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"Reduced Appendage": A Mutation Affecting Development of Pupal and Adult Appendages of the Moth, *Plodia interpunctella*

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A mutant that results in the reduced length of pupal and adult appendages was isolated from a laboratory colony of the Indianmeal moth, *Plodia interpunctella* (Hübner). "*Re*duced appendage" (*rda*) was determined to be an autosomal recessive mutation that affects the development of pupal and adult appendages during the larval/pupal molt. The *rda* mutation had no observed effect on the larval phenotype. After pupation, the appendages of *rda* were reduced in size as compared with wild-type. In addition, unsclerotized cuticle underlying the pupal appendages was exposed and the establishment of the boundary between the unsclerotized and sclerotized pupal abdominal cuticle appeared normal even though the imaginal discs of *rda* did not evaginate normally. This demonstrates that *rda* affects only imaginal discs and that the morphogenesis of structures that were not derived from the imaginal discs were not dependent on interactions with evagination of imaginal discs. Although the *rda* phenotype resulted in shorter antennae, mouth parts, legs, and wings in pupae and adults, the mutation did not affect the number of cells comprising the imaginal discs or the pupal appendages. Cell counts showed that forewing imaginal discs and pupal forewings from the *rda* mutants contained the same number of cells as did the imaginal discs and wings from the wild-type strain. Thus, *rda* appears to affect processes related to disc evagination and not cell proliferation.

Key words: imaginal discs, morphogenesis, metamorphosis, disc evagination, Indianmeal moth

INTRODUCTION

Imaginal discs in holometabolous insects are pouches of embryonic tissues that differentiate at the onset of metamorphosis (cf. Oberlander, 1985). The cells

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comprising the imaginal discs proliferate and become developmentally committed during the larval stage but remain undifferentiated. Only with the hormonal changes during the larval-pupal transformation are the imaginal discs able to realize their determined state and initiate growth to form specific adult structures.

As observed in *Drosophila melanogaster*, the competency of imaginal discs to differentiate depends on the number of cell divisions that have occurred (Mindek and Nöthiger, 1973). During the last larval instar of lepidopterans, the imaginal discs have a rapid rate of growth due to an increased rate of cellular proliferation that is stimulated by the action of 20-hydroxyecdysone (20E) (cf. Oberlander, 1985). However, the ability of imaginal discs to initiate metamorphosis following exposure to 20E in vitro changes with the age of the last instar larvae of the Indianmeal moth, *Plodia interpunctella* (Hübner). Imaginal discs from 1-day-old fifth (last) instar larvae could not respond after treatment with 20E in vitro and progress to cuticle deposition while imaginal discs from 2-day or older fifth instar larvae did respond and initiated metamorphosis (Oberlander and Silhacek, 1976; Oberlander and Lynn, 1982).

Attainment of a specific imaginal disc size is also critical to the morphology of the structure produced in the adult. In the moth, *Ephestia (Anagasta) kühniella*, the temperature sensitive mutation *kfl* limits the number of cell divisions in the imaginal wing discs (Muth, 1961). Consequently, the number of cells comprising the discs at metamorphosis is reduced and results in a normal sized adult with small wings. The number of cells in the imaginal discs can be reduced experimentally by inducing cell death. By exposing last instar larvae of the moth, *Galleria mellonella*, to X-rays, small pupal wings were induced (Meyer et al., 1980). The smaller size of the wings was the result of a reduction in the number of cells present that comprised the pupal wings.

Although the normal development of adult appendages appears to depend on the imaginal discs achieving a certain size and cell number, there are physiological conditions that can lead to the production of small adult appendages. Extirpation of the corpora allata from lepidopteran larvae before the last instar can lead to the production of precocious pupae and adults with normally proportioned, but small, adult appendages. The "normal" adult appendages are formed even though the imaginal discs have not achieved maximum size and, therefore, number of cells. The removal of the corpora allata in the penultimate instar of *Bombyx mori* resulted in small pupae at the subsequent molt (Bouhniol, 1938). In most lepidopterans, however, allatectomy of early larval instars results in larval-pupal intermediates, and the effects of the inappropriate conditions of metamorphosis result in poorly evaginated and sclerotized imaginal structures in the precocious pupae (Bouhniol, 1938; Fakuda, 1944; Piepho, 1942, 1946). Normal pupation depends on the presence of low levels of juvenile hormone during the molt as demonstrated in *Hyalophora cecropia* (Williams, 1961) and *Manduca sexta* (Kiguchi and Riddiford, 1978).

