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Anoxia hormesis following overwintering diapause boosts bee survivorship and adult performance

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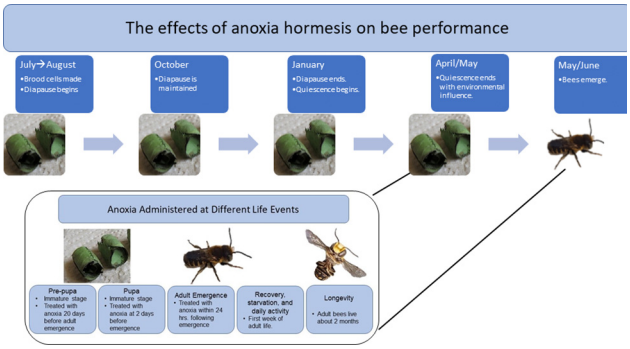
HIGHLIGHTS

- Climate related changes to overwintering in bees will be energetically draining.
- Anoxia hormesis during development improves performance and is energy neutral.
- Age dramatically impacts the effectiveness of anoxia at protecting the bees.
- Males being the most vulnerable to stress, have a higher potential benefit of hormesis.

GRAPHICAL ABSTRACT



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ABSTRACT

Insect pollination is a crucial component of our ecosystems and biodiversity, but our reliance on this ecosystem service has much broader implications. We depend on these pollination services to produce materials and food. But insect pollinators, especially bees, are in strong decline due to a plethora of factors, least of which are environmental abiotic stressors like climate change. The alfalfa leafcutting bee, *Megachile rotundata*, is the world's most managed solitary bee and is particularly vulnerable to changes in temperature. This species spends up to ten months overwintering while being exposed to the lowest temperatures of winters and the hottest temperatures of late summer. This results in usage of energy reserves prematurely and asynchronous spring emergence with their food resource. To understand the stress response of these bees and potentially boost their performance, we applied a hormetic framework where bees were exposure to different doses of anoxia (the absence of oxygen) to trigger hormesis; a low-dose stimulatory response known to lower damage and improve performance. We used hormesis on immature bees as a post-winter treatment with the goal of improving springtime performance in adults. One hour of anoxia had no negative effect on adult springtime emergence and bees were quick to recover. These bees were more active than untreated bees, as resistant to starvation, and as long-lived. Higher exposure to anoxia (3 h) was found to be mildly hormetic and 6-h exposures were detrimental. Anoxia hormesis did not represent a significant cost on the energy reserve of overwintering bees but we found that the age at which anoxia is applied will affect the effectiveness of treatment. Our data suggest that anoxia hormesis is a viable intervention to improve springtime performance in overwintering bees.

1. Introduction

Solitary bees, like the alfalfa leafcutting bee, spend a significant part of their life in their overwintering phase. In this species, females lay

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individual eggs in brood cells alongside a pollen provision before capping the cells for development (Pitts-Singer and Cane, 2011). This pollen provision is the sole food source for the developing bee larvae until it emerges as an adult the following spring/summer. The bee larva develops until it reaches the overwintering prepupal stage (Richards et al., 1987). Alfalfa leafcutting bee prepupae overwinter in a state of arrested development characterized by strong metabolic depression known as diapause (Denlinger, 2002). The shortening of day length and decreasing temperatures of mid to late summer are signals that program diapause in this species (as in many more; Denlinger, 2002), and the warming temperatures of the following spring allow the bees to finish their development before emerging as adults (Stephen and Torchio, 1961). Because these types of invertebrate arrests can last nine to ten months (Johansen and Eves, 1973; Hahn and Denlinger, 2011), that food provision provided by the mother in the summer must be enough for the bee to finish larval development, survive the long winter, and complete adult development in the spring. Drastic changes in overwintering conditions, as those predicted to occur due to climate change (Williams et al., 2014), can lead to changes in metabolism and food source phenology. It is predicted that winters will be warmer in North America, with a reduction in frequency and intensity of cold waves (Separovic et al., 2013; Marshall et al., 2020). The clear-cut effect of running out of resources during overwintering and being unable to complete adult emergence is death. The more complicated effects of this are that bees emerge early and therefore asynchronously with their food sources (CaraDonna et al., 2018). Additionally, this mismatch of flying bees and resource availability can dramatically impact the pollination services that bees provide for the ecosystem, which for this species include any production related to alfalfa as they are one of the main pollinators for this crop (Pitts-Singer, 2008).

