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Anoxia hormesis improves performance and longevity at the expense of fitness in a classic life history trade-off

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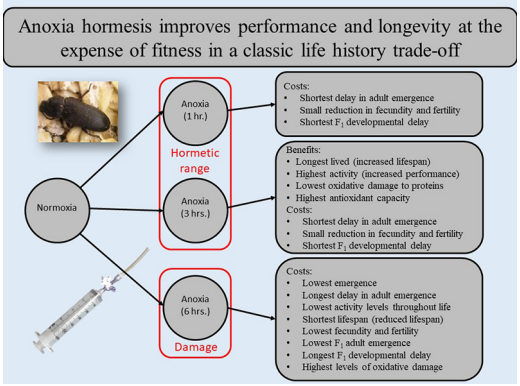
HIGHLIGHTS

- Anoxia hormesis triggers a protective response that reduces oxidative damage.
- This hormetic response improves multiple life history traits.
- These improvements come with a cost to the treated generation's reproductive output.
- The F_1 generation suffers developmental delays and incomplete adult emergence.
- These shifts in longevity and fitness represent a hormetically induced trade-off.

GRAPHICAL ABSTRACT



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ABSTRACT

Hormesis occurs as a result of biphasic dose relationship resulting in stimulatory responses at low doses and inhibitory ones at high doses. In this framework, environmental factors are often studied to understand how this exposure benefits the animal. In the current study we used anoxia, the total absence of oxygen, as the most extreme version of low oxygen hormesis. Our goal was to determine the dose, the extent of the effect, and the cost of that response in *Tenebrio molitor*. We identified that the hormetic range (1 to 3 h of anoxia) was similar to that of other insects. Individuals that were exposed to 3 h had high emergence, increased activity throughout life, and lived longer. Beetles that experienced 1 h of anoxia performed better than the controls while the 6-h group had compromised performance. These boosts in performance at 3 h were accompanied by significant costs. Treated individuals had a delay in development and once matured they had decreased fitness. There were also transgenerational effects of hormesis and F_1 beetles also experienced a delay in development. Additionally, the F_1 generation had decreased developmental completion (i.e., stress-induced developmental halt). Our data suggests that anoxia hormesis triggers a trade-off where individuals benefiting from improved performance and living longer experience a decrease in reproduction.

1. Introduction

Whatever does not kill you is said to make you stronger. This statement is meant to highlight individual resilience and organismal plasticity. However, life history theory would compel us to modify that statement into whatever does not kill you now, exerts a cost that must be paid later.

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Organisms are constrained by their resources which must be allocated for carrying out all their functions including cellular maintenance, development, defense, and reproduction (Maynard Smith et al., 1985). It is this allocation of resources over time that provides a framework for the concept of trade-offs (Maynard Smith et al., 1985), and the organism's priorities on certain life history traits at different times. The most fundamental life history trade-off that an animal can bring about is between how long it lives and its reproductive output (Stearns, 1989). This central idea of trade-offs is tied to the fact that energy is limited because resources are limited (Boggs, 1981), and therefore the role of the mitochondria and the production of reactive oxygen species (ROS) becomes central to life history trait evolution (Dowling and Simmons, 2009).

The burden of ROS production through respiration is shared by vertebrates and invertebrates; however, the burden is much heavier for high energy consuming organisms (Lutz, 1992). ROS are not inherently bad and are a necessary part of signaling and immune responses in eukaryotes (Matsuzawa et al., 2005; Suzuki et al., 2011). Controlled ROS production takes on important physiological roles especially in regulating cellular redox homeostasis; eukaryotic organelles may even differ in redox environments and may not always be in equilibrium with the environment of the cytosol (Schafer and Buettner, 2001). This results in distinct mechanisms for the regulation and maintenance of redox homeostasis that have adapted and evolved within these cellular compartments (Ayer et al., 2012). Furthermore, low levels of ROS are generated across different cell types and tissues as required signaling (Dizdaroglu, 1992; Junkunlo et al., 2016) and immune-related functions (Arefin et al., 2014; Dubovskii et al., 2010).

By lowering oxygen concentration in the cell, mitochondria enter a state of defense in preparation for resulting ROS production through the upregulation of protective genes such as antioxidants and molecular chaperones (Hermes-Lima et al., 1998; Turrens, 2004; Giraud-Billoud et al., 2019; Berry and López-Martínez, 2020). These defensive products are then readily available when excessive free radical production occurs due to oxygen reperfusion (Calabrese et al., 2012). Biologically active molecules, such as antioxidants, are then available to protect against this and other stressors; potentially more severe ones like radiation (López-Martínez and Hahn, 2012, 2014; Findsen et al., 2013). This physiological phenomenon has been fundamental throughout evolutionary history as it was necessary for organisms to adapt under extreme environmental conditions (Hermes-Lima and Zenteno-Savín, 2002). However, during periods of environmental stress, ROS are disproportionally generated which can lead to oxidative stress and damage (Halliwell and Gutteridge, 1993). The cellular targets of ROS include lipids, proteins, and DNA/RNA. Damage to these targets results in dysfunctional proteins, frail membranes, and breakages of DNA stands (Halliwell and Gutteridge, 1993). Most organisms experience high oxidative stress during and after periods of environmental stress resulting from various sources, often experiencing simultaneous combinations of multiple stressors and repeated exposures (Hermes-Lima and Zenteno-Savín, 2002; Benoit et al., 2010; Marshall and Sinclair, 2012). Organisms possess adapted inducible antioxidants mechanisms to deal with the short and long-term effects of oxidative damage by detoxifying reactive intermediates and preventing the resulting oxidative damage (Hermes-Lima et al., 2001; Hermes-Lima and Zenteno-Savín, 2002).

The concept where low dose exposures are stimulatory/beneficial, while high dose ones of the same agent are inhibitory/detrimental, is termed hormesis (Calabrese and Baldwin, 2000, 2001). This biphasic response has the shared observation, among hundreds of hormesis studies, that exposure to low levels confers protection against a more challenging high-level event (reviewed by Calabrese and Blain, 2005; Calabrese et al., 2007; Calabrese, 2016a). This adaptive response serves to create rapid acclimation that improves resistance/tolerance to benefit the organism by enhancing short-term survival (Calabrese et al., 2007; López-Martínez and Hahn, 2012; Berry and López-Martínez, 2020). Low level exposure to diverse stressors can induce a physiological conditional hormetic response that will increase organismal performance during or after low temperature, high temperature, and radiation; among others (Berry and López-Martínez, 2020). Subsequent bouts of stress result in different physiological responses compared to single episodes (Benoit et al., 2010; Marshall and Sinclair, 2010; Marshall and Sinclair, 2012), and this additional exposure can lead to preparation for future events (Visser et al., 2018). Although this biological dose response is fundamental to evolutionary adaptation, little is known about the cost that organisms must endure for the short and long-term benefits of this type of protective acclimation.

