Radiation-Induced Pathology in the Metamorphosis of the Mexican Fruit Fly (Diptera: Tephritidae)[1,2]

Donald B. Thomas and Guy J. Hallman

USDA Agricultural Research Service, Kika de la Garza Subtropical Agriculture Research Center, 2301 S. International Blvd, Weslaco, TX 78596 USA

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Abstract Late third instars of the Mexican fruit fly, *Anastrepha ludens* (Loew), exposed to ≥20 Gy of gamma radiation are able to pupariate normally but do not emerge as adults. Contrary to expectations, the imaginal discs were undamaged and metamorphosis to the pupal stage proceeded normally. Rather, development was arrested at the transition from cryptocephalic to phanerocephalic pupa, a process which requires vigorous muscular contractions. Protein metabolism during metamorphosis was found to be retarded in irradiated but living individuals relative to non-irradiated controls. These results suggest that a general radiation sickness is the cause of developmental arrest and eventual death of the pharate insect rather than a specific tissue atrophy.

Key Words *Anastrepha ludens,* Mexican fruit fly, Gamma radiation, metamorphosis, pathology

The irradiation of perishable commodities has been approved by the U.S. Food and Drug Administration and is now being used as a quarantine treatment against foreign insect pests (Hallman 1998). While the dosage required to kill an insect outright is often quite high, much lower doses are functionally efficacious by causing sterility in the adults or developmental arrest in immatures. Arrest and death may not be immediate, as in other kinds of disinfestation treatment, but will ensue at some later phase in the life cycle, typically at the larval-pupal or pupal-adult transformation (Grosch 1962).

It is a postulate of radiation biology that all significant levels of cell death from ionizing radiation results from damage to the DNA molecule (Von Sonntag 1987). A corollary of this postulate is that radiation-induced pathology is most typically manifest in tissues which are in rapid cell growth (Grosch 1962, 1973). Damage to the DNA molecule leads to chromosome breakage, and chromosome breakage and misrepairs interfere with mitotic division, thus inhibiting tissue growth. The affected organs appear atrophied relative to normal, non-irradiated tissues. Perhaps the best known example is the sterility principle wherein the gonads fail to develop fully (Walder and Calkins 1992), do not produce gametes (Steiner et al. 1962) or produce gametes containing lethal mutations (LaChance and Leverich 1962).

Specific anatomical lesions have also been identified in the somatic tissues. These

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are the lesions associated with developmental arrest. When the larval stages of holometabolous insects are exposed to ionizing radiation the ability to pupate is impaired. In the case of dipterans, there is evidence that the organs which produce or regulate the hormones which control pupariation are damaged. Bourgin et al. (1956) found that irradiation of late third instars of *Drosophila melanogaster* Meigen delays pupariation. By histological examination they were able to confirm radiation damage to the ring gland. The ring gland secretes ecdysone, the hormone that induces pupariation in the absence of juvenile hormone (Fraenkel et al. 1972). The function of the ring gland is regulated by the central nervous system, specifically by the supra-esophageal ganglion. Rahman et al. (1990) found that 20 Gy of gamma radiation applied to the eggs or first-instar larvae of *Ceratitis capitata* (Wiedemann) inhibits mitotic division of the cells in this ganglion. Because of this damage the ganglion was measurably atrophied in the third stadium compared to that in normal larvae.

Sivasubramanian et al. (1974) demonstrated that the timing of the application of radiation is critical. When larvae of *Sarcophaga bullata* Parker are irradiated earlier than about 36 h prior to pupariation, pupariation is inhibited. But, if the radiation is not applied until the last 36 h prior to pupariation a normal puparium is formed. They concluded that competence of the ring gland and central nervous system must be established by about 36 h prior to the end of the third larval stadium in this species, and thus, the capacity to pupariate is not affected by irradiation at this stage.

Although irradiation of the late third instar does not inhibit pupariation, adults do not emerge. Benschoter and Telich (1964) found that only 15 Gy applied to 10-d-old larvae of the Mexican fruit fly, *Anastrepha ludens* (Loew), causes 99.9968% mortality during the intra-puparial stage. Failure of adult eclosion following irradiation of late third instars has been reported in all tephritid fruit fly species tested (Balock et al. 1966, MacFarlane 1966, Jona and Arzone 1979, Bustos et al. 1993). Yet, while these studies demonstrate the utility of irradiation as a quarantine treatment, none have sought to identify the cause of death. To answer that question we subjected late third instars of *A. ludens* to gamma radiation at incremental doses. We then examined the morphological and physiological status of the intra-puparial stages for the purpose of determining the cause of developmental arrest.

