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RESEARCH ARTICLE

Short-term anoxic conditioning hormesis boosts antioxidant defenses, lowers oxidative damage following irradiation and enhances male sexual performance in the Caribbean fruit fly, *Anastrepha suspensa*

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SUMMARY

Most organisms are repeatedly exposed to oxidative stress from multiple sources throughout their lifetimes, potentially affecting all aspects of organismal performance. Here we test whether exposure to a conditioning bout of anoxia early in adulthood induces a hormetic response that confers resistance to oxidative stress and enhances male sexual performance later in life in the Caribbean fruit fly, *Anastrepha suspensa*. Anoxic conditioning of adults prior to emergence led to an increase in antioxidant capacity driven by mitochondrial superoxide dismutase and glutathione peroxidase. When exposed to gamma irradiation, a strong oxidative stressor, males that received anoxic conditioning had lower lipid and protein oxidative damage at sexual maturity. Anoxia conditioning led to greater male sexual competitiveness compared with unconditioned males when both were irradiated, although there was no effect of anoxia conditioning on mating competitiveness in unirradiated males. Anoxia also led to higher adult emergence rates and greater flight ability in irradiation-stressed flies while preserving sterility. Thus, hormetic treatments that increased antioxidant enzyme activity also improved male performance after irradiation, suggesting that antioxidant enzymes play an important role in mediating the relationship between oxidative stress and sexual selection. Furthermore, our work has important applied implications for the sterile insect technique (SIT), an environmentally friendly method of insect pest control where males are sterilized by irradiation and deployed in the field to disrupt pest populations *via* mating. We suggest that hormetic treatments specifically designed to enhance antioxidant activity may produce more sexually competitive sterile males, thus improving the efficacy and economy of SIT programs.

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INTRODUCTION

Oxidative stress is a pervasive challenge and has been implicated as a crucial factor mediating a range of organismal life history traits from longevity (Buttemer et al., 2010) and senescence (Beckman and Ames, 1998; Lipton, 1999) to reproduction (Metcalfe and Alonso-Alvarez, 2010) and sexual selection (von Schantz et al., 1999; Dowling and Simmons, 2009; Monaghan et al., 2009; Costantini et al., 2010a). Many stressors generate oxidative stress in animals including: low and/or high temperature (Storey and Storey, 2010; An and Choi, 2010), freezing (Joanisse and Storey, 1998; Hermes-Lima and Storey, 1993; Hermes-Lima et al., 1998), dehydration (Franca et al., 2007; Clark et al., 2009; López-Martínez et al., 2009; Benoit, 2010; Rizzo et al., 2010), ultraviolet (López-Martínez et al., 2008; Meng et al., 2010) and gamma irradiation (Peng et al., 1986; Datkhile et al., 2009), anoxia-reperfusion (Hermes-Lima and Storey, 1996; Hermes-Lima et al., 1998; Hermes-Lima et al., 2001) and hypoxia-reperfusion (Jamieson et al., 1986; Hermes-Lima et al., 2001). Stress from the accumulation of reactive oxygen species (ROS) and the resulting oxidative damage can have long-lasting effects on organismal performance, and organisms experience multiple bouts of oxidative stress throughout their lifetime, with some of these bouts involving simultaneous combinations of multiple stressors (Metcalfe and Alonso-Alvarez, 2010; Benoit et al., 2010; Teets et al., 2011; Marshall and Sinclair, 2011). Thus, an inducible and strong antioxidant response is crucial for organismal performance across many contexts, and organisms employ a suite of biologically active molecules that breakdown ROS.

Here we test two related hypotheses: (1) that exposure to a mild stressor will increase the activity of antioxidant enzymes and lower damage after exposure to an extreme oxidative stressor, ionizing radiation, and (2) that these treatments will lead to increased male sexual performance. These hypotheses are rooted in physiological conditioning hormesis (Calabrese et al., 2007), which occurs when organisms are exposed to sub-lethal stresses that induce physiological changes that lead to enhanced performance, such as greater longevity or stress resistance in later in life (Costantini et al., 2010b; Le Bourg, 2011). One type of hormetic conditioning response that has been intensively studied is rapid stress hardening, wherein a brief exposure to a stressor will very quickly lead to enhanced resistance to more severe applications of that stressor or cross-resistance to other stressors (Chen et al., 1987; Bayley et al., 2001; Benoit et al., 2009; Elnitsky et al., 2009; Chidawanyika and Terblanche, 2011). In this study, we hypothesized that a brief hormetic conditioning treatment, specifically, a short exposure to anoxia, would rapidly increase antioxidant levels in Caribbean fruit flies *Anastrepha suspensa* (Loew 1862) – hereafter caribflies – prior

to exposure to irradiation and reduce post-irradiation oxidative stress by inducing the highly conserved hypoxia-reperfusion response (Hermes-Lima and Zenteno-Savín 2002; Kim et al., 2008). Furthermore, we expected that flies exposed to treatments that enhance antioxidants early in adulthood would have greater downstream performance after exposure to irradiation stress later in life.

Our hypothesis is inspired by a flurry of work in recent years exploring linkages between oxidative stress, mate choice and carotenoid-based sexual ornamentation (Dowling and Simmons, 2009; Monaghan et al., 2009; Metcalfe and Alonzo-Alvarez, 2010; Costantini et al., 2010a). These studies have largely focused on the roles played by carotenoids as mediators of the relationship between oxidative stress and sexual selection due to their conspicuous roles in sexual ornamentation, yet carotenoids represent a small proportion of the complete antioxidant defenses of animals and the roles of non-carotenoid antioxidants in sexual selection remain largely unknown (Costantini, 2008; Costantini and Moller, 2008; Monaghan et al., 2009; Horak and Cohen, 2010). By testing whether hormetic treatments that increase the activity of endogenous antioxidant enzymes also increase male sexual performance after exposure to oxidative stress, our work begins to address this crucial gap in our knowledge of the relationship between antioxidant capacity and sexual selection.

Our work also has implications for improving an important tool in sustainable reduced-pesticide agriculture, the sterile insect technique (SIT). SIT is an area-wide pest suppression method that works by overwhelming wild females with sterile males, thus yielding inviable offspring. SIT has been particularly important for controlling infestations of invasive fruit flies in the US and Europe, where the public demands reduced pesticide use (Klassen and Curtis, 2005). Male insects for SIT are sterilized by exposure to ionizing radiation, typically from a gamma source or X-rays, causing randomly distributed double-strand breaks in genomic DNA. Chromosomal breaks are the result of both direct energy transfer to DNA and through secondary DNA damage caused by ROS produced during irradiation (von Sonntang, 1987). ROS generated during and after irradiation continue to induce other forms of cellular damage that ultimately lead to poor mating competitiveness of irradiated males compared with non-irradiated males (Calkins and Parker, 2005; Nestel et al., 2007). For example, irradiated male Mediterranean fruit flies (medflies) show reduced flight performance, female attraction and mating success compared with unirradiated males (Hooper, 1971; Ohinata et al., 1977; Nestel et al., 2007). Furthermore, many tephritid fruit flies, like caribflies and medflies, engage in highly competitive lek mating, wherein males aggregate together and display to attract females (Robinson et al., 2002). Thus sexual selection on male attractiveness is strong in these species, providing an opportunity to test the role of male oxidative status and endogenous antioxidants on sexual selection. However, if somatic cellular oxidative stress resulting from irradiation can be reduced by conditioning hormesis treatments, it may be possible to prevent some of the deleterious effects of irradiation sterilization on male sexual performance.

For more than 30 years it has been known that placing insects in anoxic or hypoxic conditions prior to and during irradiation leads to greater post-irradiation male performance without sacrificing sterility (Calkins and Parker, 2005). For example, irradiation of medflies in nitrogen increased male mating success and longevity (Hooper, 1971; Zumreoglu et al., 1979), while still preserving sterility (Hallinan and Rai, 1973). Even though irradiation in hypoxia or anoxia was shown to have performance benefits for sterilized males, its application is sparse and a comprehensive physiological mechanism for the observed performance benefits has not been proposed.

