Low oxygen atmosphere enhances post-irradiation survival of *Trichoplusia ni* (Lepidoptera: Noctuidae)

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Abstract

Phytosanitary irradiation (PI) is increasingly being used for disinfesting fresh commodities from insect pests of quarantine concern. The development of generic doses of irradiation that can be applied to broadly control all pests within a particular family, order, or even all insects across commodities and packaging types could facilitate greater use of PI. Many commodities are stored in controlled or modified atmospheres that are low in oxygen to preserve commodity quality and extend shelf life, but low-oxygen environments have been shown to affect radiotolerance in some insects and more work is needed to understand the impact of this oxygen-effect on radiotolerance for both the development of generic doses and to increase the acceptance of irradiation as a treatment by the fresh commodity industry. Here we show that irradiation of cabbage looper, *Trichoplusia ni* (Hübner) (Lepidoptera: Noctuidae), in anoxic atmospheres (0 kPa aPO_2) increases radiotolerance compared to irradiation in normoxic atmospheres (21 kPa aPO_2) last instar larvae, true pupae and mature pupae (pharate adults) when considering the emergence of healthy-looking, viable adults as the critical metric. Of the 3 stages irradiated, the last instar larvae were most susceptible to irradiation, followed by pupae, with pharate adults 24–48 h prior to emergence (sometimes referred to as late pupae) being the most tolerant stage. When pharate adults were irradiated in anoxia, healthy-looking adults emerged at absorbed doses of 784–789 Gy whereas no healthy-looking adults emerged at the same doses in normoxia. Effects of anoxia on reproduction of irradiated female pharate adults were subtle. A few F_1 larvae hatched at doses estimated to be 585–591 Gy, suggesting that more work is needed to determine whether 400 Gy is an adequate generic dose to control late pupae of *T. ni*.

Key Words: cross-tolerance; fertility; oxygen-effect; phytosanitary irradiation; radiotolerance

Resumen

La irradiación fitosanitaria (IF) es una táctica en desarrollo utilizada para desinfestar la mercancía fresca para plagas de cuarentena. De gran uso para IF es el desarrollo de una dosis genérica de irradiación la cual pueda ser aplicada ampliamente para el control de plagas en una familia, orden, o para todos los insectos indiferentemente de la mercancía o el tipo de embalaje. Mucha mercancía es almacenada en atmosferas controladas o modificadas las cuales utilizan bajo oxígeno para preservar la calidad de la mercancía, pero medio ambientes de bajo oxigeno tienen la capacidad de afectar la radio tolerancia de algunos insectos y más investigación es necesaria para entender el impacto del efecto de oxígeno en la radio tolerancia ante el desarrollo de dosis genéricas. Aquí demostramos que la irradiación bajo condiciones de anoxia (0 kPa aPO_2) de larvas de ultimo instar, pupas verdaderas y pupas en avanzado estado de desarrollo (adultos farados) del gusano medidor de la col, *Trichoplusia ni* (Hübner) (Lepidoptera: Noctuidae), aumenta substancialmente la radiotolerancia comparado con gusanos irradiados en condiciones de normoxia (21 kPa aPO_2) cuando se considera la aparición de adultos con apariencia saludable como la medida critica. De las tres etapas de desarrollo que fueron irradiadas las larvas de ultimo instar fueron las más susceptibles, seguidas por las pupas verdaderas. Los adultos farados (pupas en el último estado de desarrollo cuando las estructuras del adulto son visibles a través del tegumento) fueron los más tolerantes. Adultos con apariencia normal emergieron en doses de 784–789 Gy cuando fueron irradiados en anoxia pero no hubo ninguna aparición de adultos irradiados en esta dosis en normoxia. El efecto de irradiación en anoxia en las hembras fue bien sutil. Pocas F_1 larvas aparecieron en dosis de 585–591 Gy, lo cual sugiere que más investigación es necesario para determinar si 400 Gy es un dosis genérica adecuada para el control de *T. ni*.

Palabras Clave: tolerancia cruzada; fertilidad; efecto de oxígeno; irradiación fitosanitaria; radiotolerance

Phytosanitary Irradiation (PI) can prevent the spread of invasive pest insects in agricultural products and is a very promising tool for facilitating regional and international movement of agricultural commodities (Hallman et al. 2010). PI has several advantages over methyl bromide or other pesticide treatments that are restricted in some export markets. Chiefly, these advantages are that PI is easily applied to commodities after packaging and there is low possibility for resistance evolving due to the mode of action damaging DNA and inducing insect sterility (Heather & Hallman 2008).

A current barrier to the widespread implementation of PI is the development of generic radiation doses that would effectively kill or sterilize all pests across commodities regardless of commodity packaging. A generic dose of 150 Gy has been approved for larvae of all species of Tephritid fruit flies by both the United States Department of Agricul-

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ture Animal and Plant Health Inspection Service (USDA-APHIS) and the International Plant Protection Convention (IPPC 2011). In addition, 400 Gy has been approved by USDA-APHIS as a generic dose that can be applied to all insects except Lepidoptera pupae and adults (Hallman et al. 2013). Development of additional generic doses < 400 Gy will contribute greatly to preserving commodity quality, while helping to prevent irradiators with high dose uniformity ratios (DUR) from exceeding the current limit of 1,000 Gy for PI implemented by both the United States Food and Drug Administration (FDA) and the Food Standards Commission of Australia and New Zealand (APHIS 2006; Follett 2009; Hallman 2011; FSANZ 2015). However, developing additional generic doses will require validating generic doses in the context of all of the factors that a commodity, and thus the insects within it, may experience.

Hallman et al. (2010) proposed 5 main factors that could affect the efficacy of PI: low oxygen, developmental stage, host, dose rate, and temperature. Many commodities are stored and shipped in either low-oxygen controlled atmospheres or modified atmosphere packaging that results in a low-oxygen environment to preserve commodity quality and extend shelf life (Yahia 1998; Toivonen et al. 2009). Considering that many fresh commodities are shipped long distances before reaching the point of final sale, the industry demands that phytosanitary treatments are compatible with the use of modified atmosphere packaging and other methods for maintaining product quality and shelf life. A further advantage of packing commodities into modified atmosphere bags for producers is that these bags also prevent further infestation of fresh commodities after a phytosanitary treatment is applied. Despite the many potential benefits of modified atmosphere packaging for commodity quality, irradiation of insects in very low oxygen environments (~1–2 kPa atmospheric partial pressure of oxygen—aPO_2) or completely anoxic environments (0 kPa aPO_2) has been shown to increase radiotolerance of insects in dozens of studies, both in the context of PI and in the context of the Sterile Insect Technique (SIT) (rev. by Hallman et al. 2010; López-Martínez & Hahn 2012; López-Martínez & Hahn 2014). Yet, many commodities are packaged in modified atmospheres with only moderate levels of hypoxia (3–10 kPa aPO_2 relative to normoxia 21 kPa aPO_2; Yahia 1998; Toivonen et al. 2009). Uncertainly about the levels of oxygen needed to affect insect radiotolerance prompted USDA-APHIS-PPQ to enact a temporary policy wherein before and during PI the use of Modified Atmosphere Packaging (MAP) creating atmospheres less than 18 kPa aPO_2 surrounding fresh fruits, vegetables and cut flowers is prohibited. Considering the benefits of modified and controlled atmospheres for product quality and downstream pest exclusion along with the desire of the fresh commodity industry to use modified atmosphere packaging, it is necessary to determine the degree to which modified and controlled atmospheres may alter the efficacy of generic doses for PI.