Materials and Methods

All insects used in these experiments were obtained from the experimental colony maintained at the USDA-ARS laboratory at Weslaco, TX. The majority were reared on a standard fruit fly diet of rehydrated carrot powder and torula yeast (Spishakoff and Hernandez-Davila 1968) in a room maintained at 26.7 ± 2°C. The late third instars were removed from the diet and irradiated at age 10 d or held as controls. For electrophoretic analysis of protein profiles, larvae were reared in grapefruit. This step was taken to eliminate the confounding effects of the protein rich lab diet. Larval development is slower in grapefruit compared to lab diet, hence, these larvae were treated and/or analyzed at age 20 d.

Radiation treatments. Late third instars were subjected to discrete doses of gamma radiation in five replicates from different rearing batches. Radiation was applied with a ^{137}Cs source (Husman model 521A, Isomedix, Inc., Whippany, NJ) which was delivering a calculated centerline dose rate of about 0.38 Gy · sec^{-1} at the time of this research (see, Anon. 1998). The irradiator has a triangular array of ^{137}Cs source rods around the irradiation chamber. The insects were held in 0.5-liter paper

containers placed inside of a perforated, stainless steel cylinder (50 cm length, 11.4 cm inside diam, 2 mm thick wall). The cylinder enters the irradiation chamber moving parallel to the source rods and along their longitudinal axis via a rotating stainless steel, lead-lined drum until they are in the center of the array of rods. After the appropriate amount of time to deliver the desired dosage, the insects continue through the array until they emerge from the bottom of the irradiator. The target absorbed dosages were 6, 8, 10, 14, 20, 50, 75, 100, 150, and 200 Gy. Reference standard dosimetry was done with radiochromic film (Gafchromic MD-55, ISP Technologies, Inc., Wayne, NJ) read with a spectrophotometer (Milton Roy Spectronic 401, Ivyland, PA) at 510 nm. Confidence limits for delivered dosages was 95.6-108.8%. Thus, for a target dosage of 6 Gy actual dosage was 5.7-6.5 Gy.

An equal number of untreated larvae from these same rearing batches was retained as controls. Immediately following irradiation the larvae were returned to the laboratory and placed, along with the controls, in cups containing slightly moistened vermiculite and maintained in environmental chambers set to the same conditions described below for the phenology studies. Pupariation tended to ensue immediately except for certain irradiated larvae as discussed below. After 6 d the puparia were dissected to determine the morphological stage of the irradiated insects compared to the control insects. A total of 593 of these puparia was dissected.

Phenology of metamorphosis. Non-irradiated puparia were dissected in order to determine the normal course of intra-puparial development. At 10 d of age, larvae from the standard diet were placed in plastic cups containing slightly moistened vermiculite for pupariation. The cups were checked hourly for newly-pupariated individuals. Newly-formed puparia were removed to separate Petri dishes and labeled with the hour and date of pupariation, and placed in an environmental chamber at a constant temperature of 23 ± 2°C, 75 ± 5% RH, 12:12 DL. At selected increments of time following pupariation, puparia were dissected to determine the phenology of normal metamorphic development. All observations were made with a variable magnification dissecting microscope. A total of 219 puparia was dissected and the stage of development recorded in accordance with the terminology of Snodgrass (1924). Eclosion time was recorded for a total of 1,100 puparia.