We propose that anoxia exposure treatments prior to and during irradiation improve male performance by inducing the hypoxia-reperfusion response, a physiological conditioning hormesis response that increases antioxidant capacity, thus decreasing or preventing oxidative stress. Specifically, we tested whether anoxia exposure can increase antioxidant capacity in early caribfly adults and characterized which antioxidant enzymes were involved in this process. We also tested whether anoxic conditioning could lower oxidative damage in irradiation-stressed and unstressed caribflies. Furthermore, we tested whether anoxia conditioning treatments that induced increased antioxidant activity also yielded enhanced male mating competitiveness and other performance measures in both unstressed and irradiation-stressed flies. We show that a brief anoxic conditioning treatment rapidly enhances antioxidant activity and lowers oxidative stress damage due to irradiation, while mediating sexual interactions in caribflies. We conclude that endogenous antioxidant enzymes are likely playing an important role in sexual selection in this highly competitive lek-mating species, and we identify antioxidant enzymes as a critical suite of mechanisms that can be manipulated to improve the SIT.

MATERIALS AND METHODS

Animal rearing

Larvae of the Caribbean fruit fly, *A. suspensa* (caribflies), were obtained from a colony maintained by the Florida Department of Agriculture and Consumer Services (FDACS) in Gainesville, FL, where caribflies are reared as part of an area-wide sterile insect technique pilot program. Larvae and pupae collected at FDACS were kept in our laboratory at 24°C and 85% relative humidity under long day conditions (14 h:10 h light:dark) in an incubator (Percival Scientific, Perry, IA, USA). After adult emergence, flies were kept in a temperature- (25°C) and humidity-controlled (60% relative humidity) room in standard 30×30×30 cm insect cages with unlimited access to food (3:1 sugar:yeast hydrolysate paste) and water.

Irradiation treatments

For SIT, *A. suspensa* flies are normally irradiated 2 days prior to adult emergence during pharate adult development while confined to the puparium; these pre-emergence adults are commonly referred to as pupae. Pupae were irradiated 2 days prior to emergence using a Gammacell Cs^{137} irradiator (GC45, Ottawa, ON, Canada) with a dose rate of 8.948 Gy min^{-1} at the Florida Accelerator Services and Technology facility within the Division of Plant Industry of FDACS. Several hundred fly pupae in polypropylene bags were placed in the center of the irradiation cylinder to ensure dose uniformity. Every 3 months, FWT-60 radiochromic film (Far West Technology, Goleta, CA, USA) or Gafchromic HD-810 film (International Specialty Products, Wayne, NJ, USA) was used to verify that the dosimetry administered to the pupae remained accurate. Three sets of dosimeters were placed among the bagged pupae (top, bottom and middle) and read 24 h after irradiation for dose verification (supplementary material Table S1). All pupae used in these experiments were confirmed to be alive *via* candling using a light table prior to treatment. Irradiation for most experiments was performed at 70 Gy (7 min 49 s exposure), which is the established dose for achieving 100% sterility in *A. suspensa* (Burditt et al., 1975). For the high-range dose response experiment, 200 and 300 Gy (22 min 21 s and 33 min 32 s of exposure, respectively) were used

because they encompass the range of doses required for sterility in other SIT programs around the world. A dose of 400 Gy (44 min 42 s of exposure) was used as an extreme treatment because it was expected to yield high mortality. Pupae were irradiated in one of three atmospheric treatments at normal room temperature: (1) in the presence of oxygen [normoxia (Nx)], (2) in the absence of oxygen in a nitrogen atmosphere [anoxia (Ax)] after an hour-long anoxia pre-treatment or (3) irradiation in normoxia after the 1 h anoxia pre-treatment [anoxia plus reperfusion (AxR)]. The anoxia non-irradiated group was kept in anoxia for an additional 8 min after the 1 h pre-treatment to simulate the anoxia period of the irradiated (Ax70) individuals.

The anoxia pre-treatment consisted of placing the pupae in polypropylene bags and flushing the bag with nitrogen for 1 min, more than long enough to displace the volume of oxygen in the bag. The bags were then sealed and placed inside a second bag, nitrogen flushed and kept sealed for 1 h. Preliminary experiments showed that 1 h of anoxia was harmless to pupae and led to a strong increase in antioxidant capacity prior to and after reperfusion; therefore, we used the 1 h treatment for the rest of the experiments. Animals in normoxia treatments were sealed in a similar polypropylene bag that was heavily perforated to allow air exchange. The pupae in the anoxia-irradiation treatment were then irradiated in the sealed bags and were not reperfused with oxygen until after irradiation was completed. The anoxia reperfused pupae (AxR) were transferred from sealed nitrogen bags after the 1 h treatment and placed in perforated bags, just like normoxia treated pupae, allowing air flow and respiration during irradiation. From this additional treatment, only adult emergence was recorded in order to explore any additional effect of irradiating in anoxia after the hour-long pre-treatment. All other anoxia treatments involved both the hour-long pre-treatment and irradiation in anoxia. After irradiation, a subset of pupae from each replicate were immediately frozen in liquid nitrogen while the rest were allowed to recover and emerge in insect cages and were sampled at a later time. The treatments used for this set of experiments were: normoxia and no radiation (NxNr), anoxia and no radiation (AxNr), normoxia and 70 Gy (Nx70), and anoxia and 70 Gy (Ax70). The treatments in the high-dose experiments were: normoxia and no radiation (NxNr), anoxia and no radiation (AxNr), anoxia plus reperfusion and no radiation (AxRNr), normoxia and 200 Gy (Nx200), anoxia and 200 Gy (Ax200), anoxia and 200 Gy plus reperfusion (AxR200), normoxia and 300 Gy (Nx300), anoxia and 300 Gy (Ax300), anoxia and 300 Gy plus reperfusion (AxR300), normoxia and 400 Gy (Nx400), anoxia and 400 Gy (Ax400), and anoxia and 400 Gy plus reperfusion (AxR400). Anoxia exposure in the high-dose experiment was adjusted so the anoxia treatments received a uniform anoxia dose of 1 h 44 min, based on the exposure required for the Ax400 group.

Antioxidant activity

Pupae were collected at 0, 0.5, 2, 4, 8, 24 and 48 h after 1 h of anoxia to quantify the effect of the exposure on total antioxidant capacity and enzyme activity; similar controls were taken from normoxia groups. Pupae were frozen in liquid nitrogen and stored at –70°C until assayed. Trolox-equivalent antioxidant capacity (TEAC) was measured using the 2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical cation decolorization assay (Re et al., 1999), and compared against a Trolox standard curve. Three pools of five pupae each (~50 mg) per treatment were homogenized using a Fast Prep 120 bead homogenizer (Qbiogene Inc., Carlsbad, CA, USA) with 2 mm zirconia beads (Biospec Products Inc., Bartlesville, OK, USA) in PBS and centrifuged at 5000 ***g*** for 5 min at 4°C. This homogenate was diluted to a concentration of 2 mg protein ml^{-1} and combined with a solution of 7 mmol l^{-1} ABTS and 2.45 mmol l^{-1} potassium persulfate (Sigma-Aldrich, St Louis, MO, USA) that had rested in the dark at 25°C overnight. Antioxidant capacity was measured at 734 nm precisely 10 min after the addition of the ABTS solution to the diluted insect homogenate. Samples were quantified using an eight-point Trolox standard curve [0–150 µmol l^{-1} (ml^{-1})] and are expressed as Trolox equivalents per milligram of soluble protein.

