular structure to the closely related sibling species, and that divergence increases with increasing phylogenetic distance. For this reason the sex peptide, after being transferred to the female genital tract during copulation, reduces receptivity and increases oviposition only in virgin females belonging to the same species group and subgroup. Even though studies were hitherto limited to a small number of the secretory components, it is evident that the accessory gland proteins play a key role in reproductive success of the fruit fly by changing female sexual behavior, supporting sperm transfer, storage and displacement. Thus, genes encoding the accessory gland proteins are apparently under strong evolutionary selection.
Key words. *Drosophila*; accessory gland; reproduction; sexual behavior; sperm displacement; evolution.

The genetics and evolution of mating systems in insects have been the research subjects of many biologists[62]. Results of these studies indicate that the reproductive genes are targets of strong natural selective forces. The accessory glands (paragonia) of male *Drosophila* (fig. 1), as in many other insects[23], are known to play a primary role in mating; their secretory products are essential for transfer, storage and utilization of the sperm[22]. With the advance of modern biochemical and molecular genetic techniques, it is now possible to characterize individual accessory gland proteins as well as to dissect the invididual genes encoding them. In the following, I shall at first summarize briefly what we know about the molecular structure and gene regulation of these secretory proteins, and then focus more on their reproductive and evolutionary significance. Since to date only a small number of the accessory gland proteins have been subjected to a detailed analysis, an extensive interpretation must await further progress (see recent reviews by Kubli[33,34]).

I. Molecular structure and gene regulation

Convincing evidence that the male accessory gland secretion has immediate effects on mated female *Drosophila* is provided by a detailed study of the sex peptide (SP) in our laboratory (see Chen et al.[16]). Following its transfer to the female during copulation, the female fly becomes nonreceptive to remating by courting males and exhibits a rapid increase in egg-laying. Both mating responses induced by SP have been confirmed by analysis of ectopic expression in transgenic flies[2] and injection of the synthetic peptide into virgin females[55].

The 36 amino acid SP is encoded by a single copy gene located at chromosomal site 70A. Post-translational modifications consist of hydroxylation of five proline residues at positions 9, 13, 15, 17, 19 and one as yet unidentified modification of isoleucine at position 14. The two cysteines at positions 24 and 36 appear essential, probably due to the formation of an intrachain disulfide bridge at the C-terminal end.

Based on cDNA cloning, it is clear that SP is synthesized as a 55 amino acid precursor with a hydrophobic signal sequence of 19 residues at its N-terminus. Genomic DNA analysis has shown that the primary transcript contains 266 bp with a single intron of 65 bp[60]. The transcription is organ- and cell-specific, and starts shortly before eclosion of the male fruit fly.

From their recent experiments designed to elucidate the reacting mechanism(s) of the sex peptide, Kubli and associates suggest that there is probably only a single type of receptor molecule in one or several tissues (see Kubli[34]). In support of this suggestion, the same critical concentration (0.6 pM/female) and similar dose response curves elicit both post-mating reactions (i.e. suppression of receptivity and stimulation of egg-laying). Furthermore, injection of SP into *dunce* females failed to elicit the suppression of receptivity, but the defective response could partially be rescued by using heat-shocked transgenic females containing the hsp-*dunce* construct[21]. In addition, SP stimulates in vitro synthesis of the juvenile hormone JHB3 in corpus alla-