While diapause represents a significant reduction in energetic costs due to metabolic suppression, the diapause program is not energetically neutral. Lipid oxidation (i.e., triglyceride usage) is the main form of energy behind diapause and other types of dormancies (Hahn and Denlinger, 2011), but these insects display an inherent cold hardiness associated with overwintering diapause (Denlinger and Lee, 2010). This cold hardiness requires the use of amino acids and carbohydrates to carry out synthesis of necessary products. Cryoprotectants, stress proteins (heat shock proteins), immune genes, antioxidant enzymes, and enzymes involved in metabolism and membrane restructuring are just some of the products that remain active and responsive during this overwintering stage (Denlinger, 2002; Robich et al., 2007; Michaud and Denlinger, 2007; Sim and Denlinger, 2011; King and MacRae, 2015). As environmental conditions unpredictably vary, so will the expression and activity of many gene products that are components of diapause (Ragland et al., 2010, Williams et al., 2016).

In the early stages of diapause, this aerobic hypometabolism state is centrally controlled by the brain and temperature changes, outside the specific token stimuli that break diapause, do not affect metabolism. However, diapause termination ends this central control over metabolic suppression in the middle of winter and the bee continues its overwintering hypometabolic state in a stage called quiescence (Kostál, 2006). Metabolic suppression during quiescence is externally maintained and as temperatures rise, the animal slowly resumes development. These changes in temperatures may cause development to resume and halt countless times during this quiescent stage. Given that this quiescence can last several months (3 to 5), the available nutrient pools in the animal may be used up and adult development compromised. Thus, these predicted temperature increases will likely induce a change in energy usage, post-diapause performance, and the bee's ability to respond to environmental stressors.

Varying environmental conditions have led insects to evolve various mechanisms to deal with environmental stressors such as heat and anoxia (Yocum et al., 2006; Harrison et al., 2006). Some of these mechanisms, like heat shock proteins, can be energetically costly and add to the expense of overwintering (Feder and Hofmann, 1999; Rinehart et al., 2007). However, whether an environmental event is considered stressful or beneficial rests on the magnitude/dose of that event. This concept where high doses are detrimental and low doses are protective is termed hormesis (Calabrese et al., 2007). Hormesis elevates the animal's defenses when exposed to low doses and these defenses allow the animal to maintain higher levels of performance following a challenging event (López-Martínez and Hahn, 2012; López-Martínez et al., 2021). In insects one of the most studied types of hormesis, aside from chemical stress (Cutler, 2013; Guedes and Cutler, 2014), is a temperature preconditioning response known as rapid cold hardening (RCH; Berry and López-Martínez, 2020). During RCH, biochemical changes confer protection from freezing temperatures and boost post stress fecundity and longevity (Denlinger and Lee, 2010). It is possible that diapausing insects experience random bouts of RCH during the course of the entire winter and the subsequent spring, but this has not been empirically tested. The application of hormesis during development is known to improve multiple metrics of performance upon adult emergence. Flies and moths treated during their developmental stages have increased flight and mating performance and they live longer while accumulating lower levels of oxidative damage (López-Martínez and Hahn, 2012; López-Martínez et al., 2014).

One of the better studied hormetic responses is low-oxygen (hypoxia or anoxia), which is the basis of the preparation for oxidative stress hypothesis (POS; Hermes-Lima et al., 1998, Giraud-Billoud et al., 2019). Under POS, the mitochondria prepare for oxygen reperfusion by signaling the elevation of antioxidant and other biochemical defenses during and after the period of exposure to low oxygen. These defenses help repair any damage from the low-oxygen exposure and often protect the animal from additional damage and potential lifelong impacts (Dowling and Simmons, 2009). The animals have higher survival and experience improved performance due to this POS mechanism (Geihs et al., 2020; Berry and López-Martínez, 2020). In insects, anoxia has garnered much attention because invertebrates often survive hours at physiological relevant temperatures where vertebrates only survive minutes (Berry and López-Martínez, 2020). The beneficial effects of anoxia hormesis include increased flight ability (willingness to fly and distance/time flying), mating success early in life (López-Martínez and Hahn, 2012; López-Martínez et al., 2014) and later in life (López-Martínez and Hahn, 2014), living longer (López-Martínez and Hahn, 2014), better offspring performance (López-Martínez et al., 2016a), and improved recovery from additional bouts of anoxia (Visser et al., 2018). Given the connection between the POS hypothesis and anoxia hormesis, we wondered whether anoxia might have the potential for improving performance in an overwintering context in solitary bees.