We have isolated a mutant strain of *P. interpunctella* that has a phenotype similar to that of the X-ray induced reduced appendage structures described in *G. mellonella*. Analysis of the mutant strain offered an opportunity to examine the processes that result in the production of normal imaginal structures from imaginal discs. To define the mutation, we determined the phenotypic effect on

the cellular composition and morphology of the imaginal structures and assessed the genotype of "*re*duced *a*ppendage" (*rda*).

MATERIALS AND METHODS

The *P. interpunctella* wild-type and mutant colonies were reared according to Silhacek and Miller (1972) in a 16 h:8 h light-dark cycle at 30°C and 70% relative humidity. Genetic crosses were made between true-breeding mutant and wild-type moths. The progeny of P_1 and F_1 backcrosses were classified by phenotype and sex. Multiple single-pair matings were made for each cross. The results of the phenotype distributions for each cross were analyzed statistically by the Chi-square test for goodness of fit. Those distributions with one degree of freedom were computed with the Yates correction for continuity for two cell tables.

The number of cells comprising the forewing imaginal discs in 4-day fifth instar larvae (last instar larvae spun into cocoons) and pharate pupae (5-day fifth instar larvae) were determined by the technique described by Martin (1982). The imaginal discs were dissected from larvae removed from cocoons into saline (Weevers, 1966). Each disc was transferred into a 15 µl drop of 0.35 M citric acid on a silanized slide and allowed to stand for 10 s. The disc was dissociated into single cells by "strumming" the tip of a tungsten needle in the drop. Strumming of a needle was accomplished by holding one end of a needle in a fixed position and then picking the needle with a second so that the tip of the first needle vibrated rapidly. This action caused a vigorous stirring of the drop, and the discs were dissociated within 30–60 s. An aliquot of the drop was transferred to a Neubauer Hemocytometer (Picker International, Highland Heights, OH), and the cells were allowed to settle for 2 min. The cells were viewed using phase-contrast optics and counted. The mean number of cells in a disc was compared using a Student's *t*-test for two means.

The number of cells present on the surface of forewings of white pupae was determined by counting nuclei. The forewings were removed from *rda* or wild-type pupae (less than 4 h after pupation) in saline (Weevers, 1966) and then fixed in 4% (W/V) depolymerized paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 1 h and then transferred to fresh fixative for 12 h at 4°C. The forewings were washed five times for 1 h in 0.1 M phosphate (pH 7.4), 1% azide, and 1% Triton X-100 (PBAT) at 24°C and placed in PBAT at 4°C overnight. The nuclei were stained with DAPI (10 µg/ml PBAT) (4',6-diamidino-2-phenylindole; Molecular Probes, Eugene, OR) for 5 min and then mounted in 10% glycerol, 0.5 M NaCl, 20 mM Tris (pH 9.0) 0.1% n-propylgallate. The forewings were viewed and photographed with an Olympus BHS microscope equipped with a BH2-RFC reflected light fluorescence attachment with a DAPI/FITC/Texas Red filter set (Chroma Technology Corp., Brattleboro, VT).

The number of nuclei per square millimeter of forewing surface was determined by counting the number of nuclei per unit area using a Jandel Scientific digitizer and SigmaScan software (Jandel Scientific, Sausalito, CA). The total surface area of *rda* and wild-type pupal wings was determined from mounted portions of the pupal cases that included the entire wing surface. The pupal wings were mounted in 60% glycerol and viewed and photographed using a

Zeiss SR microscope. The total forewing surface area was determined from the photomicrographs using a digitizer and SigmaScan software.

RESULTS

rda Phenotype

The *rda* mutant arose as a spontaneous mutation in a *white-eye* (*we*) strain of the Gainesville laboratory colony. After segregation from *we* to a homozygous *rda* strain, the *rda* phenotype was described. The *rda* mutation resulted in shortened appendages in the pupal and adult stages and included the antennae, maxillary palps, legs, and wings (Fig. 1).