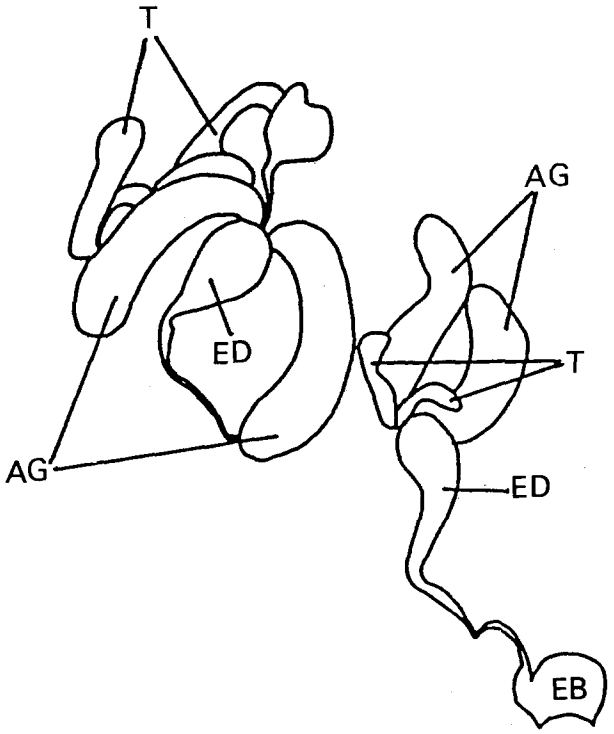

Figure 1. Internal reproductive organs of the wild-type of male *Drosophila melanogaster* (left), and the interspecific hybrid of *D. melanogaster* male × *D. simulans* female (right). The hybrid has rudimentary sterile testes, but otherwise normal functional organs. AG, accessory gland; T, testis; ED, ejaculatory duct; EB, ejaculatory bulb. (Modified from Reding[50], and see also Hadorn[25]).

tum from *Drosophila* females 3–4 days after eclosion[43]. Thus, both the nervous and endocrine systems are probably involved in the SP-induced reproductive behavioral changes.

Several male accessory gland genes in *D. melanogaster* have also been isolated and characterized by M. Wolfner and collaborators (see Wolfner[69]). The gland consists of two types of morphologically distinguishable secretory cells: the main and the secondary cells[3]. In a more recent study, Kalb et al.[31] disrupted the main cell function by introducing into the male fly a construct containing the coding sequence of diphtheria toxin subunit A (DTA) and the promoter of the gene *Acp95EF*. This gene, formerly named *mst316F*, is expressed only in the main cells[20]. At adequate levels of toxin expression the transgenic flies have no main cell-specific protein products, but spermatogenesis is not blocked. In mating experiments sons of *tudor* mothers with normal seminal fluid but without sperm are used for comparison. From observation of the mated females it is concluded that for short-term suppression of receptivity only main cell secretions are needed, whereas for long-term suppression of remating both main cell secretion and sperm appear to be essential. The latter is also true for the stimulation of oviposition. This is consistent with the finding that SP is synthesized only in the main cells of the accessory gland[60]. But the mechanism underlying the interaction of sperm and secretory proteins is still unknown.

The gene *Acp26Aa* (formerly named *msP355a*) encodes a basic protein of 264 amino acids with a region of sequence similar to the egg-laying hormone (ELH) of *Aplysia*, and the tightly linked gene *Acp26Ab* (formerly named *msP355b*) produces a small acidic protein of 90 amino acids[44]. The two proteins are synthesized in both secretory cell types and their synthesis increases following copulation. After transfer to the female only the larger protein undergoes specific cleavage in the genital tract, and both proteins rapidly enter the hemolymph[45]. The two proteins may possibly function as signals or triggers in the mating response of the female. The similarity of amino acid sequence of the cleaved *Acp26Aa* fragment to ELH of *Aplysia* suggests its probable involvement in the increase of egg production.

By using P-element mediated enhancer traps, Betram et al.[6] conclude that there are accessory gland genes expressed specifically in the main or secondary cells or in both, and their regulation following mating differs in the two cell types. Gene expression is activated in main cells by mating, whereas that in secondary cells remains constant.

In *D. funebris* two accessory gland proteins have been isolated. The protein PS-1 consists of two 27 amino acid peptides with either valine or leucine at position 2 (see Baumann et at.[5]). Injection of PS-1 into virgin females results in reduction of female receptivity. More recently, the presence of another polypeptide consisting of 63 amino acids has been identified in the accessory gland secretion of the same species (Schmidt et al.[54]). Sequence analysis showed the presence of six cysteines. Based on the position of this amino acid as well as the peptide resistance to trypsin digestion, it appears to be a protease inhibitor. In fact, the study of substrate specificity demonstrated its inhibition of acrosin (a trypsin-like protease located in the acrosomal vesicle at the sperm head[68]) by 95%. There is as yet no molecular genetic study of the gene structure or synthesis of these two regulatory peptides.