In the context of PI, most studies of the effects of low-oxygen atmospheres on radiotolerance focus on whether irradiated juvenile stages can emerge as healthy-looking adults, a commonly accepted metric for successful phytosanitary treatment (Hallman et al. 2010). Studies done in the context of SIT as well as PI, however, have shown that other aspects of adult performance including dispersal ability, adult longevity, and mating competitiveness are affected by irradiation in low-oxygen environments. Even fecundity and fertility can be increased by irradiation in anoxic or near anoxic atmospheres compared to insects irradiated in normoxia at the same dose (Hallman 2004, 2005; Calkins & Parker 2005; Mutika & Parker 2006; Hallman & Phillips 2008; Hallman & Hellmich 2009; López-Martínez & Hahn 2014; López-Martínez et al. 2014). While adult sterility can be an acceptable outcome for PI because it prevents pest establishment, the interception of healthy-looking live adults at ports or the detection of healthy-looking adults caught in routine monitoring traps can reduce the perceived efficacy of PI as a treatment (Hallman et al. 2013). More information about the degree to which irradiation of pests in low-oxygen environments consistent with those commonly used in modified or controlled-atmosphere commodity storage affect post-irradiation pest survival and reproduction are clearly needed.

Here we test whether exposure to an anoxic controlled atmosphere (i.e., 100% nitrogen) prior to and during irradiation affects survival to adulthood and fecundity of a common, foliar-feeding lepidopteran pest, *Trichoplusia ni* (Hübner) (Lepidoptera: Noctuidae). We chose anoxia because it represents the most extreme low oxygen atmosphere a pest could be exposed to during treatment or storage. To determine the most tolerant stage and whether anoxia affected survival to adulthood, we exposed 3 immature stages, last-instar larvae, pupae, and pharate adults of *T. ni* to a series of irradiation doses in normoxia or anoxia. In a 2nd experiment, we exposed the most radiotolerant immature stage, pharate adults, to a series of greater doses to determine whether irradiation in anoxia increases adult emergence and reproduction. Last, we exposed pharate adults to very high doses of ionizing radiation to determine whether adult emergence or reproduction could be prevented completely. Our goals for these studies were to confirm the most tolerant stage in *T. ni* and to estimate a range of doses at which irradiation in anoxia had effects on emergence of normal-looking adults as well as fecundity and fertility. It was not our goal to specifically define a minimum dose for control of any particular life stage.

Materials and Methods

REARING *TRICHOPLUSIA NI* CABBAGE LOOPERS

Cabbage loopers were taken from a colony reared at the United States Department of Agriculture's Agricultural Research Service's Center for Medical, Agricultural and Veterinary Entomology (USDA-ARS-CMAVE) in Gainesville, Florida. Eggs, larvae, and pupae were maintained in 500 mL cups on a pinto-bean artificial diet (Guy et al. 1985) in an incubator (Percival Scientific, Perry, Iowa, USA) at 24 °C and 75% RH under long d conditions (14 L:10 D). After treatment and prior to emergence, larvae and pupae were transferred to 2 L plastic bucket cages or 500 mL plastic cups and kept in a temperature (25 °C) and humidity (60% RH) controlled rearing room. Upon emergence, adults were supplied a 2% sugar and 2.25% honey solution ad libitum.

IRRADIATION TREATMENTS

Last instar larvae, pupae (3-4 d after pupal formation, before pharate adult apolysis), and pharate adult moths late in their development at approximately 1-2 d prior to adult emergence (sometimes referred to as late pupae) were irradiated using a Gammacell 1000 ^{137}Cs irradiator (GC45, Ottawa, Ontario, Canada) at a dose rate of 8 Gy/min at the Florida Accelerator Services and Technology facility within the Division of Plant Industry of Florida Department of Agriculture and Consumer Services (FDACS) in Gainesville, Florida. Immature (larvae or early/late pupae) moths were contained in polypropylene bags and placed in the center of the irradiation cylinder to ensure dose uniformity. To compare our target dose with the dose delivered, we used Gafchromic HD-810 film (International Specialty Products, Wayne, New Jersey, USA; uncertainty 5.6% at 95% confidence level) by placing 2 dosimeters per bag (top and bottom). Dosimeters were read 24 h after irradiation at 600 nm on a FWT-100 radiachromic reader with a computer controlled densitometer (Far West Technology, Inc., California, USA). The radiachromic reader was calibrated against film irradiated at a National Institute of Standards and Technology facility (NIST, Gaithersburg, Maryland, USA) between 50 Gy–400 Gy. Target doses and delivered doses were recorded for treatments within each experiment with doses up to 400 Gy (Table 1a). Actual delivered doses were within 10% of target

Table 1. Target doses and actual received doses up to 400 Gy as estimated with Gafchromic HD810 film, all estimated values ± 5.6% uncertainty (**A**). Target doses and estimated received doses up to 800 Gy were determined by alanine pellet dosimetry, and all estimated values have an uncertainty of ± 3.5% (**B**).

1A. Film Dosimetry—Target and absorbed doses

Target Dose	D_{max}	D_{min}	D_{max}/D_{min}
0 Gy	0	0	–
50 Gy	56	53	1.06
100 Gy	105	100	1.05
150 Gy	180	177	1.02
200 Gy	215	212	1.01
300 Gy	301	300	1.00
400 Gy	411	411	1.00

1B. Alanine Dosimetry—Target and absorbed doses

Target Dose	D_{max}	D_{min}	D_{max}/D_{min}
0 Gy	0	0	–
200 Gy	195	192	1.02
400 Gy	392	387	1.01
600 Gy	591	585	1.01
800 Gy	789	784	1.01

doses for all treatments with the exception of 150 Gy where the delivered dose ranged between 177–180 Gy across all replicates. Because our film dosimetry system was not calibrated for doses above 400 Gy, we were not able to quantify the absorbed doses for our 600 and 800 Gy target doses at the time of treatment. However, after the treatments were completed we gained access to an alanine-pellet dosimetry system that we used to estimate the relationship between the target doses and delivered doses up to 800 Gy (Table 1b). Alanine pellet dosimeters were purchased from Far West Technology, (#FWT-50-10, Lot T030901; uncertainty 3.5% at 95% confidence level). The dosimetry system (Bruker BioSpin E-scan, Billerica, Massachusetts, USA) used to read the pellets was calibrated against alanine pellet dosimeters irradiated between 50–1,000 Gy at a NIST facility (Gaithersburg, Maryland, USA). Although we were not able to quantify the actual doses delivered to pharate adult cabbage loopers in the 2 experiments where we used 600 and 800 Gy, we expect that the alanine dosimetry done after the experiments were completed is representative of the absorbed experimental doses. We do note that the alanine dosimetry estimates are slightly lower for each target dose than our film dosimetry, so all absorbed doses reported in the test up to 400 Gy are taken from the film estimates in order to be the most guarded.