Protein analysis. Samples (treatment and controls) were prepared by dissecting the insects from the puparia and grinding each individual in a TenBroeck tissue grinder in 0.5 ml of 0.002M Tris-$MgCl_2$ buffer, pH 7.5, plus 20 µl of 1 mM EDTA. The solution of ground insect was double centrifuged at 16,000 × g for 10 min at 4°C. The supernatant was decanted and 5 µl of bromophenol blue tracking dye (0.2%) added. A 50-µl portion of each decanted sample was combined with 50 µl of running buffer containing 5% SDS, 5% glycerol and 10% 2-mercaptoethanol and boiled for 5 min. Four µl of each sample, along with molecular weight standards, was then applied to separate lanes of a precast 12.5% SDS-polyacrylamide homogeneous gel and run for 75 volthours at 15°C (10 mA, 3 W, actual running time 25 min) on a Phastgel electrophoresis unit. Following electrophoresis, the gel was fixed with acetic acid and stained with Coomassie blue (Pharmacia Biotech 1990). Each lane was then scanned with a laser densitometer (LKB UltroScan XL, Pharmacia Biotech, Piscataway, NJ) for detection of polypeptide bands. The densitometer was linked to a computer with a gel scanning program, ImageMaster (Pharmacia Biotech), which automatically determined molecular weight by comparison with the standards, and quantity of material in each band as a proportion of total protein present.

Results

Metamorphosis. Metamorphic development in tephritids has been described by Snodgrass (1924) for *Rhagoletis pomonella* Walsh, and by Rabossi et al. (1992) for *Ceratitis capitata* (Wiedemann). The sequence of events in the Mexican fruit fly differs from those species in detail, mainly in the duration of the different stages. For example, metamorphic development in the Mediterranean fruit fly, from pupariation to adult eclosion, requires 310 h at 23°C, but 480 h in the Mexican fruit fly at the same temperature. The developmental sequence at constant 23°C is illustrated schematically in Fig. 1. It is notable that the true pupal stage occupies only about 100 h of the

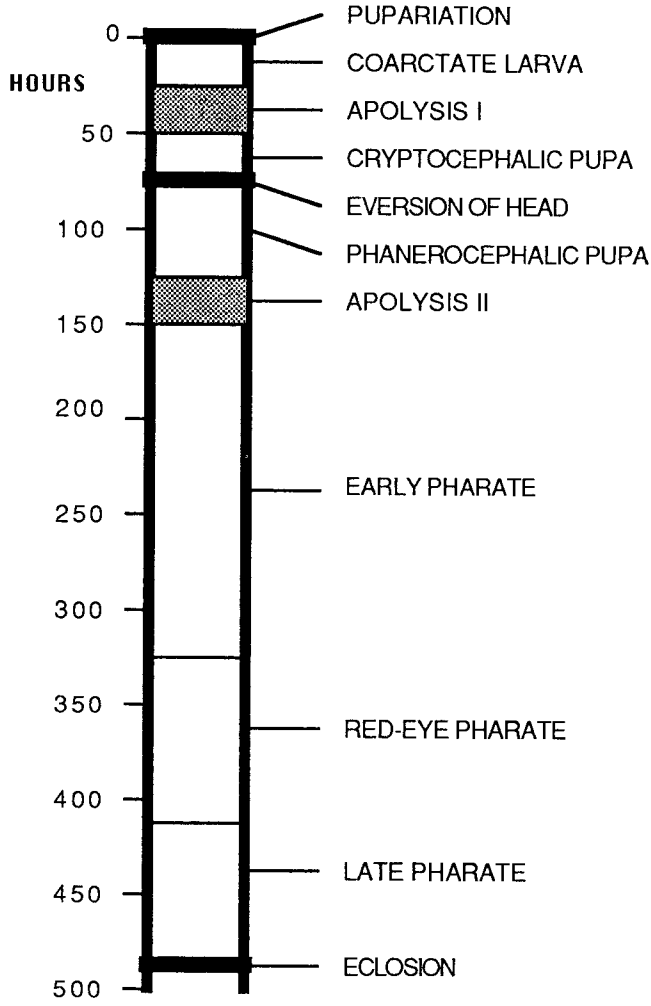

Fig. 1. Phenology of metamorphosis in the Mexican fruit fly at 23°C.

nearly 500 h that the insect is inside the puparium. Unlike the Caribbean fruit fly, *Anastrepha suspensa* (Loew), there is no difference in eclosion time between the males and females (Sivinski and Calkins 1990). At constant 23°C males emerged at a mean 483 h (n = 508) while females emerged at a mean 482 h (n = 592), post-pupariation.

At pupariation the third instar contracts into an oblong, oviform shape with the larval head (the two anteriormost segments) invaginated. The larval cuticle becomes sclerotized by irreversible deplasticization followed by melanization (Zdarek and Fraenkel 1972) over a period of 2 to 3 h. This stage is called a coarctate larva, also a prepupa (Whitten 1957). Puparia dissected at 17 h post-pupariation were still in this stage. Those dissected at 21 h post-pupariation had begun apolysis. Rabossi et al. (1992) also reported apolysis initiated in the medfly at 20.5 h post-pupariation.