Superoxide dismutase (SOD) activity was measured using the SOD assay kit from Cayman Chemical (Ann Arbor, MI, USA). We separated out the cytosolic SOD (Cu-ZnSOD) from mitochondrial SOD activity (MnSOD) according to the manufacturer's protocol by centrifuging the initial homogenates (pools of five pupae per treatment) at 10,000 ***g*** for 15 min at 4°C to remove the mitochondrial fraction prior to mitochondrial lysis. The values for this assay are presented as SOD units per milligram of soluble protein, where one unit of SOD is defined as the amount of enzyme required for 50% dismutation of the superoxide radical.

Catalase activity was measured using the Aebi (Aebi, 1983) catalase assay with modifications by Wheeler et al. (Wheeler et al., 1990). An assay reagent mixture composed of 250 mmol l^{-1} phosphate buffer, methanol and 44 mmol l^{-1} hydrogen peroxide was added to the homogenized sample (groups of five pupae per treatment) and incubated for 20 min. A solution of 22.8 mmol l^{-1} Purpald in KOH (Fisher Scientific, Fair Lawn, NJ, USA) was added, and the mixture was incubated for an additional 20 min. Finally, a 65.2 mmol l^{-1} potassium periodate (Sigma-Aldrich) and KOH solution was added to the assay wells, and after 5 min the absorbance was read at 550 nm. Because the product of the reaction in this assay is formaldehyde, catalase activity was quantified using a seven-point formaldehyde standard curve [0–75 µmol l^{-1} (ml^{-1})]. Data are presented as units per milligram of soluble protein, where one unit is the amount of known enzyme that yields 1 nmol of formaldehyde per minute at 25°C.

Glutathione peroxidase (GPx) activity was measured using the GPx assay kit from Cayman Chemical according to the manufacturer's protocol. By monitoring the absorbance of the samples with the enzyme mixture at 340 nm for 5 min, the slope was used to calculate enzyme activity quantified against a GPx standard curve. GPx activity is presented as GPx units per milligram of soluble protein, where one unit is the amount of the enzyme required to oxidize 1 nmol of NADPH per minute at 25°C.

Oxidative damage

We quantified two metrics of lipid peroxidation, one measuring the early effects of oxidative stress (lipid hydroperoxides) and the other measuring one of the end products of this damage pathway (malondialdehyde). Lipid hydroperoxides were measured using a commercially available kit from Cayman Chemical following the manufacturer's guidelines. This method begins with a chloroform:methanol extraction that precipitates proteins, thus eliminating contamination of protein carbonylation products in estimates of lipid peroxidation. After extraction and separation of the organic and aqueous phases, the assay was performed in chloroform to decrease potential interactions of hydrogen peroxides with ferrous ions, therefore avoiding an overestimation of lipid hydroperoxides. The data are presented as µmol l^{-1} per milligram of soluble protein.

The main aldehyde product of lipid peroxidation, malondialdehyde (MDA), was measured using the thiobarbituric acid reactive substances test (TBARS). The assay performed was

adapted from those previously described (Uchiyama and Mihara, 1978; Ohkawa et al., 1979). Groups of five male flies per treatment were homogenized in RIPA buffer with EDTA (Fisher Scientific). One aliquot of the homogenates was treated with 10% trichloroacetic acid and incubated in ice to precipitate the proteins out of the homogenate, eliminating protein carbonyl contamination, whereas the other aliquot was used to quantify protein content for standardization. After centrifugation at 2200 ***g*** for 15 min in 4°C, the supernatant was combined with a 0.67% (w/v) thiobarbituric acid solution, and the samples were placed on a heating block at 95°C for 1 h. The samples were then allowed to rapidly cool to 25°C (in an ice bath) and were centrifuged (2200 ***g*** for 5 min) prior to being dispensed into a 96-well plate and read at 532 nm. Quantification of MDA used an eight-point MDA standard curve [0–50 μmol l^{-1} (ml^{-1})] and data are presented as μmol l^{-1} of MDA per milligram of soluble protein.

Oxidation of proteins was measured using the method established by Levine et al. (Levine et al., 1990). This method consists of using an excess amount of 2,4-dinitrophenylhydrazine (DNPH) (Fisher Scientific) to extract the carbonyls, trichloroacetic acid to precipitate them, and repeated washes in a 1:1 ethanol:ethyl acetate solution to extract the remaining excess DNPH. The resulting protein precipitate was diluted in 6 mol l^{-1} guanidine hydrochloride. The samples were then read at 370 nm against sample blanks processed in HCl. A nine-point BSA standard curve (0–2 mg ml^{-1}) and protein blanks were used to quantify protein concentration and standardize the results. Male flies were homogenized in a 5% sulfosalicylic acid solution. Data are presented as nmol mg^{-1} soluble protein.

Organismal performance

Adult emergence (eclosion), sex ratio, flight ability and longevity under stress tests were performed in accordance with the International Atomic Energy Agency (IAEA) guidelines for sterile mass-reared tephritid fruit flies (FAO/IAEA/USDA, 2003). Eclosion was monitored by placing one treated pupae per well in a 96-well plate with lid (five replicate plates per treatment; Fisher Scientific) and allowing the flies to emerge, noting the emergence percentage and sex ratio. Flies that fully emerged from the puparium and were able to completely extend their wings were counted as eclosed. Partially emerged flies and flies with malformed wings were scored as failure to emerge. Flies that did not emerge were counted as dead. Flight ability was tested by placing 100 pupae inside a paper ring in a Petri dish. The Petri dish was covered with a black PVC cylinder measuring 10 cm in height and 8.9 cm in diameter, with the inside coated with talcum powder to encourage flying rather than walking out of the tubes; flies that were found outside the tubes were scored as having flown. An additional tube with Petri dish that contained no pupae was placed inside the cage to estimate the number of flies flying back into the tubes, allowing us to adjust the final flight ability percentages to include fly-backs. Flight ability was tested in the absence of food and water. Three replicate cages per treatment were used for flight ability tests. Longevity under stress involved tracking how many of the flies were still alive 48 h after emergence without having access to food or water. The data are presented as percent eclosion, male:female ratio, percent flyers and percent survival at 48 h.

Sexual performance

To assess the effects of irradiation and the anoxia treatment on male sexual performance, we used assays where untreated females were provided males from two different treatments and we recorded which male mated successfully (Pereira et al., 2010). We separated virgin flies no later than 5 days after emergence because sexual maturation in caribflies can be completed within 7 or 8 days (Calkins et al., 1988). Flies were age synchronized in all the treatments and mating trials were always conducted 10, 11 or 12 days after adult emergence. Pupae were dusted with orange, blue or green fluorescent powder (DayGlo, Cleveland, OH, USA) to identify their treatment group, and as adult flies emerged the pigments were retained inside the sutures of the head and were visible under an ultraviolet light source. Powder colors were alternated between the treatments to ensure that no color bias occurred, even though a lack of bias has been previously demonstrated (Serghiou, 1977). Virgin untreated females and virgin treated and untreated males were transferred to separate cages 3 to 5 days after emergence and were kept separated until mating trials were performed. Two males from pair-wise combinations of the four treatments were placed in a wire mesh cage measuring 8.5 cm in height and 7 cm in diameter, and an untreated female (normoxia and no radiation) was added to the cage to start the trial. Because sexual display in this species involves both male competition and female choice (Dodson, 1982; Burk, 1983), we first placed both males in the cage and allowed them to acclimate for 20 to 30 min. Females were then introduced and we monitored them while they displayed and until they successfully mated. In addition to scoring mating success in each trial, the time between the introduction of the females and the beginning of mating (the period of latency to mating) was recorded for all four comparisons. Females were replaced if they did not participate in courtship display after 1 h (well beyond the average period of latency to mating) and/or if they were actively egg-dumping. The males that successfully mated with females were then collected, and their heads were examined under UV light to identify their treatment. Two groups of 15 cages were set each day for 3 days (N=15, for each of six replicates). The experiment was then repeated the following week with a different male–male comparison until all four comparisons (NxNr *vs* Nx70, Nx70 *vs* Ax70, NxNr *vs* Ax70 and NxNr *vs* AxNr) had been performed in three separate weeks using three different cohorts.

