Research Article

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Critical PO_2 as a diagnostic biomarker for the effects of low-oxygen modified and controlled atmospheres on phytosanitary irradiation treatments in the cabbage looper *Trichoplusia ni* (Hübner)

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Abstract

BACKGROUND: Phytosanitary irradiation is a sustainable alternative to chemical fumigants for disinfesting fresh commodities from insect pests. However, irradiating insects in modified atmospheres with very low oxygen (<1 kPa O_2) has repeatedly been shown to increase radioprotective response. Thus, there is a concern that modified atmosphere packaging could reduce the efficacy of phytosanitary irradiation. One hurdle slowing the widespread application of phytosanitary irradiation is a lack of knowledge about how moderate levels of hypoxia relevant to the modified atmosphere packaging of most fresh commodities (3–10 kPa O_2) may affect phytosanitary irradiation treatments. Therefore, we hypothesize that critical PO_2 (P_{crit}), the level of oxygen at which an insect's metabolism becomes impaired, can be used as a diagnostic biomarker to predict the induction of a radioprotective response.

RESULTS: Using the cabbage looper *Trichoplusia ni* (Hübner), we show that there is a substantial increase in radiation resistance when larvae are irradiated in atmospheres more hypoxic than their P_{crit} (3.3 kPa O_2). These data are consistent with our hypothesis that P_{crit} could be used as a diagnostic biomarker for what levels of hypoxia may induce radioprotective effects that could impact phytosanitary irradiation treatments.

CONCLUSION: We propose that the relationship between P_{crit} and radioprotective effects could allow us to build a framework for predicting the effects of low-oxygen atmospheres on the efficacy of phytosanitary irradiation. However, more widespread studies across pest species are still needed to test the generality of this idea.

Keywords: phytosanitary irradiation; modified atmosphere packaging; radioprotection; critical PO_2

1 INTRODUCTION

Due to increased global trade, insect pests are able to spread further and faster than ever before, directly affecting agricultural production and food security.[1,2] Global trade of fresh fruits and vegetables poses substantial risk for spreading invasive agricultural pests, especially insects. Phytosanitary disinfestation measures facilitate trade in commodities by eliminating or killing pests of quarantine importance to reduce the risk of their introduction and establishment to new areas.[3] The most widely used phytosanitary treatments for fresh fruits and vegetables are fumigation with methyl bromide or other chemicals, and thermal treatments including cold treatment for days/weeks or short-term high-temperature treatments such as hot-water dips.[3,4] While thermal processing of fresh fruits and vegetables can be effective, thermal treatments can also have a detrimental impact on food quality.[5] Further, methyl bromide is a stratospheric ozone-depleting substance and its use is being phased out.[6] Other chemical fumigants are also toxic to non-targeted organisms[7] and can be a potential source of pesticide residues on food.[8] The need for sustainable, non-residue bearing phytosanitary

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treatment technology is crucial to ensure food security and reduce environmental pollution.[9]

One sustainable alternative to traditional treatments is phytosanitary irradiation, whereby regulated commodities are exposed to ionizing radiation from gamma rays, x-rays, or an electron beam.[9–11] Phytosanitary irradiation removes the risk of pest incursions and reduces post-harvest losses, without damaging or changing the quality of most commodities.[11,12] Phytosanitary irradiation is also an increasingly attractive treatment for growers and distributors because commodities can be irradiated after packing at the point of origin, or even after shipping at the point of arrival.[13–15] Some producers would like to combine phytosanitary irradiation treatments with controlled or modified atmosphere packaging that preserves the quality and increases the shelf life of fresh commodities. Modified atmosphere packaging that maintains a low-oxygen environment during transport and storage slows the rate of plant respiration and delays ripening, providing a higher value product to the marketplace.[10] Modified atmosphere packaging also acts as an additional pest-proofing layer preventing pests from re-infesting a commodity after a phytosanitary treatment. However, this combination of phytosanitary irradiation with low-oxygen modified atmosphere packaging is of concern to regulators because severe hypoxia (<1 kPa O_2) is known to increase insect radioprotective responses and decrease radiation-induced mortality in a wide range of insects.[3,11,12,16] For example, survival of the cabbage looper moth *Trichoplusia ni* (Hübner) was increased from 0% adult emergence when last-instar caterpillars were irradiated at 100 Gy in normoxia (the partial pressure of O_2 in ambient normal air at sea level is 20.9 kPa) to more than 80% adult emergence when caterpillars were irradiated at the same dose in extreme hypoxia <0.1 kPa O_2.[17]