Once the ecdysial membrane has formed, the pharate insect separates from the puparium by the infusion of ecdysial fluid between the two. The last step in apolysis I is the detachment of the larval pharyngeal armature. This highly sclerotized apparatus, the mouth hooks, remain within the inverted head. As late as 49 h post-pupariation some dissected pupae could not be removed without tearing of the cephalic tissues, indicating that the pharyngeal apparatus had not yet completely detached. Once the pharyngeal apparatus is detached the pharate insect can be dissected and removed from its puparium with minimal physical disruption. Separation of the pupal body from the puparium is never absolutely complete, as the pupal tracheae remain necessarily connected to the exterior via the larval pygidial spiracles. At this stage the body is differentiated externally into two tagmata, the abdomen and thorax. The pupal head is still invaginated. The precursors of the legs and wings are visible on the ventral side of the thorax at the anterior end of the insect. Dissected specimens had reached this phase as early as 44 h post-pupariation. This phase, called the cryptocephalic pupa, lasts for only about 24 h.

At 70 to 80 h post-pupariation the insect transforms from a cryptocephalic to a phanerocephalic pupa by vigorous muscular contractions which force fluid from the abdomen into the thorax and head, causing evagination of the head, inflation of the head, and extension of the legs and wings (Fraenkel 1938). The process, referred to as "pupal ecdysis" by some authors (e.g., Zdarek and Friedmann 1986), takes about 30 min. As a consequence of the eversion of the head the larval pharyngeal armature is disgorged from the cephalic invagination and displaced ventrally where it becomes adpressed to the wall of the puparium. The legs become fully extended with the tarsomeres reaching nearly to the apex of the abdomen. At this stage the pharate pupal insect resembles the adult externally in having three tagmata: the head, thorax, and abdomen.

Mortality and radiation dose. Consistent with the findings of Benschoter and Telich (1964) no adults emerged from treatment lots receiving doses of ≥20 Gy. Even at a level of 6 Gy, the lowest dosage tested, there was 74% non-eclosion. In all of the radiation treatment groups there were some larvae that did not pupariate. These individuals would live for several days before eventually dying, most likely from lack of sustenance. This phenomenon was not seen in the control groups. In mass rearing there is a 1 to 2 d spread in maturation, and evidently, these larvae had not yet been hormonally entrained to pupariate at the time of radiation treatment, in accordance with the observations of Sivasubramanian et al. (1974).

Developmental arrest. Puparia from irradiated larvae were dissected at 6 d post-pupariation. At 6 d 98% of the control insects had developed to at least the phanero-

cephalic pupal stage with the head fully inflated and the legs extended to their full length reaching the tip of the abdomen (Fig. 2a). The data in Table 1 show a weak trend of earlier developmental arrest with increasing radiation dose. With a dose of ≥20 Gy the insects never become pharate adults. However, even at the highest dose, 200 Gy, nearly 70% of the treated insects developed to the cryptocephalic pupal stage before arrest. At the lowest radiation doses tested (6-8 Gy), most of the treated insects developed successfully to the phanerocephalic stage and appeared to be morphologically normal.

At dosages of 10 Gy and above the majority of individuals failed to develop past the cryptocephalic stage. Most of these died with the head everted but not fully inflated (Fig. 2b). Snodgrass (1924) applied the term "microcephalic pupa" to describe this stage in development. Discoloration indicated that these insects were dead at the time of dissection, and not just in arrest at the time they were examined.

Protein metabolism. Yulo-Nazarea et al. (1991) found that irradiated larvae of the oriental fruit fly, *Dacus dorsalis* Hendel, fail to develop a protein that appears in normal larvae just prior to pupariation. Lescano et al. (1994) reported that non-irradiated pupae of the Queensland fruit fly, *Bactrocera tryoni* (Frogatt), develop a protein that is not detectable in irradiated pupae. However, the differences in the latter study, and likely the former, were associated with the fact that the irradiated insects were dead. We wished to compare irradiated but still living insects with the untreated controls in the pharate stage. For our comparisons we therefore used insects subjected to a marginally lethal dosage of 12 Gy.