According to Ohashi et al.[46] in *D. suzukii* the male accessory gland synthesizes an ovulation stimulating substance (OSS) which has a minimum size of 35 amino acids. In a more recent investigation Schmidt et al.[56] isolated an accessory gland product from the same species that contains 41 amino acids and exhibits a high homology to SP in *D. melanogaster*. The major difference is the insertion of five additional amino acids after position 7 of the *melanogaster* SP sequence.

II. Reproductive significance

Insect reproduction in general

The different insect species have reproductive systems which vary greatly in both their morphology and react-

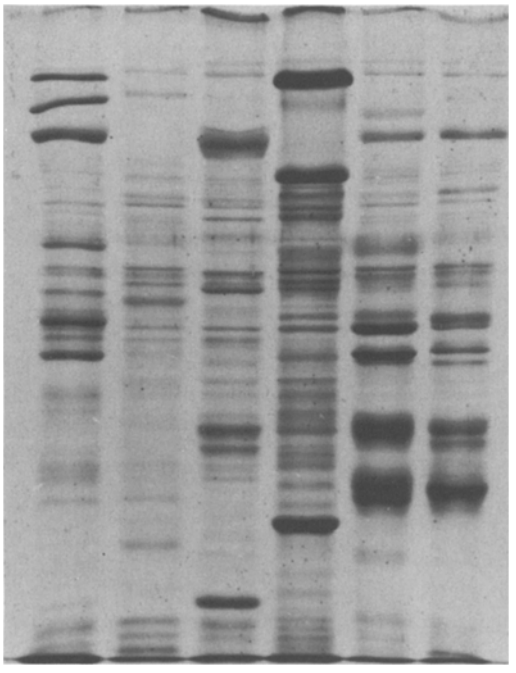

Figure 2. Species-specific patterns of *Drosophila* accessory gland proteins separated on 10% SDS gel. Dm, *D. melanogaster*; Db, *D. buskii*; Df, *D. funebris*; Dh, *D. hydei*; Dn, *D. nigromelanica*; Dv, *D. virilis*. (From Chen, Stumm-Zollinger, and Caldelari[15]).

ing mechanisms. However, one common feature is the key role of the accessory glands in males (see Chen[12]). Their gland protein patterns are strictly species-specific (fig. 2). In most insects the secretory products serve to produce the spermatophore for sperm transfer and sperm activation. For example, in the mealworm *Tenebrio molitor* the individual structural components can be traced to their synthesis in specific gland cells[26]. In the silkmoth *Bombyx mori* a reaction cycle to produce energy for sperm activation through arginase in the spermatophore has been analyzed in detail by Osanai and colleagues[47].

In *Drosophila*, as in other higher dipterans, the sperm are directly deposited in the female genital tract during copulation. But the structural protein components of the spermatophore may still be synthesized. Following transfer to the female, they may undergo proteolysis and the resulting amino acids may serve as general metabolites for nutritional purpose, synthesis of yolk in the fat body and ovary, or production of somatic tissue proteins[8,41]. Based on non-equilibrium pH gel electrophoresis (NEPHGE) we estimated that the accessory gland secretion in *D. melanogaster* contains a minimum of 85 different proteins (see fig. 3, and Stumm-Zollinger and Chen[58,59]). As mentioned above, only a few of these have so far been studied in more detail. The two major effects of female receptivity and oviposition following mating are clearly initiated by the sex peptide, but their persistence needs probably the presence of sperm[31]. As yet there is no information about the nature of the so-called sperm factor suggested earlier by Manning[40]. In general, it appears that the complexity of reproduction in *Drosophila*, as in many other insects, requires the coordinated action of a large number of accessory gland proteins. Their specific functions are, as yet, still unknown.