In this study we investigated whether anoxia hormesis would improve performance in adult bees. We established a dose response curve and determined the length of anoxia required to stimulate a protective response based on previous work (López-Martínez and Hahn, 2012; López-Martínez et al., 2014; López-Martínez et al., 2016b). We hypothesize that a hormetic dose would improve multiple metrics (survival, activity, and longevity) of bee performance and that this effect would be age specific. We also hypothesized that macronutrient usage would be strongly impacted by hormesis. Here we report that one hour of anoxia hormesis improves multiple metrics of bee performance without negatively impacting macronutrients reserves following overwintering diapause in alfalfa leafcutting bees.

2. Methods

2.1. Bee maintenance

Alfalfa leafcutting bee prepupae, *Megachile rotundata*, purchased from JWM Leafcutters (Nampa, ID) were maintained in an incubator at 6 °C in the dark (Percival Scientific, Perry, IA, USA). These conditions allowed us to keep the bees in the post-diapause quiescent stage they were purchased. Prior to treatment, bees were moved to a Percival

incubator under long day (15L:9D) photoperiod and 60 ± 5% relative humidity. The temperature was kept at 29 °C during day time hours and 25 °C during night. It took ~21 days for the prepupae to complete development and emerge as adults under these conditions. Bees were treated as prepupae, pupae, or adults and kept in the same 29 °C incubator under the same conditions after treatment. Treated bees were only used for one of the experiments described below.

2.2. Anoxia treatments

Bees were treated to one of four anoxia treatments: 0, 1, 3, or 6 h of no oxygen (anoxia). In the control treatment (Ax-0) bees experienced normal oxygen levels (normoxia). The lengths of the anoxia treatments were chosen based on previous work with anoxia in four other insect species where we found the hormetic dose to be between 1 and 3 h of anoxia (López-Martínez and Hahn, 2012, López-Martínez et al., 2014, López-Martínez et al., 2016b; De La Torre and López-Martínez unpub). These treatments were applied only once to the bees at different life stages (prepupa, pupa, or adult). The immature overwintering/diapausing prepupae are the stage that resumes development in the spring. In the lab we mimic the onset of spring by transferring them from 6 to 29 °C. Prepupae were treated 20 days before adult emergence (1 day after the transfer to spring-like conditions). Pupae are immature bees that have undergone additional development towards adulthood but are not fully developed adults. These pupae were treated 2 days before adult emergence (~19 days after transfer to spring-like conditions) at a stage called pharate adult. Adult bees already emerged from their brood cells and were treated within 24 h of emergence.

To carry out the anoxia treatments, groups of prepupae, pupae, or adults were placed in 30- or 60-ml syringes (Covidien Ltd., Dublin, Ireland) that were flushed with pure nitrogen gas for two minutes to completely displace all oxygen as previously shown (López-Martínez and Hahn, 2012). The syringes were sealed using a three-way stop cock (Cole Palmer Inc., IL, USA). To ensure the precision of the treatments, individual syringes with sealed anoxic bees were place in polyethylene bags, flushed with nitrogen, and heat sealed to provide a second barrier from oxygen. Control normoxic bees were placed in heavily perforated 30- or 60-ml syringes to allow normal air flow. With the exception of the bees used in the recovery experiments, treated bees were placed in 473 ml cups after treatment for an undisturbed two-hour oxygen reperfusion recovery. Adult bees were then used in the experiments below, while immature bees were left in their individual brood cells and allowed to emerge undisturbed. Adult bees were fed Pro-sweet (Mann Lake Ltd., Hackensack, MN, USA) diluted with water (1:1) ad libitum.