Irradiation was done in 1 of 2 atmospheres, i.e., in the presence of oxygen (nx = normoxia) and the in the absence of oxygen (ax = anoxia). Anoxia was our controlled atmosphere treatment known to induce radiotolerance in flies and moths (Curtis & Langley 1972; Zumreoglu et al. 1979; Calkins & Parker 2005; Nestel et al. 2007; López-Martínez & Hahn 2012; López-Martínez et al. 2014). Radiation treatments for our experiments consisted of the following target doses (exposure times): 50 Gy (6 min and 8 s), 100 Gy (12 min and 16 s), 150 Gy (18 min and 24 s), 200 Gy (24 min and 32 s), 300 Gy (36 min and 48 s), 400 Gy (49 min and 4 s), 600 Gy (73 min and 36 s), and 800 Gy (98 min and 8 s). We chose this wide array of doses in hope of encompassing the range that includes 100% male and female sterility and 100% mortality. The 50, 100 and 150 Gy series was designed to test the radiotolerance of each likely stage present in commodities. The 200, 300, and 400 Gy series was chosen because 400 Gy represents the generic PI dose that has been largely accepted by USDA for all insects except Lepidoptera pupae and adults (Hallman et al. 2013). We recognize that 600 and 800 Gy target doses are outside of the target dose range suggested for some commodities because it approaches the 1,000 Gy maximum limit of absorbed doses under commercial conditions proposed for PI in fresh food by the FDA, especially for irradiation facilities without high dose uniformity ratios. However, our previous work shows that the protective benefits of low-oxygen hormesis are stronger at greater doses (López-Martínez & Hahn 2012) and we decided to extend our work to these greater doses after observing substantial adult emergence at a target dose of 400 Gy in some treatments. Having confirmed that pharate adults were the most tolerant stage (Fig. 1), we used only this stage in experiments with target doses above 150 Gy.

Our modified atmosphere treatment, 1 hr of anoxia in pure nitrogen followed by irradiation in nitrogen, was achieved by heat-sealing insects in a polypropylene bag that had been flushed with nitrogen for 2 min. We have successfully applied this treatment multiple times in the past (López-Martínez & Hahn 2012; López-Martínez & Hahn 2014; López-Martínez et al. 2014) and chose it for our experiments here because it represents the most extreme low-oxygen environment. The bag containing the moths was placed in a 2nd bag that was also nitrogen flushed and heat sealed. Moths in the normoxia treatments were sealed in identical bags that had been perforated to allow air exchange. Because the greater doses of irradiation would produce insects that had been exposed to longer bouts of anoxia, we adjusted our exposures to anoxia for all anoxia treatments to be as long as the longest anoxia exposure within an experiment. For example, the 800 Gy group spends 2.63 h in anoxia (1 h of preconditioning followed by 1.63 h of irradiation), thus the 0 and 600 Gy groups in that experiment had their anoxia treatment times adjusted to 2.63 h in anoxia. For experiments with highest target doses of 150 and 400 Gy, the adjusted anoxia times were 1 h and 18 min and 1 h and 49 min, respectively, for all doses within that experiment.

EMERGENCE ASSAYS

Groups of 10 to 15 caterpillars, pupae, or pharate adults were sealed in bags and irradiated. After treatment, individuals were allowed to recover and complete development in 2 L plastic bucket cages. A week after treatment, emergence or death was assessed for each treatment. Moths were counted as successfully emerged if they were able to exit the pupal skin successfully and their wings were completely and correctly formed. Hereafter these moths are referred to as "normal" in appearance because their physical appearance was like that of unirradiated control moths (Fig. 1A, C). Moths that were not able to exit the pupal skin were counted as dead. However, we also noticed a third class of moths that successfully emerged from the pupal skin, but were clearly abnormal, typically having malformed wings and lighter colored scales. Hereafter these moths are referred to as "abnormal" (Fig. 1B). We tracked this 2nd category of moths because moths with malformed wings can still be alive and present for d on shipping containers and produce. Thus, we estimated emergence in 2 ways. First, we show data counting only those moths classified as "normal" as *emerged* and then we consider "total emergence" as the sum of both "normal" and "abnormal" moths that had eclosed. Each treatment was replicated 2 or 3 times within each experiment depending on moth availability. All experiments were repeated at least 2 times using different cohorts of moths. The 0, 600 and 800 Gy experiments were repeated 3 times.

FECUNDITY AND FERTILITY ASSAYS

Because pharate adults are the most radiation-tolerant stage, they were used to assess effects of irradiation in anoxia on reproduction. Irradiation was done 1–2 d before adult emergence was expected to occur. Fecundity (number of eggs laid) and fertility (percent of eggs hatched) were assessed by placing each individual pair of moths in a 500 mL plastic

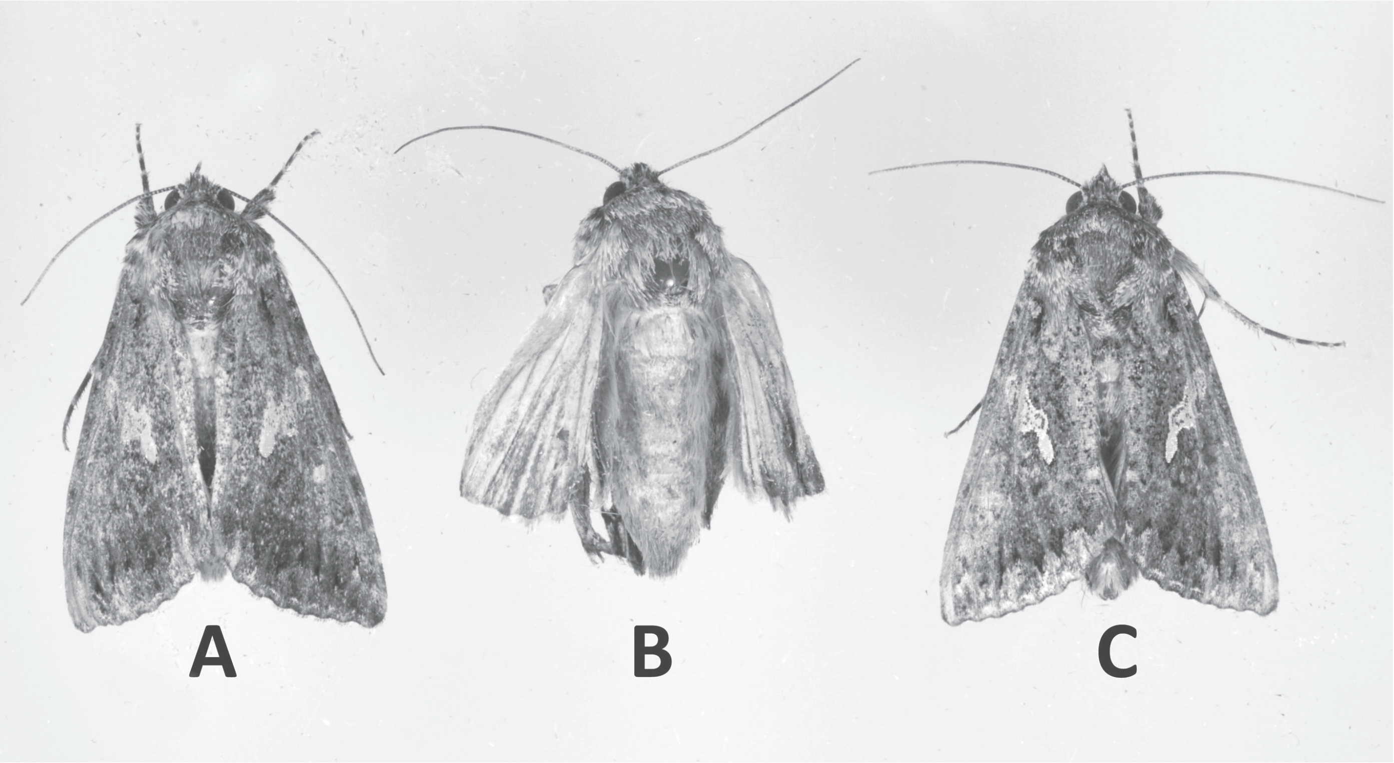

Fig. 1. Unirradiated *Trichoplusia ni* adults (A) and those that were irradiated in normoxia at 600 Gy as pharate adults (B) had distinct morphological phenotypes, i.e., the former normal-appearing in appearance and the latter abnormal-appearing. We saw substantial prevention of the induction of the irradiation-damaged phenotype when the pharate adults were given an anoxia pre-treatment and irradiated in anoxia at 600 Gy (C). Photograph courtesy of Lyle Buss.

cup fitted inside with a brown-paper coffee filter lining as an oviposition substrate. Each irradiated individual was paired with a non-irradiated individual of the opposite sex. Each of the cups was inverted over another cup to provide the sugar:honey food solution through a wick connecting both cups. The cups were placed in a temperature (25 °C) and humidity (60% RH) controlled room. Preliminary experiments showed that moths from this colony completed oviposition after 6 d of adulthood. Therefore, 7 d after treatment, adults and the food wick were removed from the cups and the eggs laid on the coffee filters were transferred to an incubator (Percival Scientific, Perry, Iowa, USA). Eggs were then kept for 7 additional d under long day conditions (14 L:10 D) under 24 °C and 85% RH to assess fertility by egg hatching.