Control of sexual behavior

Prior to copulation, *Drosophila* males exhibit a series of courting behaviors, including tapping the female abdo-

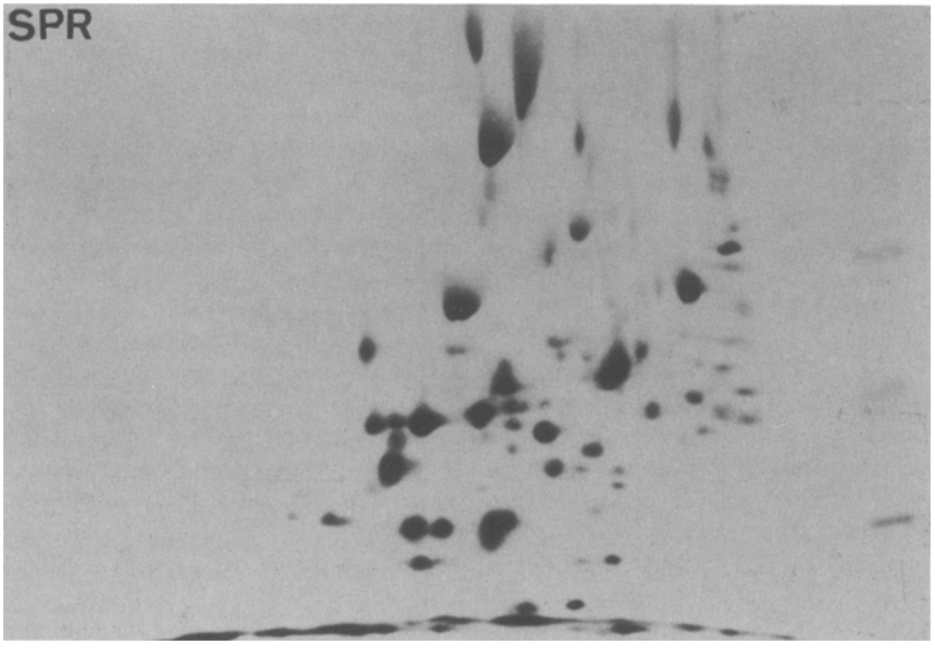

Figure 3. Two-dimensional pattern of labeled secretory proteins (SPR) in male accessory gland of *D. melanogaster*; separated by non-equilibrium pH gradient gel eletrophoresis (NEPHGE). (From Schmidt, Stumm-Zollinger, and Chen[53]).

men, vibrating their wings to produce the love song, licking the female genitalia, and attempt to mate[9]. The female's response and readiness for mating are expressed by moving more slowly, spreading both wings, and opening the vaginal plates. These complex courtship behaviors are age-, species- and sex-specific (see a recent review by Tompkins[65]). For example, newly emerged young flies do not show courtship behaviors until they are sex mature at the age of 1–2 days[64]. In *D. melanogaster* wing vibration is performed only by males, whereas in *D. buskii* this is done by both males and females[7]. From their studies of gynandromorphs Tompkins and Hall[66], as well as Szabad and Fajszi[61], conclude that bilateral groups of female genotype cells in the dorsal-anterior brain are essential for receptivity to mate with male flies. Based on her extensive analysis of single gene mutations, Tompkins[63,64] has well documented the essential roles of olfactory, visual, acoustic and contact stimuli in the sexual behavior of *Drosophila*. The control of sexual behavior also includes the synthesis of pheromones[30]. In view of such complexity, it is indeed astonishing that the female receptivity is turned off immediately following mating or injection of SP.

III. Evolutionary roles

Phylogenetic relationships

Electrophoretic studies have repeatedly shown that the accessory gland protein patterns in male *Drosophila* are highly species-specific (see Chen[11] and Chen et al.[15]). Even within a single species, polymorphic variants are of common occurrence. Whalen and Wilson[67] estimated that in *D. melanogaster* about half of the major protein components show polymorphism, and genetic evidence indicates that such variations are not artefacts resulting from the experimental procedure. More recently, the detailed phylogenetic DNA sequence analysis by Aguadè et al.[1] showed that the *Acp26Aa/b* gene regions have high levels of amino acid replacement and divergence of cleavage sites in polymorphic alleles of *D. melanogaster* and its sibling species. The authors discussed the probable presence of multiple common ancestors and emphasized the possible role of selection in the gene regions studied by them.