Oxygen manipulation is one of the commonly studied hormetic environmental non-chemical treatments (Calabrese, 2016b), with anoxia showing the most robust protective effect (Campbell and López-Martínez, 2022). The benefits are attributed to a decline in metabolic processes (Van Voorhies, 2009; Krivoruchko and Storey, 2010), which in insects are characterized by the reduction in the critical partial oxygen pressure and metabolic rate, halting of oxidative phosphorylation in the electron transport chain, and an increase in glycolysis (Kölsch et al., 2002; Campbell and López-Martínez, 2022). For birds and mammals, these metabolic responses can be catastrophic; only being capable of surviving short periods without oxygen at physiological temperatures (Hermes-Lima and Zenteno-Savín, 2002). Insects normally experience functional hypoxia during respiration, making them far more tolerant of oxygen deprivation due to evolutionary adaptations; physiological and morphological changes in the tracheal system, metabolic reorganization, and suppression of activity, feeding, and growth (Harrison et al., 2006). Insects are not immune to the damaging effects of ROS, and are particularly vulnerable to oxidative damage because of phase transfusion in their tracheal system that delivers oxygen to tissues at a much higher partial pressure (i.e., higher oxygen concentration) than vertebrates (Bradley, 2007). Tolerance of anoxia varies among species (Wegener and Moratzky, 1995) and throughout life stages (Van Voorhies, 2009; Visser et al., 2018; Campbell et al., 2019), and these adaptions are attributed to the differing environmental conditions where insects evolved and survived (Wegener, 1993; Visser et al., 2018).

Anoxia hormesis radically improves performance across animal groups (Berry and López-Martínez, 2020), but given limited resources and physiological limitations, there must be a cost to this improvement. The central tenet of life-history theory is that organisms cannot optimize all aspects of fitness when resources are limited (Stearns, 1989, 1992; Zera and Harshman, 2001; Monaghan et al., 2009). In *Drosophila*, infection-induced hormesis leads to increased survivability and fecundity, but at the expense of reduced immune function. These pathogen-challenged flies have higher survival and reproduction rates, but also experience higher susceptibility to additional infections (McClure et al., 2014). Thus, it seems reasonable that hormesis resulting in mild improvements to performance or survival would have low or nearly undetectable costs. Meanwhile, when hormesis enhances multiple life history traits, a clear cost must exist.

In the present study, we investigated the cost of hormesis on the development of treated individuals and their untreated offspring. The cost of hormesis is a crucial factor of the hormetic response that has been widely understudied as most studies have focused on the benefits to the organisms. We first identified a hormetic range for anoxia in our model the mealworm beetle, *Tenebrio molitor*, and then investigated multiple life history traits, across two generations, to elucidate the broad benefits and specific organismal costs of anoxia hormesis. We chose *T. molitor* as our anoxia model due to their high tolerance to low-oxygen which likely results from their grown-dwelling lifestyle (Loudon, 1988; Greenberg and Amos, 1996). Using a model with high tolerance to oxygen disruptions will allow us to carefully characterize the benefits and costs of such a response. We hypothesized that the improvements in organismal performance normally associated with anoxia hormesis would come at a quantifiable cost if enough traits were measured, and expected those cost to be associated with reproduction (López-Martínez et al., 2016a). Tracking post-treatment development, activity, longevity, and reproductive output in treated individuals allowed us to quantify their cost of hormesis. By looking at F_1 development, we identified the transgenerational cost of parental hormesis. We present evidence that anoxia hormesis improves performance across multiple life history traits, but that enhancement comes at a cost to the parental generation that represents a classic life history trade-off.

2. Methods

2.1. Colony rearing and maintenance

The Comparative Stress Physiology Lab has maintained a colony of *Tenebrio molitor* beetles since 2014. Mealworms used to establish and repopulate the colony came from Rainbow Mealworms (Compton, CA, USA). Mealworms were reared in a temperature (25 ± 0.5 °C), relative humidity (55 ± 5 %), and light controlled (14 h: 10 h) incubator (Percival Scientific, IA, USA). Their diet consisted of a 1:4 mixture of ground powdered milk and oats. All beetles (larvae, pupae, and adults) were provided whole oats (Honeyville Inc., Ogden, UT, USA) as a media for burrowing and given potato slices (Russet) once weekly. Beetle cages consisted of food grade polystyrene containers (Pioneer Plastics, KY, USA) customized with mesh lids to ensure air flow. Larvae and adult colonies were kept in large population cages (31.27 cm × 23.02 cm × 10.16 cm) while the pupae were kept in small, cubed ones (9.53 cm × 9.53 cm × 7.78 cm). All cages were customized with drilled holes to maintain normal atmospheric oxygen levels (normoxia), while preventing beetle escape.

2.2. Experimental rearing

Pupae were sorted daily between 8:30 and 9:30 a.m. to ensure that individuals in each experimental cohort were of the same age. Pupae showing signs of molting into adults were excluded from all experiments and added back to the rearing colony. Under our laboratory conditions, beetles emerge as adults within 7–8 days post pupation. All experimental groups were kept under the same conditions as the rearing colony.

2.3. Anoxia treatments

Beetles were treated two days prior to adult emergence during the period of pharate adult development (referred hereafter as pupae). 30–90 pupae were placed in 60 ml syringes (Medline Industries, IL, USA) that were customized for normoxia with perforated holes or left intact for anoxia. For consistency, and to allow direct comparisons with previous work, all treatments started between 9:30 and 10:30 a.m. To achieve the designated anoxia dose, syringes were flushed with N_2 gas for 2 min to displace all oxygen from the environment and then sealed via a three-way stop cock (Cole Parmer Inc., IL, USA). This method allows for the complete removal of oxygen without having any detrimental dehydration effects on the insects (Visser et al., 2018). Syringes were then placed in polyethylene bags, flushed with additional N_2 and heat sealed to ensure a second complete anoxic (0 % oxygen) environment buffer. We hypothesized that the hormetic range for this species would occur between 0 and 4 h due to their evolved high tolerance for low oxygen conditions shared among many beetle species (Loudon, 1988; Greenberg and Amos, 1996) and our own preliminary findings. Once the designated anoxia dose (1, 3, or 6 h) was achieved, pupae were placed into emergence cages (9.53 cm × 9.53 cm × 7.78 cm) where they underwent oxygen reperfusion and anoxia stress recovery undisturbed. Control groups (0 h) were also placed in syringes and heat-sealed bags (with holes to allow normal air flow) and left undisturbed in normoxic (Ax0) conditions prior to their placement in emergence cages.