Because of the radioprotective response induced in insects irradiated in extreme hypoxia, the International Standards for Phytosanitary Measures number 28 (ISPM 28) Phytosanitary Treatments for Regulated pests states that phytosanitary irradiation treatments are not acceptable for commodities stored in modified atmospheres.[18] The only exception to this general rule is for an approved treatment against the Oriental fruit moth *Grapholita molesta* (Busck), where irradiation at a minimum absorbed dose of 232 Gy in severely hypoxic environments (<1 kPa O_2) has been approved.[18] Similarly, from 2012 to 2018 the USDA-APHIS Plant Protection and Quarantine prohibited the use of modified atmosphere packages creating atmospheres of <18% O_2 surrounding fresh fruits, vegetables, and cut flowers, before and during a phytosanitary irradiation treatment.[19] However, the USDA-APHIS Plant Protection and Quarantine Treatment Manual was recently updated to allow phytosanitary irradiation to occur in modified or controlled atmospheres with greater than or equal to 10% O_2.[20] Although the radioprotective effects of extreme hypoxia (<1 kPa O_2) are well documented, the low-oxygen controlled or modified atmospheres used for most commodities include much milder hypoxia, typically between 3 and 10 kPa O_2.[21] Only four studies to our knowledge have documented the effects of intermediate levels of hypoxia on radiation sensitivity. Follett *et al.*[13] used three different modified atmosphere packaging bags to test the effects of 1–4, 3–8, and 11–15 kPa O_2 on phytosanitary irradiation of larvae of the melon fly (*Bactrocera cucurbitae* Coquillett). There was a trend towards greater numbers of larvae irradiated in the lowest hypoxic bags (1–4 kPa O_2) surviving to adulthood, but none of the larvae irradiated in modified atmosphere packaging bags had statistically significantly greater survival than larvae irradiated in normoxia when treated at 50 Gy. At the 150 Gy generic dose for fruit fly larvae, no larvae survived to adulthood after irradiation in any of the modified atmosphere packaging treatments.[14] Similarly, Follet *et al.*[22] showed that the effect of hypoxia treatment between 3.2% and 15.4% O_2 by commercial modified atmosphere packaging bags did not enhance the radioprotective response in *Drosophila suzukii* (Matsumura) treated at 60 Gy. Srimati *et al.*[23] showed that hypoxia treatments of 7.2–9.6% O_2 along with 8.6–26.7% CO_2 atmospheres generated by commercial modified atmosphere packaging bags actually acted as an additional stressor. Rather than enhancing radioprotection in the oriental fruit fly *Bactrocera dorsalis* (Hendel), larvae irradiated in mangos under these moderately low O_2 and high CO_2 conditions had an even lower LD_{99} radiation dose than larvae irradiated in normoxic conditions. However, because both oxygen and carbon dioxide levels were altered by the commercial modified atmosphere packaging bags in the three studies described above, the relative contributions of low oxygen levels *versus* high carbon dioxide levels to changes in susceptibility to radiation are unclear. Condon *et al.*[17] exposed last-instar cabbage looper caterpillars to a range of controlled hypoxic environments including <0.1, 5, 10, 15, and 21 kPa O_2 (normoxia) prior to and during irradiation at a range of doses from 0 to 200 Gy, while keeping CO_2 levels less than 2 kPa over the trials. They found that at an intermediate dose of 100 Gy cabbage looper larvae irradiated in <0.1 kPa O_2 had ~82–85% survival to adulthood, larvae irradiated at 5 kPa O_2 had ~20–25% survival to adulthood, and those irradiated at 10, 15, and 21 kPa O_2 (normoxia) did not survive to adulthood. Thus, moderate hypoxia at 5 kPa O_2 had a small radioprotective effect that was only a fraction of the radioprotective effect observed at <0.1 kPa O_2.[17] When treated at the higher radiation dose of 200 Gy all cabbage looper larvae irradiated in atmospheres of 5–21 kPa died, but there was still ~15–20% survival to adulthood in caterpillars irradiated at <0.1 kPa O_2.[17] Given that only four studies have been published on irradiation in atmospheres with intermediate levels of hypoxia (2–15 kPa O_2), more work is clearly needed to determine the extent to which modified atmosphere packaging may alter the efficacy of phytosanitary irradiation treatments, as well as the levels of oxygen at which radioprotective effects may be induced.

When considering what levels of oxygen may impart radioprotective effects, we know that insects can sense alterations in oxygen partial pressures even at 18 kPa O_2 *vs* normoxia (21 kPa O_2).[24,25] Insects may have some physiological responses to moderate hypoxia, but the most substantial changes in insect metabolism occur when insects encounter more severe hypoxic conditions in the range of 4–6 kPa O_2.[24,25] This suggests that the increased radioprotection observed in insects under severe hypoxia (<1 kPa O_2) may not be of concern when irradiation occurs at mild (10–18 kPa O_2) or moderate (5–10 kPa O_2) levels of hypoxia. If increased radioprotection does not occur until very severe hypoxia, regulations could be further revisited to allow phytosanitary irradiation to be used in modified atmospheres under lower levels of oxygen. Thus, there is a critical need to determine what levels of oxygen will increase insect radioprotection and determine the radiation doses needed to control insect pests irradiated in commodities held in modified atmosphere packaging.

One way to determine the level of oxygen that can increase insect radioprotection is to perform radiation dose–response experiments under a commodity-relevant range of O_2 levels for each target insect species, as has been done for the Oriental fruit moth.[18,26] However, performing such dose–response experiments for many pest species would be very time consuming and difficult to complete rapidly for new/emerging pests. Understanding the mode of action of hypoxia on radioprotective responses is needed to reduce empirical testing across pest insect species. Here we suggest a physiologically informed framework that we believe will allow more rapid prediction of what levels of hypoxia in controlled or modified atmosphere packaging will induce a radioprotective response.

In general, insects deal well with exposure to hypoxic environments down to 8–10 kPa O_2, losing little in the way of growth, feeding, or reproductive performance.[25,27] Furthermore, insects can also successfully survive and thrive after exposure to hours or days of <5 kPa O_2, levels of hypoxia that would be deadly for mammals.[25,27,28] While insects can sense moderate hypoxia, triggering small but detectable cell-signaling responses at 18 kPa O_2 compared to normoxia (21 kPa O_2), insects typically do not mount a dramatic physiological response to hypoxia until they hit their critical PO_2 (P_{crit} hereafter).[25] P_{crit} is defined as the atmospheric oxygen partial pressure (PO_2) at which oxygen becomes limiting for metabolism, typically estimated by a hypoxia-induced decrease in CO_2 production.[25] Below this oxygen level, insects switch to anaerobic metabolism and also induce a series of cellular protective mechanisms that may decrease the insect's sensitivity to radiation.[24,25,29,30] Published reports of insect P_{crit} range between 0 and 10 kPa, with most species falling between 0.5 and 6 kPa.[31] Our working hypothesis is that substantial radioprotective effects are induced when an insect reaches its P_{crit} (Fig. 1).

If substantial radioprotective effects are only induced when an insect reaches its P_{crit}, we propose that P_{crit} could be used as a diagnostic biomarker to indicate the levels of oxygen that may be of concern to the effective application of phytosanitary irradiation. In this study, we used the cabbage looper moth, *T. ni* (Hübner) (Lepidoptera: Noctuidae), as a model insect to test our hypothesis. We estimated P_{crit} in late-instar larvae, and then tested for correspondence between P_{crit} and the radioprotective response by measuring successful adult emergence after irradiating larvae under a series of different levels of O_2 from normoxia (21 kPa O_2) to functional anoxia (<0.1 kPa O_2). We use final-instar larvae because this is the most tolerant life stage of lepidopteran pests likely to be found in commodities.[12] We show that cabbage looper larvae irradiated in hypoxic atmospheres below their P_{crit} (~3.3 kPa O_2) have much greater survival to adulthood at intermediate radiation doses than larvae irradiated in hypoxic atmospheres above their P_{crit}, consistent with our notion that P_{crit} may be used as a biomarker for the level at which O_2 radioprotective effects are induced. We then review the literature on insect P_{crit} estimates, showing that most fall between 0.5 and 6 kPa O_2. We suggest that if P_{crit} does indeed indicate at what level of O_2 radioprotection is induced, then irradiation in modified and controlled atmospheres that contain greater than 6 kPa O_2 should have little impact, if any, on the efficacy of phytosanitary irradiation treatments.