STATISTICAL ANALYSES

Each of the emergence/survival experiment was replicated 2 or 3 times within a cohort of moths and was performed at least 2 separate times using different cohorts of moths. The fecundity and fertility experiments were also repeated and replicated the same way. Data were analyzed using 2-way ANOVAs with radiation dose and atmospheric treatment as main effects, followed by Tukey's HSD or linear contrasts to compare groups of interest in JMP 9.1 (SAS Institute, Cary, North Carolina, USA).

Results

EMERGENCE EXPERIMENTS

During the late larval stage, anoxia had a substantial effect on emergence of normal appearing adults across irradiation doses ($F_{7,24} = 38.4$; $P_{\text{oxia treatment}} < 0.0001$, $P_{\text{dose}} < 0.0001$, $P_{\text{oxia treatment x dose}} = 0.0002$; Fig. 2A). When late larvae were irradiated in anoxia with 50 Gy (53–57 Gy absorbed), adult emergence was not different from unirradiated controls (~72% emerged), whereas only 24% of adults successfully emerged when late larvae were irradiated with 50 Gy in normoxia. Similarly, when considering total adult emergence—emergence of both normal- and abnormal-appearing adults—few did so when late larvae were irradiated in normoxia with 100 Gy (100–106 Gy range of absorbed doses), and none emerged after irradiation with our 150 Gy target dose (177–180 Gy range of absorbed doses). When late larvae were placed in an anoxic environment prior to irradiation with both target doses, 100 Gy and 150 Gy ($F_{7,24} = 29.0$; $P_{\text{oxia treatment}} < 0.0001$, $P_{\text{dose}} < 0.0001$, $P_{\text{oxia treatment x dose}} < 0.0001$; Fig. 2B), a greater percent of total adult emergence occurred.

The adult emergence patterns following the irradiation of pupae were very similar to those described above following the irradiation of late larvae wherein a greater percent of normal-appearing adults emerged from anoxia-irradiated pupae than from their normoxia-irradiated counterparts ($F_{7,24} = 30.0$; $P_{\text{oxia treatment}} < 0.0001$, $P_{\text{dose}} < 0.0001$, $P_{\text{oxia treatment x dose}} = 0.0003$; Fig. 2C). Anoxia had no effect on the total number of both normal and abnormal appearing adults that emerged from true pupae irradiated with the lowest dose (50 Gy), but it did in the case of those irradiated with the 100 Gy and 150 Gy (177–180 Gy absorbed) doses ($F_{7,24} = 38.4$; $P_{\text{oxia treatment}} < 0.0001$, $P_{\text{dose}} < 0.0001$, $P_{\text{oxia treatment x dose}} < 0.0001$; Fig. 2D).

For pharate adults, irradiation in anoxia had only a marginally detectable effect, manifested in some reduction of emergence of normal-looking adults at the 100 Gy target and 150 Gy target (177–180 Gy absorbed) doses ($F_{7,16} = 3.0$; $P_{\text{oxia treatment}} = 0.0544$, $P_{\text{dose}} = 0.0549$, $P_{\text{oxia treatment x dose}} = 0.0998$; Fig. 2E). There was no detectable effect of anoxia on the total emergence of normal- and abnormal-appearing adults when doses up to 177–180 Gy were applied to pharate adults ($F_{7,16} = 0.43$; $P_{\text{oxia treatment}} = 0.8089$, $P_{\text{dose}} = 0.5967$, $P_{\text{oxia treatment x dose}} = 0.7944$; Fig. 2F). Thus we confirmed pharate adults as the most radiation-tolerant stage.