2.4. Emergence

Post treatment adult emergence (eclosion) was observed and recorded every 24 h (between 10:30 and 11:30 a.m.) until all pupae in each experimental replicate had fully emerged or died. Adults that emerged with severe phenotypic deformities that prevented normal adult function (i.e., incomplete exoskeleton, malformed wings, and/or incomplete sclerotization) were counted and recorded as non-emerged but removed from additional performance experiments as these deformities decreased performance (i.e., shorter lifespans and no mating). All pupae that successfully emerged were then placed in larger rectangular cages (18.73 cm × 9.37 cm × 7.62 cm or 29.85 cm × 12.38 cm × 6.19 cm) where they mated and laid their eggs.

2.5. Daily activity

A locomotor activity monitor (LAM; Trikinetics, Inc., MA, USA) was used to quantify and compare the overall activity of adult beetles. This monitor is specifically designed for flies, and therefore it was slightly modified for larger walking insects. The glass tubes used to house the insects were lined internally with mesh to provide better traction for the beetles and prevent death from overturning on their backs, as our preliminary data showed. Rubber stoppers, drilled with air flow holes, were used to contain each beetle inside the monitor tube while providing sufficient airflow. A daily activity baseline was established over several weeks using beetles of different ages to record undisturbed activity over a period of 48 h. We focused on young beetles (two weeks post treatment) and old beetles (two months) for our measurements. Two-week-old beetles were chosen as the young treatment because full sclerotization can take more than a week in some individuals and therefore they are not fully active yet. Two-month-old beetles were used as our old treatment because of the sufficient decline in survivorship recorded in preliminary studies. All daily activity experiments were run in a Percival incubator under the controlled conditions mentioned above. We compared overall beetle activity across treatment and age. Multiple groups of six biological replicates per treatment were assessed over multiple 48-hour periods. The data is presented as average movement per minute (i.e., the average number of times a beetle moved across the tube in 1 min).

2.6. Longevity

After treatment, pupae were allowed to emerge, and longevity was assessed weekly by monitoring survivorship until all individuals in all treatment cohorts died of natural senescence. Additional longevity cages were set up to collect samples for biochemical analysis. In these cages, survivorship was also assessed weekly to ensure large enough sample sizes for biochemical analysis. Longevity counts were done on the same day of each week in the midday hours (11:30 a.m.–1:30 p.m.). Longevity data is presented as percent survivorship by week.

2.7. Fecundity and fertility

Following the 24-hour anoxia recovery period, pupae from each treatment group were sexed and separated into small emergence cages (9.53 cm × 9.53 cm × 7.78 cm). Males were marked with metallic marker on their darkened exoskeleton while females remained unmarked. Oats were provided as a medium for adult beetles to walk around and mate, as we previously found that the presence of food substrate made reliable egg collection possible. Sexually mature adults were randomly assigned to mating trials. A 1:1 sex ratio was set up for each mating trial cage with three to five replicate cages/treatment. Each 1:1 sex ratio cage included five mating pairs and all ten beetles in each cage came from the same treatment. After two weeks, all adults were removed from the mating cages and the eggs were counted to assess fecundity. Although most eggs were glued to the bottom surface of the cage, the medium from each cage was sifted to remove oats from feces and ensure that all eggs were captured. All media was analyzed using microscopy at different resolutions to ensure total egg collection. Hatched larvae, within a week post oviposition, were then sifted into a wide plastic container (34.6 cm × 21 cm × 12.4 cm; Sterlite, MA, USA) and counted as our measure of fertility. Fecundity data is presented as the average number of eggs laid and fertility data is presented as the percent egg hatching.

2.8. F_1 adult emergence

Mealworms that hatched from the parental generation were reared until they developed into pupae. F_1 pupae were separated and placed in small

cages (7.32 cm × 7.32 cm × 2.69 cm) until adult emergence occurred. Successful emergence was recorded based on the same criteria established for the parental generation. Emergence was monitored and counted weekly during a nine-month period. Data is presented as percent F_1 adult emergence. Because some individuals never reached adulthood, the entire data set is presented as percentage of developmental stage reached.

2.9. Sampling

Sampling for each biochemical marker was taken at four time points following anoxia: 24 h, 1 week, 1 month, and 2 months after exposure ended. At 24 h, 1 week, and 1 month following treatment, five replicate experiments were used as the sampling population. Each experimental cohort ranged from 30 to 60 pupae per treatment. Not all anoxia treatment groups, and experimental cohorts survived up to two months, so the sampling population size at this age was limited to just three replicates. Twenty-four hours post oxygen reperfusion, control and treated samples were flash frozen in liquid nitrogen and stored at −80 °C until assayed. Samples at 1 week, 1 month, and 2 months post emergence were also treated in this fashion.

2.10. Oxidative damage

Oxidation of proteins was measured by quantifying the amount of protein carbonylation across treatments and ages. Whole pupae or adult beetles were individually homogenized using 2.0 mm dia. zirconia beads (BioSpec Products, OK, USA) and a microtube homogenizer (BeadBlaster24; Benchmark Scientific, NJ, USA). After centrifugation, the supernatants of the homogenates were assayed with the 2,4-dinitrophenylhydrazine (DNPH) method (Levine et al., 1990). A nine-point BSA standard curve (0–2 mg/ml) was used to standardize the results and quantify protein carbonyl concentration. Data is presented as protein carbonyls per mg of soluble protein (μmol/mg protein). Lipid peroxidation was quantified by measuring the main aldehyde product of lipid oxidation; malondialdehyde (MDA; Uchiyama and Mihara, 1978, Ohkawa et al., 1979). Individual whole pupae or adult beetles were homogenized as described above, treated with trichloroacetic acid to remove proteins, and combined in a 1:1 ratio with a thiobarbituric acid (TBA) solution. Samples were read at 532 nm along with an eight-point MDA standard curve (0–50 μmol/ml) for quantification. Data is presented as MDA per mg of soluble protein (μmol/mg of protein).

2.11. Antioxidant activity

To quantify the effect of treatment on total antioxidant activity, the ABTS radical cation decolorization assay was performed (Re et al., 1999). Whole beetles were individually homogenized as described above, diluted, and combined with the free radical solution, and read at 734 nm. An eight-point Trolox standard curve (0–150 μmol/ml) was used to quantify the total antioxidant capacity of each sample. Data is expressed as Trolox equivalents per milligram of soluble protein (μmol/mg of protein).

2.12. Statistical analysis

Datasets with normal distributions (TEAC) were analyzed using two-way ANOVAs followed by a Tukey's HSD analysis. F_1 emergence data had a Johnson SI distribution and after transformation was analyzed with an ANOVA. Emergence, daily activity, median lifespan, lipid peroxidation, and protein carbonyl data were analyzed with a generalized lineal model to fit the specific distribution (Weibull, exponential, or gamma) followed by a Tukey's HSD and linear contrast analysis to separate treatment effects. Fecundity, fertility, and F_1 hatching data were analyzed using a mix model followed by Tukey's HSD. Longevity and emergence by day data were analyzed using proportional hazards models.

