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Anatomy and Histology of the Male Reproductive Complex of the Onion Maggot Fly, *Delia Antiqua*, (Diptera: Anthomyiidae) Including Some Comparisons With *D. Platura* and *D. Radicum*

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ANATOMY AND HISTOLOGY OF THE MALE REPRODUCTIVE COMPLEX OF THE ONION MAGGOT FLY, *DELIA ANTIQUA,* (DIPTERA: ANTHOMYIIDAE) INCLUDING SOME COMPARISONS WITH *D. PLATURA* AND *D. RADICUM*

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ABSTRACT

In *Delia antiqua* (Meigen) (Diptera: Anthomyiidae), the male reproductive complex is composed of a pair of testes, paired vas deferens connecting the testes to the anterior ejaculatory duct, and a pair of paragonial (accessory) glands. Each *D. antiqua* paragonial gland consists of a single layer of secretory epithelial cells surrounded by a thin sheath of muscle tissue. The paragonial cells appear to be largely homogeneous in form, however a minor number of cells exhibit unique staining characteristics distinct from the main cells of the gland. This is preliminary evidence for a secondary cell type as has been found for *Drosophila* and *Aedes* paragonial glands. In contrast to the testis and vas deferens, where most of the growth occurs during the pupal stage, the *D. antiqua* paragonial glands expanded markedly due to secretory accumulation during the first days of adult life. Based on histochemical analyses, the paragonial secretion contained abundant protein, with evidence of glycoprotein. The reproductive complex in all three *Delia* species (*D. antiqua, D. radicum* (Bouche) and *D. platura* (Meigen)) appears similar, with the exception of size differences and timing of paragonial secretory accumulation and sperm maturation. Paragonial glands of *D. radicum* were the largest in both length and width, and only this species possessed abundant sperm upon eclosion. Of the three species, *D. radicum* appears most capable of mating immediately after eclosion based on the histology of its reproductive complex, which is consistent with biochemical and behavioral observations made earlier in this laboratory.

In addition to sperm, semen of male insects in various insect orders contains factors known to influence mating and oviposition (Leopold 1976, Chen 1984, Kalulenas 1992). This phenomenon is particularly well studied in the Diptera, where the active substances have been dubbed "sex peptides" (Gillott 1988). These sex peptides may function for chemical "mate guarding" in some species by reducing the females' receptivity to additional mating attempts by male flies, which may result in female monogamy. An additional effect as an ovipositional stimulant is often seen. The source of sex peptides is the paragonial gland system in mosquitoes and fruit flies (as reviewed by Chen 1984) as well as in onion maggot flies (*Delia antiqua* (Meigen)) (Spencer et al. 1992).

In contrast to behavioral characterizations, paragonial glands have been anatomically or histologically characterized for relatively few Dipteran species: *Musca domestica* (Leopold 1970), *Drosophila* (as reviewed by Chen 1984) and *Aedes* (Dapples et al. 1974) are the primary exceptions. The study reported here broadens the number of species for which detailed ontogeny and maturation data are available and provides evidence for the development of secretory capability that can be correlated with existing behavioral data (Spencer et al. 1992, 1995, 1997, Spencer and Miller 2002). In addition, comparisons will be drawn between *D. antiqua* and two closely related species, the cabbage maggot fly,

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Delia radicum (Bouche) and the seed corn fly *Delia platura* (Meigen). Some data on electrophoretic similarity of enzymatic loci already exist for these *Delia* species (Harris et al. 1986). Additional data on biochemical relatedness has been collected via reciprocal injections of paragonial secretions among *Delia* species, which resulted in mating inhibition and oviposition stimulation (Spencer et al. 1997). Collectively, these three species may serve as a general model for *Delia* flies, so we can expand our understanding of this economically important genus.