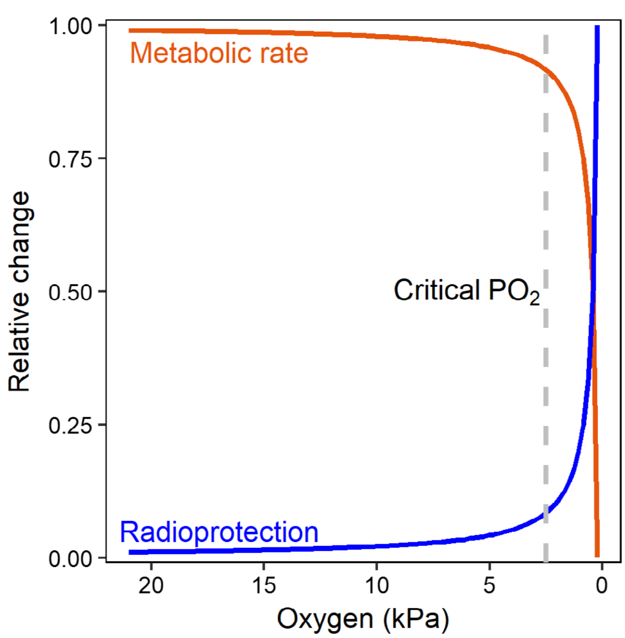

Figure 1. Hypothetical correlation between insect critical PO_2 (P_{crit}; dashed grey line) and radiation sensitivity. P_{crit} is the O_2 level at which insects decrease their metabolism as estimated by measuring their rates of CO_2 production (orange line). We predicted that oxygen levels lower than the P_{crit} will induce a robust increase in radioprotection (blue line).

2 MATERIALS AND METHODS

2.1 Insects

Cabbage looper larvae were sourced from a colony at the United States Department of Agriculture's Agricultural Research Service Center for Medical, Agricultural and Veterinary Entomology (USDA-ARS-CMAVE), in Gainesville, FL, USA. Larvae were reared in 32-well trays (4 × 4 × 2.5 cm; Frontier Agricultural Sciences, Newark, DE, USA) on a pinto bean artificial diet with two larvae per well at 24 ± 1 °C under a 16:8 (light:dark) photoperiod.[32] Larvae used for both P_{crit} and irradiation experiments were late fifth-instar wandering larvae (~12 days after hatching).

2.2 Critical PO_2

We measured the P_{crit} of 27 larvae from two temporal cohorts (10 females and 17 males across both cohorts). We used a programmable ROXY-4 gas mixing system (Sable Systems International, North Las Vegas, NV, USA) to gradually reduce the atmospheric partial pressure of oxygen (aPO_2) while simultaneously recording the CO_2 production of the larvae and excurrent O_2 on a FOXBOX gas analyzer system (Sable Systems International). We used a similar experimental system to Klok *et al.*[33] where the ROXY-4 regulated the aPO_2 inside an 18-L carboy from which air was pumped to the animal chamber and gas analyzers. Briefly, aPO_2 was regulated by the ROXY-4 controlled from a laptop by mixing N_2 (Airgas, Jacksonville, FL, USA) and atmospheric air inside the carboy. The air inlet port of the pump was connected to the carboy by a Bel-va-line tube and air the mixture was pumped from the carboy to the mass flow valve that regulated the flow to 50 mL min^{-1} and then passed through a Drierite-Ascarite II-Dririte scrubber to remove water vapor and atmospheric CO_2 from the incurrent air. CO_2-free air then passed through a small humidifier (damp cotton balls) to prevent desiccation stress during the P_{crit} trial. The gas passed through the animal chamber and then to a magnesium perchlorate [$Mg(ClO_4)_2$] scrubber to remove the water vapor before the FOXBOX. Gas passed through a filter to the CO_2 analyzer port of the FOXBOX and then through an $Mg(ClO_4)_2$ and Ascarite II scrubber to remove the water vapor and CO_2 prior to passing though the oxygen fuel cell inside the FOXBOX.

For each trial, the aPO_2 ramp had seven phases: (i) 20 min baseline recording with an empty chamber; (ii) 20 min recording with an insect inside the chamber at ambient aPO_2; (iii) aPO_2 was decreased from ambient atmospheric concentration of O_2 in steps of 0.75 kPa O_2 per minute to 10 kPa O_2; (iv) aPO_2 decreased 0.2 kPa O_2 per minute until approx. 1 kPa O_2; (v) O_2 was held below 0.5 kPa O_2 for at least 20 min; (vi) the carboy was opened and nitrogen regulation disabled so the aPO_2 rapidly returned to ambient oxygen concentration during which organismal gas exchange was recorded for another baseline for at least 20 min; (vii) the larva was removed from the chamber and another approx. 20 min baseline was recorded (see Fig. 2). When an insect was placed or removed from the chamber, the system was flushed for 2 min with ambient air before a new recording started. Each larva was weighed before and after the P_{crit} trial to determine both initial mass and mass lost from desiccation stress. Throughout the trial, data were recorded on a laptop with Expedata 1.7.3 (Sable Systems International).

To determine the P_{crit} we used a macro in the Expedata program from Klok *et al.*[33] The first two macro steps transform the data from ppm to ml/min and add markers to the Expedata file at every 1 kPa O_2 point between 20 and 0 kPa. The CO_2 trace is smoothed and then integrated by time in seconds. This transformation is the absolute difference sum of the CO_2 trace – the successive sum of absolute differences between data points at all prior time points.[34] A linear regression was applied between the absolute difference sum and experimental time so the CO_2 residual can be estimated. Then we plotted the CO_2 residuals *versus* experimental time, which amplified the dynamic variability of the recorded CO_2 release. So, the O_2 tension where CO_2 residual peak appeared is the estimated P_{crit} for each insect. A representative data output for O_2 level, CO_2 level, and the residuals for CO_2 for a single caterpillar is shown in Fig. 2 and the estimated P_{crit} is 2.97 kPa.