3. Results

3.1. Emergence

Emergence was recorded as successful if the adult showed no phenotypic deformities and was able to completely exit the pupal exoskeleton. There was no difference between controls, one, and 3 h of anoxia exposure (Ax0 = 96.56 ± 1.33 %, Ax1 = 90.6 ± 1.71 %, and Ax3 = 95.14 ± 2.28 %); but 6 h of anoxia exposure resulted in a decrease (87.07 ± 1.76 %) in adult emergence (Fig. 1A; $\chi^2 = 18.95$ $p = 0.0003$).

3.2. Emergence by day

Under normoxia, pupae emerge a week after pupation and most individuals (99 %) emerge by day 3. Following anoxia, it was observed that there was a delay in this emergence period, which has been previously recorded in response to radiation (López-Martínez and Hahn, 2012). For controls (Ax0), 66.9 % of all beetles emerged on days 1 and 2 (Fig. 1B). The hormetic range of anoxia in this species was determined to be between 1 and 3 h of exposure. After 1 h of anoxia exposure (Ax1), 95.5 % of pupae emerged on days 2 and 3, with only 3.2 % emerging on day one. Following 3 h of exposure (Ax3), 97.9 % of beetles emerged between days 2 and 3

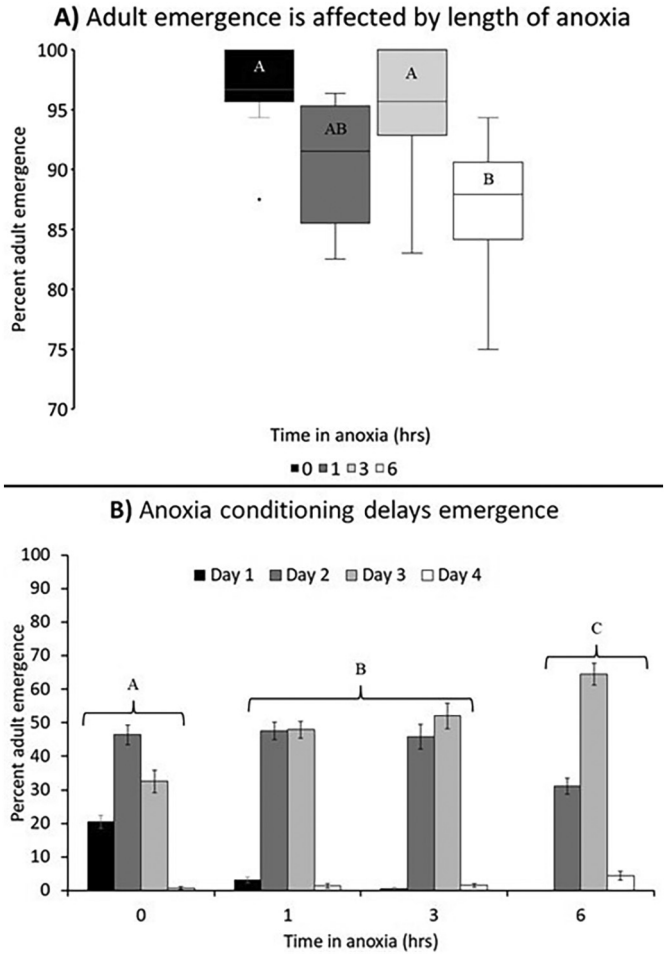

Fig. 1. A) There was no difference in emergence between Ax0 (control), Ax1 (1 h), and Ax3 (3 h) treatments, but Ax6 (6 h) exposure resulted in a 10 % decrease from Ax0. Statistical differences are indicated by letters based on a Tukey's HSD following a generalized regression analysis. B) Exposure to anoxia significantly delayed development. For Ax0, the majority of adult emergence occurs in the first two days. In the hormetic range 95 % (Ax1) and 98 % (Ax3) of emergence occurs on days 2 and 3. For Ax6 beetles, ~67 % emerged on day 3. Statistical differences indicated by letters based on risk ratios from a proportional hazards fit analysis.

with <1 % emerged on day 1. At our highest dose, 6 h of anoxia (Ax6), we saw the most severe delay in development where 64.4 % of the adults emerged on day 3 and no beetles emerged on day 1 (Fig. 1B; $\chi^2 = 50.9$, $p < 0.0001$).

3.3. Daily activity

Beetle daily activity at youth was different among the treatments (Fig. 2A; $\chi^2 = 47.9$, $p < 0.0001$), with the Ax0 and Ax3 beetles having similarly high levels of activity ($p = 0.07$). Ax6 beetles were the least active at about a quarter the activity recorded for controls. Beetles that experienced 3 h of anoxia were more active than beetles that only experienced 1 h ($p = 0.0001$). Two months after treatment, the effects of anoxia hormesis are more pronounced in beetles treated to 3 h of anoxia as they are more active than the controls and the Ax6 beetles (Fig. 2B; $\chi^2 = 24.19$, $p < 0.0001$). At this later age, Ax0 beetles are as active as Ax6 ones but with similar activity to the Ax1 group ($p = 0.18$). When comparing across age, two-month-old anoxia hormesis beetles (Ax3) are 3× more active than young Ax6 ones and have the highest activity measured ($\chi^2_{model} = 91.27$, $p_{model} < 0.0001$; $\chi^2_{treatment} = 54.08$, $p_{treatment} < 0.0001$; $\chi^2_{age} = 14.86$, $p_{age} = 0.0001$; $\chi^2_{treatment*age} = 10.56$, $p_{treatment*age} = 0.014$).

3.4. Longevity

Ax0 adults lived 14 weeks under laboratory conditions. Adults exposed to anoxia in the hormetic range (1 to 3 h) lived longer than the controls (Fig. 2C; Ax1 16 weeks and Ax3 18 weeks, $\chi^2 = 122.12$, $p < 0.0001$). Exposure beyond this hormetic threshold resulted in a significant decline in lifespan. Lifespan of adults treated with 6 h of anoxia was on average 11 weeks. We use median lifespan to convey survival prospects and to compare across previously published work. Ax3 had the longest median lifespan at 7.5 weeks (Fig. 2D; $\chi^2 = 48.03$, $p < 0.0001$). The median lifespan of our controls (Ax0) was 6.8 weeks and was not different from Ax1 (6.4 weeks; $p = 0.25$). Ax6 beetles had the shortest median lifespan at 5.5 weeks.