In an analysis of functional species-specificity, accessory gland secretions from *D. funebris*, *D. buskii*, *D. hydei*, *D. nigromelanica* and *D. virilis* were injected into virgin females of *D. melanogaster* and showed no stimulation of oviposition (Chen et al.[15]). Similarly, reciprocal injection of PS-1 from *funebris* and SP from *melanogaster* into their virgin females has no effect on either receptivity and oviposition[4,16]. This is not surprising since *funebris* belongs to subgenus *Drosophila*, whereas *melanogaster* belongs to subgenus *Sophophora*. On the other hand, secretions from sibling species belonging to the *melanogaster* subgroup exhibit no species-specificity in eliciting the two post-mating responses. In accord with this, analyses of amino acid sequence in SP of all sibling species revealed a high homology[14,54]. *D. suzukii* is an exception; though it belongs to another subgroup, its sex peptide is equally good at eliciting both post-mating reactions in *melanogaster* virgins and vice versa[56]. The reason for this is that the *suzukii* peptide has a high homology to *melanogaster* SP in both N- and C-terminal ends, in spite of its insertion of five additional amino acids. The functional domain at the C-terminus has been demonstrated[55].

Owing to the high similarity of their polytene chromosome banding patterns, *D. simulans*, *D. mauritiana* and *D. sechellia* are designated as homosequential species[36,37]. Biochemical studies of the allozymes and the larval proteins indicate that the genetic distances between these sibling species are even shorter than each of them to *D. melanogaster* (Gonzalez et al.[24]). The search for structural homology of the sex peptide in the fruit fly provides an additional tool in our understanding of the phylogenetic relationships between the species as well as the process of speciation[13,55].

Sperm displacement

In natural populations of *Drosophila* remating of mated females is not rare. This can be due to insufficient transfer of semen in the first copulation, or when the mated female fly is taken by force by the second male. Analysis of the progeny of such double-mated females indicate that the sperm of the second male are used first (see Fowler[22]). Apparently, there is no homogeneous mixing of the sperm from both males in the storage organs (seminal receptacle and spermathecae). Consequently, offspring production of such double-mated female flies does not follow a coordinated progressive sperm utilization of both males.

For laboratory experiments eye color mutants such as *cn*, *bw* or bw^D have often been used to simplify identification of progeny genotype. Thus male flies of the wild-type or mutant can be utilized as the first or second male in double mating. Results from this type of experiment designed to test sperm displacement (or sperm competition) suggest that genes encoding accessory gland proteins in the second male might mediate an 'attack' component, whereas those in the first male a 'defense' component (see Clark et al.[17]). In studies employing the two males in reciprocal order, the magnitude of both components in each genotype can be statistically estimated. Of special interest is the finding that the 'attack' or 'defense' values are correlated with certain polymorphic alleles of candidate genes encoding the accessory gland secretory proteins.

In a further study of the importance of sperm displacement related to reproductive success, Clark et al.[18] carried out an extensive analysis of variations of the

displacement process in a total of 152 lines of *D. melanogaster* from natural populations. The screened lines were made homozygous for the second and/or third chromosomes. Their assay also included scoring of the progeny of *cn,bw* females mated sequentially to *cn,bw* and tested males, or in the reverse order. According to their findings there are large variations in the ability to displace resident sperm in the mated female (attack component) as well as the ability to resist displacement (defense component) by the subsequent sperm. In most lines nearly all progeny, in some lines only half, are fathered by the second male. Again males with the ability to resist displacement by subsequent sperm are associated with certain polymorphic alleles of genes encoding the accessory gland secretory proteins (*Acp26Aa/b*, *Acp29B*, *Acp36DE* and *Acp53E*). Apparently these are candidate genes involved in the sperm displacement process.

According to Clark et al.[18] there is no correlation between the male's ability to displace resident sperm and that to resist being displaced. They interpret this as being due to two different mechanisms underlying the attack and defense components. Their interpretation is in agreement with the fact mentioned above, that only the defense component is associated with polymorphic alleles of the main cell-specific genes. Unfortunately we know virtually nothing about the mechanism which mediates sperm displacement.