2.3. Emergence

To monitor the effect of age on treatment survival to adulthood, bees treated as prepupae or pupae were individually placed in 24-well plates (Corning Life Sciences, Corning, NY, USA). Five replicate plates were used for each treatment (0, 1, 3, and 6 h of anoxia), and the experiments was replicated at least three times (i.e., 15 plates of 24 bees each/treatment). The plates were monitored daily until adult emergence began. Because adult emergence typically occurs over the course of eight days for this species (Pitts-Singer and James, 2005), we also monitored daily emergence and sexed the bees once they came out. Data are presented as percent bee emergence per day by sex.

2.4. Starvation resistance

Overwintering bees commonly do not have immediate access to food upon adult emergence, thus we decided to test the effect of anoxia on newly emerged bees in the absence of food. Three to five groups of treated adult bees (~15 bees/group, ~45 bees/treatment) were placed in 946 ml plastic cages without food but with access to water. These cages were placed in an incubator as described above. Bee survivorship under starvation was recorded every 24 h. Dead bees were removed daily, sexed, and counted. Data are presented as percent adult bee survival by day and by sex.

2.5. Recovery and daily activity

Insects go into an anoxic comma when oxygen is removed from their immediate environment and must undergo a period of recovery before resuming normal function (Rodgers et al., 2010). To determine the length of this period of recovery in treated bees, groups of 12 bees (6 females and 6 males) were placed in vials (Genesee Scientific, San Diego, CA, USA). These vials were closed with a 3D printed fitted lid, containing a micro mesh, that prevented the bees from escaping but that allowed for adequate air exchange. These vials were placed in activity monitors (Trikinetics, Inc., MA, USA). Four activity monitors, one for each treatment, ran simultaneously for 8 h immediately following the end of the anoxia treatment. The experiment was replicated seven times. Data is presented as time to recovery per treatment. Given our previous work on heat shock affecting bee activity for several days following the treatment (Hayes and López-Martínez in press), we wanted to test whether anoxia exposure affected bee activity beyond the recovery period. Groups of 10 bees (5 females and 5 males) were place in vials as described above and placed in the activity monitors. Four monitors, one per treatment, ran simultaneously and recorded movement for a 24-h period. The experiment was replicated 5 times. A period of 3 h of average daytime activity was used to quantify activity levels as bees were completely inactive at night. Bee survivorship was tracked, and the data was adjusted to reflect any death occurring during the 24-h period. Data is presented as average movement/min/bee.

2.6. Longevity

Following treatment and recovery, groups of 20 to 25 bees, that were treated as pupae or adults, were place in 2.37 l plastic cages with mesh tops. Pro-sweet solution was supplied as food in 8 ml glass vials with a dental wick. The bee cages were checked for mortality three times a week. Dead bees were removed, sexed, and counted. Food was refilled as needed to ensure food was not a limiting factor. Data is presented as percent survivorship over time by sex.

2.7. Energy biochemistry

Macronutrients (carbohydrates, lipids, and proteins) were quantified in treated prepupae to determine whether different lengths of anoxia affected nutrient stores differently for an immobile non-feeding developing bee. Bees were sampled prior to treatment (prepupae), one week after treatment (early pupae), two weeks after treatment (mid pupae), and three weeks after treatment (on the day of adult emergence). Carbohydrates were quantified using the Van Handel anthrone method (Van Handel, 1985a). Three bees per treatment were used for each assay and the assay was repeated three times. Bees were individually homogenized in 2% sodium sulfate using 2.0 mm dia. zirconia beads (BioSpec Products, OK, USA) in a microtube homogenizer (BeadBlaster24; Benchmark Scientific, NJ, USA). Methanol was added to the homogenate and the extracted solution was heat concentrated. Potassium hydroxide and ethanol were used to remove chitin, a structural polysaccharide that can skew glycogen quantification. An anthrone solution was then added and following a heating step, color changed was read at 625 nm. A five-point glucose standard curve (0-2 mg/ml) was used to determine sugar concentration and samples were standardized by fresh weight. Data are presented as mg of glucose/mg of bee weight.