2.3 Radiation sensitivity across atmospheres

We used a fully factorial experimental design for O_2 and radiation treatments. There were five concentrations of O_2 (0, 2.5, 5, 10, and 21 kPa) combined with four radiation doses (0, 50, 80, 100, and 150 Gy), yielding 25 different treatments in total. Each of the radiation sensitivity experiments was replicated with four different temporal cohorts and two replicate jars of eight larvae for each cohort, so there were 64 larvae for each treatment (2 jars × 8 larvae × 4 cohorts = 64 larvae) and 1280 larvae were treated in total (64 per treatment × 25 treatments = 600 larvae treated in total).

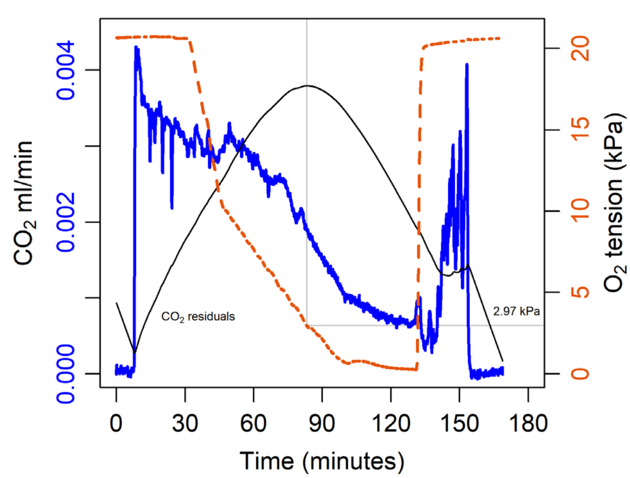

Figure 2. Representative recording of a single *Trichoplusia ni* larva critical PO_2 trial. CO_2 production of the larva declines (blue solid line) as O_2 (orange dashed line) declines due to more and more nitrogen being added to ambient air as part of the gas-mixing program. When normoxia is restored CO_2 production quickly recovers. An approximately 20 min baseline is recorded at the beginning and end of the trial with an empty insect chamber to account for background instrument readings (cut off in this figure to just a few minutes before and after the trial). The O_2 tension where the CO_2 residual peak (solid black line) appears is the estimated P_{crit} for the insect. The estimated P_{crit} of this larva was 2.97 kPa.

Prior to irradiation, eight larvae were placed inside two stacked polystyrene Petri dishes (60 × 15 mm, Fisher Scientific, Hampton, NH, USA) inside a ~473-mL polypropylene jar (Paper Mart, Orange, CA, USA) that could be purged with specific atmospheres and sealed. Two three-way, leur-lock valves (Cole-Parmer, Vernon Hills, IL, USA) were glued into the lid of the jar to allow the chambers to be flushed with the appropriate modified atmosphere gas. The lid was sealed with high-vacuum grease (Dow Corning Corporation, Midland, MI, USA). Preliminary trials showed that up to eight caterpillars could be used in each jar over the trial period without significantly altering the controlled atmosphere (unpublished data). Petri dishes were modified to have mesh bottoms to allow ample gas exchange and were kept in the middle of the jar using a metal mesh holder at the location in the irradiation chamber where the radiation field is the most consistent for the irradiator used in this study (details below).

Each plastic jar chamber was flushed with a treatment atmosphere: <0.1 kPa O_2 (anoxia), 2.5 kPa O_2, 5 kPa O_2, 10 kPa O_2, or 21 kPa O_2 (normoxia from ambient air). We used industrial N_2 (Airgas) for the <0.1 kPa O_2 treatment and gas was passed through a Drierite and Ascarite II scrubber to eliminate any water or CO_2. For the hypoxia treatments (2.5, 5, and 10 kPa O_2), we used certified gases mixed by Airgas traceable to N.I.S.T. gas mixture standards (analytical uncertainty ±2%). Chambers with insects sealed inside were flushed with the treatment gases for 15 min at >2000 mL min^{-1} from the gas mixture cylinders (chamber volume ~473 mL). After flushing, chambers were sealed by turning off the leur-lock valves, and insects were acclimated to the treatment atmosphere for 1 h prior to irradiation. All insects were sealed inside the chambers for a total of 2 h before returning them to normoxia.

To verify that the insects did not substantially alter the target atmosphere during the experiment, 5 mL samples from each chamber were taken at the time the chambers were sealed and after 2 h on the day of irradiation. Atmospheric samples were injected against a N_2 baseline and analyzed using the FOXBOX system. The oxygen concentration of the treatment atmospheres was found to have differed less than 1 kPa compared to the original concentration over the 2 h sampling period.

Chambers were gamma-ray irradiated at the Cancer and Genetic Research Complex (CGRC) at the University of Florida in Gainesville, FL, USA. Target doses of 0, 50, 80, 100 and 150 Gy were applied using a Gammacell 1000 137Cs irradiator (GC45, Ottawa, Ontario, Canada) at a dose rate of 8.8 Gy/min, thus higher doses required longer exposure. Gafchromic HD-V2 film dosimeters (1 cm × 1 cm) (uncertainty below 2%; Ashland, Covington, KY, USA) were individually placed in small paper envelopes and affixed on both top and bottom of the Petri dishes. Alanine pellet dosimeters (uncertainty 3.5%; lot T030901, Far West Technology, CA, USA) were also used with the film dosimeters side by side in some randomly chosen treatments to confirm the absorbed dose. We read film with a DoseReader 4 spectrophotometer (Radiation General Instrument Development and Production Ltd, Budapest, Hungary) with the wavelength at 590 nm amber light ~24 h after irradiation. Alanine pellets were read later at the National Center for Electron Beam Research, Texas A&M, College Station, TX, USA. Absorbed doses of radiation were calculated from the mean of the film dosimeters on the top and bottom of each Petri dish irradiated at each target dose. Calculated mean absorbed doses and their standard deviations from film dosimeters were 49.6 ± 2.8 Gy (range 43.1–56.3 Gy), 81.4 ± 2.7 Gy (range 74.31–86.6 Gy), 102.9 ± 3.4 Gy (range 94.4–108.9 Gy), and 151.9 ± 4.2 Gy (range 141.0–161.2 Gy). Calculated mean absorbed doses and their standard deviations from alanine dosimeters were 50.8 ± 1.17 Gy (range 49.0–53.0 Gy), 81.5 ± 2.12Gy (range 80.0–83.0 Gy), 104.1 ± 2.02 Gy (range 101–107 Gy), and 152.7 ± 2.34 Gy (range 149.0–158.0 Gy).