We found no differences in comparisons of 5-d-old pupal insects, however, a significant difference was seen in the late pharate adult stage. In all higher Diptera the

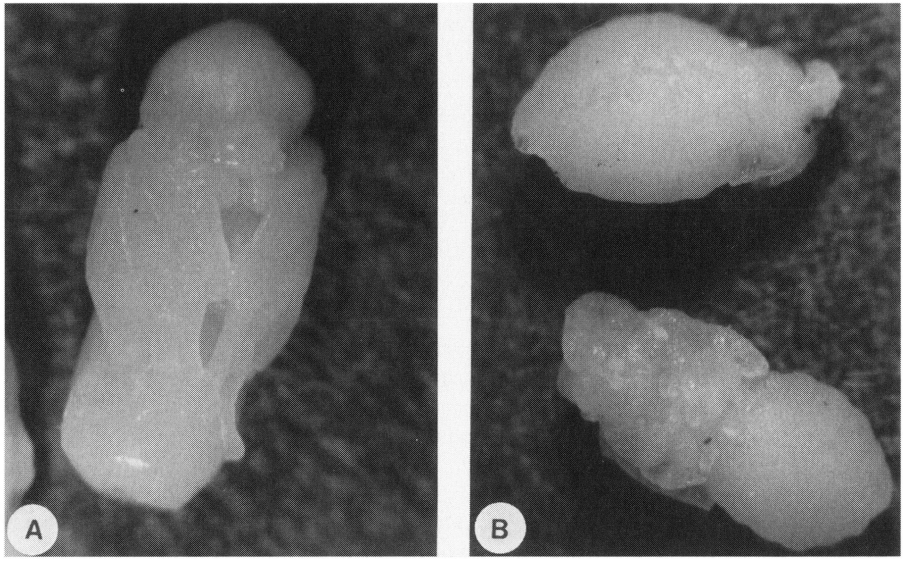

Fig. 2. (A) Normal phanerocephalic pupa. (B) Microcephalic pupae; the head has everted but not inflated.

Table 1. Stage of development achieved at 6 d post-pupariation following application of incremental doses of radiation to late third instar Mexican fruit fly larvae

Dosage (Grays)	Percent in stage			
	Coarctate	Cryptocephalic	Microcephalic	Phanerocephalic
0 (n = 57)	0	0	2	98
6 (n = 45)	0	0	11	89
8 (n = 55)	2	2	58	38
10 (n = 68)	4	1	60	34
14 (n = 123)	2	2	81	15
20 (n = 110)	1	5	93	1
50 (n = 25)	16	32	52	0
75 (n = 15)	0	87	13	0
100 (n = 50)	10	88	2	0
150 (n = 25)	1	24	0	0
200 (n = 35)	29	71	0	0

hemolymph of the third instar accumulates a hexameric protein called the larval storage protein (LSP) (Levenbook 1985). The LSP is anabolized during metamorphosis to provide material for the adult structures (Telfer and Kunkel 1991). It dominates the electrophoretic profile of the late third instar but is absent or diminished by the time the adult ecloses. In our SDS gels the LSP resolves into its constituent polypeptide subunits of approximately 80 kD (Fig. 3). Katsoris and Marmaras (1979) reported three polypeptide subunits in the LSP of *Ceratitis capitata.* Kefaliakou-Bourdopoulou et al. (1981) reported two polypeptide subunits in *Dacus oleae* (Gmelin). The latter condition is found in *Anastrepha ludens,* as shown in Figs. 3 and 4a,b & c. The twin peaks (9 & 10) in Figure 4a show the dominance of the two LSP polypeptides in the 10-d-old third instar. The two polypeptides have estimated molecular weights of 84.6 ± 0.8 and 79.4 ± 0.6 kD (mean ± SE, n = 26). Figure 4b shows the reduction of the putative LSP (peaks 5 & 6) 19 d later at the end of the puparial stage by which time the insect has become a pharate adult ready for eclosion. Yet, in Fig. 4c, the 19-d-old irradiated insect still has elevated amounts of LSP (peaks 7 & 8). In both the irradiated and control insects the eyes were red from the deposition of pteridine eye pigments. Pteridines are a byproduct of purine metabolism and their presence indicates that the insect, though irradiated, is still living and physiologically active. Fig. 3 shows the electrophoretic profile of these 19-d-old insects. For direct comparison in Fig. 3 lanes 5 and 6 are the 19-d-old controls showing greatly reduced amounts of the polypeptides which are the strongest bands (indicated by the arrow) in the irradiated insects (lanes 1-3). The LSPs are still present in elevated amounts indicating that the irradiated insects have failed to anabolize much of their available protein resource during development.