Sterility

We tracked the number of eggs laid (fecundity) and number hatched (fertility) to assess the effects of the anoxia pre-treatment on sterility at the 70 Gy dose. Ten replicate groups of five treated (Ax70) or five untreated (NxNr) males were placed in cages with food and water, and constant access to five untreated non-sterile females (NxNr). An oviposition substrate was provided using agar egging domes. Plastic trays were used to create 2% agar spheres (Fisher Scientific) that were colored with food dye (Yellow no. 5 and no. 6) to enhance oviposition (Sharp et al., 1975) and tightly wrapped in parafilm to simulate fruit skin rigidity (Boller, 1968). Agar domes were placed in the morning of the tenth day of adult life and were replaced every 24 h for 5 days. Eggs were only collected in the first week of oviposition as it has been shown in fruit flies that egg load decreases over the reproductive lifetime (Prokopy and Boller, 1970). A week after removal from the mating cages, the agar domes were slowly melted in hot water and carefully sieved. Eggs were counted and scored as hatched or unhatched.

Statistical analyses

All biochemical experiments were conducted with three replicates per treatment, where each replicate was a pool of five individual flies, and all assays were standardized by total soluble protein (mg ml^{-1}). To assess the repeatability of our results and increase our statistical power, each replicated experiment was also repeated

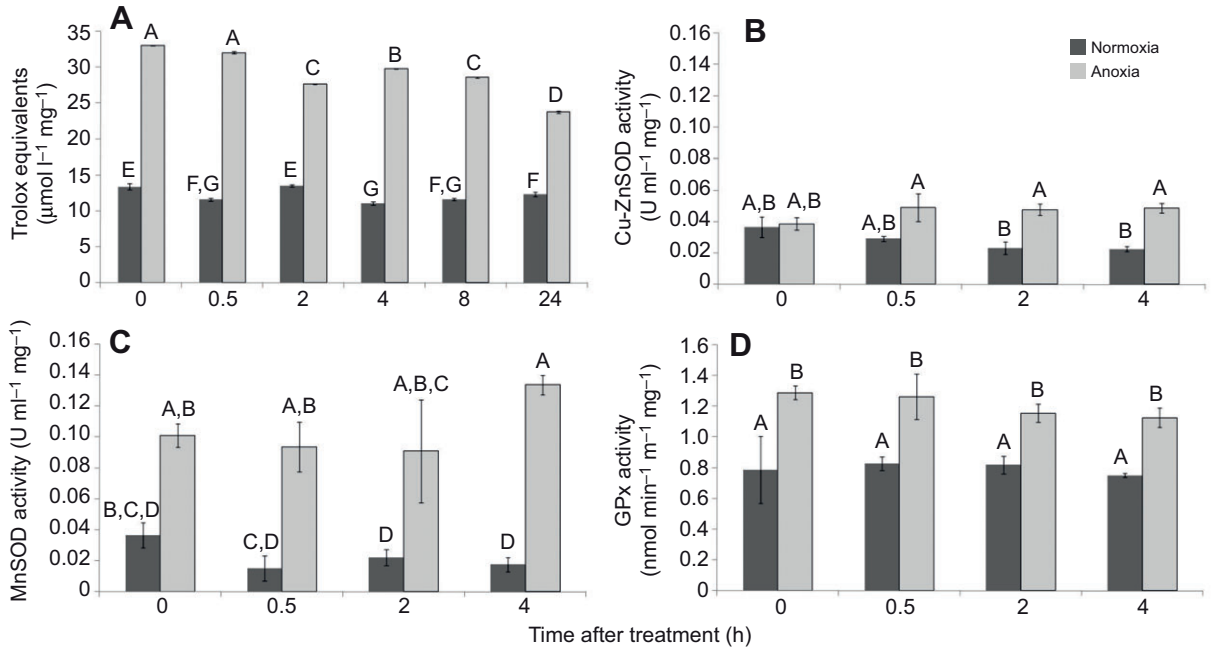

Fig. 1. Antioxidant capacity in *Anastrepha suspensa*. (A) Elevated total antioxidant capacity was correlated with increased activities of (B) cytoplasmic superoxide dismutase (Cu-ZnSOD), (C) mitochondrial superoxide dismutase (MnSOD) and (D) glutathione peroxidase (GPx). This increase in antioxidant activity is apparent by the end of 1 h of anoxia treatment and the increase is still evident 24 h after treatment. Groupings are based on two-way ANOVAs (A–C) or one-way ANOVA (D) followed by Tukey's HSD test.

three times with different cohorts of flies (i.e. experimental blocks), with the exception of the glutathione peroxide and lipid hydroperoxides assays, which were repeated only twice. Initial analyses included this block term to account for possible differences between experiments repeated on successive cohorts of flies. However, we never detected an effect of experimental block and thus experimental blocks were combined and the block term was eliminated from the final models. For ANOVAs, treatment × time (two-way) or treatment × time × radiation (three-way) interactions were also considered and retained in models when significant (P<0.05). Tukey's honestly significant difference (HSD) corrections for multiple comparisons were then used within each ANOVA model to separate experimental groups. When there were multiple interactions between model terms (i.e. three-way interactions), we also applied specific linear contrasts to ask whether anoxia treatment yielded lower damage than animals treated in normoxia following irradiation stress at the time of reproductive maturity (day 10). Each biochemical assay of antioxidant activity was performed on a separate sample and was analyzed with two-way ANOVA with experimental treatment (normoxia/anoxia) and time (after treatment) as explanatory variables. Each biochemical assay of oxidative damage was performed on a separate sample and was analyzed with three-way ANOVA with experimental treatment, irradiation treatment (0 or 70 Gy) and time as explanatory variables. Organismal performance and sterility experiments were analyzed using ANOVAs (one-way or two-way) followed by Tukey's HSD. Male mating performance was analyzed using logistic regression.

RESULTS

Antioxidant activity

Total antioxidant capacity was increased by more than twofold in the caribfly pupae after 1 h of anoxic conditioning and this increase was maintained for 24 h ($F_{11,24}$=2145.25, model P<0.0001, treatment P<0.0001, time P<0.0001, interaction P<0.0001; Fig. 1A), but declined until there was no difference at 48 h ($F_{1,4}$=0.3046, P=0.6104, data not shown). This increase in total antioxidant capacity was associated with increases in MnSOD and GPx but not catalase. Cu-ZnSOD activity was not elevated after 1 h anoxic conditioning compared with normoxia (Fig. 1B), but it was higher in the anoxic-conditioned pupae than normoxia-treated pupae during recovery ($F_{7,16}$=5.1828, model P=0.0031, treatment P<0.0001, time P=0.8375, interaction P=0.0964). MnSOD activity increased immediately following the anoxia treatment and remained high during recovery ($F_{7,16}$=10.131, model P<0.0001, treatment P<0.0001, time P=0.4217, interaction P=0.3090; Fig. 1C). Catalase activity did not increase in response to anoxia ($F_{7,16}$=0.3996, model P=0.8888, treatment P=0.7172, time P=0.5937, interaction P=0.8771; data not shown). GPx activity was also substantially elevated in response to anoxia treatment and remained high for at least the 4 h recovery period after anoxia exposure ($F_{7,12}$=4.2792, model P=0.0136, treatment P=0.0002, time P=0.7539, interaction P=0.8906; Fig. 1D). Overall, anoxia pretreatment led to higher total antioxidant capacity, substantially greater MnSOD activity, and greater GPx activity. This led us to predict that anoxia-pretreated individuals would suffer less oxidative damage due to irradiation.

