Developmental Arrest in Mexican Fruit Fly (Diptera: Tephritidae) Irradiated in Grapefruit

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ABSTRACT When holometabolous insect larvae are exposed to a radiation treatment, morbidity or mortality are typically manifested during a major developmental transition, usually a transition involving ecdysis. Thus, early instars fail to develop into later instars or the later instars fail to pupate or pupariate. Over a range of sublethal doses of gamma radiation (increments of 0, 15, 20, 25, and 30 Gy) applied to third-instar Mexican fruit flies, *Anastrepha ludens* (Loew) (Diptera: Tephritidae), infesting or implanted in grapefruits, *Citrus paradisi* Macfayden, survival decreased with increasing dose. At all radiation doses, the majority of treated larvae arrested development at pupal ecdysis, the transformation from a cryptocephalic to a phanerocephalic pupa. More than 96% of treated larvae died at, or before, reaching this transition at the highest dose tested (30 Gy). Contrary to expectations, the radiation treatment did not cause atrophy of the imaginal tissues, a result that we attribute to apoptosis.

KEY WORDS irradiation, *Anastrepha ludens*, phytosanitation, apoptosis

Ionizing radiation is used to disinfest imported commodities potentially hosting invasive pest species. Tephritid fruit flies are an especially serious threat because the larval stages occur within fruit, and their detection on inspection is thus problematic. As a phytosanitary treatment, irradiation is especially useful for perishable commodities that do not withstand temperature or fumigation based treatments. One perceived disadvantage of irradiation is that acute mortality of the targeted pest does not result from this treatment. Rather, development is arrested such that irradiated larvae are incapable of becoming viable adults (Hallman 1999). The problem with delayed versus acute mortality is that any pests detected on inspection of the treated commodity are often still alive. According to the USDA Treatment Manual (APHIS–PPQ 2010) the "endpoint" of an irradiation treatment against a targeted pest is the inability to emerge or fly. A better understanding of the attributes associated with radiation morbidity could enhance our ability to assess the risk of failure associated with a given treatment protocol. At presently recommended quarantine level dosages, 50–150 Gy, fruit fly larvae irradiated as late third instars are capable of pupariating, but adults do not emerge from these puparia (Heather and Hallman 2008). In previous in vitro studies in which third instars of the Mexican fruit fly, *Anastrepha ludens* (Loew) (Diptera: Tephritidae), were exposed to incremental doses of radiation (Thomas and Hallman 2000), it was determined that development was arrested at the pupal ecdysis; the stage where the cryptocephalic pupa metamorphoses to a phanerocephalic pupa. This result was somewhat contrary to expectations. The primary damage induced by radiation is breakage of the chromosomes (Kaplan and Morgan 1998, Olive 1998). Chromosome breakage impairs cell division. Hence, tissues undergoing rapid or continuous cell division and growth are most affected by irradiation (Grosch 1973, Bakri et al. 2005), the classical example being atrophy of the gonads (Proverbs 1969, Walder and Calkins 1992). During the metamorphosis of higher dipterans most of the larval cells are autolyzed with key exceptions, the most notable of which are the imaginal discs, the epithelial cells that grow to form the tissues of the adult (Chapman 1982). Hence, any carryover of damage from the application to the larval stage should by all expectations involve the imaginal discs, and if that be true, the insect should fail to metamorphose to the imaginal stage (Grosch 1962). Yet, in our studies, arrest and eventual death ensued from the radiation, but only after, rather than before, development of the imaginal tissues. Because the imaginal organs (head, thorax, legs, and wings) had developed, but did not evaginate, we hypothesized that the observed impairment had

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resulted from damage to the tissues responsible for the evagination, specifically, the caducous musculature.