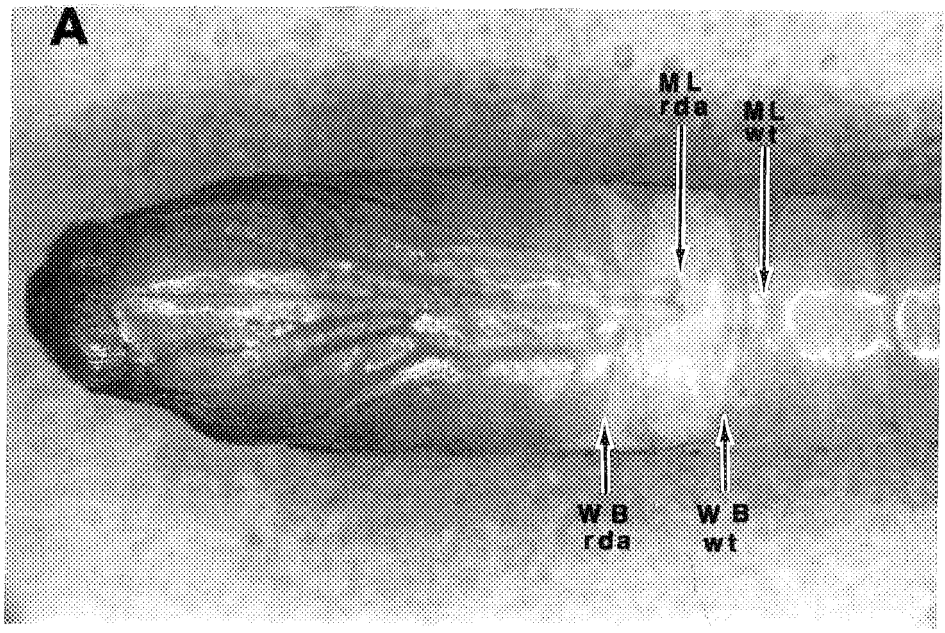
The shortened appendages in the pupae resulted in the exposure of unsclerotized abdominal cuticle that would normally underlie the wing structures. Longitudinally, the exposure of the unsclerotized abdominal cuticle extended from the first abdominal segment, where the border of the *rda* wings stopped, to the middle of the fourth abdominal segment, where the border of wild-type wings would normally be located. Laterally, the border of the hind wings extended to the line, where the wild-type normally covered. The greatest area of exposure was in the region of the posterior wing border, where the length of the wing was reduced by 50%, but the width of the wings appeared similar to that of the wild-type.

The lengths of the adult appendages for *rda* mutants were statistically significantly shorter than the appendages of wild-type adults (Table 1). The magnitude of the reduction in appendage length ranged from a maximum of 50% for the wings to a minimum of 16% for various leg segments as compared with the wild-type phenotype. In the case of the wings, the shortened *rda* phenotype also resulted in an inability to inflate the wings following adult eclosion. During the post-eclosion period of wing inflation, the *rda* mutants attempted but could not increase the size of the wings. Instead of inflating, the wings often developed diverticula that protruded from the surface of the wings.

The mutation also affected the operation of the genitalia, although there were no observable changes in the sizes of the external male genital structures (i.e., aedeagus and claspers). The effect of the mutation on the genitalia resulted in a low mating efficiency; only 50% of the single-pair matings involving either homozygous *rda* male or female parents produce offspring. Part of the low efficiency results from the failure of some of the moth pairs to uncouple after copulation which interferes with egg deposition by the female.

In contrast to the pupal and adult appendages, no differences in the sizes of the appendages between larvae of the *rda* and wild-type strain were observed. The lack of observable effects on the larval appendages suggested that this mutation only affects the growth and/or development of the adult appendages from the imaginal discs. This hypothesis is also supported by the observation that *rda* had no effect on non-imaginal disc structures since total body mass and the overall body length were statistically not significantly different between *rda* and wild-type moths (Table 1).