Oxidative damage

Quantities of lipid hydroperoxides were low just after emergence (day 1) across all treatments and increased by the time of male sexual maturation (day 10; Fig. 2A). At the time of sexual maturity (10 days), males had substantially less lipid hydroperoxide damage when treated with anoxia and irradiated (70 Gy) than males irradiated in normoxia ($F_{6,33}$=50.41, model P<0.0001, time P<0.0001, treatment P=0.7891, radiation P=0.1764, treatment × time P=0.0003; linear contrast, P<0.0001; Fig. 2A). Consistent with the lipid hydroperoxide pattern, at day 10, males had substantially less

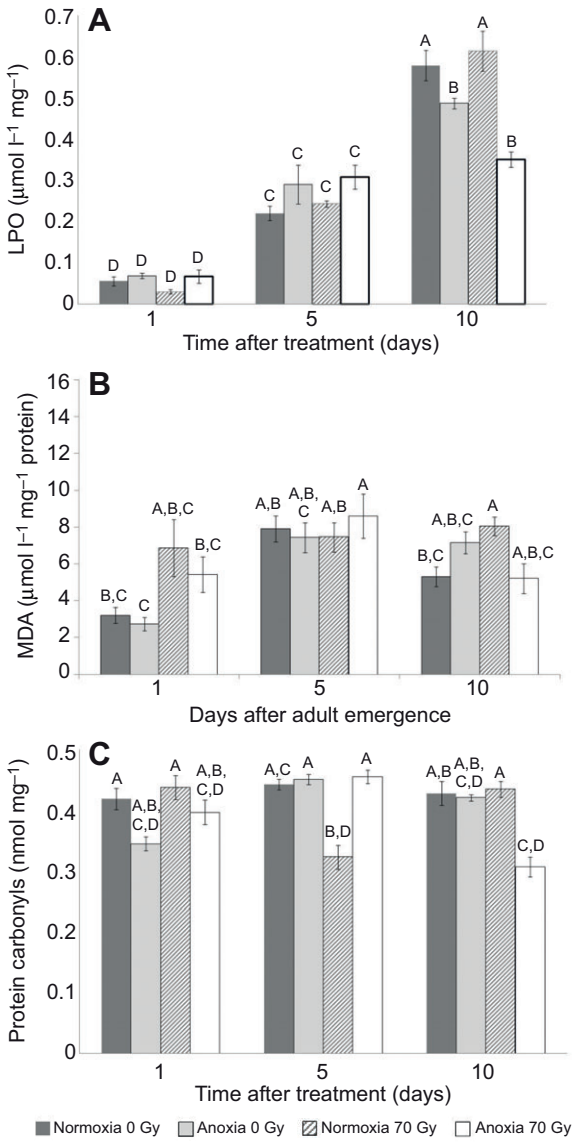

Fig. 2. Oxidative damage in *Anastrepha suspensa*. (A) Lipid hydroperoxides at the time of mating were significantly lower in flies irradiated in anoxia. (B) Another measure of lipid oxidation damage (lipid peroxidation) reveals that anoxia-70 Gy flies had lower damage than normoxia irradiated individuals, but comparable damage to non-irradiated individuals. (C) Post-irradiation protein oxidative damage was also lowest for the anoxia-70 Gy group. Groupings are based on three-way ANOVAs followed by Tukey's HSD test.

TBARS when treated with anoxia and irradiated than males irradiated in normoxia ($F_{9,87}$=5.31, model P<0.0001, time P<0.0001, treatment P=0.782, radiation P=0.0151, treatment × time P=0.2743, treatment × radiation P=0.6518, treatment × time × radiation P=0.0313; linear contrast, P=0.0252; Fig. 2B). Also at 10 days, males had substantially less protein carbonyls when treated with anoxia and irradiated (70 Gy) than males irradiated in normoxia ($F_{9,98}$=5.71, model P<0.0001, time P=0.037, treatment P=0.040, radiation P=0.1033, treatment × time P=0.253, treatment × radiation P=0.3385, treatment × time × radiation P=0.0002; linear contrast, P<0.0001; Fig. 2C). Overall, although some patterns of oxidative damage had complex interactions between treatments and time, all three measures of oxidative damage were detectably lower at the time of sexual maturation in anoxia-irradiated males than in those irradiated in normoxia. Thus, our anoxia treatment did indeed have a hormetic effect in irradiated males, blunting oxidative damage at the time of sexual maturity.

Organismal performance

There was no effect of anoxia pretreatment or irradiation on adult emergence at 70 Gy, the standard target dose for sterility ($F_{3,24}$=0.1358, model P=0.9377, treatment P=0.9376, radiation P=0.5729, interaction P=0.7875; Fig. 3A). Anoxia treatment did improve the flight ability of irradiated pupae compared with normoxia-irradiated pupae, but anoxia treatment had no detectable effect on flight performance in unirradiated individuals ($F_{1,15}$=7.4718, P=0.0154; Fig. 3B). Anoxia had no effect on the sex ratio of irradiated flies but a 10% increase in the ratio of females to males was noted for the non-irradiated anoxia group ($F_{3,16}$=8.1419, P=0.0016; Fig. 3C). Adults emerging from pupae irradiated in anoxia survived substantially longer in the absence of food and water than adults from pupae irradiated (70 Gy) in normoxia, a trend also present in unirradiated flies ($F_{3,7}$=27.1652, model P=0.0003, treatment P=0.0001, radiation P=0.814, interaction P=0.0024; Fig. 3D).

The hormetic effects of anoxia before and during irradiation were even more pronounced under the greater oxidative stress caused by higher radiation doses ($F_{11,24}$=556.914, model P<0.0001, treatment P<0.0001, radiation P<0.0001, interaction P<0.0001; Fig. 4A). Although adult emergence decreased precipitously with increased radiation (200, 300 and 400 Gy) doses in normoxic animals, even high doses of irradiation did not alter adult emergence success in pupae exposed to 1 h of anoxic pre-treatment and then irradiated in anoxia (Fig. 4A). Pupae exposed to 1 h of anoxia pre-treatment followed by reperfusion and irradiation in normoxia had substantially higher emergence than pupae kept in normoxia, but lower adult emergence than pupae pretreated and irradiated in anoxia, suggesting that both anoxic pretreatment and irradiation in anoxia provide benefits for oxidative stress resistance, particularly at higher doses.

Flight ability decreased in a dose-dependent manner with increased exposure in normoxia-irradiated flies, but anoxia substantially rescued flight ability even at high radiation doses ($F_{7,16}$=9.3717, model P<0.0001, treatment P=0.0023, radiation P<0.0001, interaction P=0.0437; Fig. 4B). Sex ratio was also heavily affected by radiation dose and the males were more radiation sensitive than females (Fig. 4C). The average sex ratio in this caribfly colony is ~1:1 male:female, and as radiation exposure increased so did male mortality ($F_{3,8}$=100.835, P<0.0001). Anoxia treatment in the absence of irradiation also led to a slight drop in male emergence (38% male and 62% female), but anoxia treatment substantially blunted the effects of high doses of irradiation on male mortality and male emergence was substantially greater at high irradiation doses in anoxia-treated groups ($F_{7,16}$=11.5572, P<0.0001; Fig. 4D).