To further test this hypothesis, we obtained a data set under a different experimental regimen. A potential influence on the aforementioned results was that they were obtained in vitro, that is, with insects reared on an artificial diet and irradiated in an artificial medium. It is known that there are nutritional effects on fitness and sensitivity to radiosterilization (Langley and Abasa 1970, Rull et al. 2007). Also, fruit flies treated in vitro are often controlled with lower radiation doses than those treated in fruit (Hallman and Worley 1999, Follett and Armstrong 2004). Hence, in this test the radiation was applied to grapefruits infested with Mexican fruit fly larvae at a range of doses around the expected minimum lethal dosage. We also used simulated infestations, by implanting diet reared larvae into fruit, a technique used in some phytosanitary research (Hallman and Thomas 2010). We hypothesized that if the damage to the caducous musculature was not the critical lesion, then the point of developmental arrest might shift in response to the different experimental conditions.

Materials and Methods

The insects used in this study were obtained from a laboratory colony maintained at the USDA–ARS facility at Weslaco, TX, by using the methods of Spishakoff and Hernandez-Davila (1968). The colony has been in culture since 1997 with flies originating in Nuevo León, Mexico.

Irradiation Source and Dosimetry. The radiation machine (Husman model 521A, Isomedix, Inc., Whippany, NJ) uses gamma rays from ^{137}Cs in a sealed containment chamber located at the USDA–APHIS Mexican Fruit Fly Rearing Facility at Mission, TX. It emitted a dose rate of $\approx$40 Gy min^{-1}. Reference standard dosimetry was done in 1996 with the Fricke system and routine dosimetry was done with radiochromic film (Gafchromic MD-55, ISP Technologies, Inc., Wayne, NJ) placed in areas of the load (center and edges) with the most extreme dose readings. Dosimeters were read with a spectrophotometer (Spectronic 401, Milton Roy, Ivyland, PA) at 600 nm. The target dosages applied in these tests were 15, 20, 25, or 30 Gy. Depending on the position of the fruit within the housing cylinder, the actual absorbed doses varied from -3 to $+28\%$ around the target doses, with means $\approx$16% greater than target doses.

Fruit Infestation. Cohorts of 50 'Rio Red' grapefruit, *Citrus paradisi* Macfayden, harvested fresh from the USDA grove near Weslaco, TX, were placed in a screen cage (1.2 by 0.8 by 0.5 m) with $\approx$2,000 Mexican fruit fly adults for 1.5 h. Fruit were selected for uniformity in apparent ripeness and used within 24 h of harvest. Subsequent to exposure for oviposition, the fruit were held at 26.5 $\pm$ 0.5°C for 2 wk, sufficient time to allow all or most of the larvae to become late third instars, but before they began emerging from the fruit. Fruit were periodically opened to ensure that this stage had been reached at which time they were transported to the radiation facility. For each replicate, 25 fruit were irradiated and five fruit were not irradiated as controls.

For comparison, 30 fruit were artificially infested by implanting 25 third instars that had been reared on diet into the center of the fruit. The larvae were inserted into 10-mm-diameter holes bored to the center of the fruit, a plug from the bore was then replaced and the hole sealed with hot-melt glue. After 1 d at 26.5 $\pm$ 0.5°C, 25 of these implanted grapefruits were transported along with the infested grapefruits to the radiation facility. The five remaining grapefruits artificially infested in this manner were held as untreated controls. There were three to five replicates of each dose–infestation mode combination with total numbers of third instars tested per combination ranging from 585 to almost 3,800. An advantage of this method in which the larvae were implanted is that sample sizes were uniform. In contrast, sample size within infested fruit was uncontrolled and thus varied considerably.

Autopsy of Puparia. On the day after irradiation, all fruit were opened and the larvae placed in plastic containers (230 ml) with moist vermiculite for further development. When all adult emergence was completed in the controls, the remaining puparia were opened to determine the stage of development achieved. The stage of development was scored with the aid of a dissecting microscope in accordance with anatomical criteria defined by Snodgrass (1924) as modified by Rabossi et al. (1992). The objective of the autopsy was to assign each individual to one of the defined stages of metamorphic development. Within the puparium six successive intrapuparial stages were distinguishable.