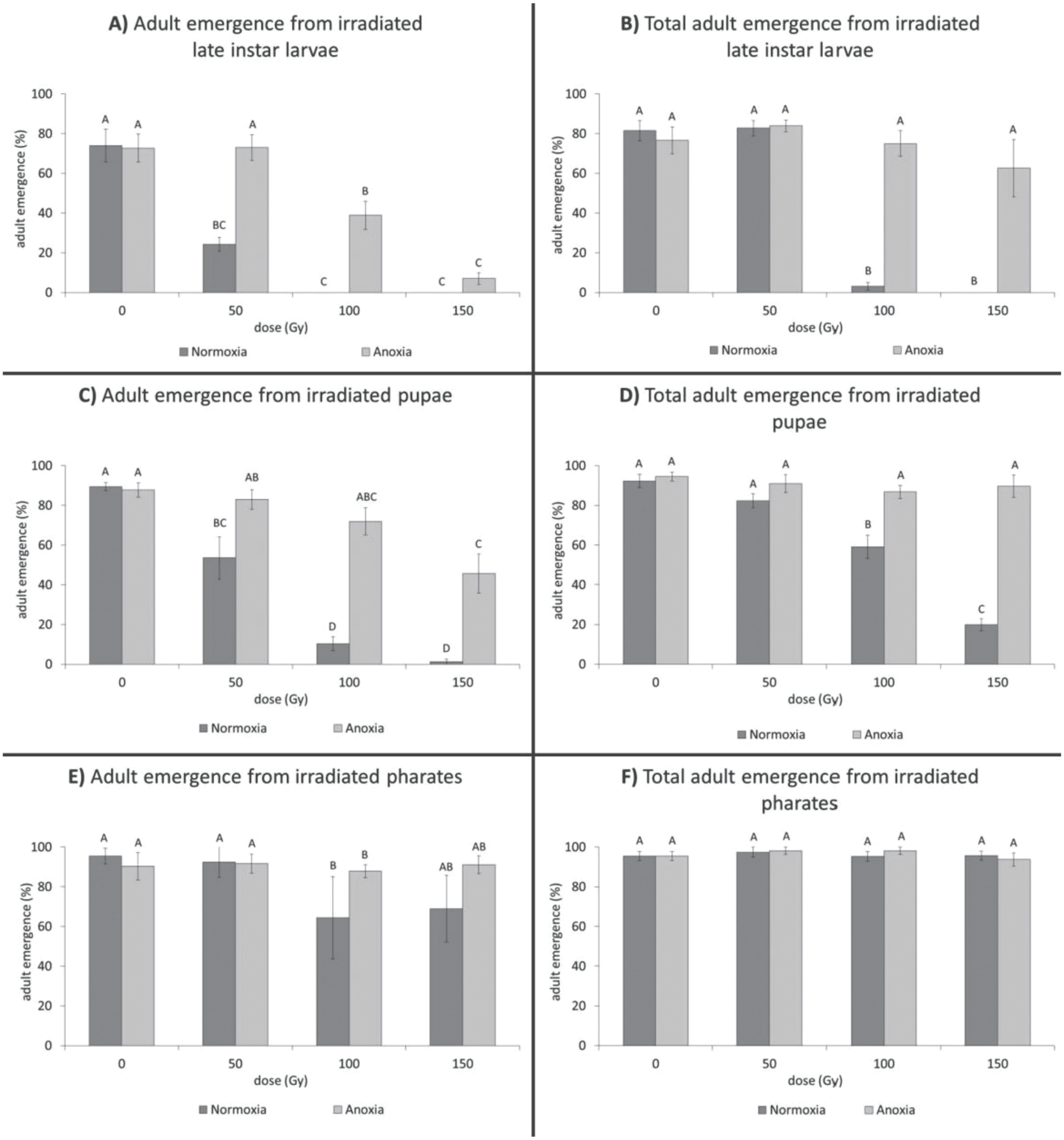

Fig. 2. *Trichoplusia ni* larvae irradiated in normoxia were vulnerable to small radiation doses, but the induction of abnormal-appearing adults were partially prevented when larvae were irradiated in anoxia (A). When percent emergence of both normal- and abnormal-appearing adults are considered, then the prevention effect of anoxia is even stronger (B). Also pupae were very vulnerable to small radiation doses in normoxia (C) with a strong rescue effect apparent for both phenotypes (normal- and abnormal-appearing) when pupae were irradiated in anoxia (D). Pharate adults had the highest tolerance to small radiation doses and anoxia had no effect on percent emergence at the doses used in this experiment (E). Total emergence from treated pharate adults of both phenotypes was around 96%, the same as unirradiated moths (F). Letters within each panel denote groups that differed by ANOVA at $P < 0.05$ after Tukey's HSD correction for multiple comparisons.

We further tested pharate adults with greater target doses including 200, 300 and 400 Gy in 1 experiment, and a follow-up experiment with even greater target doses of 600 and 800 Gy. Even though the percent emergence of normal-appearing adults appeared to decline slightly with dose, an effect of dose was not clearly detectable, and there was no effect of irradiation on percent emergence in anoxia vs. normoxia up to an absorbed dose of 411 Gy ($F_{7,16} = 1.2$; $P_{\text{oxia treatment}} = 0.7658$, $P_{\text{dose}} = 0.0713$, $P_{\text{oxia treatment x dose}} = 0.9994$; Fig. 3A). Similarly, percent

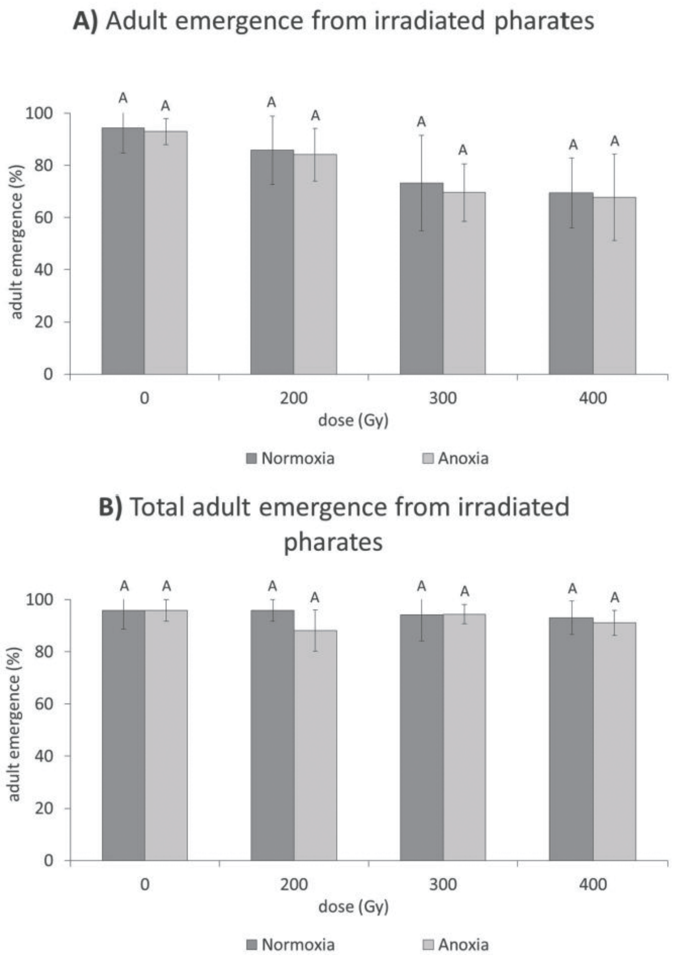

Fig. 3. Percent adult emergence was not affected by irradiation of pharate adults with 200, 300 or 400 Gy in normoxia (A). Anoxia had no significant effect on percent emergence at these doses. Total emergence of both normal and abnormal phenotypes was on average 94%, and almost the same as that of unirradiated moths (B). Letters within each panel denote groups that differed by ANOVA at $P < 0.05$ after Tukey's HSD correction for multiple comparisons.

total adult emergence (both normal-and abnormal-appearing adults) was not affected by either irradiation dose or anoxia treatment up to 411 Gy ($F_{7,16} = 0.32$; $P_{\text{oxia treatment}} = 0.4988$, $P_{\text{dose}} = 0.8281$, $P_{\text{oxia treatment x dose}} = 0.8319$; Fig. 3B). We did observe a substantial decline in the percent emergence of normal-appearing adults at the 600 and 800 Gy target doses (estimated 585–591 Gy and 784–789 Gy absorbed, respectively; $F_{5,23} = 60.3$; $P_{\text{oxia treatment}} < 0.0001$, $P_{\text{dose}} < 0.0001$, $P_{\text{oxia treatment x dose}} < 0.0001$; Fig. 4A).

In our large-dose experiment with 784–789 Gy, no pharate adults from the normoxia-irradiated group emerged as normal-appearing adults, but in the anoxia-irradiated group more than half of the pharate adults emerged as normal-appearing, a substantial prevention effect at this large dose (Fig. 1C). Percent total adult emergence (both normal- and abnormal-appearing adults) from pharate adults irradiated with our 600 Gy target dose—estimated 585–591 Gy absorbed—did not differ between those irradiated in normoxia and those irradiated in anoxia, but at the 800 Gy target dose (784–789 Gy absorbed) percent total emergence was less from normoxia-irradiated than from anoxia-irradiated pharate adults. This further emphasized the impact of anoxia on the efficacy of irradiation on percent adult emergence ($F_{5,23} = 5.9$; $P_{\text{oxia treatment}} = 0.0702$, $P_{\text{dose}} = 0.0312$, $P_{\text{oxia treatment x dose}} = 0.0044$; Fig. 4B).