3.5. Fecundity and fertility

Anoxia exposure resulted in a decline in egg production for all treated beetles (Fig. 3A; $F_{3,3} = 235.01$, $p < 0.0001$). Controls (Ax0) averaged 19.47 ± 0.24 eggs/female. Ax1 averaged 14.22 ± 0.21, Ax3 had 15.18 ± 0.27 and Ax6 had 9.04 ± 0.37 eggs/female. Ax6 beetles had the strongest reduction in fecundity; 53 % fewer eggs. We recorded an additional cost of anoxia on egg hatching that was significantly lower than Ax0 fertility of 93.44 ± 0.78 % (Fig. 3B; $F_{3,3} = 251.52$, $p < 0.0001$). Fertility dropped to 80 ± 0.72 % for Ax1 and 82.95 ± 3.32 % for Ax3. Once again, the most dramatic drop in reproduction was in beetles exposed to 6 h of anoxia where the percentage of hatched eggs was 53.85 ± 1.97 %. When we considered both fecundity and fertility, the reduction in reproductive output was 37.44 % for Ax1, 30.78 % for Ax3 and 73.23 % for Ax6.

3.6. F_1 adult emergence

Emergence of the untreated F_1 generation adults followed a similar pattern as the treated parental generation where Ax6 beetles had the lowest adult emergence (Fig. 4A, $F_{3,3} = 47.074$, $p < 0.0001$). But there was no difference in adult emergence between the control and beetles in the hormetic range (Ax0 vs Ax1 $p = 0.25$, Ax0 and Ax3 $p = 0.17$). Emergence for controls averaged 96.69 ± 1.13 % but Ax6 F_1 emergence was only 66.85 ± 2.76 %. Additionally, a delay in development was also observed in the F_1 generation. Although mealworms were allowed ample time (nine months; twice as long as needed) to develop into adults, a greater percentage of the anoxia treated groups did not successfully transition into adulthood (Fig. 4B; $F_{3,8} = 19.919$, $p = 0.0005$). In the controls, 68.85 ± 1.13 % of mealworms made it to adulthood during that time. For anoxia, adult

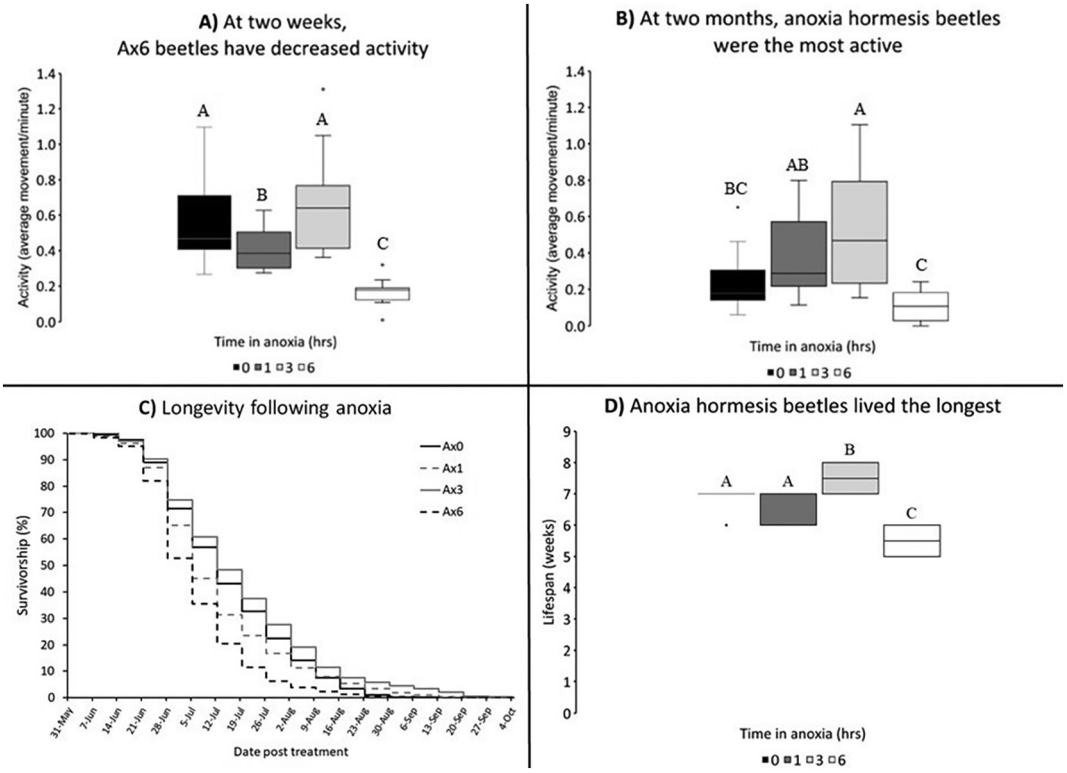

Fig. 2. A) Young beetle activity, over a 48-hour-period, showed that hormesis (Ax3; 3 h) and control (Ax0) beetles have the greatest activity, while Ax6 (6 h) beetles are the least active. B) At old age, Ax6 beetles were the least active and Ax3 beetles had the highest activity. C) Hormesis beetles lived the longest; a 30 % increase over the controls while Ax6 beetles had the shortest lifespan. D) Median lifespans for Ax0 (6.8 wks.) and Ax1 (6.4 wks.) were not different from each other. Ax3 beetles outlived all experimental treatments (7.5 weeks). The lowest median lifespan (5.5 wks.) occurred in the Ax6 treatment. Letter groupings are based on Tukey's HSD.

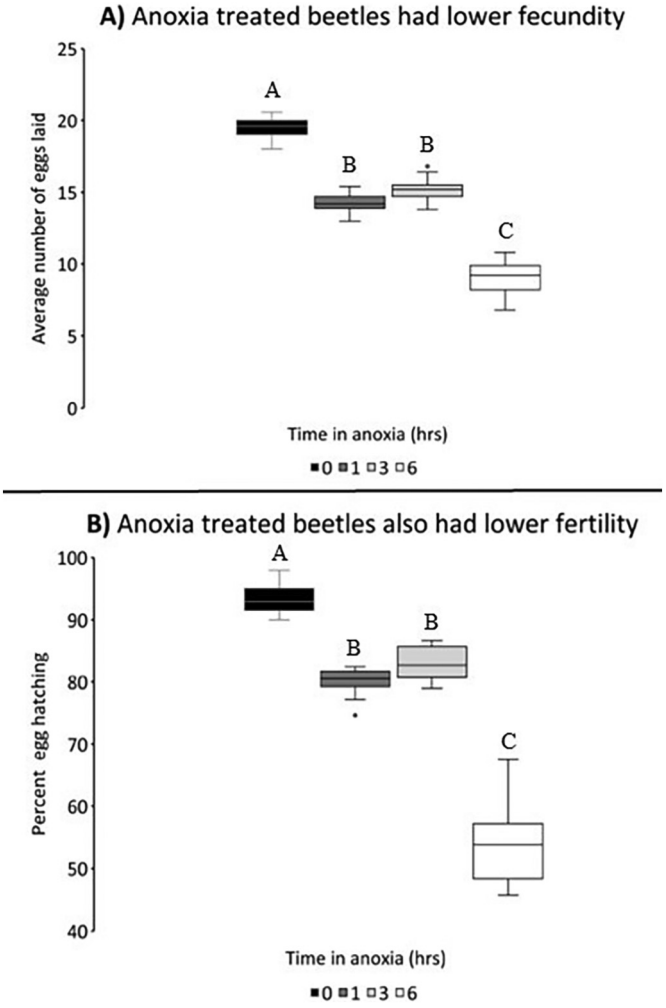

Fig. 3. A) There was a decrease in the number of eggs laid (fecundity) in the hormetic range (1 and 3 h of anoxia) compared to controls (Ax0). Fecundity was lowest in Ax6 (6 h) where egg production dropped by 50 %. B) There was also a decrease in the number of eggs hatched (fertility) in the hormetic range. Fertility was the lowest in Ax6, which dropped by 42 %. Statistical differences are indicated by Tukey's HSD following a mix model analysis.