1. The coarctate larva is the stage that immediately follows pupariation but precedes the larval/pupal ecdysis wherein the pupal integument once formed, separates from the puparium, except at the spiracles (Whitten 1957).
2. The cryptocephalic pupa is the succeeding stage of development following the larval/pupal apolysis but wherein only the thorax and abdomen are visible because the head is still invaginated. The legs and wings are visible but are not inflated beyond the thorax.
3. The phanerocephalic pupa is the next stage wherein the head of the pupa has completely evaginated and the appendages have attained their full extension. Zdarek and Friedman (1986) called this process the "pupal ecdysis," and it is triggered by ecdysone. Inflation is accomplished by peristaltic contractions of the caducal musculature of the abdomen, forcing hemolymph into the head and thorax.
4. The early pharate adult is the succeeding stage wherein the adult integument has developed and separated (apolysis) from the integument of the pupa. This separation is most easily observed at the extremities of the limbs.
5. The mid-pharate adult is distinguished by the deposition of pteridines in the eyes, whereas the early pharate adult eye is unpigmented.

Table 1. Incidence (mean percentage ± SD) of each developmental stage at arrest after irradiation of fruit implanted with larvae

	Control	15 Gy	20 Gy	25 Gy	30 Gy
Dead larvae	9.5 ± 2.7a	4.6 ± 0.8b	5.5 ± 0.1b	6.8 ± 1.8ab	6.2 ± 0.9b
Coarctate	1.6 ± 0.8a	2.0 ± 1.3a	2.6 ± 0.8a	4.1 ± 4.6a	1.9 ± 1.6a
Cryptocephalic	1.4 ± 0.5a	58.3 ± 19.8b	76.9 ± 1.4b	78.3 ± 3.2b	90.7 ± 2.6c
Phanerocephalic	2.0 ± 1.4a	2.2 ± 1.3a	3.3 ± 3.6a	2.8 ± 2.1a	1.0 ± 1.3a
Early pharate	1.4 ± 1.4a	1.1 ± 0.6a	0.8 ± 0.6a	0.6 ± 0.3a	0.1 ± 0.1a
Mid-pharate	0.7 ± 0.8a	0.7 ± 0.2a	0.4 ± 0.6a	0.6 ± 0.5a	0.1 ± 0.1a
Late pharate	0.6 ± 0.6a	3.2 ± 2.3b	4.2 ± 2.0b	3.1 ± 1.6b	0.1 ± 0.1a
Partly eclosed	2.4 ± 1.7a	11.2 ± 10.5a	3.7 ± 0.5a	2.1 ± 0.8a	0.0 ± 0.0a
Adults	80.4 ± 4.5a	16.4 ± 7.9b	4.4 ± 4.1c	1.4 ± 1.1c	0.0 ± 0.0c
Mortality	19.6	83.6	95.6	98.6	100

Means within a row followed by the same lowercase letter are not significantly different at $P = 0.05$.

6. The late pharate adult is distinguished by the presence of melanized setae on the body, whereas setae are not apparent in the mid-pharate adult. This is the stage targeted for radiosterilization in sterile insect technique programs because the adult tissues, with the exception of the gonads, have completed development.

Individuals found to have died in the transition of one stage to the next were scored as having achieved only the preceding stage. Linear regression was used to measure the degree of correlation between dosage and mortality. Statistical comparisons of the incidence of given stages at arrest between treatments and controls was made by pairwise *t*-test and one-way analysis of variance (ANOVA). Comparisons between treatment means at different dosages were made with two-way ANOVA. Abbott's correction (Abbott 1925) was used for calculating mortality. Probabilities of *t* and *F* were calculated with NCSS statistical software (NCSS, Kaysville, UT).