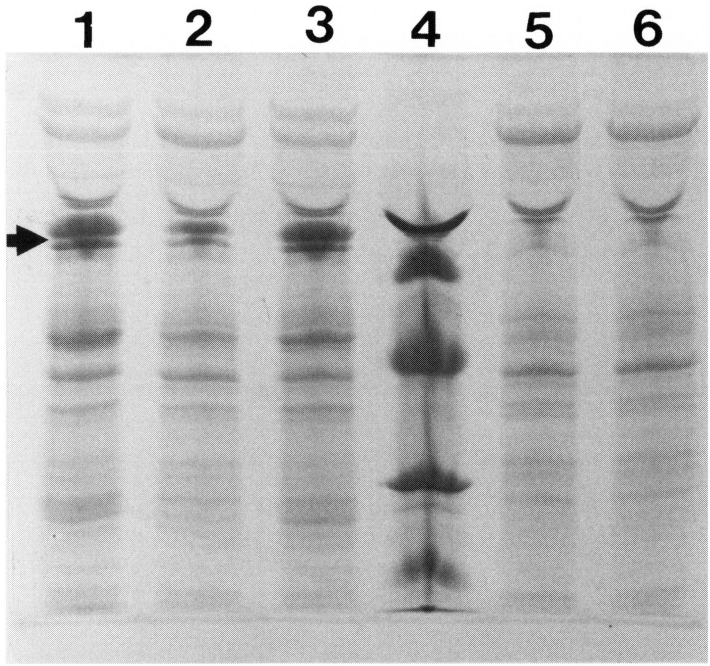

Fig. 3. Homogenous 12.5% SDS gel following electrophoresis and Coomassie blue staining showing polypeptide bands of individuals at red-eyed pharate adult stage. Lanes 1, 2, 3: irradiated 12 Gy; lane 4: molecular weight standards; lanes 5, 6: non-irradiated. Arrow indicates putative LSP polypeptides at 79-85 kilodaltons.

Discussion

Our investigations revealed that irradiated late third instars are able to pupariate but succumb during the transition from the cryptocephalic to the phanerocephalic pupal stage. The process of head eversion is a salient event in metamorphic development. Evagination and inflation of the head and expansion of the thoracic appendages is accomplished by a hydraulic increase in hemocoel pressure through peristaltic contractions of the abdominal muscles (Zdarek and Friedman 1986). Evidently, the absorption of ionizing radiation during the late larval stage weakens the pupa in some manner such that it is unable to sustain the vigorous muscular contractions necessary to evaginate and inflate the head, wings and legs.

The muscles which produce the peristaltic contractions during head eversion are the intersegmental abdominal muscles. Most, but not all, of the larval musculature degenerates and histolyses during the prepupal stage beginning immediately following pupariation, according to Crossley (1968). However, in the higher Diptera, unlike other holometabolous insects, the pupal abdomen consists of the larval epidermis without intervening mitoses (Whitten 1976). Along with the epidermis some of the musculature persists for the express purpose of accomplishing the head eversion.