After treatment, each larva was placed individually in a 32-well rearing tray kept at 24 ± 1 °C and allowed to pupate. Approximately 1 week after irradiation, insects began to emerge as adult moths. Adult emergence was recorded over 5 days and this measure was used to score the effect of irradiation in different atmospheric treatments. Emergence was scored as successful when an insect detached completely from the pupal cuticle with fully formed wings. Inability of a pest to emerge or fly is a generally acceptable outcome for phytosanitary irradiation treatments.[35]

2.4 Statistical analysis

To analyze critical PO_2 data, we used an ANCOVA with sex and cohort as fixed effects and initial mass of the insect as the covariate. ANCOVA was performed in JMP Pro (v. 11.0, SAS Institute, NC, USA). Significance was determined at $P < 0.05$ for all analyses.

Emergence data were analyzed via a bias reduced binomial generalized linear model,[36] in R[37] with the *brglm* library.[38] Absorbed radiation and oxygen atmosphere were treated as categorical fixed effects. Because random effects are currently not implemented in the *brglm* package, cohort and jars in each cohort were also fitted as fixed factors. We determined the best-fit model and significance of the fixed effects via analysis of deviance and AIC, and the factors cohort and jars were removed from the final model. After the final model was determined, we performed a *post hoc* multiple comparisons test to compare emergence data between different oxygen levels at each of the radiation treatment levels.

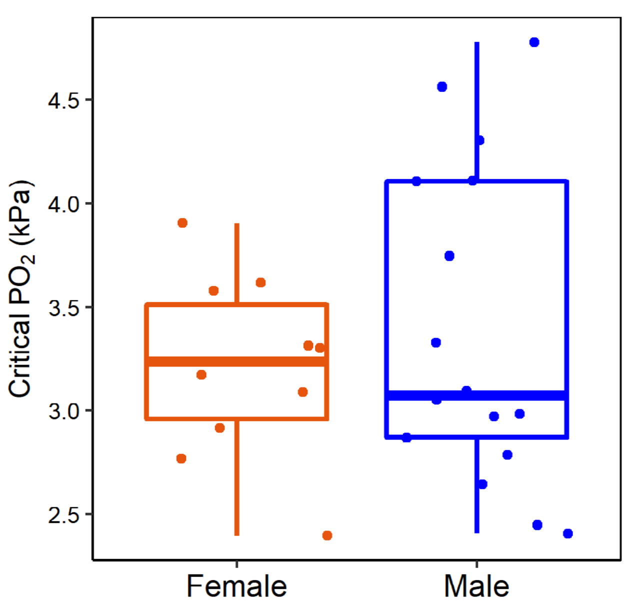

Figure 3. Critical PO_2 (P_{crit}) values of last instar larvae of *Trichoplusia ni* are not affected by sex.

To estimate the effective dose needed to prevent the emergence of 95% insects under different O_2 (ED_{95}), we fitted a model with dose as a continuous numeric factor for each O_2 treatment by the *brglm* library and the ED_{95} under each O_2 treatment was estimated by both logit and probit link families with 95% confidence intervals. The effect of O_2 treatment on ED_{95} was analyzed by one-way ANOVA with *post hoc* Tukey's HSD tests. Data are available from the Dryad Digital Repository: https://doi.org/10.5061/dryad.v15dv41sn

3 RESULTS AND DISCUSSION

Phytosanitary irradiation is an increasingly attractive treatment for commodity growers because fresh commodities can be treated in modified atmosphere packaging to enhance the shelf life (i.e. irradiated in low oxygen). Currently, the use of phytosanitary irradiation with modified atmosphere packaging is restricted to use only in packaging that keeps atmospheres at greater than 10% O_2, compared with 21% O_2 in normal air, because of concerns that exposure to anoxia or severe hypoxia may induce radioprotection and reduce the efficacy of phytosanitary irradiation.[12] However, while some commodities are packaged with very low O_2 (<1 kPa), most commodities are packaged at moderate levels of O_2 (3–10 kPa, relative to 21 kPa ambient air),[39] levels that may not be low enough to induce radioprotection. Despite this, most previous studies have largely focused on comparisons between normoxia and anoxia or extreme hypoxia.[26,30,40–44] This leaves a knowledge gap about how intermediate levels of hypoxia affect insect radioprotection and phytosanitary irradiation treatment efficacy, highlighting the critical need to determine what levels of O_2 induce insect radioprotection. Thus, we aimed to investigate whether P_{crit} could serve as a diagnostic biomarker to predict the point at which O_2 induces radiotolerance and below which the use of phytosanitary irradiation in modified atmosphere packages should be seriously considered.

3.1 Estimating critical PO_2

Critical PO_2 is the level of atmospheric O_2 that limits an organisms' aerobic metabolism. Most insects do not need high PO_2 to maintain normal metabolic processes because their gas exchange systems are capable of delivering sufficient O_2 to cells at low ambient PO_2. Thus, the critical PO_2 is a predictable metabolic parameter that is generally similar across different insect taxa. Approximately 80% of the published insect P_{crit} estimates fall between 1 and 6 kPa.[25] The P_{crit} of *T. ni* larvae was 3.31 kPa O_2 (95% CI: 3.05–3.56 kPa O_2, $N = 27$) in our study (Fig. 3), well within the expected range. Critical PO_2 was not affected by the initial mass of the larva (0.27–0.35 mg, $F_{1,1} = 0.16$, $P > 0.05$) or sex ($F_{1,1} = 0.01$, $P > 0.05$), and there was also no detectable difference in P_{crit} between larvae from two temporally separated cohorts ($F_{1,1} = 0.01$, $P > 0.5$).