Lipid quantification was done using the Vanillin protocol (Van Handel, 1985b). Five bees per treatment were homogenized for each assay and the assay was repeated three times. Bees were individually

homogenized in 1:1 chloroform-methanol as described above. The supernatant was treated with sulfuric acid and then combined with the vanillin-phosphoric acid solution. Samples were read at 625 nm and a 6-point (0-8 mg/ml) lipid standard curve was used to quantify total lipid content. Samples were standardized using fresh weight. Data is presented as mg of lipids/mg of bee weight. Protein content was quantified by measuring protein homogenates at 280 nm (Stoscheck, 1990). Five bees per treatment were individually homogenized in PBS as described above and the homogenate was used for protein quantification. Protein data is presented as mg of protein/mg of total bee weight.

2.8. Statistical analysis

All statistical analyses were carried out using JMP Pro 15. Percent daily emergence, of prepupae and pupae, and percent starvation survival for 0, 1, 3, or 6 h of anoxia were analyzed using proportional hazards models; a survival analysis that examines the change in the hazard rate over time. The length of period for anoxia recovery in minutes for bees exposed to 0, 1, 3, or 6 h of anoxia was analyzed using a generalized linear model (GLM) followed by linear contrast analysis to tease out treatment effects. Bee activity (movements/min/bee) after 0, 1, 3, or 6 h of anoxia was analyzed using one-way ANOVAs with a Tukey's post hoc analysis. Percent survivorship over time (longevity) data for bees subjected to 0, 1, 3, or 6 h or 0 or 1 h of anoxia were analyzed using proportional hazards models. Weight data (mg) for bee prepupae, pupae, or adults used in the macronutrient experiment after exposure to anoxia (0, 1, 3, or 6 h) were analyzed using one-way ANOVAs with a Tukey's post hoc analysis. Macronutrient content (mg/mg) and percent loss (sugar, glycogen, lipid, protein) were analyzed using generalized linear models (GLMs) followed by Tukey's post hoc or linear contract analysis to separate out treatment effects. Due to our replication strategy, multiple replicates on the day of the experiment and multiple replicates over time, a block term was originally added to each statistical test. Upon determination that there were no day (block) effects, this was removed from the final analysis to simplify the output. For reference and to avoid excessive values in the text, the supplementary full statistical analysis output can be found in Table S1.

3. Results

3.1. Emergence

Bees treated to 3 or 6 h of anoxia as prepupae, or pupae had lower emergence than the controls and those treated to 1 h (Fig. 1A; $p_{treatment} = 0.008$). Bees further along in development, pupae, had a higher percentage of emergence in all treatments (Fig. 1A; $p_{age} = 0.0097$). Anoxia treatment affected development and emergence in treated male prepupae but had no effect on female prepupae ($p < 0.0001$). When we analyze the emergence data for prepupae by sex, we find that only males treated with three hours of anoxia emerged, on average, earlier than the rest (Fig. 1B; $p_{treatment} = 0.03$). Anoxia treatment had no effect on adult emergence for those individuals treated later in life, as pupae, and the only difference noted was that males emerged before females, as expected in this species (Pitts-Singer and Cane, 2011, Fig. 1C; $p_{sex} < 0.0001$). A comparison of the daily emergence of females treated as prepupae with those treated as pupae reveals that age at treatment affects emergence but length of anoxia treatment does not ($p_{age} < 0.0001$). When we compare prepupae- and pupae-treated males we see a different pattern where both age and the interaction between age and dose affects daily emergence ($p_{age} = 0.0004$; $p_{treatment*age} = 0.01$). The main distinction here is that males treated as prepupae with 3 h of anoxia emerge earlier, but this accelerated development was not seen in pupae treated with the same dose.