MATERIALS AND METHODS

Sources of Insects and Rearing Methods. Onion maggot flies *(D. antiqua)* were collected from a field population in Grant, Michigan and have been maintained in laboratory culture since 1986 as described by Havukkala and Miller (1987). Seedcorn maggot flies *(D. platura)* were obtained from Dr. J. W. Whistlecraft of the Agri-Food Canada Research Center in 1993 and were maintained in the laboratory on soaked lima beans. Two cultures of cabbage maggot flies *(D. radicum)* were used in this study: one was obtained from Dr. Whistlecraft (above), and a second was obtained from Dr. Erich Stadler at the Swiss Federal Research Station for Fruit-Growing, Viticulture and Horticulture (Wadenswil). Both cultures were maintained on rutabaga slices (Spencer et al. 1997). All *Delia* flies were maintained at 22± 2°C, 35±10% RH and under a (L:D) 16:8 hr photocycle.

Histological methods. Reproductive complexes of male *D. antiqua*, including testes and paragonial glands, were dissected from intermediate (mid) pupae (defined in this study as those pupae where only the eyes were clearly visible through the puparium) and from late pupae (near eclosion, with eyes, legs and wings clearly visible). Newly eclosed adult males were segregated from females and held in group culture at 22±2°C until dissection.

Animals were anesthetized with carbon dioxide or by exposure to cold and dissected in *Delia* saline (Spencer et al. 1992). Measurements of fresh accessory complexes (10 per stage) were made using an ocular micrometer. Tissues were fixed for histological study in either 10% buffered formalin (Fisher, Pittsburgh, PA) or in 3% gluteraldehyde (Sigma, St. Louis, MO) freshly prepared in *Delia* saline. Tissues were dehydrated through increasing alcohol concentrations to xylene and embedded in TissuePrep 2 (Fisher). Slices were cut at 6 µm on an rotary microtome, and stained with one of the following: Gill's triple strength hematoxylin (Sigma) and eosin or Cason's trichrome for structural features, Coomassie blue or Fast green (trichloroacetic acid omitted) for proteins, or Alcian blue for acidic mucopolysaccharides (all methods from Kiernan, 1990). Periodic acid-Schiff's base staining (PAS) for carbohydrates was performed with an EM Diagnostics kit (Fisher). Structural features on serially sectioned tissue were measured with an ocular micrometer. Twenty measurements were taken from no fewer than five individual glands.

Analysis of variance was performed on the data using the JMP statistical program (SAS Institute 1995). Comparisons of means was accomplished with the Tukey - Kramer's HSD statistic.

RESULTS AND DISCUSSION

Male Reproductive System. The reproductive complex of male *D. antiqua* is composed of a pair of testes, paired vas deferens connecting the testes to the anterior ejaculatory duct, and a pair of paragonial glands. The paragonial glands deposit their secretions into the anterior ejaculatory duct lateral to the vas deferens attachment (Fig. 1). Each paragonial gland of *D. antiqua* consists of a single layer of epithelial cells surrounded by a thin sheath of muscle tissue. Upon dissection into saline, random muscular pumping of the paragonial glands was seen frequently. Secretions accumulate in the lumen of the gland during adult life.

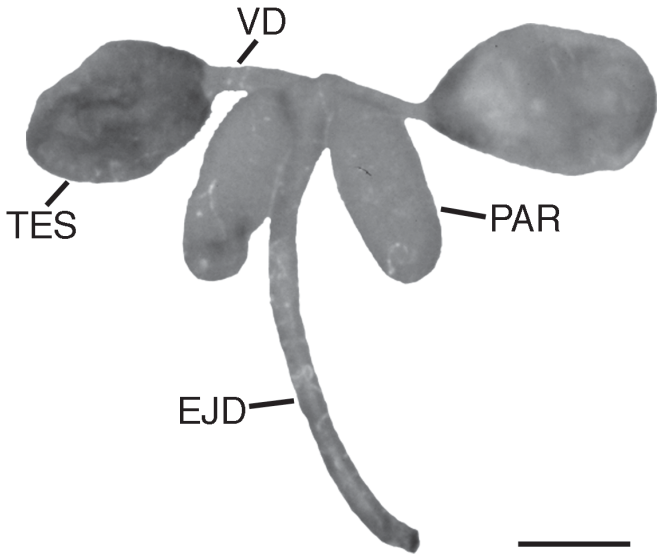

Fig. 1 Digitized image of the reproductive accessory complex from 7-day adult male *Delia antiqua*. Scale bar is equal to 200 µm. EJD, ejaculatory duct, PAR, paragonial gland, TES, testis, VD, vas deferens.