To determine if the *rda* mutation affected imaginal disc growth by cell proliferation in excess of cell death, the number of cells in forewing imaginal discs from late larvae and early pupae were counted. Cell counts were made at three developmental stages: before metamorphosis (i.e., 4-day fifth instar lar-



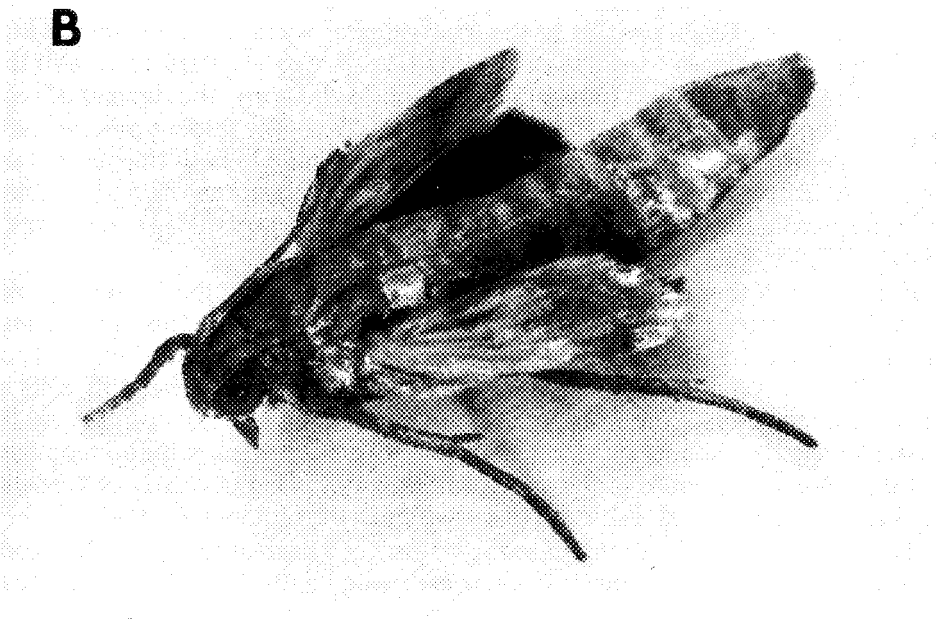


Fig. 1. Phenotypic characters of *rda* in (**A**) a female pupa and in (**B**) an adult female. The lower arrows in A point to the edge of the wing border (WB) for the *rda* and to where the wing border for the wild-type (wt) would normally be found. The upper arrows in A point to the end of the metathoracic leg (ML) for the *rda* and to where the end of the leg for the wild-type would normally be found.

TABLE 1. Comparison of the Appendage Length of the *rda* Phenotype With the Wild-Type Phenotype of the Indianmeal Moth

	Wild-type	*rda*
	Mean (SE)	Mean (SE)
Body mass of prepupae	11.9 (0.4)	10.7 (0.7)
Length (mm) of adult body part		
Whole body	7.7 (0.1)	7.0 (0.4)
Antennae	4.1 (0.1)	3.0 (0.2)[a]
Forewing	6.1 (0.1)	3.1 (0.1)[a]
Hind wing	5.2 (0.1)	2.6 (0.1)[a]
Femur	4.4 (0.2)	3.7 (0.2)[a]
Tibia	5.3 (0.1)	4.1 (0.2)[a]
Tarsa	4.7 (0.2)	3.7 (0.2)[a]

[a]*t*-test for two means; significantly different from wild-type at the 0.05 level.

vae), after initiation of metamorphosis (i.e., pharate pupae [5-day fifth instar larvae]), and after the larval/pupal molt (i.e., white pupae). The mean number of cells comprising forewing imaginal discs from the 4-day fifth instar *rda* larvae and pharate pupae was statistically not different from those of discs from wild-type of the same stages (Table 2). However, the number of cells in the discs increased significantly from the 4-day larvae to the pharate pupae for both the *rda* and wild-type phenotypes.

The number of cells present in the forewings of white pupae from *rda* and wild-type phenotypes was also determined. Although the surface area of the pupal forewing of *rda* was only 90% of the wild-type wing, the density of cells was greater than that in the wild-type (Table 2). Thus, the total number of cells per wing surface was the same at the white pupal stage in both the *rda* mutant and the wild-type strains. The total number of cells in the forewing had doubled from approximately 60,000 cells in the pharate pupal larval stage to approximately 157,000 cells in the white pupae.