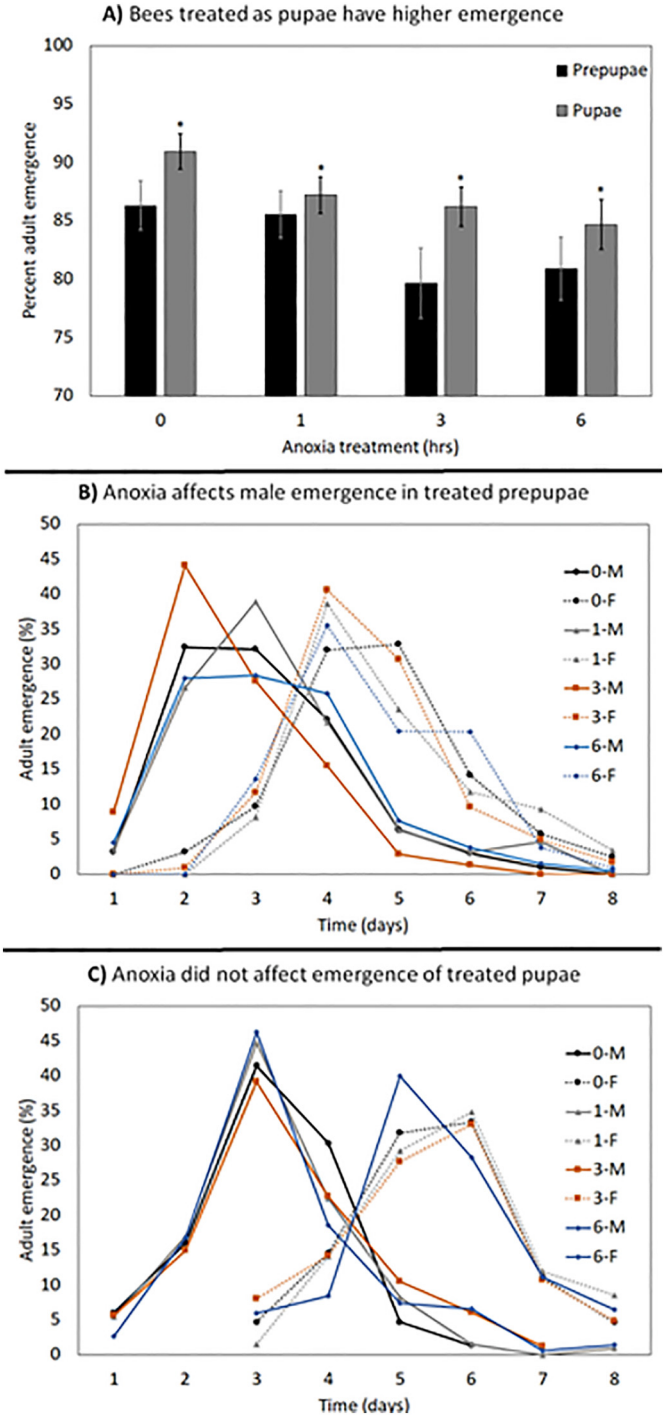

Fig. 1. A) Adult emergence of bees treated to 0, 1, 3, or 6 h of anoxia as prepupae was negatively affected by the treatment while older pupae were not. B) In prepupae, 3 h of anoxia speed up development in males but not females. C) In pupae, anoxia treatment had no effect on the remaining development and adult emergence in either sex.

3.2. Starvation resistance

Most of the treated adult bees died five days into the starvation experiments, with just a handful of bees surviving to the 6th day. The Ax6 group had the most dramatic response to anoxia and over 95% of all bees were dead by day 3 post treatment; the only difference noted for females in response to starvation (Fig. 2A; $X^2 = 13.35$, $p = 0.0039$). Control (Ax0) males survived starvation at the same rate as Ax1 males, but Ax3 males died faster than both and Ax6 males had