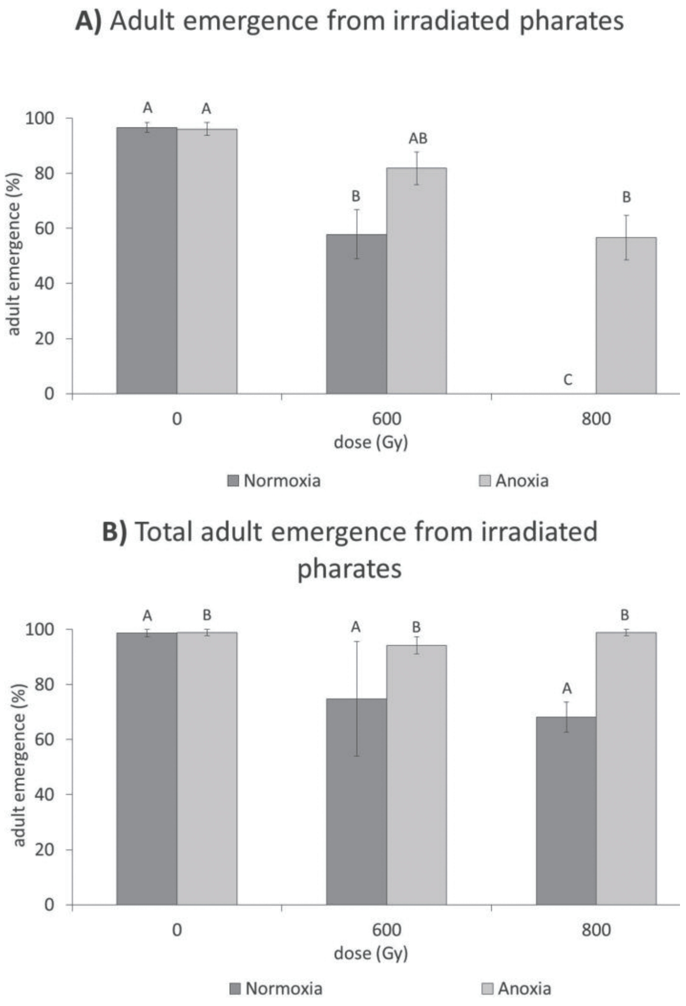

Fig. 4. With normoxia the percent emergence of normal-appearing adults from irradiated pharates was reduced to less than 60% at 600 Gy and none emerged successfully at 800 Gy (A). Anoxia had a strong effect in preventing abnormal-appearing adults that was very pronounced with 800 Gy of irradiation (A). When abnormal-appearing adults were taken into account nearly 75% emergence occurred in the 800 Gy-normoxia treatment group (B). Letters within each panel denote groups that differed by ANOVA at $P < 0.05$ after Tukey's HSD correction for multiple comparisons.

FECUNDITY AND FERTILITY

The number of eggs laid by females that were irradiated as pharate adults declined in a dose-dependent manner, and irradiation in anoxia had no significant effect on fecundity at target doses of 150, 300, or 400 Gy (absorbed doses 177–180 Gy, 300–301 Gy and 411 Gy, respectively; $F_{7,32} = 6.7$; $P_{\text{oxia treatment}} = 0.4777$, $P_{\text{dose}} < 0.0001$, $P_{\text{oxia treatment x dose}} = 0.03$; Fig. 5A). However, anoxia in the absence of irradiation had a deleterious effect on fecundity; females that were held in anoxia as pharate adults but were not irradiated laid fewer eggs than females that were kept normoxic and not irradiated (linear contrast anoxia vs. normoxia at 0 Gy = 0.0043). The percent hatching of eggs oviposited by irradiated females decreased in a dose-dependent manner up a 411 Gy absorbed dose ($F_{7,32} = 5.6$; $P_{\text{oxia treatment}} = 0.9584$, $P_{\text{dose}} = 0.0004$, $P_{\text{oxia treatment x dose}} = 0.006$; Fig. 5B). Again, anoxia had a strong negative effect on female fertility in the absence of irradiation (linear contrast anoxia vs. normoxia at 0 Gy = 0.0185; Fig. 5B). Percent fertility of anoxia treated pharate adult females was greater than of normoxia-treated counterparts when both were irradiated with 177–180 Gy (linear contrast = 0.006; Fig. 5B), but no prevention of the induction of sterility by anoxia was noted at 300 Gy or 400 Gy target doses.

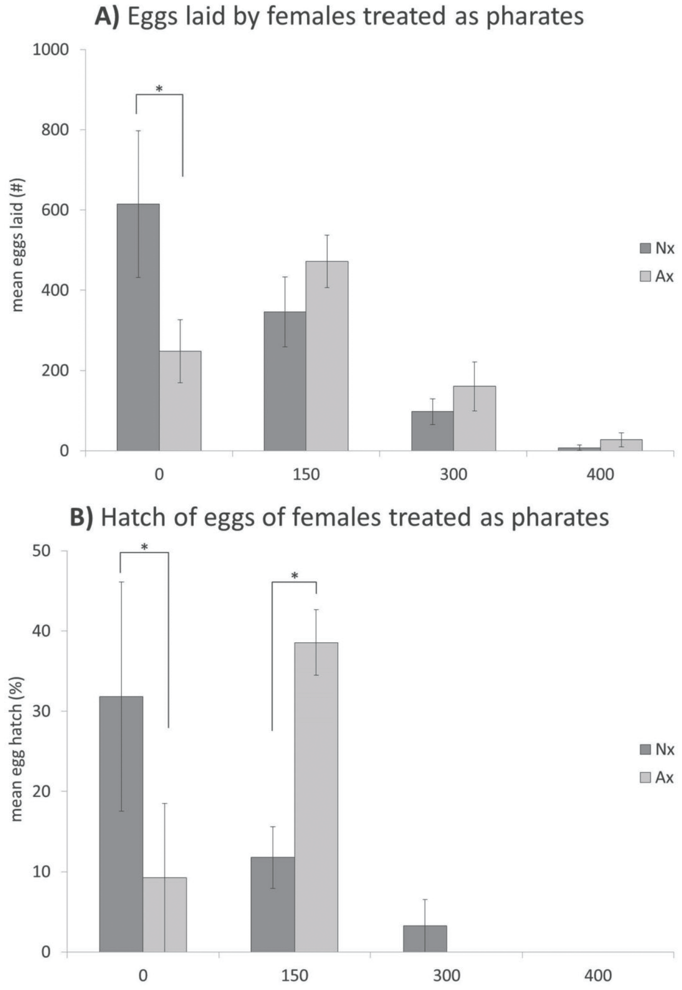

Fig. 5. Both fecundity (A) and fertility (B) of females irradiated as pharate adults decreased in a dose-dependent manner. No eggs of females irradiated with 400 Gy hatched (B). Anoxia in the absence of irradiation suppressed both fecundity (A) and fertility (B); but when anoxia was combined with 150 Gy of irradiation, fertility was largely restored (B). When normoxia- and anoxia-treated groups differed within a particular dose at $P < 0.05$ after a linear contrast was marked with an asterisk (*).