Because the general reproductive anatomy of all three species is very similar, primary emphasis is given to *D. antiqua* in the following analysis. Differences between the three species will be summarized near the end of this report.

Testicular Development. Ocular measurements of the testes from all *D. antiqua* stages examined are listed in Table 1. The majority of the testicular volume is established before the intermediate pupal stage (eyes visible through the puparium). Only a 12% increase in testicular length (with no change in width) was seen among the stages investigated in this study. Growth of testicular tissue preceded sperm development. Numerous differentiated sperm were not detected until around day 5 of adult life. While the absolute onset of sperm maturation was not determined, it was clear that *D. antiqua* flies did not emerge with a significant number of mature sperm present in their testes. This is consistent with behavioral studies in which *D. antiqua* males tend to become sexually active and routinely successful in mating after day 3 of adult life (McDonald and Borden 1995, Spencer et al. 1995).

Vas Deferens Development. In contrast to the testes and the paragonial glands of *D. antiqua*, the vas deferens that connects the testes to the anterior ejaculatory duct underwent no significant changes during the developmental period covered in this study (Table 1). The tissue itself did not appear to be secretory and likely functions merely as conduit for the sperm to the ejaculatory duct.

Paragonial Gland Development. Of all the structures characterized in this study, the paragonial glands showed the most dramatic changes. In measurements of whole freshly-dissected glands, both paragonial length and width increased significantly between the intermediate pupal and 7 day adult stage

Table 1. Sizes of the components comprising the male reproductive complex in *Delia antiqua*.

Stage	Mean size (micrometers ± S.E.[1])				
	Testis length	**Testis width**	**Vas deferens length**	**Paragonial gland length**	**Paragonial gland width**
Intermediate pupae	426 ± 11[2]	265 ± 11[2]	241 ± 10[2]	200 ± 6b3	67 ± 3c[3]
Late pupae	435 ± 11	261 ± 6	233 ± 9	212 ± 6b	68 ± 2c
0 day adult	453 ± 18	272 ± 12	233 ± 13	300 ± 11a	88 ± 4b
5 day adult	445 ± 12	258 ± 8	223 ± 20	293 ± 26a	103 ± 6b
7 day adult	476 ± 11	265 ± 7	226 ± 14	330 ± 9a	128 ± a

[1]n = 10 individuals.
[2]Means in this column were not significantly different at $P < 0.05$.
[3]Means sharing a letter within a column were not significantly different at $P < 0.05$ in the Tukey-Kramer HSD test.

(Table 1). One increase in length and width took place around eclosion, and substantial gains occurred during adult development, especially in paragonial width. Given that the flies studied were virgin males isolated from females, it is not surprising that paragonial secretions accumulated over time without evidence of emissions. This was further documented in the histological study below.

Histological examination of tissue stained with hemotoxylin and eosin revealed several interesting changes in morphology during the course of paragonial gland maturation (Table 2). Although the lateral margins of individual epithelial cells could not be determined, some information was gained about the size of cells through their large and clearly defined nuclei. Height of the single cell layer also was measured. Over the first seven days after eclosion, the size of nuclei present in the paragonial gland remained constant, with the exception of a significant lengthening (major axis) of the nuclei by day 7 compared to day 0 of adult life. Minor axis size (width) did not change significantly during this period. In contrast, nuclear spacing (as estimated by the average distance between the edges of adjacent nuclei) increased steadily and significantly during the first 7 days of post-eclosion development. Mean cell height declined approximately 63% in a linear fashion between eclosion and 7 day adult glands for males isolated from females (linear regression formula was mean cell height = 17.0 - 0.94 age in days, $r^2 = 0.99$).

No mitotic figures were observed in adult paragonial tissue, apparently because the majority of the necessary mitoses occurred in the pupal stage. We noted that the cells flattened and the nuclear distances increased by 450% during the adult stages, suggesting the epithelial layer was stretching to accommodate the secretory accumulation.