Harshman and Prout[28] have also provided evidence that the seminal fluid is responsible for sperm displacement in *Drosophila*. In the first series of experiments they used semen from two different sterile mutants. Males from the maternal effect mutant *tudor*[38] have no sperm but normal accessory glands. The second group of sterile males is from a transgenic strain with a construct containing the promoter of the gene *Acp95EF* and the coding sequence of subunit A of DTA (see Kalb et al.[31]). The main cells in the accessory gland are killed by production of diphtheria toxin, and the absence of sperm is probably due to the presence of toxin in the testes. Thus the males are deficient in both seminal fluid and sperm.

Virgin females (Oregon-R) were first mated with fertile males, separated, and after four days again mated with sterile males. From the number of progeny after the second mating the residual sperm from the fertile male in the female storage organs can be calculated. The results show that mating with *tudor* males which produce the normal amount of seminal fluid gives rise to distinctly less progeny than does mating with the DTA transgenic males which are deficient in main cell secretion.

In a further series of experiments the second copulation with fertile males was interrupted immediately, 2 or 4 minutes after the initiation of mating. In *D. melanogaster* it is known that for the first five minutes only seminal fluid but no sperm transfer occurs[3]. The results show clearly a stronger suppression of the remaining progeny at 2 and 5 minutes than by immediate interruption.

The general conclusion from the work discussed above is that the accessory gland secretion is involved in the process of sperm displacement. The precise mechanism as well as the candidate genes encoding the specific secretory protein components must await further study.

Life span of mated females

It is known that virgins of *Drosophila* usually have a longer life span than mated females[48]. This phenomenon appears to be related to the increase of oviposition which, as discussed above, is one major female response following copulation. One reasonable conclusion is that egg production requires energy. This is in agreement with experiments designed to reduce ovarian activity by either X-ray irradiation[35] or selection of aged adults[39,51]. Both procedures lead to an extension of female longevity. Thus all these results suggest that egg production takes place at maternal cost and accelerates female death.

One significant point in the study of Patridge et al.[48] is that egg-laying and earlier death of mated *Drosophila* females are reversible. For example, following removal of the males both oviposition and life span of the mated flies are similar to those of virgins of the same age. In other words, there is in fact no acceleration of ageing. It is possible that mated and virgin females lay eggs of different qualities. David[19] reported that eggs laid by the two types of females are different in length. Furthermore, probably only the mated flies release certain essential nutritional elements from the somatic tissues to the eggs, and altered hormonal and other metabolic activities lead to a higher death rate. Of further interest is their observaion that females continuously exposed to males have a shorter longevity than intermittantly exposed females. In the latter case the female flies were kept for two days without males followed by one day with two males[49]. As pointed out by the authors, there are several possible explanations of this exposure effect: first, premating damage to the female caused by some substances produced by the male during courtship; second, a direct injury of the female by the mating action; third, certain detrimental components in the ejaculate may reduce the life span of the mated female. The seminal fluid is know to be a complex mixture derived from the accessory gland, the ejaculatory duct and the ejaculatory bulb[12]. An analysis of the metabolic effects of individual components involved appears promising.

Very recently, Chapman et at.[10] reported convincing evidence that the cost of mating is not due to the receipt of sperm, but owing to the transfer of secretory protein components from the accessory gland. Their experimental design has been mentioned in the previous section.

Virgin females of *D. melanogaster* were mated either to males of a transgenic strain containing the construct of a main cell-specific promoter and the coding sequence of DTA[31], or to sons of the *tudor* mutant. The transgenic males have no main cell products and lack sperm due to the presence of toxin, whereas the mutant males produce normal accessory gland secretion but no sperm, due to the absence of germ line cells. Intact wild-type males serve as mating controls, and males with microcauterized genitalia as non-mating controls. All virgin females were kept intermittently for two days with experimental males (one set with DTA males, one set with *tudor* males), and for one day with intact normal males or microcauterized males as controls. The results are clear: females kept with DTA males having no main cell products lived on average for 29 days, whereas those kept with *tudor* males having normal main cell products had an average life span of only 21 days. Thus, the cost of mating is due to the transfer of main cell secretion of the accessory gland, and not due to the presence of sperm. It is clear that there is no difference in female survival between the two sets of non-mating controls. In a second experimental series using DTA flies with different degrees of main cell deficiencies, a dose-dependent effect of the cell-specific products could be observed.