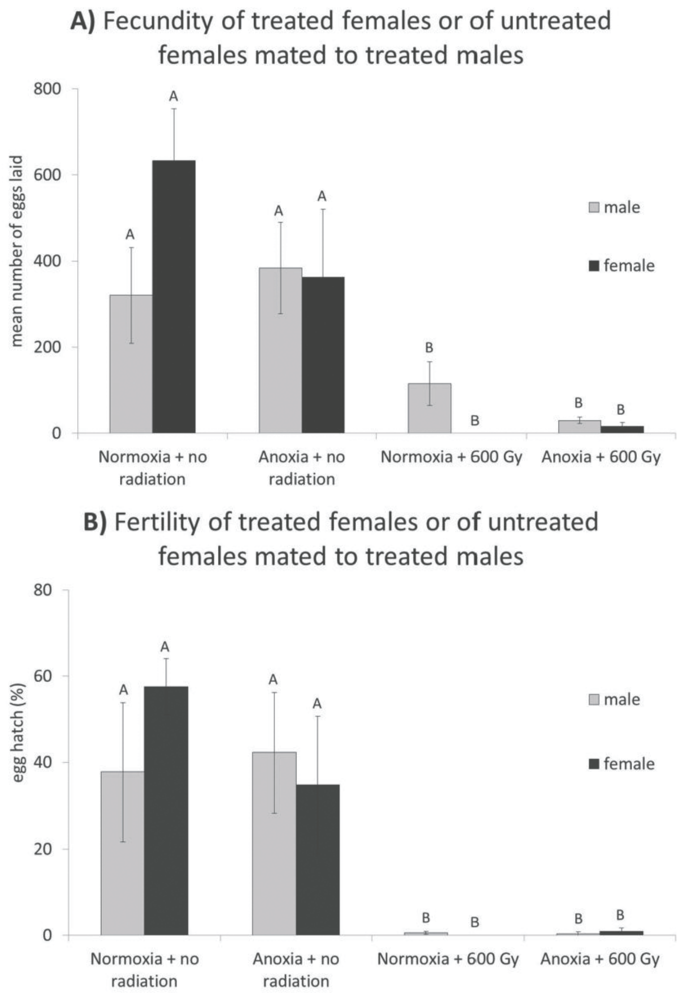

Fig. 6. Fecundity (A) and fertility (B) were greatly reduced when either adult males or females were irradiated with 600 Gy. In the normoxia + 600 Gy treatment, treated males were only 0.6% fertile, and treated females were totally infertile. However in the anoxia +600 Gy treatment, treated females were still 0.9% fertile (B). Letters within each panel denote groups that differed by ANOVA at $P < 0.05$ after Tukey's HSD correction for multiple comparisons.

Because male Lepidoptera are known to require high doses of irradiation to induce loss of fertility (Carpenter et al. 2005), we did not assess male-specific fertility and fecundity in our experiments at smaller doses. At the greatest target doses, i.e., 600 Gy and 800 Gy, we assessed the fecundity and fertility of both males and females. No individuals that were treated at 784–789 Gy as pharate adults produced any eggs, regardless of sex or atmospheric treatment, so we limited our analysis to those treated at 585–591 Gy. Fecundity in comparison to the control was massively reduced when either males or females were irradiated at 585–591 Gy and mated with an untreated individuals ($F_{7,32} = 6.3$; $P_{\text{oxia treatment}} = 0.2899$, $P_{\text{dose}} < 0.0001$, $p_{\text{sex}} = 0.5311$, $P_{\text{oxia treatment x dose}} = 0.5953$, $P_{\text{sex x dose}} = 0.1113$, $P_{\text{oxia treatment x sex}} = 0.3716$, $P_{\text{oxia treatment x dose x sex}} = 0.0991$; Fig. 6A). The effect of an atmospheric treatment during irradiation is not readily apparent when correcting for multiple comparisons, but a directed linear comparison showed that unirradiated females exposed to anoxia had lower fecundity than those exposed to normoxia, with a 42% reduction in the number of eggs laid. This substantial reduction is consistent with our observation in the previous experiment (linear contrast = 0.0429; Fig. 5B). Percent fertility was strongly affected by irradiation at 585–591 Gy, leading to nearly 100% sterility in both males and females, with no apparent sparing effect of anoxia ($F_{7,32} = 6.0$; $P_{\text{oxia treatment}} = 0.5263$, $P_{\text{dose}} < 0.0001$, $P_{\text{sex}} = 0.6587$, $P_{\text{oxia treatment x dose}} = 0.4957$, $P_{\text{sex x dose}} = 0.6538$, $P_{\text{oxia treatment x sex}} = 0.3462$, $P_{\text{oxia treatment x dose x sex}} = 0.3085$; Fig. 6B). Nevertheless ~4% of F_1 larvae did hatch out of 81 eggs at 585–591 Gy when females were treated in anoxia, but no eggs were even laid when females were treated in normoxia. When considering males treated at 585–591 Gy and mated to unirradiated females, ~0.6% of 576 eggs hatched in the normoxia treatment and ~0.5% of 149 eggs hatched in the anoxia atmospheric treatment. We did not track fertility and fecundity in the 784–789 Gy treatment group because irradiation-induced deformities prevented moths from mating and laying eggs.

Discussion

Phytosanitary irradiation (PI) is an alternative to fumigants that can facilitate trade by preventing the spread of invasive insect pests in fresh commodities. The outcome for a traditional fumigation-based phytosanitary treatment is death of any insects associated with the commodity. However, successful treatment with irradiation only requires that quarantine pests cannot become established in a new area. Thus PI may have several successful outcomes beyond dead insects including live insects that will not successfully complete development (sometimes referred to as "wigglers") or insects that will complete development and be reproductively sterile (Hallman 2008; Follett 2009; Hallman 2011). Here we show that irradiation in anoxic atmospheres enhances radiotolerance of larvae, pupae, and pharate adults of the cabbage looper *T. ni.* This result shows that the commodity atmosphere should be considered when developing phytosanitary irradiation doses for this pest, and reinforces the idea that the atmosphere within which a commodity is held before and during irradiation should be considered carefully when developing generic doses more generally.

As expected, we found that radiotolerance increased with developmental stage such that last-instar caterpillars were the least radiation-tolerant stage tested, followed by true pupae that were just slightly more tolerant, and pharate adults were by far the most tolerant stage. Irradiation in anoxia increased the proportion of normal, healthy-appearing adults emerging at any particular dose across all 3 life stages. No healthy looking adults emerged from last instar larvae irradiated in normoxia with doses above 100 Gy. However, pre-conditioning and irradiating last instar larvae in anoxia prevented abnormal development and allowed healthy appearing adults to emerge at doses up to 180 Gy. Irradiation does not normally kill immature insects immediately; rather irradiation typically acts to prevent completion of development with developmental arrest occurring in life stages subsequent to the one irradiated (Thomas & Hallman 2011). If irradiation damage is not severe enough insects may continue to develop to the point of adult emergence, but emerge as malformed adults that are ecologically inviable because of their inability to disperse, mate, and/or successfully reproduce. When considering both normal-looking adults and those adults that successfully emerged but appeared abnormal, the prevention effect of anoxia on radiation damage was even greater. However, abnormal adults were easily distinguishable due to severe malformations of their wings (Fig. 1). While abnormal animals had successfully emerged from the pupal cuticle into adulthood, preliminary trials in the lab suggested that these individuals were not capable of coordinated movement and could not fly and disperse. Thus, these abnormal individuals pose little risk of establishing pest populations.

Effects of anoxia on prevention of irradiation-induced damage and the emergence of normal-looking adults were particularly apparent when considering the pharate adult stage. Numerous previous studies have shown that pharate adult moths are less susceptible to developmental disruption by irradiation because they have already completed most of adult development, so it was not surprising that many normal-looking adults emerged at doses up to 411 Gy (Hallman & Phillips 2008; Follett & Snook 2012; Hallman et al. 2013). However, we were surprised to find that more than half of individuals irradiated at 585–591 Gy in normoxia and more than ¾ of those irradiated at 585–591 Gy in anoxia emerged as normal-looking adults. Perhaps even more surprising was that roughly half of the pharate adults irradiated at 784–789 Gy in anoxia emerged as normal-looking adults (Fig. 4A). No individuals emerged as normal-looking adults at 784–789 Gy delivered in normoxia, but more than half successfully emerged as abnormally formed adults (Fig. 4B). In a commercial setting, where large pallets of commodities may be treated in irradiators with a large dose-uniformity ratio, minimum doses above 400 Gy are typically not recommended because some portions of the commodity may receive doses in excess of the current 1,000 Gy limit imposed by the United States Food and Drug Administration and the Food Standards Commission of Australia and New Zealand for fresh fruits and vegetables (APHIS 2006; Follett 2009; Hallman 2011; FSANZ 2015). Thus, prevention of the emergence of normal-appearing adults does not seem an appropriate success metric for PI when pharate adult lepidopterans that have completed most of their development are present. Because the pharate adult stage occurs within the pupal cuticle, it can be difficult to distinguish highly radiotolerant late pharate adults (sometimes referred to as late pupae) from true pupae and early pharate adults, both of which should be much more susceptible to disruption of development by irradiation (Hallman et al. 2013).