The P_{crit} estimate in our study was determined by calculating the CO_2 residuals from the regression of the absolute difference sum of the CO_2 change over experimental time. While this approach differs slightly from the most prevalent methods in the literature that have previously directly used CO_2 emission data, this residual approach was developed by Klok *et al.*[33] to eliminate several of the shortcomings of estimating P_{crit} directly from CO_2 emission. The direct emission approach to measuring P_{crit} estimates the breakpoint where CO_2 emission transitions from being independent of atmospheric PO_2 to being dependent on atmospheric PO_2 because CO_2 emission should ideally be constant and not significantly affected by atmospheric PO_2 higher than the P_{crit}. However, some insects do not show a clear biphasic response of CO_2 exchange to decreasing atmospheric PO_2, making it difficult to estimate P_{crit} in some situations, such as some of our trials in *T. ni*. Figure 2 shows a representative recording of a critical PO_2 trial where the larva's CO_2 production declines in response to the declining atmospheric PO_2 flushed through the chamber across experimental time. This decreasing pattern makes it difficult to clearly identify the breakpoint of CO_2 emission by just using the CO_2 emission rate (blue line in Fig. 2). By using the residuals of CO_2 sum change (black line in Fig. 2) over the experimental time course we were able to more precisely predict when CO_2 emission began to decrease substantially. From the CO_2 residuals, the breakpoint of CO_2 emission can be precisely estimated as a peak in the CO_2 residuals along the declining portion of the curve. The P_{crit} was estimated as 2.97 kPa for the individual shown (Fig. 2). Another benefit of using this residual-based approach to estimate P_{crit} is that all of the procedures, including the gradient decreasing atmospheric PO_2, data acquisition, and data analysis, are automated using a macro in the ExpeData software.[33] Using an automated workflow for data analysis can decrease inadvertent mistakes by different operators and makes estimates of P_{crit} easy to perform by researchers without a background in insect respiratory physiology. Thus, we recommend this method be used as a standard way to estimate insect P_{crit} for future applications.

3.2 Adult emergence after irradiation under hypoxia

The probability of emergence as an adult moth was significantly affected by both oxygen atmosphere ($z = 3.3$, $P = 0.001$) and absorbed radiation ($z = -10.4$, $P < 0.001$) (Fig. 4). With increasing radiation, emergence declined across all O_2 treatment groups and no insects successfully emerged at 150 Gy in the O_2 treatments from 2.5 to 21 kPa, and only 25% emerged when treated

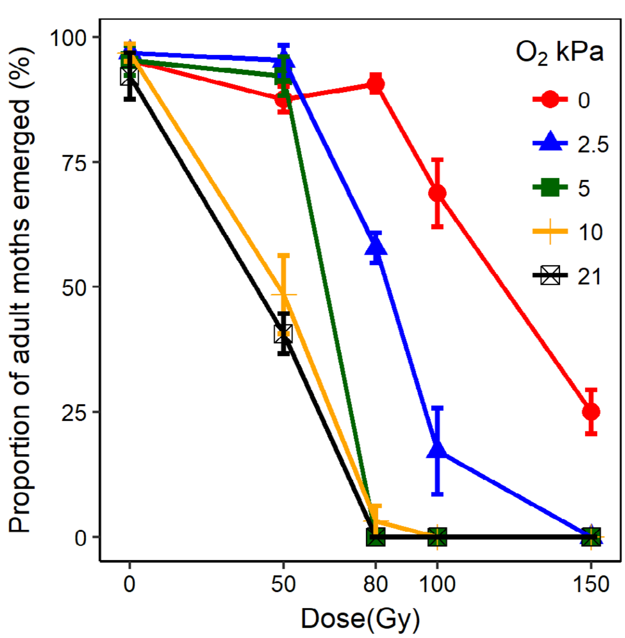

Figure 4. Low oxygen increased radioprotection at higher doses with the two lowest oxygen atmospheres, those below the *Trichoplusia ni* P_{crit}, inducing the greatest protective effects. Data presented are based on the coefficients from a bias-reduced binomial general linear model with oxygen atmosphere and absorbed radiation, and their interaction fitted. Bars represent standard errors.

with <0.1 kPa O_2 at 150 Gy. We also detected a significant interaction between PO_2 and absorbed radiation ($z = -7.364$, $P < 0.001$). At 50 Gy, insects irradiated in <0.1, 2.5, and 5 kPa O_2 had significantly higher emergence than insects irradiated under 10 and 21 kPa O_2. At 80 Gy, insects irradiated in <0.1 kPa O_2 showed the highest emergence followed by insects irradiated in 2.5 kPa O_2, with almost no insects emerging when irradiated under 5, 10, or 21 kPa O_2 (one adult emerged from the 10 kPa O_2 treatment at 80 Gy). At 100Gy, insects irradiated in <0.1 kPa O_2 showed significantly more emergence than all the other treatments, again insects irradiated in 2.5 kPa O_2 had intermediate emergence, and no insects emerged when irradiated at 5, 10 or 21 kPa O_2. Thus, these data at 80 and 100 Gy are consistent with our hypothesis that atmospheres with less O_2 than the P_{crit} would induce radiation resistance. At 150 Gy, only 25% of irradiated larvae successfully emerged in the <0.1 kPa O_2 treatment (significantly different from 0%), which was clearly different from all the other atmospheric treatments where no individuals emerged. These data clearly show that extreme hypoxia, bordering on functional anoxia (<0.1 kPa O_2), induces a greater radioprotective response than a hypoxic treatment just under P_{crit}.

Our results are similar to those reported in two previous *T. ni* studies from our laboratory.[17,41] In these studies, we also found detectable effects of functional anoxia enhancing radioprotection. However, there are notable differences between our current study and these two previous studies. Here we found that insects irradiated in functional anoxia (<0.1 kPa O_2) showed higher adult emergence for all radiation doses than in those two studies. At 50 Gy, our data in this paper showed significantly higher emergence when larvae were irradiated under 0 or 5 kPa O_2 than observed previously. In contrast, no significant difference was found between 5 and 10 kPa O_2 at 50 Gy in Condon *et al.*[17] Condon *et al.*[17] also noted significantly higher adult emergence after radiation in 5 kPa O_2 compared to 10–21 kPa O_2 at 50 Gy, attributed to one unusual cohort of caterpillars.