A subset of the flies that failed to emerge as adults was unable to extend their wings after emergence because of irradiation damage, and thus those flies were counted as failure to emerge. Another group of pupae died after irradiation and/or prior to emergence, and mortality increased with radiation exposure in the normoxia treatment groups ($F_{11,47}$=490.2233, model P<0.0001, radiation P<0.0001, interaction P<0.0001; supplementary material Fig. S1A). Post-irradiation mortality for all four anoxia-irradiated groups was low and unaffected by irradiation dose ($F_{3,16}$=0.3952, model P=0.7582, radiation P=0.7582; supplementary material Fig. S1B). Pupae that were reperfused and irradiated in oxygen after anoxia conditioning (anoxia plus reperfusion) had higher mortality after irradiation than those irradiated in anoxia ($F_{7,31}$=13.3199, model

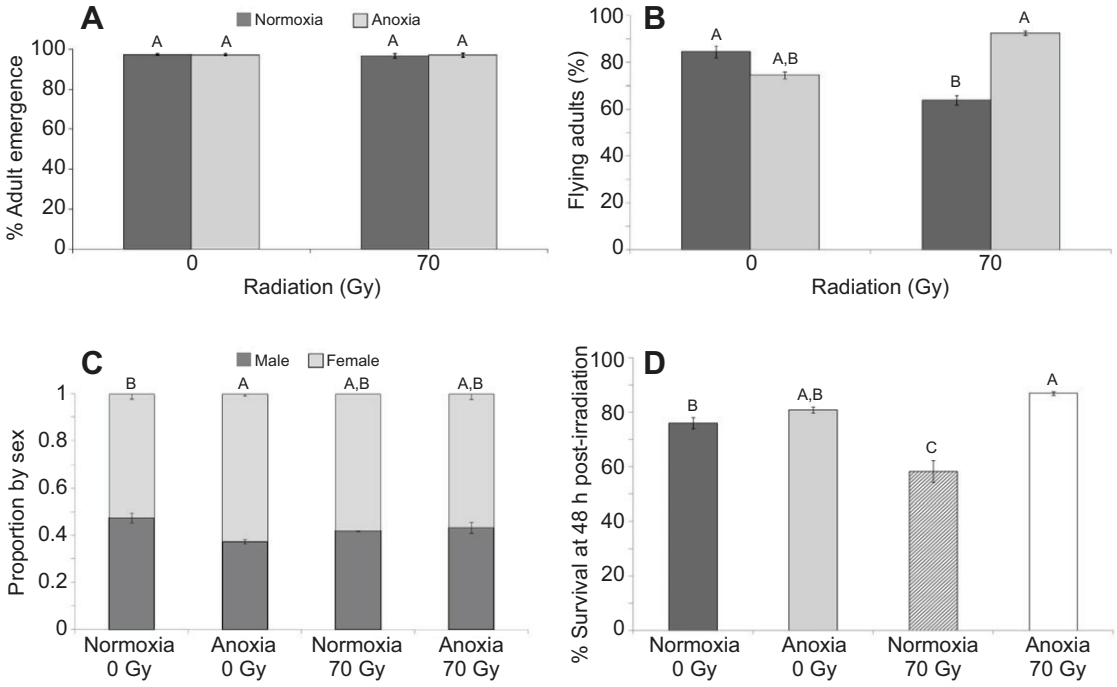

Fig. 3. Organismal performance at the standard irradiation dose (70 Gy). (A) Neither anoxia nor irradiation altered adult emergence. (B) One hour of anoxia exposure led to better post-irradiation flight performance. (C) Anoxia affected the sex ratio of emerging flies that were not irradiated but had no effect on irradiated groups. (D) Anoxia-irradiated flies had higher survival at 48 h in the absence of food and water. Groupings are based on one-way ANOVAs followed by Tukey's HSD test.

P<0.0001, radiation P=0.0004, interaction P<0.0001; supplementary material Fig. S1B). Mortality for the anoxia reperfusion groups increased with irradiation dose.

Sexual performance

Anoxia treatment improved male mating success when males were exposed to irradiation stress, but there was no effect of anoxia treatment on mating performance in unirradiated males. Normoxia-irradiated males mated poorly when competing against non-irradiated males (χ^2=36.8297, P<0.0001, d.f.=1; χ^2_{block}=0, P>0.05; Fig. 5A). Anoxia treatments that enhanced antioxidant defenses improved male mating success after exposure to irradiation stress (χ^2=31.7178, P<0.0001; Fig. 5B). There was no difference in mating performance between non-irradiated males and anoxia-irradiated males (χ^2=0.0909, P=0.763; Fig. 5C), reinforcing the hypothesis that anoxia conditioning rescues the deleterious effects of irradiation on male sexual performance. Anoxia conditioning had no effect on mating success when males were not exposed to irradiation (χ^2=0.1740, P=0.6766; Fig. 5D). Beyond male–male competition for mating, there were no effects of anoxia or irradiation treatment on the period of latency to mating across all treatments ($F_{3,82}$=0.1252, P=0.945; supplementary material Fig. S2).

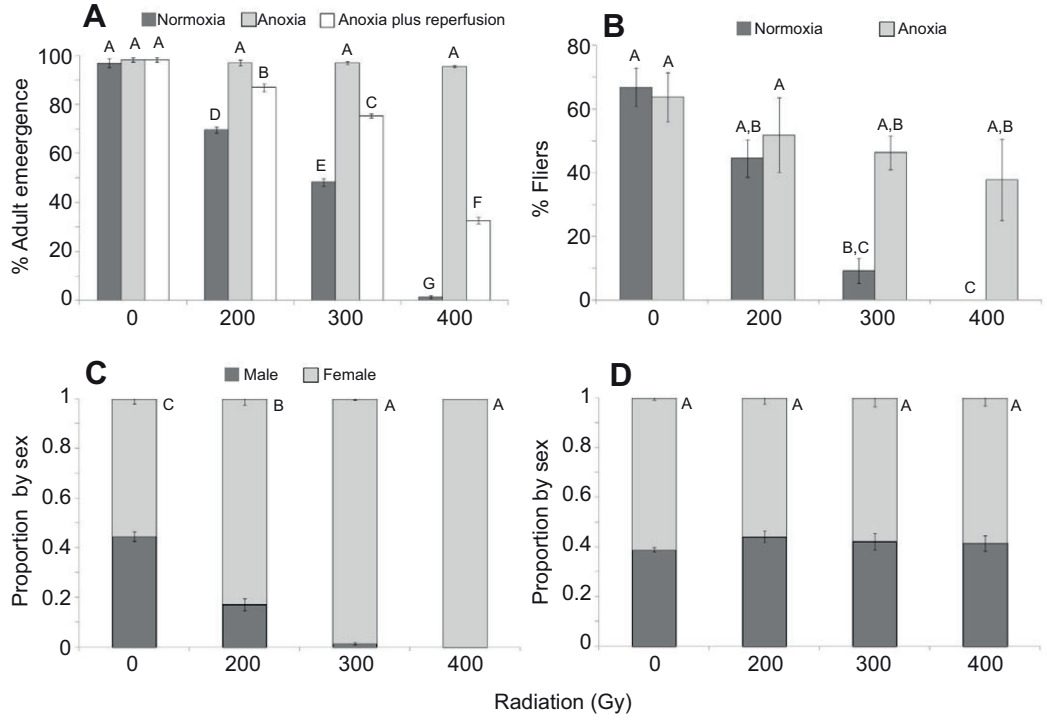

Fig. 4. Performance of *Anastrepha suspensa* at high irradiation doses. (A) Adult emergence, (B) flight ability and (C) male survivorship decreased in a radiation dose-dependent manner for flies irradiated in normoxia, but all of these performance effects were rescued by the anoxia pre-treatment and irradiation in anoxia. (D) Flies that were also irradiated in anoxia in addition to the pre-treatment had lower mortality that those reperfused and irradiated in normoxia. Groupings are based on two-way ANOVAs followed by Tukey's HSD test (A,B) or on one-way ANOVAs followed by Tukey's HSD test (C,D).