To test the hypothesis that gland size increase was due to secretory accumulation, we calculated the volume of the entire gland, of the cell layer and of the secretion (the lumen) by approximating the general gland shape as an ellipsoid sphere. For total gland volume, the external measurements were used. Cell heights were subtracted from measurements of the glands, and the volume of the remaining central area of the gland was considered to be secretion volume. The cell heights were taken from Table 2 and supplemented with preliminary calculations of the intermediate pupal stage cell height (mean = 15.6µm, n = 8), late pupal cell height (mean = 18.9µm, n = 8) and 5 day adult height (12.3µm, estimated from the cell height regression line). The cell volume was taken to be the difference between the total and the secretory volumes. The results of these calculations indicate that while the cell volume increases only slightly after eclosion, the secretion volume increases dramatically and accounts for the majority of size increase in the paragonial glands (Fig. 2). By day 7 of adult life, the size of the glands from the precisely staged flies used in this study approximated paragonial gland size of adult virgin males randomly selected from open culture (data not shown). We conclude from these data that secretory accumulation levels out at approximately day 7 of adult life if males are isolated from females. This finding contrasts with *Drosophila* where rate of synthesis of some accessory gland proteins declines around day 3 of adult life in the absence of mating (Monsma et al. 1990).

The amount of secretory material doubles between newly eclosed flies and 5 day adult flies (Fig. 2), and there is evidence that the titer of active ingredients also increases during early adult life. When homogenized glands were injected into virgin females, the 1 day old glands were not as effective (at 1/10th male equivalent) as the 3 day adults in stimulating oviposition and inhibiting remating (Spencer et al. 1995). It is unclear if the difference between newly eclosed males and 3-day males is due only to the amount of secretion or some difference in the biochemical synthetic patterns of the glands.

Table 2. Cell height, nuclear size, and nuclear spacing for paragonial glands of variously aged *Delia antiqua* males.

		Mean size (micrometers ± SEM[1])		
Adult age	**Cell height**	**Major nuclear axis**	**Minor nuclear axis**	**Space between adjacent nuclei**
0-24 hr	16.8 ± 0.4a [2]	8.2 ± 0.2b [2]	6.7 ± 0.2 [3]	1.6 ± 0.5c [2]
48-72 hr	14.6 ± 0.7a	8.7 ± 0.2ab	7.2 ± 0.2	3.4 ± 0.3b
7 day	10.3 ± 0.7b	9.4 ± 0.3a	7.3 ± 0.3	8.9 ± 0.6a

[1]n = 20 measurements.
[2]Means sharing a letter within a column are not significantly different at $P < 0.05$ in the Tukey-Kramer HSD test.
[3]Means in this column were not significantly different at $P < 0.05$.

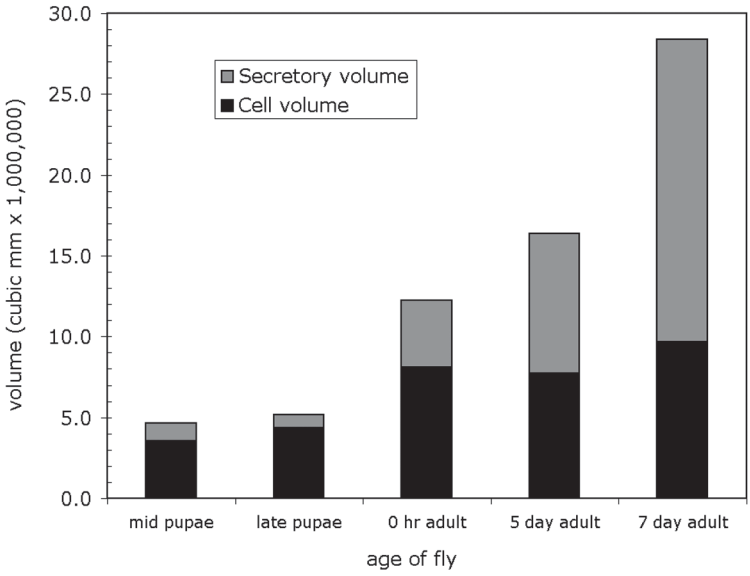

Fig. 2 Comparison of total cell and secretion volumes for the paragonial glands of male *Delia antiqua* flies. Values were calculated using the mean values for cell height and gland size at each age.