These differences among the three studies all done by our laboratory on the same insect species may be due to a number of factors, including the different sources of radiation used, radiation dose rate, differences in the colony used, or small differences in the handling done by people in each experiment. López-Martínez *et al.*[41] used a different gamma-ray irradiator, with a dose rate of 8.0 Gy/min, to the one used in this report with a dose rate of 8.8 Gy/min, and Condon *et al.*[17] used an electron beam accelerator to produce x-rays with a radiation dose rate of 25 Gy/min. Dose rate is thought to be a factor that could potentially influence the efficiency of phytosanitary irradiation, although the data supporting this are scarce and there is no evidence of the dose rate significantly affecting phytosanitary irradiation efficiency.[12,19] Hallman *et al.*[12] hypothesized that an increased dose rate leads to increased irradiation efficacy because it overwhelms radiation repair mechanisms. A faster dose rate has previously been shown to be harmful to insects based on male reproductive development[45] and larval or pupal development,[12] yet no significant effects of dose were found in either survival rate or egg hatching in previous studies.[12] The comparisons of our own studies using *T. ni* suggest that the higher dose rate is less harmful to the insects when other factors are assumed to be the same. However, these studies were also carried out using the same colony but in different years and by different laboratory personnel, and the absorbed dose showed greater variation in Condon *et al.*[17] than in the other two studies, possibly due to wider process control tolerances in the configuration of the particular x-ray irradiator used, leading to a greater range of absorbed doses and a poorer dose-uniformity ratio than the two studies performed with gamma-ray irradiators. Thus, the effects of radiation source and dose rate on the efficiency of phytosanitary irradiation warrant further investigation.

3.3 Correlation between P_{crit} and radioprotection

The aim of our current study was to find a diagnostic biomarker to predict radioprotection induced in insects under hypoxic levels of O_2. Increased radioprotection by hypoxia can be explained by physiological conditioning hormesis,[46] which occurs when organisms are exposed to sublethal stresses that induce physiological changes, leading to enhanced performance. Most insects exhibit relatively large safety margins for oxygen delivery, allowing them to maintain normal metabolism under moderate hypoxia.[25] For example, in *Drosophila melanogaster*, resting adult metabolic rate is nearly constant between 21 and 3 kPa atmospheric PO_2, then linearly decreases by approximately 10-fold in comparison to normoxia, when oxygen levels are reduced from 3 and 0.1 kPa atmospheric PO_2.[47] Reduced mitochondrial activity induced by hypoxia may produce fewer reactive oxygen species (ROS), and this has been hypothesized to increase insect tolerance to radiation.[16] Meanwhile, hypoxia also induces increased antioxidant activity that may subsequently lower ROS levels and subsequently decrease oxidative damage.[16,30] Hypoxia also activates multiple downstream stress response signaling pathways such as heat shock proteins[27] that have been shown to be up-regulated when insects were exposed to ionizing radiation.[48,49] Thus, atmosphere-induced radioprotection is likely caused by a multifaceted physiological response. Evidence of the multifaceted nature of the insect physiological response to hypoxia at P_{crit} is seen in a metabolomics study of the false codling moth, *Thaumatotibia leucotreta* (Meyrick).[50] The P_{crit} of false codling moth larvae is 4.5 kPa. When atmospheric treatment groups were analyzed by a multivariate Partial least squares discriminant analysis (PLS-DA) score, the anoxia treatment (<0.1 kPa O_2) was considerably different from PO_2 groups >5 kPa, with a 2.5 kPa O_2 treatment falling in between. This pattern of metabolomic change occurring only near and below the P_{crit} supports the idea that P_{crit} is a physiological threshold that may predict physiological changes that could lead to increased radiotolerance. Clearly more work is needed to characterize the cellular, biochemical, and physiological mechanisms that increase radiation resistance in modified atmospheres.

To assess the extent to which different levels of O_2 both higher and lower than P_{crit} may affect radioprotection, we assessed changes in the ED_{95} for adult emergence calculated across radiation doses within each atmospheric treatment (Fig. 5). The results estimated by both logit and probit regression showed that the ED_{95} was significantly affected by O_2 treatment (logit: $F_{4,15} = 71.03$, $P = 1.45\text{e-}09$; probit: $F_{4,15} = 60.79$, $P = 4.31\text{e-}09$). There was no difference in the ED_{95} across atmospheres between 5 and 21 kPa O_2 (Tukey's HSD, $P > 0.05$ for all the comparisons). However, the ED_{95} sharply increased at 2.5 kPa O_2 to 122.79 Gy (111.79–133.78 Gy) (logit regression) or 126.84 Gy (115.28–138.40 Gy) (probit regression) ($P < 0.001$ for all the comparisons). Maximum radioprotection was observed when O_2 was <0.1 kPa, in which the estimated ED_{95} values increased even more precipitously [217.26 Gy (187.24–247.31 Gy) (logit) or 220.09 Gy (188.89–251.30 Gy) (probit)].

The range of atmospheric O_2 that induces robustly increased radioprotection is therefore below 5 kPa O_2 and higher than 2.5 kPa O_2, directly coincident with our estimated P_{crit} value of 3.31 kPa O_2 (95% CI: 3.05–3.56 kPa O_2). This coincidence between P_{crit} and the levels of O_2 that induced radioprotection is consistent with our hypothesis that radioprotective effects are induced at or below P_{crit}. We hypothesize that P_{crit} can be used as a marker to predict the levels of O_2 at which insects will robustly increase their radioprotection. This concordance between P_{crit} and radioprotection has, to date, only been tested in our study and clearly more studies are needed to confirm the extent to which this pattern holds across insects. However, if the relationship between P_{crit} and radioprotective effects is shown to be more general, it could provide an efficient way to evaluate the radioprotective response and could be used to promote the use of phytosanitary irradiation combined with modified atmosphere packages.