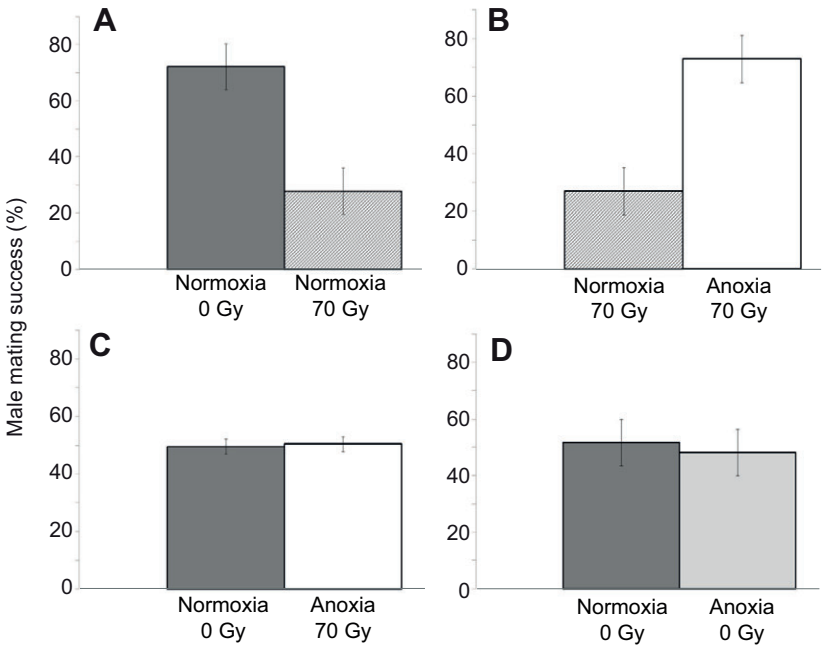

Fig. 5. Sexual performance of *Anastrepha suspensa*. (A) Non-irradiated males were more successful at mating than males irradiated in normoxia. (B) However, anoxia-irradiated males mated significantly more than normoxia-treated and irradiated males without the antioxidant boost. (C) Anoxia did allow irradiated males to mate with equal likelihood to those males not irradiated. (D) Anoxia conditioning treatment had no effect on mating success for unirradiated males. Sexual performance data were analyzed using logistic regression.

Even though our anoxia treatment substantially improved several metrics of male performance, anoxia-treated males remained sterile ($F_{1,8}$=6635.25, P<0.0001; Fig. 6A). In addition to being effectively sterilized, untreated females that mated with anoxia-treated sterile males showed a substantial reduction in fecundity (~40% fewer eggs laid) compared with females paired with non-treated fertile males ($F_{1,8}$=8.5838, P=0.019; Fig. 6B).

DISCUSSION

Anoxia conditioning of late-stage caribfly pharate adults led to: (1) a rapid and substantial increase in antioxidant capacity and (2) greater performance after irradiation stress, including greater male mating success. The increased antioxidant capacity response to 1 h of anoxia exposure was maintained for 24 h, but had diminished by 48 h. Several metrics of oxidative damage were lower in anoxia-treated and irradiated flies at the peak of sexual maturity 10 days after adult emergence (12 days after the conditioning event) than in flies irradiated without anoxia treatment. Thus, exposure to anoxic hormetic conditioning ameliorated the effects of irradiation stress early in adulthood, yielding both short-term benefits for adult emergence and flight ability in young adults and long-term benefits on male mating performance later in life.

As expected from the highly conserved low oxygen-reperfusion response (Hermes-Lima and Zenteno-Savín, 2002), anoxic conditioning led to a substantial increase in antioxidant capacity in pre-emergence adult caribflies. This strong and persistent increase in total antioxidant capacity was associated with substantial increases in both MnSOD and GPx. Cu-ZnSOD did not increase by the end of the anoxia treatment, but activity was higher in anoxia-treated individuals during the recovery period compared with individuals that did not undergo anoxic conditioning (Fig. 1B). The superoxide anion is produced in the mitochondria and MnSOD represents the first line of defense prior to ROS leakage into the cytosol once respiration resumes. Oxygen reperfusion and the resumption of respiration can lead to an increase of superoxide anions in the cytosol, perhaps driving increased elevation of Cu-ZnSOD. The increased activity of GPx during anoxia and the consistent activity thereafter correlate with the production of hydrogen peroxide resulting from the dismutation of superoxide. We did not observe an increase in catalase activity during anoxia or after reperfusion, but perhaps the concentration of hydrogen peroxide was not high enough to warrant elevated levels from what is already high catalase activity in caribflies compared with other flies (Grubor-Lajsic et al., 1996; Forcella et al., 2007).

Anoxic conditioning treatments that enhanced antioxidant activity in pre-emergence adults also improved several metrics of early adult organismal performance in irradiation-stressed individuals and later-life male mating performance. However, antioxidant-boosting anoxic conditioning did not enhance any metric of performance in unstressed individuals, an observation that is consistent with physiological conditioning hormesis. Higher antioxidant levels have been associated with greater mating competitiveness and sexual selection in numerous taxa (Peters et al., 2004; Catoni et al., 2008; Pérez-Rodríguez, 2009; Costantini et al., 2010a; Metcalfe and Alonzo-Alvarez, 2010), although this work has typically focused on diet-derived carotenoids rather than endogenous antioxidants. Caribflies have a highly competitive lek-mating system with strong potential for sexual selection on male condition (Dodson, 1982; Burk, 1984; Sivinski, 1989; Pereira et al., 2010), so oxidative stress is expected to be deleterious to male mating success. In the field, we expect that adult caribflies are exposed to many situations that induce oxidative stress, from heat and desiccation to the high metabolic costs of courtship in a lek. Male caribflies take 8–10 days to become sexually mature, but our experiments to date have focused on fully developed adults prior to emergence, and anoxia-induced increases in antioxidant capacity disappeared within 48 h, around the time of adult emergence, a full 10 days before sexual maturation. Enhancing antioxidant capacity with a bout of anoxia or by other means during the period of sexual maturity could potentially enhance male mating success. Determining whether increased antioxidant enzyme capacity enhances male mating competitiveness in caribflies in the context of natural stressors less severe than irradiation, as suggested in vertebrates (Peters et al., 2004; Catoni et al., 2008; Costantini et al., 2010a), will require further experimentation manipulating antioxidant capacity and oxidative stress during adulthood prior to and during the peak of sexual

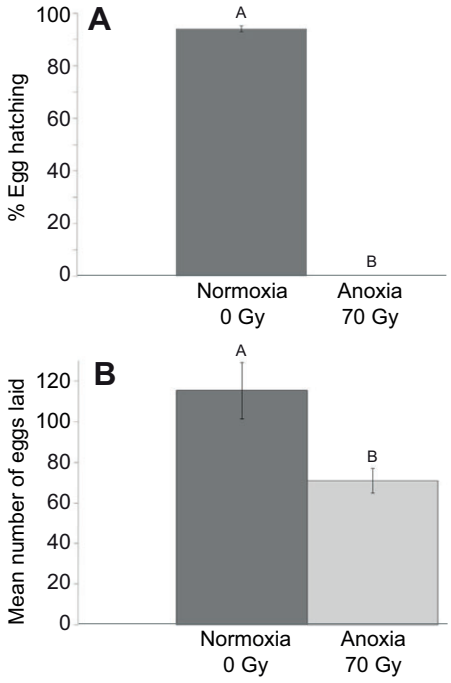

Fig. 6. Sterility in *Anastrepha suspensa*. (A) Fertility was not affected by the anoxia pre-treatment, as 0% of eggs laid by anoxia-irradiated flies hatched compared with 94% hatching for non-irradiated flies. (B) Sterile individuals had lower fecundity, on average a decrease in egg production of 38%. Groupings are based on one-way ANOVAs followed by Tukey's HSD test.

maturation. However, it is possible that conditioning hormesis in the pupal stage might prime future responses to stressors at a later age, providing additional defense (Le Bourg et al., 2004; Le Bourg, 2005). Our results do support the idea that endogenous antioxidant enzymes can play crucial roles in sexual selection in the face of oxidative stress.

Beyond understanding the role of antioxidants and conditioning hormesis in sexual selection more broadly, our results have direct applications in the context of the SIT, where irradiation is used to induce sterility but also diminishes male sexual performance. Irradiation in anoxia has long been known to boost post-irradiation male sexual performance in caribflies (Sharp et al., 1975) and other tephritid fruit flies (Hooper, 1971; Wakid, 1973; Zumreoglu et al., 1979). This association between anoxia during irradiation and greater post-irradiation performance has led some authors to recommend that all SIT facilities seal insects in airtight containers and allow them to become hypoxic/anoxic prior to irradiation to take advantage of conditioning hormesis (Schwarz et al., 1985; Fisher, 1997; Bakri et al., 2005). However, to our knowledge, no mechanistic hypothesis for the positive effects of anoxia exposure on performance had previously been evaluated.