Even though there were equivalent numbers of cells in the forewings, the organization of the cellular patterns around scale stem cells on the wing surface of the white pupae was not as clear in the *rda* as was apparent for the wild-type strain (Fig. 2). Whether the lack of organization, apparent in the scale cells of the *rda* mutant pupae, affects the adult scale production was not critically examined. However, in the adult *rda* moths, scales were present on all of the appendages, but the coloration pattern of the scales on the wings was not clearly discernable because of the structural distortion of the wings in the mutant (Fig. 1B).

To determine if the *rda* mutation was the result of a decreased juvenile hormone titer, wandering fifth (last) instar larvae were treated with 50 µg of methoprene in 1 µl acetone. All of the larvae showed the *rda* phenotype in the pupal stage.

rda Genetics

Genetic crosses between moths that had the *rda* phenotype always produced the *rda* phenotype. The sex ratio of the offspring was 1:1 (data not shown).

TABLE 2. Number of Cells in the Forewing Imaginal Discs From Last Instar Larvae and in the Pupal Forewing Surface From *rda* and Wild-Type Indianmeal Moths

	Stage of development				
	4-day fifth larvae	Pharate pupae	White pupae		
	No. cells/disc	No. cells/disc	No. cells/mm^2	Wing surface area (mm^2)	No. cells/wing surface
	Mean (SE)	Mean (SE)	Mean (SE)	Mean (SE)	Mean (SE)
Wild-type	50,080 (3,370)	65,280 (4,030)[a]	19,833 (944)	3.88 (0.07)	78,817 (3,240)
rda	47,370 (4,950)[b]	57,730 (3,310)[a,b]	22,589 (741)[c]	3.47 (0.05)[c]	78,382 (2,572)[b]

[a] *t*-test for two means; significantly different from the previous developmental stage at 0.05 level.
[b] *t*-test for two means; not significantly different from wild-type at 0.05 level.
[c] *t*-test for two means; significantly different from wild-type at 0.05 level.

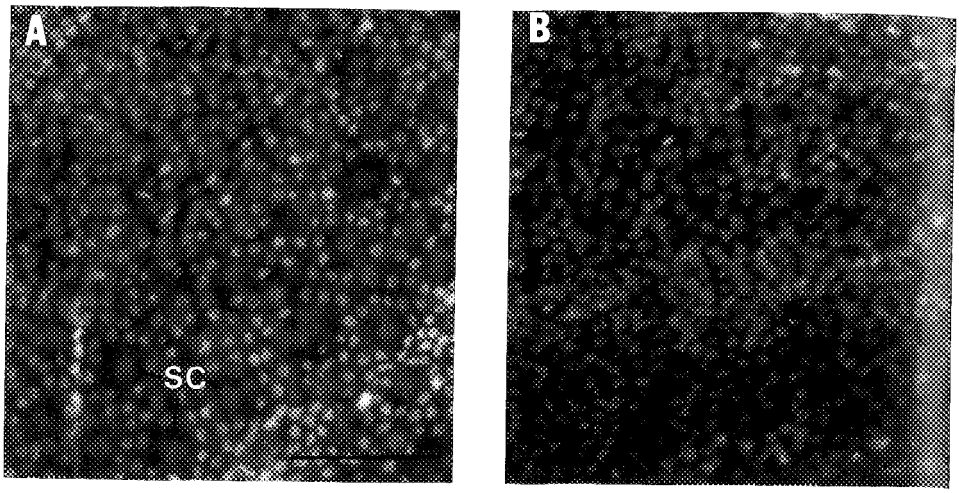


Fig. 2. Scale stem cell patterning in forewings of wild-type and *rda* white pupae. The micrographs show DAPI stained nuclei in portions of whole-mounted forewings of wild-type (**A**) and *rda* (**B**) white pupae. The pattern of scale stem cells (SC) and surrounding epidermal cells can be clearly observed in the forewing of the wild-type but only poorly in the *rda*. Magnification bar = 50 μm.