Because 400 Gy has been proposed as a general PI dose for all insects (Hallman 2011) and as a dose adequate for even lepidopteran pupae and adults (Hallman et al. 2013), we treated *T. ni* at that level. However, a 411 Gy absorbed dose was not enough to induce much mortality in pharate adults. Even when we separated abnormal from normal-appearing adults, the emerged moths had not accumulated sufficient radiation damage to suffer clear and visible physical defects. There was no protective effect of anoxia evident at the 411 Gy absorbed dose either, probably because there was not substantial somatic damage to detect such an effect. Beyond failing to induce acute mortality in *T. ni*, an absorbed dose of 411 Gy may even have other effects as it does in the codling moth *Cydia pomonella* (L.), where a 400 Gy treatment in the context of SIT led to an increase in lifespan in codling moth males and females (White & Hutt 1970), further demonstrating the radiation resistance of pharate adults. At our highest doses, estimated to be 585–591 Gy and 784–789 Gy absorbed respectively, both irradiation and irradiation in anoxia had a strong effect on mortality and survival (Fig. 4A). Our largest target dose was 800 Gy (784–789 Gy absorbed) because it was the dose at which we achieved 0% normal emergence for pharate adults irradiated in normoxia. The protective effect of anoxia when irradiating pharate adults was strongest at our 784–789 Gy dose where more than 50% of those moths irradiated in anoxia emerged as normal-looking adults even though no normal-appearing adults emerged after pharate adults were irradiated in normoxia with the same dose. There was a high proportion (68%) of normoxia-irradiated moths that emerged in the abnormal category (Fig. 1C), but these moths were so damaged that they could not walk normally, could not fly, and were unable to mate.

We targeted female fertility and sterility at the 200, 300, 400 Gy levels because lepidopteran females are generally rendered sterile at lower doses than males, if irradiation precedes oocyte maturity (Hallman 1998; Bakri et al. 2005). The fertility of males is of minor concern in the case of quarantine pests if females in the commodity are sterilized. Sterility can be achieved in either of 2 ways, i.e., the treatment induced total infecundity or total infertility. In our first experiment, which involved only irradiating female pharate adults, ~3% out of 487 eggs in the normoxia 300–301 Gy absorbed dose treatment hatched into F_1 larvae, but no F_1 larvae emerged out of the anoxia 300–301 Gy absorbed dose treatment or from either normoxic or anoxic atmospheric treatments at the 411 Gy absorbed dose. These data appear to be consistent with the suggestion that 400 Gy may be a good generic dose to control lepidopteran pupae in commodities if preventing the hatching of F_1 larvae is the desired outcome (Hallman et al. 2013). However, we did observe a few F_1 larvae hatching from both females and males irradiated as pharate adults at our 585–591 Gy dose across atmospheric treatments. Why we observed no F_1 larvae hatching from female pharate adults irradiated at 411 Gy in 1 experiment and then did see a few (~4%) eggs hatching when female pharate adults were irradiated at 585–591 Gy is unclear. These 2 experiments were performed several months apart with different cohorts of pharate adult moths and perhaps some aspect of the rearing or handling process was inadvertently changed between these 2 experiments. Furthermore, our estimates of F_1 larval hatching throughout are very likely biased downward because there was poor survival to hatching in F_1 larvae that were neither irradiated nor treated with anoxia as controls (only 30% F_1 egg hatch in the first experiment and 40% in the 2nd experiment). Perhaps the great mortality prevented viable eggs from hatching into F_1 larvae in the first set of experiments. We believe that this great mortality in the controls among these studies was due to problems regulating humidity within the egg-laying chambers. Thus, our estimates of F_1 larval survival are overly conservative and if large numbers of pharate adults were treated we expect to observe even greater F_1 larval hatching, but we do not know whether development of the latter into subsequent instars may occur. Additional work is needed to properly evaluate the dose of radiation needed to sterilize pharate adults of *T. ni* when both male and female parents are irradiated at a particular target dose, as would occur in a quarantine situation.

In conclusion, irradiating in anoxia not only increased the survival of pharate adult *T. ni* at high radiation doses, it also improved their physical appearance after emergence. Most anoxia-treated and irradiated moths were physically indistinguishable from unirradiated moths at doses up to 600 Gy. The effects of anoxia on radiotolerance were large in our study, as found in many others (rev. by Hallman et al. 2010). This substantial effect of anoxic atmospheres on insect radiotolerance motivated USDA-APHIS-PPQ to enact a temporary policy that prohibits the use of PI in atmospheres of less than 18 kPa aPO_2. However, when considering the potential for effects of modified atmosphere packaging or controlled atmosphere storage on the efficacy of PI it is important to consider that some commodities may be stored in very low-oxygen atmospheres that approximate anoxia (< 1 kPa aPO_2), but most commodities do not tolerate highly hypoxic conditions well over long time periods (Yahia 1998; Toivonen et al. 2009). Many commodities packaged in modified atmosphere plastic films or controlled atmospheres are at moderately low oxygen levels to preserve commodity quality (3–10 kPa aPO_2 relative to normoxia 21 kPa aPO_2; Yahia 1998; Toivonen et al. 2009). These intermediate levels of oxygen both serve to promote product quality and to enhance shelf life of many fresh commodities. Because irradiation can be done after commodities have been packaged in modified or controlled atmosphere films, PI is a very promising treatment if it could be combined with these methods of packaging that are in high demand by the industry. Yet, we know little about how intermediate levels of hypoxia commonly present in these modified and controlled atmospheres may affect insect radiotolerance and therefore treatment efficacy.

To our knowledge, Follett et al. (2013) is the only study to compare the efficacy of irradiation in mildly hypoxic atmospheres. Late stage larvae of the melon fly, *Bactrocera cucurbitae* (Coquillett) (Diptera: Tephritidae), were artificially infested into papayas and infested fruits were placed into 1 of a series of modified atmosphere bags that produced very hypoxic (1–4 kPa aPO_2), moderately hypoxic (3–8 kPa aPO_2), or mildly hypoxic (11–15 kPa aPO_2) atmospheres and subsequently were irradiated at a sublethal dose (50 Gy). There was a small effect of irradiation in the very hypoxic atmosphere on survival at a 50 Gy target dose, but subsequent work showed no atmospheric treatment effect at 150 Gy in melon fly or at 100 Gy in the medfly, *Ceratitis capitata* (Wiedemann) (Diptera: Tephritidae) when each was treated in the very hypoxic atmosphere bags (Follett et al. 2013). This work suggests that very hypoxic environments will have no effect on the utility of 150 Gy as a generic dose for tephritid fruit flies if the criterion is total mortality. However, tephritid fruit fly larvae feeding inside fruits may already be in a mildly hypoxic atmosphere. The atmosphere surrounding host fruits may have little impact on internal feeding insects, but atmosphere may be more important for inducing radiotolerance in surface-feeding insects. Additional studies of the effects of moderate, commodity relevant levels of hypoxia on radiotolerance across a range of insects are needed to produce generalities about the potential for impacts of controlled or modified atmospheres on the efficacy of generic doses in PI.

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