We propose that the increase in antioxidants following anoxic conditioning improves male performance in caribflies by increasing antioxidants and decreasing oxidative damage incurred as a result of irradiation. We show that males flies receiving 1 h of anoxic conditioning followed by irradiation in anoxia as pre-emergence adults at the standard dose for SIT, 70 Gy, have less oxidative damage than males kept in normoxia at the time of sexual maturity (10 days). Specifically, males given the anoxic conditioning followed by irradiation in anoxia had substantially less lipid peroxidation and fewer protein carbonyls than males irradiated in normoxia without pre-treatment. Thus, lower levels of oxidative damage in the anoxia-treated group were correlated with greater mating success, increased flight ability and increased survival under stress compared with males exposed to normoxia throughout the irradiation process. The benefits of anoxic conditioning followed by irradiation in anoxia are even more apparent at higher irradiation doses. Anoxic conditioning followed by irradiation in anoxia rescued 100% of the irradiation-induced mortality in the 400 Gy treatment group. Furthermore, higher percentages of the anoxic conditioned flies were able to fully emerge and extend their wings, enabling successful flight at the very high 300 and 400 Gy doses. Survival at these high doses is of particular interest because current phytosanitary treatments are being developed involving hypoxic and anoxic controlled atmosphere environments prior to irradiation at high doses (Hallman, 1999; Hallman et al., 2010). These reduced oxygen quarantine treatments have the potential of triggering conditioning hormesis in insects, allowing them to survive the higher doses used in phytosanitary irradiation. Despite improving performance and decreasing oxidative damage, anoxia-treated male flies were still sterile. This finding reinforces the idea that damage to nuclear DNA that fragments chromosomes that cause sterility can be separated from somatic cellular damage that affects performance, providing potential new avenues for using antioxidants to improve post-irradiation male sexual performance and thus the efficacy and economy of SIT.

Our experiments suggest that there is a synergistic effect of anoxic conditioning with irradiation in anoxia. Indeed, we chose the combined treatment because it fits with standard protocols in SIT facilities (FAO/IAEA/USDA, 2003) and is therefore the most likely rapid conditioning and stress treatment combination to be implemented and used. However, when we conditioned pre-emergence adults with 1 h of anoxia and then reperfused them with oxygen just prior to irradiation, we observed levels of adult eclosion intermediate between the completely normoxic and completely anoxic treatments. Mortality in anoxia conditioned individuals increased in the high dose treatments when those irradiations were performed in normoxia (supplementary material Fig. S1B). By separating the effects of the anoxic conditioning from irradiation in nitrogen, we see that at higher doses there is a significant effect of irradiation in nitrogen beyond the effect of the anoxic conditioning. This is consistent with the idea that ROS are generated during irradiation from the splitting of gaseous oxygen and water (von Sonntag, 1987), and with the fact that irradiation in nitrogen increases survival (Curtis and Langley, 1972) and longevity (Zumreoglu et al., 1979). This increase in mortality can be directly attributed to irradiation in oxygen after the anoxia pre-treatment and can be avoided by irradiating these individuals in an oxygen-free environment. Thus the full benefit of the anoxia pre-treatment only occurs if oxygen reperfusion is delayed until after irradiation is completed. Further experiments are required to understand the individual benefits of anoxia conditioning *versus* irradiation in anoxia.

Conditioning treatments must activate a response substantial enough to have a beneficial hormetic effect when individuals are exposed to downstream stressors, but without the pre-treatments themselves becoming so stressful that they diminish performance. We found no effects of our anoxia treatments on organismal performance in unirradiated males, thus our treatment effectively blunts damage from irradiation without detectable costs to the flies. SIT programs for some other tephritid pests, such as medflies, irradiate and ship pupae in sealed bags so that they experience hypoxic or anoxic conditions for 24–36 h (FAO/IAEA/USDA,

2003), albeit at low temperatures (4°C). Like other tephritid fruit flies, caribflies pupate in the soil and their pupae are expected to regularly experience hypoxia or anoxia for several hours when inundated by rainfall (Hou et al., 2006; Montoya et al., 2008). This history of exposure to environmental hypoxia during the soil-bound immobile pupal stage may contribute to the anoxia tolerance in caribfly pupae, which are known to survive well even after 60 h of anoxia (Benschoter et al., 1981). Thus, anoxic conditioning treatments are concordant with naturally occurring hypoxia events experienced by caribflies in the field.

We expect that antioxidants play a major role in the protective effects of the anoxia treatment that increased antioxidant capacity and enhanced post-irradiation performance. However, anoxia exposure causes a coordinated multifactorial suite of biochemical changes in cells that goes well beyond increasing the activity of antioxidant enzymes, so the hormetic effects of anoxic conditioning for irradiation stress resistance may be due to multiple factors. Some authors have suggested that irradiation in anoxia reduces ROS loads from gaseous oxygen (Bakri et al., 2005), but anoxia-induced metabolic suppression and decreased production of ROS due to mitochondrial respiration may also benefit insects irradiated in anoxia (Hermes-Lima et al., 1998; Storey, 2002; Marcus et al., 2003). Trehalose serves a protective role in anoxia in yeast (Pereira et al., 2003) and *Drosophila* (Chen et al., 2002), where it can be responsible for preventing protein aggregation during long periods of anoxia. Similarly, molecular chaperones such as the heat shock proteins (Hsps) also prevent protein aggregation during periods of metabolic suppression, and represent part of the strong cellular defense mounted during hypoxia and anoxia. Hsps are involved in anoxia and oxygen reperfusion events in turtles (Milton et al., 2007) and flies (Liu et al., 2006; Lopez-Martinez et al., 2008; Michaud et al., 2011), as well as in aging (Tower, 2011), where they are thought to prevent oxidative-stress-induced protein denaturing and aggregation. In addition, multiple signal transduction pathways (Insulin, Notch, Toll/lmd, and EGF receptor) that have been associated with stress are upregulated in anoxia tolerance selection lines in *Drosophila* (Zhou et al., 2008). Molecules that may be involved include non-traditional antioxidants such as cytochrome P450 monooxygenases, metallothioneins, vacuolar ATPases and vitellogenins, among others. Thus it seems likely that there are multiple components driving the strong post-irradiation performance benefit that we attribute to anoxia, but we expect that antioxidants are a main contributor to the anoxic conditioning hormesis response we observed. To examine the specific effects of antioxidants in somatic protection from irradiation relative to other mechanisms, we plan to conduct functional tests and overexpression and loss of function experiments to explore the importance of enzymatic antioxidants in caribflies.

In summary, by taking advantage of the hypoxia reperfusion mechanism to induce physiological conditioning hormesis in caribflies, we have shown that an anoxia pre-treatment for insects commonly irradiated in the implementation of the SIT can induce higher antioxidant capacity, lower oxidative damage and increased performance, including mating success. Anoxic conditioning increased the activity of antioxidant enzymes and decreased post-irradiation oxidative damage to lipids and proteins. Male caribflies with lower oxidative damage had higher mating success after irradiation and were just as likely to mate as non-irradiated flies. Thus, our work suggests that antioxidant enzyme activity can mediate sexual selection in the context of oxidative stress. Although we have used an extreme oxidative stressor in our studies, gamma irradiation, it is possible that hypoxia/anoxia exposure during the soil-dwelling pupal and pre-emergence adult stages during rain inundation in nature may confer similar antioxidant-boosting benefits, resulting in cross-tolerance to other stressors such as heat and desiccation. Further, we propose that other hormetic environmental, chemical or genetic manipulations that enhance antioxidant capacity may improve the performance of irradiation-sterilized males, thus increasing the efficacy and economy of the SIT.

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