Reciprocal crosses between the *rda* true-breeding strain and the wild-type strain resulted in F_1 progeny with only the wild-type phenotype. Intercrosses between the F_1 progeny resulted in a 3 wild-type (*RDA*):1 *rda* phenotype ratio characteristic of monohybrid Mendelian inheritance (Table 3). Genetic crosses between moths that were heterozygous for both the *we* and *rda* genotypes segregated into a phenotypic ratio of 9 *WE RDA*:3 *we RDA*:3 *WE rda*:1 *we rda* characteristic of two independent loci (Table 4).

Reciprocal backcrosses between the F_1 progeny and the homozygous *rda* strain resulted in a 1:1 ratio of wild-type to *rda* phenotypes regardless of whether the F_1 was male or female. Reciprocal backcrosses of the F_1 progeny to the homozygous wild-type strain initially resulted in three of the progeny with what appeared to be the *rda* phenotype (Table 3). Because adults with "crumpled wings" occasionally appear in the normal wild-type culture, the genotype of these putative F_1 *rda* phenotype progeny was checked. When the putative F_1 *rda* were mated with the homozygous *rda* strain, the phenotype of the offspring were all wild-type, demonstrating that the genotype of the F_1 *rda* phenotypes was wild-type.

The mean number of progeny produced by a cross of wild-type parents was 114. The mean number of progeny resulting from each of the various crosses and backcrosses was statistically not significantly different from the number of progeny produced by the wild-types.

DISCUSSION

From results of genetic crosses, we conclude that *rda* in *P. interpunctella* is the result of the mutation of a single recessive autosomal gene and that the mutation

TABLE 3. Segregation of Phenotypes in Crosses Between *rda* Homozygotes (*rda/rda*), *rda* Heterozygotes (*RDA/rda*), or Wild-Type (*RDA/RDA*) Genotypes of *Plodia interpunctella*

Genotypes of parents		No. of crosses	Total progeny	Mean no. progeny	Observed progeny phenotype[a]		Expected progeny phenotype		χ^2[b]
♀	♂			Mean (SE)	*RDA/*	*rda/rda*	*RDA/*	*rda/rda*	
RDA/rda	*RDA/rda*	11	1,704	155 (60)	1,306	398	1,278	426	2.37[c]
rda/rda	*RDA/rda*	18	2,124	118 (59)	1,023	1,101	1,062	1,062	2.79[d]
RDA/rda	*rda/rda*	23	2,664	116 (57)	1,370	1,294	1,332	1,332	2.11[c]
RDA/rda	*RDA/RDA*	8	1,133	142 (86)	1,133	0[e]			
RDA/RDA	*RDA/rda*	14	1,266	90 (66)	1,266	0[e]			
+/+[f]	+/+	7	798	114 (67)	798				

[a]Sex ratio of progeny was near unity in all crosses.
[b]χ^2 was computed on the basis of the Yates correction for continuity for two-cell tables.
[c]Observed phenotypic ratios are not significantly different from expected ratios at the 0.01 level of probability.
[d]Observed phenotypic ratios are not significantly different from expected ratios at the 0.05 level of probability.
[e]*rda* phenotypes were checked for genotype.
[f]Wild-type strain of *P. interpunctella*.

TABLE 4. Segregation of Dihybrid *we* and *rda* Phenotypes in *Plodia interpunctella*

Genotypes of parents		No. of crosses[b]	Total progeny	Observed progeny phenotype[a]				Expected progeny phenotype				χ^2
♀	♂			*WE/RDA*	*we/RDA*	*WE/rda*	*rda/rda*	*WE/RDA*	*we/RDA*	*WE/rda*	*rda/rda*	
WE/we; RDA/rda	*WE/we; RDA/rda*	9	1,259	745	206	223	85	708.21	236.06	236.06	78.69	6.97[c]

[a]Sex ratio of progeny was near unity in all crosses.
[b]Matings were made with one to five pairs of adults per cage.
[c]Observed phenotypic ratios are not significantly different from expected ratios at the 0.05 level of probability.

had full penetrance. Even though *rda* arose spontaneously in a *we* mutant strain, *rda* was not closely linked with the *we* locus, and the two genotypes were segregated into separate homozygous stocks. The phenotype of *rda* was not similar to any other wing or appendage mutation previously described for *P. interpunctella*, *E. kühniella*, or *B. mori* (Robinson, 1971; Leibenguth, 1986).