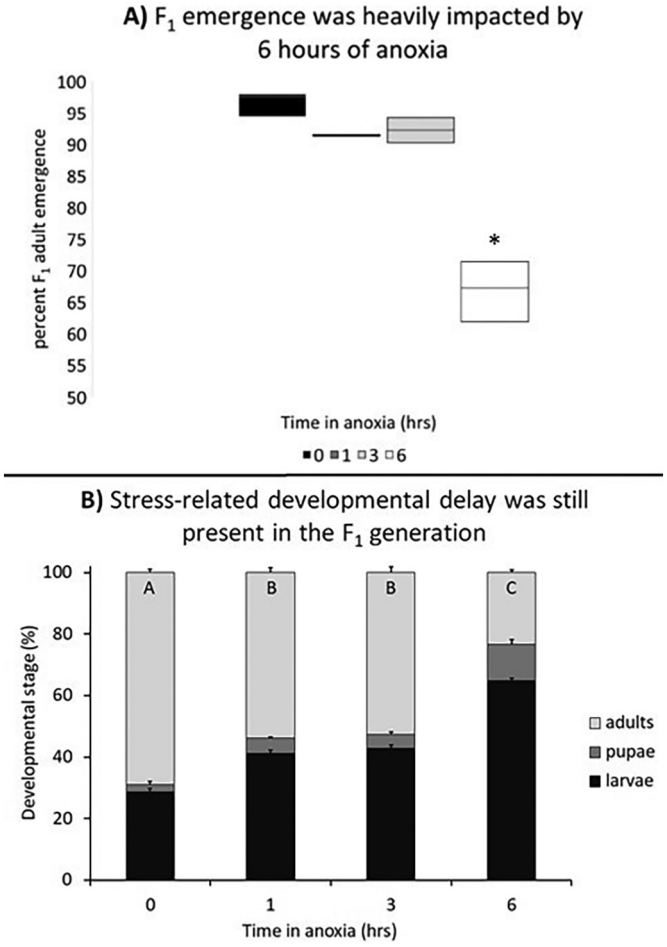

Fig. 4. A) Ax0 (control), Ax1 (1 h), and Ax3 (3 h) F_1 adult beetle emergence was not different from each other. However, a 30 % decrease in emergence of Ax6 (6 h) beetles indicates a strong negative transgenerational effect of anoxia. Statistical differences are indicated by an asterisk. B) A similar developmental delay was observed in the F_1 generation. After nine months of larval development, a percentage of larvae never made it to adulthood. In the control, 68.8 % became adults. But all beetles experiencing anoxia had a strong decline in reaching the adult stage; 53.7 % (Ax1), 52.7 % (Ax3), and 23.4 % (Ax6). Letter rankings are based on Tukey's HSD.

emergence rates for the F_1 generation were 53.72 ± 1.48 % for Ax1, 52.68 ± 1.79 % for Ax3, and only 23.45 ± 0.78 % for Ax6.

3.7. Oxidative damage

There was no difference between anoxia in the hormetic range and the normoxic control in protein carbonylation two weeks post treatment. But there was an increase in protein carbonyls recorded for Ax6 (Fig. 5A; $\chi^2 = 14.13, p = 0.0027$). At two months of age, the trend in protein carbonylation differs. Ax3 had the lowest amount of accumulated oxidative damage to proteins while Ax6 had the highest (Fig. 5B; $\chi^2 = 34.68, p < 0.0001$). Damage in Ax3 beetles was lower than that of controls ($p = 0.001$).

There was a strong increase in lipid peroxidation one week following exposure in the Ax6 beetles (Fig. 5C; $\chi^2 = 10.81, p = 0.013$), but no differences were found in the other treatments. At one-month post-treatment, a similar pattern was detected where Ax6 beetles had 4 times more oxidative damage than the rest (Fig. 5D; $\chi^2 = 20.43, p = 0.0001$). Two months after exposure, Ax6 beetles had 7.5 times the damage detected than Ax3 hormesis beetles (Fig. 5E; $\chi^2 = 23.58$, $p < 0.0001$). Throughout the experiment the amount of lipid peroxidation damage detected in the beetles experiencing hormesis was not different from background damage accumulated in the controls ($p_{\text{one week}} = 0.26$, $p_{\text{one month}} = 0.18$, $p_{\text{two months}} = 0.19$). Both treatment and age affected how much lipid peroxidation damage was accumulated ($\chi^2_{\text{model}} = 64.86$, $p_{\text{model}} < 0.0001$; $\chi^2_{\text{treatment}} = 20.43$, $p_{\text{treatment}} = 0.0001$; $\chi^2_{\text{age}} = 18.62$, $p_{\text{age}} < 0.0001$; $\chi^2_{\text{treatment*age}} = 12.17$, $p_{\text{treatment*age}} = 0.058$).

3.8. Antioxidant capacity

One week after exposure, there was no significant difference between anoxia treatments when compared to the normoxic control (Fig. 5F; $F_{3,56} = 2.25$, $p = 0.09$). A month post treatment, a significant difference among treatments was detected (Fig. 5F; $F_{3,80} = 5.7$, $p = 0.001$), where Ax3 beetles had the highest antioxidant capacity, while Ax1 and Ax6 had the lowest. Two months following treatment, a significant difference in Trolox equivalents is seen across treatments with Ax3 beetle having the highest antioxidant capacity (Fig. 5F; $F_{3,68} = 9.2$, $p < 0.0001$). When we compared the data across all treatments and age groups, we find that while age did not have an effect on antioxidant capacity, there was an interaction between age and treatment ($F_{11,204} = 5.83$, $p_{\text{model}} < 0.0001$; $F_{3,3} = 4.95$, $p_{\text{treatment}} = 0.002$; $F_{2,2} = 2.58$, $p_{\text{age}} = 0.08$; $F_{6,6} = 2.58$, $p_{\text{treatment*age}} = 0.02$). This indicates that certain treatments had age-specific responses in antioxidant capacity and a post hoc analysis indicates that Ax3 beetles increased antioxidant capacity with age while Ax6 ones had a decrease.