Histochemical Analysis of *D. antiqua* Paragonial Glands and Secretions. Several histochemical stains were used to characterize the paragonial cells and their secretory material. The presence of basophilic cytoplasmic staining in the hemotoxylin and eosin slides indicated considerable synthetic activity in the epithelial cells. Nuclei were clearly visible within the secretory cells.

Two stains were used to detect proteins in the tissues and secretions. Staining with Coomassie blue revealed that although the nuclei excluded stain to a certain extent, both epithelial cytoplasm and secretory material of the paragonial glands contained abundant and uniformly distributed protein sources. Fast green without trichloroacetic acid (as suggested in Leopold, 1970) stained secretory material more intensely than the surrounding cells. No appreciable protein accumulations were detected in testes, vas deferens or ejaculatory duct tissue, although the cells of each tissue stained positively for protein, as would be expected.

Alcian blue, a histological stain with an affinity for acidic mucopolysaccharides, has been used to indicate secretory materials in other dipteran paragonial glands (Leopold, 1970). In *D. antiqua,* no appreciable sources of acidic mucopolysaccharides were present in the paragonial glands, although some staining of testicular tissue was seen.

The paragonial gland secretion contained abundant PAS positive material as evidenced by the intense purple reaction after addition of Schiff's base during Periodic acid-Schiff's staining. Tissue may show a positive PAS reaction for several reasons: extant aldehyde linkages, glycogen deposits, or glycoprotein staining. No reaction was observed without the oxidation step, which indicates a total lack of pre-existing aldehyde linkages. Although it is unlikely that our preservation methods retained tissue glycogen, it is important to rule out glycogen presence as a cause for the intense reaction observed in the secretory material. Pre-treatment with human saliva for 1 hr at 37°C (salivary amylase destroys glycogen reactivity) did not reduce the staining intensity, which indicates that the PAS reactive material is probably not glycogen. Based on these data, we believe that there is abundant glycoprotein material in the paragonial gland secretion. This finding supports data from initial sex peptide purification attempts in *D. antiqua*, in which there was evidence of glycoprotein presence (Spencer 1994). Some of the functionally significant proteins in the accessory glands of *Drosophila* are glycosylated or contain glycosylation sites in their transcripts (Monsma et al. 1990).

In contrast, the paragonial gland cytoplasm did not show any PAS reactivity. Perhaps the glycosylation steps occur close to the time of secretion, or maybe only minute levels of secretory material exist in the cells at any one time. If the latter were true and the rate of synthesis is relatively slow, then accumulation kinetics may play an important role in preparation for successful mating. In contrast to the paragonial gland, no PAS reaction was seen in the testes, vas deferens or in anterior ejaculatory duct tissue.

Cason's trichrome stain (on formalin-fixed material) showed homogeneous secretion in the paragonial glands, except for areas of distinctive bright pink staining which were located at the distal tip of each gland. This unusual staining was noted in both accumulated secretory material and in isolated cells in the same area as the secretion. The bright pink color was detected in glands from 7-8 day adult males, but not in 0 or 4 day adults. We considered whether a difference in cellular pH could account for the unusual staining, but found that pretreatment of sections with a strong base did not diminish staining intensity.

Cason's trichrome stain (on gluteraldehye-fixed complexes) reveals striking color differences in the muscle layers around the testis, and sperm tails were stained very distinctly. In the paragonial gland, the secretion stained a rich purple, but there was a crescent of pink staining material along the distal edge of the gland and an occasional pink cell was noted. This result is consistent with the findings in the formalin-fixed material.

We conclude that most of the stains used in this study indicate that *D. antiqua* cells are of a uniform type and produce a homogeneous secretion. The exception is seen with the trichrome stains, where unusual pink staining might be related to aging of secretory material in virgin males isolated from females. Or these data might indicate a minor cell type, as has been noted for *Aedes aegypti* (Dapples et al. 1974), possibly with distinct biochemical synthetic abilities, as in *D. melanogaster* (Bertram et al. 1992). Although staining differences due to aging within the gland cannot be entirely ruled out, it seems more likely given the distribution characteristics of the unusually staining cells (broadly dispersed over the distal ends of the paragonial glands analogous to the cells in *Drosophila*) that these represent an authentic minor cell type in *D. antiqua* paragonial glands.