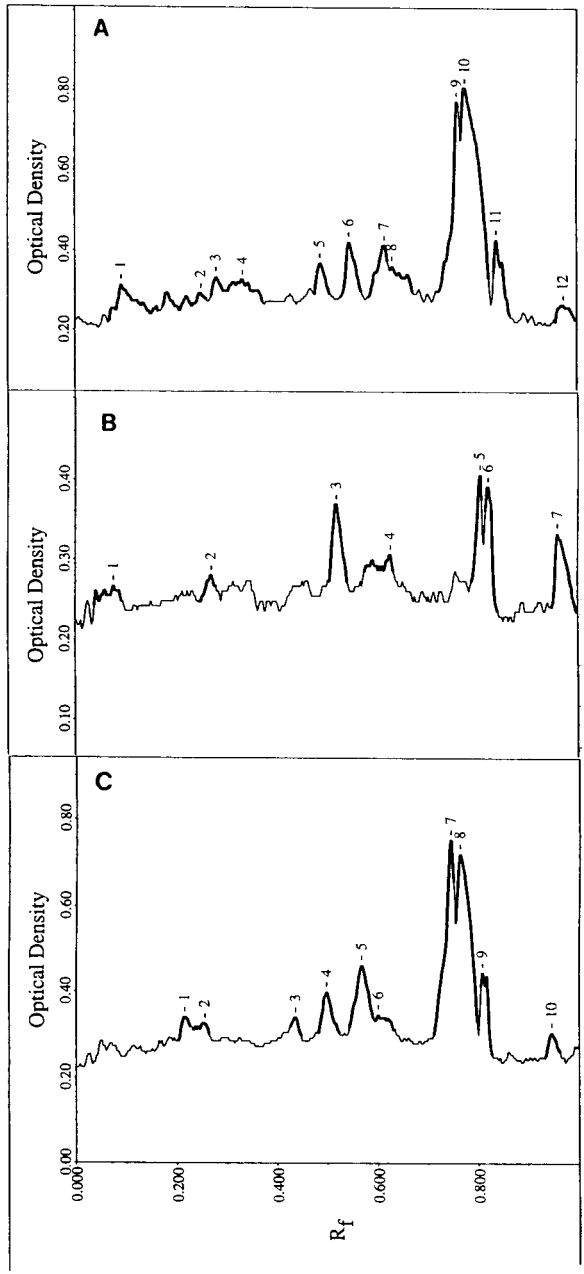

Fig. 4. Densitometric profile of electrophoretically separated polypeptides of pre-imaginal stages of Mexican fruit fly. (A) Normal third instar larva. (B) Non-irradiated pharate adult. (C) Pharate adult following irradiation in third instar. Scans B and C are from gel in Figure 3.

These so called "caducous" muscles are not histolysed but are transformed to imaginal muscles later in the pupal stage (Miyan 1989).

Radiation damage to the musculature has been reported in cases where the radiation was applied at sublethal doses to the late pupal and adult stages of Diptera. Radiosterilized flies, though viable, are often less competitive than non-irradiated flies (Hooper 1971) and the impairment has been attributed at least in part to lessened flight capacity (Sharp et al. 1975). Carney (1965) found that the wing muscle mitochondria were abnormal in their response to ATP in houseflies with radiation impaired flight ability (reduced wing beat frequency).

Our results do not accord well with the expectation that vital growing tissues are the most vulnerable to ionizing radiation (Grosch 1973). Ionizing radiation damages cells by the production of free oxidative radicals (Wallace 1998). It is widely held that the nucleus is the primary target of free radical damage and that cell death results at the divisional phase because of DNA strand breaks and misrepairs (Kaplan and Morgan 1998, Olive 1998). Thus, at the late third instar the most susceptible target for damage should have been the imaginal discs. However, Horikawa and Sugahara (1960), working with third instar *Drosophila melanogaster* Meigen, demonstrated with *in vitro* studies that the imaginal discs were actually an order of magnitude less susceptible to radiation than were the other tissues tested. This difference in radiosensitivity may be attributable to the phenomenon of apoptosis, a mechanism wherein damaged cells are selectively eliminated (Olive and Durand 1997). In our experiments the growth and differentiation of the imaginal discs into their corresponding adult structures was not detectably impeded, providing cogent evidence that the discs were not incapacitated even though the treatment dosages were much higher than the probit-9 lethal dose of 14 Gy reported by Hallman and Worley (1999).

There is no doubt that oxidative damage occurs in other parts of the cell besides the nucleus. Konings et al. (1979) found that cell membranes are susceptible to radiation induced peroxidation, and Edwards et al. (1984) report that cell microsomes are even more radiosensitive than nuclei or mitochondria.

A developmental arrest associated with a failure to inflate the pupal head and limbs, rather than the expected failure to differentiate a head and limbs, is more consistent with cytoplasmic dysfunction than it is with the nuclear damage principle. The retardation in the metabolism of the larval storage protein is likewise symptomatic of physiologic dysfunction and a general radiation sickness rather than a specific tissue atrophy. We suspect that the nuclear damage postulate, which holds well at the *in vitro* level, may not be completely applicable at the organismal level and that our results are an instance in which this is manifest.

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