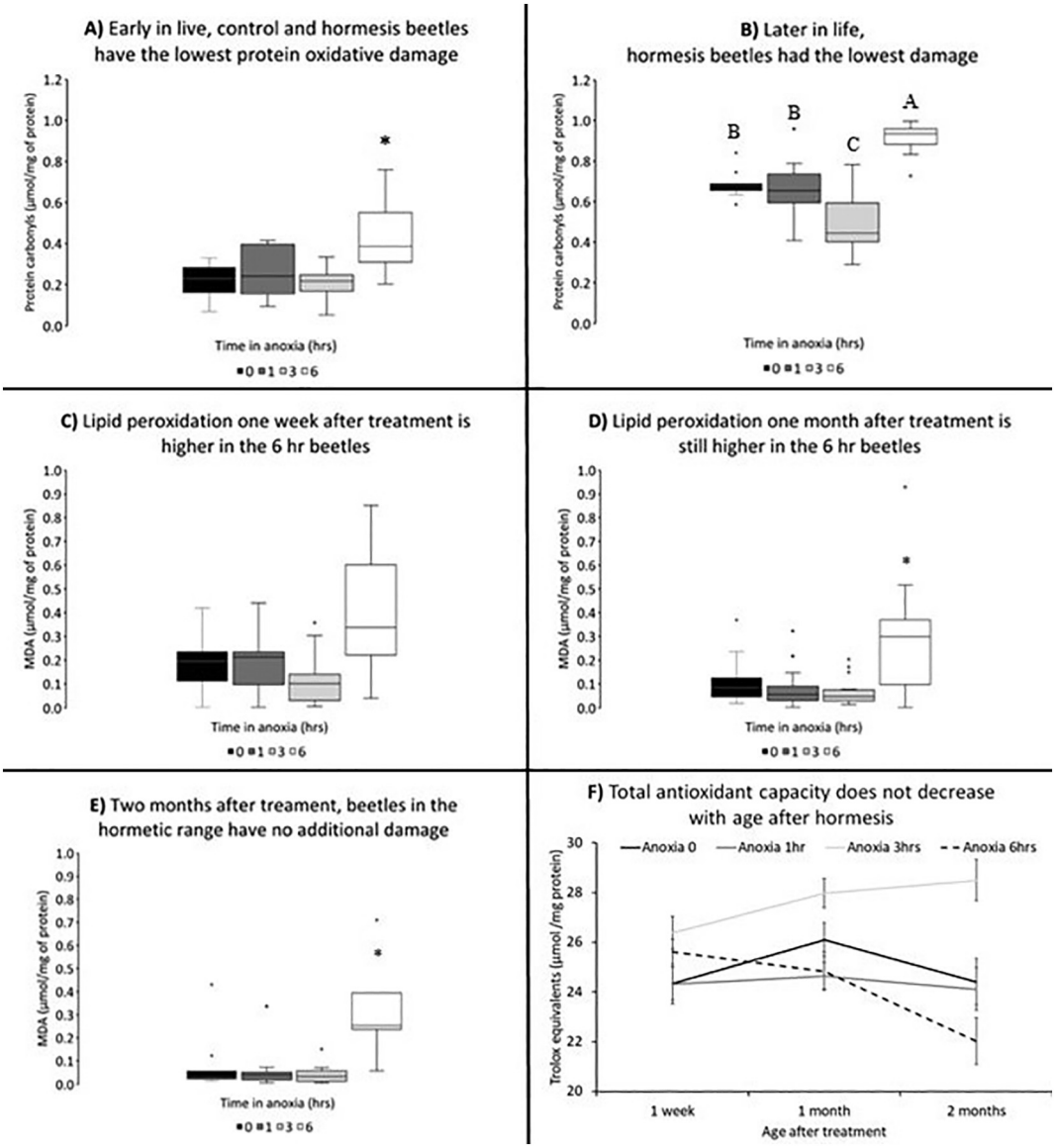

Fig. 5. A) Oxidative damage to proteins two weeks after anoxia was not different among the controls, 1 h (Ax1), and 3 h (Ax3), but 6 h (Ax6) beetles had a sharp increase in accumulated protein damage. B) At two months, Ax3 beetles had the lowest amount of protein damage. Statistical differences are indicated by Tukey's HSD. Lipid peroxidation damage was not different between Ax0, Ax1, and Ax3 at C) one week, D) one month, or E) two months after exposure. Ax6 had significantly higher levels of oxidative damage to lipids throughout the experiment. F) There was no difference in total antioxidant capacity across treatments a week after anoxia exposure. Ax3 has the highest antioxidant capacity after that. There was an interaction between treatment and age showing anoxia hormesis (Ax3) beetles increased their antioxidant capacity over time while Ax6 decreased.

4. Discussion

Many soil-dwelling and overwintering animals do so in an oxygen poor environment (Visser et al., 2018). While hypoxia is normally thought to be more common for hibernators and diapausers, soil-dwelling insects will occasionally experience low oxygen due to water submergence (spring/summer) or frozen soil (fall/winter) (Montoya et al., 2008; Storey and Storey, 2010). It is thought that strong anoxia survival mechanisms arose from the hypoxia reperfusion response as described by the preparation for oxidative stress (POS) hypothesis (Hermes-Lima et al., 1998). Under POS, cellular and mitochondrial defenses are elevated as the animal enters a hypoxic state. This elevation ameliorates the harm caused by excess free radical production upon oxygen reperfusion and resumption of normoxic mitochondrial function. While a large body of work indicates that antioxidants are involved in this POS response (reviewed by Moreira et al., 2017 and Giraud-Billoud et al., 2019), other protective genes and transcription factors have been linked to it (Giraud-Billoud et al., 2019), and there may be a substantial overlap between POS mechanisms and hormesis (Oliveira et al., 2018; Berry and López-Martínez, 2020).