Results and Discussion

At all dosages tested, there was a significant difference in the survival of the radiation treatment groups compared with controls. Adult emergence did not exceed 16.4% even at the lowest dosage (Tables 1 and 2), whereas mean adult emergence in the controls was 91.5 ± 4.4 and 80.4 ± 4.5% for the infested and implanted methods, respectively. Although the larvae infesting fruit fared somewhat better than those implanted, among the nonirradiated controls the difference was not statistically significant ($F = 4.05$; df = 1, 6; $P = 0.09$). Mortality in the treatment groups was highly correlated to dosage ($r^2 = 0.769$, slope 0.998; $t = 2.45$, df = 6, $P = 0.025$). The difference in mean survival was statistically significant ($F = 79.4$; df = 3, 3; $P = 0.02$) among dosages but not between radiation treatments (5.62 ± 8.03% for the implanted larvae and 4.48 ± 6.53% for the infested larvae) ($F = 0.03$; df = 1, 3; $P = 0.88$). Because of its importance to the issue of quarantine efficacy, the mortality rates between the two infestation techniques is discussed in greater detail in a separate communication (Hallman and Thomas 2010). The morbidity associated with developmental arrest resulting from the applied radiation is reported here.

Some larvae never pupariated, and rarely some viable adults emerged from the irradiated puparia; these insects were scored, respectively, as dead larvae or live adults. More often irradiated insects reaching the adult stage died attempting to eclose from the puparium. Such adults were scored as "partly eclosed" if they managed to burst the puparium but did not fully emerge, or died before their wings inflated.

Regardless of the dosage of radiation received, or their nutritional history, development was arrested at the cryptocephalic stage in the majority of individuals of all irradiated groups, but not the controls. Although a considerable number of larvae died, failure to pupariate was not attributable to the radiation treatment because there was no difference between the mean percentage that died as larvae in the control groups, 5.44 ± 4.40%, and the treatment groups, 5.85 ± 1.60% ($F = 0.06$; df = 1, 14; $P = 0.80$). Also, within the

Table 2. Incidence (mean percentage ± SD) of each developmental stage at arrest after irradiation of fruit infested with larvae

	Control	15 Gy	20 Gy	25 Gy	30 Gy
Dead larvae	1.7 ± 0.6a	6.8 ± 3.7b	4.2 ± 4.0ab	6.0 ± 4.8ab	4.9 ± 6.1ab
Coarctate	2.1 ± 1.4a	3.1 ± 1.7a	2.2 ± 0.7a	10.2 ± 4.5b	4.8 ± 6.9ab
Cryptocephalic	2.4 ± 1.4a	64.7 ± 20.1b	85.8 ± 1.2bc	77.5 ± 10.1bc	80.8 ± 16.9bc
Phanerocephalic	0.4 ± 0.6a	5.0 ± 4.0b	2.7 ± 2.6ab	15 ± 1.5ab	2.2 ± 3.0ab
Early pharate	0.2 ± 0.4a	1.3 ± 1.8a	1.0 ± 1.4a	0.5 ± 0.7a	0.1 ± 0.2a
Mid-pharate	0.3 ± 0.2a	2.0 ± 3.1a	0.4 ± 0.4a	0.4 ± 0.5a	0.2 ± 0.3a
Late pharate	0.7 ± 0.8a	2.9 ± 2.9a	0.9 ± 0.8a	1.2 ± 0.8a	1.0 ± 1.5a
Partly eclosed	0.9 ± 0.9a	5.6 ± 4.5b	1.3 ± 1.9ab	1.5 ± 1.3ab	0.8 ± 1.4a
Adults	91.5 ± 4.4a	9.2 ± 10.8b	1.4 ± 1.9b	1.2 ± 1.8b	0.4 ± 1.0b
Mortality	8.5	90.8	98.6	98.8	99.6

Means within a row followed by the same lowercase letter are not significantly different at $P = 0.05$.

treatment groups, there was no significant difference in failure to pupariate between the implanted larvae, 5.42 ± 1.01%, and the infested larvae, 6.27 ± 2.11% ($F = 0.49$; df = 1, 3; $P = 0.53$).