The phenotype of the *rda* mutation resulted in a decrease in the length of the pupal and adult appendages, but it was not based on a decrease in the number of cells that comprise the appendage. The same number of cells was present in the forewing of the *rda* and the wild-type of the three stages of development that were examined. The forewing imaginal discs of the wild- type pharate pupa from *P. interpunctella* contained approximately 65,000 cells, which was similar to the number of cells observed in the hind wing imaginal discs from *E. kühniella* (Pohley, 1956), a closely related pyralid moth of similar size. The rate of increase in the cell number, and therefore the relative size, of the wing imaginal discs from *P. interpunctella* wild-type strain was similar to that observed for other lepidopterans (Pohley, 1956; Williams, 1980; Kurushima and Ohtaki, 1975; Meyer et al., 1980; Kawasaki and Iwashita, 1987).

Although phenotypes, both genetic and acquired, that are similar to *rda* in *P. interpunctella* have been reported, they apparently do not affect the same mechanism(s) controlling imaginal disc development. A shortened wing mutation, *kfl*, was described for *E. kühniella* that was a temperature sensitive mutation. When last instar larvae were reared at the restrictive temperatures, *kfl* affected the development of normal sized wings from the wing imaginal discs during the larval/pupal molt by reducing the number of cell divisions in the imaginal discs (Muth, 1961). When larvae of *G. mellonella* were exposed to X-irradiation, an induced wing morphology similar to that of the *rda* mutation was observed (Meyer et al., 1980). However, the reduced length of the wings in *G. mellonella* was the result of a reduced number of cells in the forewings. In contrast, the *rda* mutation in *P. interpunctella* was not temperature sensitive (data not shown) and was not the result of reduced cell numbers in the imaginal discs.

Because the number of cells present in the imaginal discs and pupal wings is the same in the mutant and wild-type, the *rda* mutation does not involve a limitation on the divisions of the imaginal disc cells. One possible lesion that could cause this phenotype would be the result of an endocrinopathy for juvenile hormone during the last larval instar. Shortened appendages can be induced in pupae of *H. cecropia* and *M. sexta* by allatectomy of early last instar larvae (Williams, 1961; Kiguchi and Riddiford, 1978). The effect of the allatectomy can be rescued in *M. sexta* by a subsequent treatment of the larvae with a juvenile hormone analogue between the wandering and the ocellar retraction stages (Kiguchi and Riddiford, 1978). However, treatment of wandering last instar larvae of the *rda* mutant with the juvenile hormone analogue, methoprene, did not have any effect on the phenotype in the pupal stage in *P. interpunctella*.

These data show that the *rda* mutation affects the organization of the epidermal cells and scale stem cells in the pupal wings and the ability of the wings to inflate after adult eclosion by processes that are independent of juvenile hormone titers and of imaginal disc cell divisions during metamorphosis. Additionally, from the morphology of the pupae we conclude that growth and

evagination of the imaginal discs do not affect abdominal development because the unsclerotized abdominal cuticle underlying the pupal appendages and the abdominal border for the pupal wings were normal. By excluding these processes as possible causative mechanisms for the *rda* phenotype, we suggest that the mutation acts on disc evagination. In *Drosophila melanogaster*, disc evagination results from cell re-arrangements (Fristrom and Fristrom, 1975; Fristrom, 1976) and changes in cell shapes (Milner et al., 1984; Condic et al., 1991). The hormonally regulated proteolytic cleavage of apical surface glycoproteins suggests that evagination requires the separation of the apical cells from the overlying extracellular matrix (Pino-Heiss and Schubiger, 1989; Birr et al., 1990). Following the initiation of evagination, cuticle deposition begins (Fristrom and Liebrich, 1986) which again fixes the position of the epidermal cells. Biochemical or temporal disruption of any of these processes can lead to aberrant disc evagination. These mechanisms will be examined in the *rda* mutant strain of *P. interpunctella* to assess their importance to normal imaginal disc evagination in Lepidoptera.

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