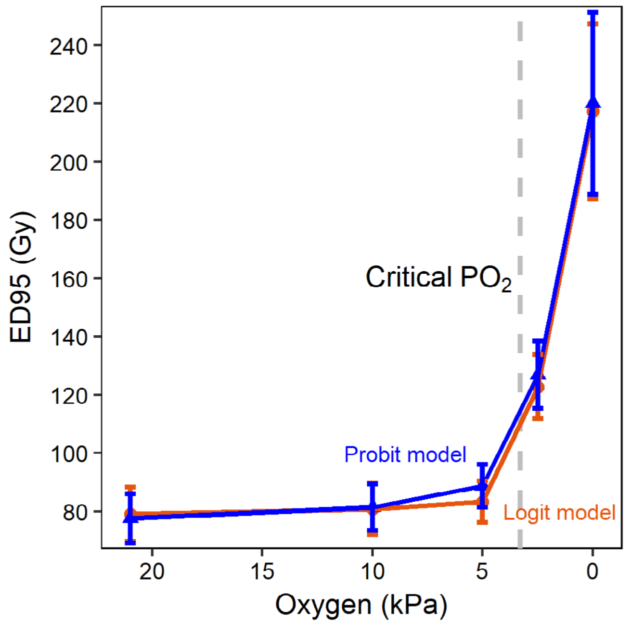

Figure 5. Radioprotection of *Trichoplusia ni* increased robustly when O_2 levels fell below their P_{crit} (dashed grey line). ED_{95} was estimated by either a probit or logit model, P_{crit} is estimated as 3.3 kPa. Bars represent 95% confidence intervals around the ED_{95} estimate.

3.4 What levels of O_2 may induce a radioprotective response that is important to phytosanitary irradiation?

Although there are many studies showing that anoxia or severe hypoxia (<2 kPa O_2) can increase insect radioprotection, to our knowledge there are only four other studies besides this one that have investigated radioprotection of moderately hypoxic atmospheres. Three of these studies have been performed by placing infested fruits in modified atmosphere bags where both O_2 and CO_2 concentrations are modified by the respiration of fruit in the bags.[14,22,23] One study placed late stage larvae of the melon fly, *B. cucurbitae* (Coquillett), into very hypoxic (1–4 kPa O_2), moderately hypoxic (3–8 kPa O_2), or mildly hypoxic (11–15 hypoxic O_2) modified atmospheres and irradiated them at a sublethal dose of 50 Gy.[23] The results showed that moderately or mildly hypoxic atmospheres did not enhance survivorship and even a very hypoxic atmosphere (1–4 kPa O_2) appeared to only increase survivorship to adulthood from 14 to 25%, but this was not a significantly detectable difference.[14] A very similar study recently showed that there was no detectable effect of hypoxia treatment between 3.2% and 15.4%, with CO_2 levels between 2.3% and 21.5% in *Drosophila suzukii* larvae treated at 60 Gy.[22] Another study in *B. dorsalis* showed that hypoxia treatment when combined with very high CO_2 increased larval mortality after radiation.[23] Srimartpirom *et al.*[23] concluded that commercial use of modified atmosphere packaging will not reduce the efficacy of the approved 150 Gy quarantine irradiation treatment for *B. dorsalis*. Combining commercially available modified atmosphere bags with radiation treatments is an excellent approach because it closely models how commodities are handled in the marketplace. Ultimately, we do need to understand how modified atmosphere packaging may affect radioprotection in real-world conditions. However, low oxygen levels generated by selective permeability of modified atmosphere packaging are also often correlated with very high levels of carbon dioxide, thus making it impossible to partition effects of hypoxia *versus* hypercapnia (high CO_2) on radiation susceptibility.

Here we have specifically altered O_2 levels while keeping CO_2 levels low to partition out the oxygen effect from any potential carbon dioxide effects with respect to radiation sensitivity (less than 1 kPa CO_2 for most of the treatments and less than 2 kPa CO_2 across all individual replicate jars). A previous study by our group examined how irradiation in a series of oxygen conditions (0.1–20.9 kPa O_2, all with less than 2 kPa CO_2) altered radioprotection of larvae and pupae of a model lepidopteran *T. ni* (Hubner).[17] These data showed that in both larvae and pharate adults (a.k.a. late pupae), severe hypoxia (<0.1 kPa) induced the greatest radioprotection, followed by a very small effect of moderate hypoxia (5 kPa) in only one of three treatments, and mild hypoxia (10 and 15 kPa) having no difference from normoxia.[17] In the present study with *T. ni*, we also showed that near anoxia (<0.1 kPa O_2) will induce the greatest radioprotection, followed by a substantial increase in radioprotection with severe hypoxia (<2.5 kPa O_2) below P_{crit} (Fig. 4). In our current study, moderate hypoxia (≥5 kPa O_2) had no detectable effect on radioprotection. Collectively, these studies show that moderate or mild hypoxia will not induce greater radioprotection of concern for phytosanitary irradiation. However, it is difficult to extrapolate a general response to different concentrations of O_2 for all insects based on five studies across four species. To determine if our hypothesis that radioprotection is only enhanced substantially by levels of low O_2 below an insect's P_{crit} is correct, we will have to study this relationship in a series of insect pests. Fortunately, P_{crit} values have been measured in more than 40 insect species,[31] giving us a roadmap to follow for testing this hypothesis. Ultimately, because controlled and modified atmospheres often include both low O_2 and high CO_2, additional studies that factorially vary both O_2 and CO_2 will be needed to tease apart what aspects of the atmosphere surrounding an insect directly affect radiation sensitivity.

4 CONCLUSIONS

Our study suggests that P_{crit} can be a diagnostic marker to predict the levels of atmospheric O_2 that may induce a radioprotective effect in insects. More testing across taxa is clearly needed, but P_{crit} has the potential to provide a simple and efficient way to evaluate risk for radioprotective effects across insects without having to perform laborious dose–response experiments, assisting regulators in developing future requirements with regard to combining phytosanitary irradiation treatments with modified atmospheres. Further research that allows a lowering of the threshold oxygen concentrations in modified atmosphere packaging that are eligible for use with phytosanitary irradiation treatments could facilitate increased trade and have substantial economic benefits for commodity producers and importers alike.

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