The process of pupariation in higher Diptera is initiated by ecdysone and involves shrinkage and melanization of the cuticle of the third instar (Zdarek and Frankel 1972). Because no cell division is involved it is entirely within expectations that the process would not be impaired by the radiation treatments. Our results in which there was no significant impairment of pupariation would seem to be contrary to that of Nation et al. (1995) with *Anastrepha suspensa* (Loew), wherein it was reported that melanization activity is reduced even at low levels of irradiation and greatly decreased at doses ≥20 Gy. However, Nation et al. (1995) irradiated first instars in their experiments. A great deal of tissue growth ensues between the first instar and the pupariation competent third instar. And although most larval tissues are already differentiated embryonically, the cells of the brain and ring gland continue to divide throughout the larval stage, and the prothoracic gland, the source of ecdysone, is mitotically active during the first instar (Madhaven and Schneiderman 1977). The reason that the late third instar is the most resistant stage to radiation is attributable to it already having completed its tissue growth and cell divisions (Sivasubramanian et al. 1974). Thus, irradiation applied to the late third instar has no effect on melanization, and for that reason the phenoloxidase test for irradiation impairment as proposed by Nation et al. (1995) would be unreliable to that extent.

A small proportion of developmental arrest occurred at the coarctate larval stage. But again, there was no significant difference between mortality in the implanted (3.47 ± 1.52%) and infested fruit (5.47 ± 3.87%) ($F = 0.58$; df = 1, 3; $P = 0.50$). There was a numerical but statistically nonsignificant ($F = 3.35$; df = 1, 14; $P = 0.09$) difference between the control (2.07 ± 1.37%) and the treatment (4.47 ± 2.93%) group mortality manifest at this stage. The low mortality rate seen at the prepupal stage is most certainly not in accord with the chromosome breakage paradigm. The coarctate larval stage is a crucial phase in metamorphosis. It is in this stage, immediately after formation of the puparium, that the larval cells die and are replaced with the imaginal tissues by proliferation of the imaginal disc cells. It is only after the pupal epidermis is completed that the larval–pupal apolysis can ensue. The latter process involves release of the ecdysial fluid into the space between the remnants of the larval epidermis, which serves as the ecdysial membrane, and the new pupal epidermis. This results in separation of the "cryptocephalic" pupal stage from the wall of the puparium (Whitten 1957, 1976; Rabossi et al. 1992). That this is accomplished even at the lethal 30-Gy dosage of irradiation is contrary to the mitotic disruption and tissue atrophy paradigm but can be explained by the phenomenon of apoptosis. Because insects do not have antibody-based immune systems, they depend on gene silencing and apoptosis for protection against infectious pathogens (Lu et al. 2004, Clem 2005). Ionizing radiation is known to induce apoptosis in insect embryos (Zhou et al. 1999). Apoptosis is a programmed cell death that eliminates surplus cells through lysis and autophagy (Song and Steller 1999). Weinkove et al. (1999) credit apoptosis for the resistance of the imaginal discs to such perturbations, i.e., the population of undamaged cells replaces the damaged cells and the tissues develop unatrophied. Horikawa and Sugahara (1960) reported that the imaginal discs of *Drosophila* are an order of magnitude more resistant to radiation than are other tissues. Unless chromosome repair mechanisms are different between the gonads and imaginal discs, an unlikely scenario, a dosage effect would have been detectable as an increase in failure to pupariate or to metamorphose to the pupal stage. The fact that no such dosage effect occurred in our experiments allows us to infer that a much more effective repair mechanism such as apoptosis was at work. Regardless of the repair mechanism the relevant finding is that the imaginal tissues developed fully and thus lethality was from a different mode.