The application of anoxia early in life triggers a change in the life history path of the beetles. Those receiving doses found to have hormetic benefits (1 and 3 h) display improvements in performance while those receiving the negative dose (6 h) suffer the detrimental effects of sub-lethal stress. We determined that 3 h of anoxia is the hormetic dose for this species and those beetles that experienced this treatment show a change in the rate of mortality and increased activity previously seen in flies (López-Martínez and Hahn, 2012) and moths (López-Martínez et al., 2014) exposed to anoxia. This change in the rate of death can be characterized as a divergent performance path where multiple metrics of organismal performance are improved following exposure and at least some of those improvements are carried into old age. This is evident as hormetic beetles live longer and maintain higher levels of activity as they aged. Their higher activity at old age correlates with lower oxidative damage to lipids and proteins and higher antioxidant capacity. Thus, it is likely that the decrease in

accumulated damage during their lifetime is what allows for increased performance later in life. This type of performance improvement late in life is referred to as improving healthspan (Salmon et al., 2010; López-Martínez and Hahn, 2014). This provides a tangible evolutionary advantage to hormesis which may help explain why this adaptive response to anoxia has persisted (Robinson, 1975; Hermes-Lima et al., 1998). A connection between anoxia, the POS hypothesis (Hermes-Lima et al., 1998), and hormesis (Calabrese and Baldwin, 2001) has been established, and this response is not limited to invertebrates but also found in snakes, frogs, turtles, and fish (Berry and López-Martínez, 2020). But it is in insects that we understand the response the best due to the range of life history traits measured in these studies. The scope of performance benefits ranges from higher treatment survival and flight to increased mating and longer life in flies (López-Martínez and Hahn, 2012, 2014) and moths (López-Martínez et al., 2014; López-Martínez et al., 2016a, 2016b). However, one commonality among anoxia hormesis studies is the mystery around the cost of this protective response.

Hormesis often leads to dramatic improvement in performance of multiple life history traits (reviewed by Berry and López-Martínez, 2020, Campbell and López-Martínez, 2022). Thus, it has become common place to expect these fascinating performance developments while knowing that improving performance in perpetuity is not possible due to limited resources. These limited resources likely trigger potential tradeoffs, and we have identified multiple traits where performance decreased as a result of hormesis. The immediate cost of hormesis rests with the concept of the oxygen debt and the need to recover from a period of lack of oxygen (Wegener, 1993; Visser et al., 2018). The cost of this recovery shifts the pattern of adult emergence presumably as the animal reallocates its limited energy reserves and more efficiently copes with anoxia recovery. This developmental delay was seen in all anoxia treatments and had been previously found in this species in response to hypoxia (Loudon, 1988; Greenberg and Amos, 1996) and following anoxia in bees (Cervantes and López-Martínez, 2022). But the developmental delay that the treated generation suffered was also present in the F_1 generation indicating that part of this cost is somehow transferred down to the offspring, which may be via damaged or temporarily modified DNA. The more daunting cost of hormesis goes beyond developmental delays and into reproductive output. Our group had previously shown that both sexes contribute to the effects (positive or negative) of anoxia (López-Martínez et al., 2016a, 2016b), but rather than focus on male-female differences this time we studied the most profound effect of treatment on fecundity and fertility; when both parents experienced the same anoxia treatment. While all anoxia exposures yielded a decline in number of eggs laid and hatched, we focused on anoxia hormesis because it was this three-hour group that experienced increased activity and longevity. The decrease in fecundity for anoxia hormesis was about 20 %, which is not trivial but potentially inconsequential since the beetles are living longer than their normoxia counterparts. When you consider egg hatching, the total reduction in reproductive output by anoxia hormesis beetles is closer to 31 %. Thus, the anoxia hormesis that allowed for higher activity and longer life dealt a cost of almost one third of their reproductive output. Furthermore, we did not recover any additional eggs laid past the point of reproductive senescence at around two months (Morales-Ramos et al., 2012). This finding indicates that even though the females lived longer, they did not have a longer reproductive period and thus were unable to offset the cost that anoxia hormesis had on their fitness.

It is that cost of reproductive output that outlines the trade-off we see in anoxia hormesis as a classic life history shift between longevity and reproduction. This trade-off is so ingrained into beetle physiology that we were able to detect it even in the context where nutrients were not limited. These beetles have been reared for multiple generations in a comfortable environment where nutrients were always in excess. During the experiments, we ensured food was available ad libitum while maintaining optimal rearing conditions. We expected that under these conditions, the beetles might continue to reproduce more after anoxia hormesis, but the trade-off was maintained, and the costs of anoxia hormesis were passed down to their offspring. We only measured developmental milestones in the F_1 generation, so it is possible that additional costs of parental hormesis on the offspring exist. It is also entirely plausible that the F_1 generation retained some of the parental's higher levels of performance as seen in moths and crickets (López-Martínez et al., 2014; Fuciarelli and Rollo, 2020). This transgenerational effect of stress was present in all anoxia treatments with a negative effect found in the Ax6 group. The F_1 generation developmental delay is an additional and unexpected cost of hormesis that potentially reduces the overall fitness of the parental and future generations. And while the identification of these cost to hormesis might seem negative because of the decrease in organismal fitness, insects experiencing anoxia hormesis have increased sexual competitiveness (López-Martínez and Hahn, 2012; López-Martínez et al., 2014) and represent the best performers in the population, giving them an edge when experiencing low-oxygen conditions and likely passing those responses to their offspring (Costantini, 2019, 2022). Therefore, we present here the strongest possible case by mating two treated individuals to each other, but it is likely that the reproductive cost would be much lower if only one parent experienced anoxia.

In conclusion, we argue that the effect and cost of hormesis depends on, at least, two factors (length of exposure and age). We present evidence that the magnitude of the hormetic response (Ax1 vs Ax3) leads to differences in the benefit and cost to the animal and that a strong hormetic response is accompanied by a strong cost. Anoxia hormesis is connected to an elevation of antioxidant defenses and a reduction in accumulated oxidative damage over the lifespan of the animal which may, in part, mechanistically explain this biological phenomenon. We expect that the type of stress involved in hormetic responses would also be important for determining the cost of the response. Anoxia combined with irradiation yields remarkable improvements in mating and longevity, but no cost was found given that the flies are sterile due to the irradiation (López-Martínez and Hahn, 2012, 2014). Similarly, investigations into low temperature hormesis in insects, rapid cold hardening, have revealed little cost outside of reduced lifespan (Berry and López-Martínez, 2020). We expect that additional work into the cost of hormesis will further elucidate costs associated with this response and a mechanism for energy reallocation and metabolic efficiency.

CRediT authorship contribution statement

- Alyssa De La Torre and Giancarlo López-Martínez: Conceptualization and design of experiments.
- Giancarlo López-Martínez: obtained the funding.
- Alyssa De La Torre: carried out the experiments
- Alyssa De La Torre and Giancarlo López-Martínez: data analysis, and wrote, revised, and edited the manuscript.

Data availability

Data will be made available on request.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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ADLT and GLM conceived the idea, designed, and carried out the experiments, data analysis, and wrote the manuscript. GLM obtained the funding. The authors wish to thank Chelsea Rodriguez and Kelsey Montoya for their assistance in the early stage of the anoxia dose response work and Jacob Pithan for reviewing the manuscript and helping with the graphical abstract. Research reported in this publication was supported by an Institutional Development Award (IDeA) from the National Institute of General Medical Sciences of the National Institutes of Health under grant number P20GM103451 (GLM), and by National Science Foundation Office of Integrative Activities RII Track-2 #1826834 (GLM).

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