The interplay between synthesis kinetics and accumulation phenomena was not the subject of this report, but it is clear that both processes will be important in preparing the fly for successful mating. We suggest *Delia antiqua* will make a good model system for investigation of paragonial gland events, both in terms of biochemical characterization and physiological effects of sex peptides on female physiology and behavior.

Comparative data within the Genus *Delia*. Anatomically, the reproductive complexes of *D. radicum* and *D. platura* closely resemble that of *D. antiqua*. Some differences between the species consisted of size characteristics of the mature reproductive systems (Table 3) and timing of sperm production and secretory accumulation events. Complete histochemical comparisons were not made between all three species and the age study undertaken for *D. platura* and *D. radicum* was not as detailed as that of *D. antiqua*.

Physically, *D. platura* has the smallest body length and mass of the three species, and that relationship holds for the size of the mature accessory structures and testes as well. The largest species, *D. antiqua,* also has the largest testes (both length and width). *Delia radicum* is of moderate size, but its paragonial glands appear to be the largest in terms of length, width and, by inference, volume. Paragonial gland sizes of *D. antiqua* and *D. platura* males were more similar to each other than to *D. radicum*. When paragonial gland secretions from *D. antiqua* and *D. platura* were reciprocally injected into virgin females of the other species, full inhibition of mating and stimulation of oviposition was seen (Spencer et al. 1997). Reciprocal injections with *D. radicum* were not as behaviorally active, suggesting a greater evolutionary distance between these species and *D. radicum*. Genetic distances calculated from electrophoretic enzyme analyses also indicate that *D. antiqua* and *D. platura* are more closely related to each other than to *D. radicum* (Harris et al. 1986). Sequence

Table 3. Size of the reproductive complex for mature males of *Delia antiqua, Delia platura* and *Delia radicum*.

	Mean size (micrometers ± SEM[1])				
Species	**Testis length**	**Testis width**	**Vas deferens length**	**Paragonial gland length**	**Paragonial gland width**
D. antiqua	476 ± 11a	265 ± 7a	226 ± 14a	330 ± 9b	128 ± 4ab
D. platura	393 ± 17b	160 ± 8b	220 ± 7a	255 ± 9c	115 ± 7b
D. radicum	406 ± 16b	148 ± 5b	130 ± 12b	440 ± 11a	153 ± 8a

[1]n = 10 individuals; means sharing a letter within a column are not significantly different at $P < 0.05$ in the Tukey-Kramer HSD test.

analyses of paragonial gland products will undoubtedly further illuminate the relationship among the *Delia* species.

Additional differences were seen in the pattern of maturation of the testis and the accessory glands between *D. antiqua* and *D. radicum*. The testes of *D. antiqua* contained few clearly differentiated sperm at eclosion and the size and secretory volume of each paragonial gland was small. These facts correlate well with the observations noted earlier that *D. antiqua* begins mating at approximately 3 days post eclosion, and is much more successful when mating at day 6 or later Spencer et al. 1995). In marked contrast, *D. radicum* testes contained abundant sperm at eclosion (also reported by Salem et al. 1989), and their accessory gland lengths were larger in the late pupal stage ($x=370 \pm 19\mu m$, $n=10$) than *D. antiqua* glands in the 7 day adult stage. For *D. melanogaster*, a positive correlation has been established between body size, accessory gland size and mating frequency (Bangham et al. 2002), but these relationships appear more complex among species of *Delia* investigated here. In our experience, young *D. radicum* males are more aggressive than the other two species and have frequently been observed mating on the first day after eclosion. Mating activity for *D. radicum* appears to peak on day 3 of adult life (Swailes 1971). Although *D. radicum* is not the largest fly of the three species investigated here, it is clear that the abundant secretion and early sperm maturation could significantly contribute to mating success. Our anatomical data suggest that mate competition may be more severe for *D. radicum* than for *D. platura* or *D. antiqua* flies.

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