It is at the transition from cryptocephalic-to-phanerocephalic pupa that the effects of the radiation treatments become manifest. Depending largely on the radiation dosage and partly on whether the larvae were infested or implanted, the arrest of development at the cryptocephalic stage ranged from 57 to 86%, thus accounting for the majority of developmental endpoints in the treatment groups. The mean treatment mortality with Abbott's correction was 75.5 ± 11.2% whereas mean control mortality was 2.0 ± 1.2%. Moreover, the mortality rate was highly correlated with dosage ($r^2 = 0.92$). By contrast the difference in the mortality rate at the pharate adult stage between the controls (2.1 ± 2.2%) and the treatments (4.1 ± 3.5%) was not significant ($F = 1.99$; df = 1, 14; $P = 0.18$). Nair et al. (1967) and Sivasubramanian et al. (1970) attributed the failure of eclosion in house flies irradiated with 10–40 Gy during the puparial stages to muscular dysfunction. The effect was highly correlated to the age of the insects postpupariation at treatment and thus to the stage of tissue differentiation, the coarctate stage being the most susceptible. That situation clearly did not apply to our experiments because there was no difference in eclosion failure (pharate adults that died attempting to emerge from the puparium) between the controls (1.80 ± 1.57%) and treatments (3.02 ± 3.10%) ($F = 1.0$; df = 1, 14; $P = 0.33$) and was negatively correlated to dosage ($r = -0.79$). Thus, all significant treatment mortality was attributable to whatever lesions induced failure of the pupal ecdysis.

Although scored as a cryptocephalic pupa, the a priori-defined stage of development, many of the autopsied insects were actually "microcephalic," the term applied by Snodgrass (1924) to describe the transitional stage between the crypto- and phanerocephalic pupa. The transition requires a vigorous pumping of hemolymph from the abdomen into the head causing the latter to evaginate (Zdarek and Friedman 1986). The peristaltic contractions of the

abdomen are accomplished by the "caducous" musculature. The caducous muscles are holdovers from the intersegmental muscles of the larval abdomen (Crossley 1968, Miyan 1989). One might suppose that the heads failed to evaginate because they were incompletely developed (atrophied) because of insufficient cell division. But, if that were the case, one would have expected a strong or at least detectable dosage effect. Alternatively, if the evagination failure is in fact attributable specifically to damage to the caducous musculature, the weakening of these muscles must be from other than disruption of mitosis. Impaired flight capacity is often reported as a side effect in radiosterilized insects (Rull et al. 2007), and in that regard Carney (1965) and Bhakthan and Nair (1972) traced impairment to swelling of sarcosomes and subnormal mitochondrial activity in the flight muscles of irradiated house flies. More specifically, Sohal and Dubey (1993) found that the functional lesion was at the house fly muscle mitochondrial membrane and was due to attack by highly reactive oxidative radicals, i.e., radiation poisoning.

Insects in developmental arrest would eventually die of starvation. However, it should be noted that individuals arrested at the cryptocephalic or microcephalic stage died before pigmentation of the eyes and setae ensued. This suggests that death came sooner and was probably attributable to a general radiation poisoning with damage extending beyond dysfunction of the caducous muscles. In Thomas and Hallman (2000), we demonstrated a sharp reduction in storage protein metabolism during metamorphosis even in those pharate insects that survived the radiation treatments. The different nutritional regimes tested in the present experiments may have had a slight effect on the mortality rate, but there was no shift in the point of developmental arrest.

The metamorphosis of the higher Diptera is unique in that it occurs within a puparium with the pupal head invaginated. Also, unlike other holometabolous insects the pupa is a blend of imaginal and larval tissues. Hence, studies on a cyclorrhaphan species provide a unique demonstration of tissue specific pathological effects from irradiation, in this case failure of the caducous musculature (larval tissue) and the anterior tegmina (imaginal tissue). As in other radiation studies, our results provide a further example that tissue damage and capacity for repair is demonstrably stage specific and should be considered when applying irradiation as a control method.

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