Another interesting observation was that mated females which also received full main cell products by exposure to *tudor* or intact males had a lower hatching rate than recipients of seminal fluid lacking the main cell products. Similarly, the egg fertility of the mated females was lower compared to those exposed intermittantly to microcauterized, non-mating males. These results indicate, as suggested by the authors, that the main cells synthesize components which eliminate or destroy part of the sperm stored in the mated females. In other words, the secretory components of the accessory gland are at least in part involved in the phenomenon of sperm displacement. As discussed in section III above, Clark et al.[18] reached the same conclusion from their analysis of polymorphic alleles in natural populations.

VI. Suppression of pest insect population

Many economically important pests are insects which infect animals as well as plants and may cause, in cases of large populations, serious damages to agriculture and forest systems. Menn and Hallingworth[42] estimate that world crop losses due to pests, including post-harvest losses due to insects, amount to over 50% of production. As tools for pest insect control, insecticides (i.e. DDT) have played a dominant role. But owing to their high toxicity, and in particular growing insect resistance, investigators soom became aware of the limits to the use of such chemically synthesized compounds.

Recent research, for reasons just mentioned, has become increasingly interested in finding less toxic and more easily metabolized agents. As a means of biological control Rosen[52] has proposed the use of pheromones and sterilized males, the development of resistant plants, and the utilization of natural pest enemies raised on ecological basis to minimize the damage. In principle, biological control by irreversible sterilization and related ideas, as previously suggested by Knipling[32], is doubtless the most promising strategy, but our knowledge of the reproductive mechanisms of many insect species is still very limited.

As in *Drosophila*, mating of pest dipteran insects is likewise under regulatory control of the male accessory gland or homologous organs (see Ito and Yamagishi[29]; Harris and Miller[27]). Recently, Spencer et al.[57] reported that in the onion fly (*Delia antiqua*), a monocoitic dipteran, injection of extract from the male paragonial gland into virgin females resulted in the suppression of receptivity and the induction of oviposition. Their data show that the rate of egg-laying is comparable to that following mating (14.5 eggs/female/day) and that the injected flies refuse remating during their experimental period of more than 15 days. Thus it is reasonable, as suggested by the authors, that the active paragonial components (apparently including the sex peptide) can be utilized as a biorational sterile agent for insect control.

The use of biological agents for insect control has, however, several problems which must be solved prior to their application. First, from our experience the sex peptide of *D. melanogaster* is active in suppressing receptivity of virgin females only within closely related sibling species, obviously due to their high structural homology (see Chen[13] and Schmidt et al.[56]). In other words, sequence analysis of the purified peptide and the test of its species-specificity appear to be essential. Second, for the large scale control of pest insect populations, chemical synthesis of SP is desirable. The *melanogaster* SP has only 36 amino acids. If the peptide molecule is large and complex, both the activity and stability of the synthetic product will be more problematic. Third, one convenient way to introduce the active agent into the virgins is by feeding; the 'sterile' females are then released. But a peptide in the digestive tract needs a special design to protect it from undergoing proteolysis by the digestive enyzmes during uptake.

In *D. melanogaster* we have produced permanently sterile females by germ line transformation[2]. A construct containing the SP coding sequence and the enhancer of the yolk protein gene is introduced into the female genome by using the P-element. After eclosion the transgenic female flies, due to ectopic expression of the SP gene, do not accept courting males and thus remain unmated. Of course, *Drosophila* is the only insect whose genetics is well known. But many pest insects are also

dipterans, and the production of permanently sterile females by using similar transgenic techniques may be considered as a potential strategy for reproductive control of pest insects.

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