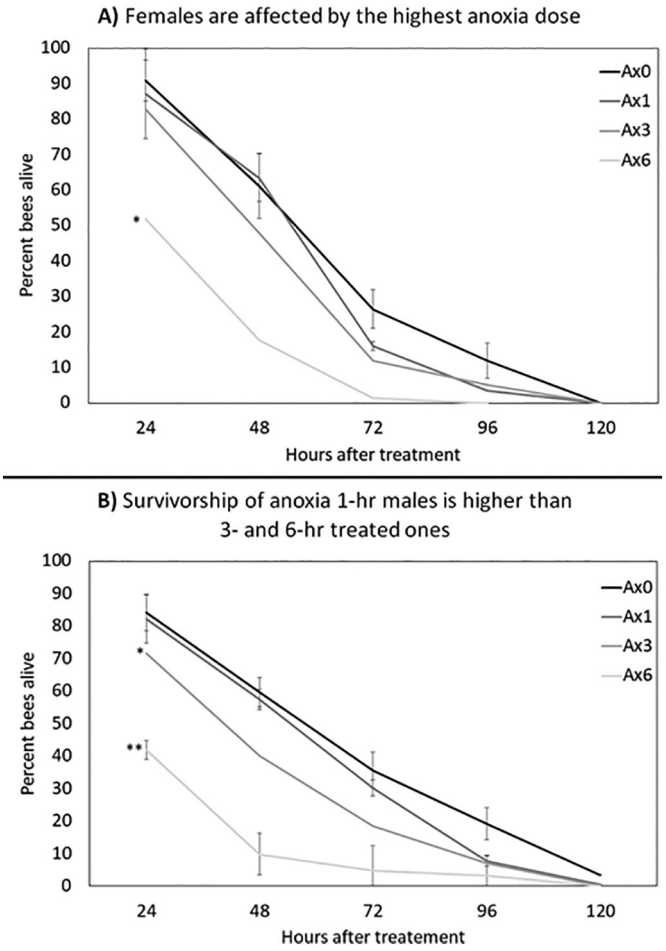

Fig. 2. A) 1 h of anoxia did not affect starvation resistance in female bees, but those treated to 6 h of anoxia had lower survivorship ($p = 0.0039$). B) Ax1 male survivorship was not different from controls (Ax0), but Ax3 and Ax6 males each had lower survivorship under starvation ($p < 0.0001$). Data is presented as average and ± SE. Statistically significant differences are marked by * or **.

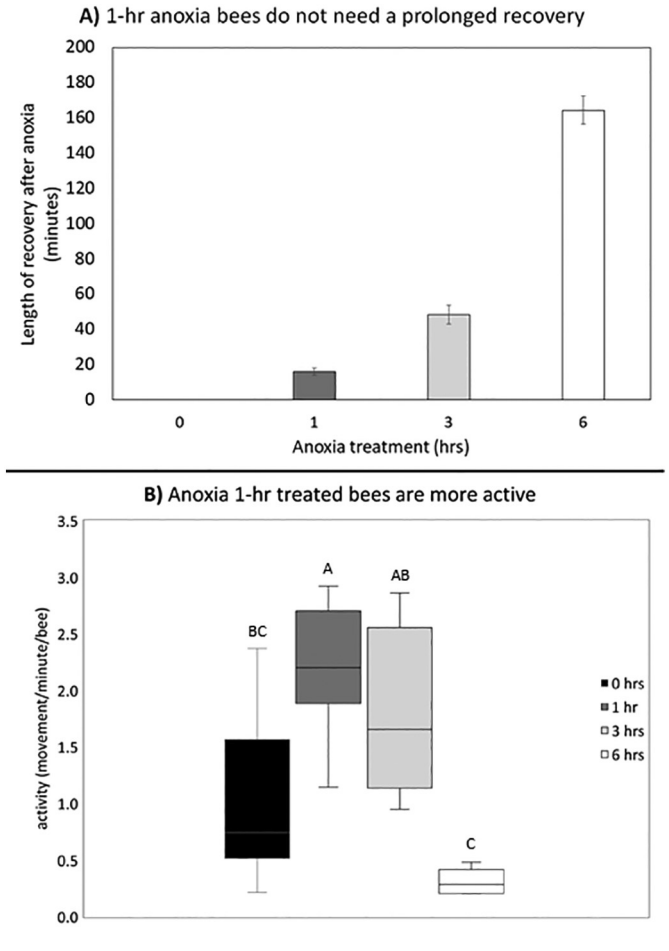

Fig. 3. A) The recovery time needed to resume average mobility following anoxia treatment (anoxic comma) increased with increased length of exposure. Data is presented as average and ± SE. B) Bee activity was higher for Ax1 bees than control (Ax0) and Ax6 bees ($p < 0.0001$). Bees exposed to 3 h of anoxia had activity levels similar to control (Ax0) and Ax1 bees. 6-hour bees were the least active.

the lower survivorship under starvation (Fig. 2B; $X^2 = 69.24$, $p < 0.0001$).

3.3. Recovery and daily activity

The time bees took to recover from anoxia was related to the length of the anoxia exposure (Fig. 3A; $X^2 = 149.47$, $p < 0.0001$). Ax6 bees took the longest time to recover from treatment at ~164 min, ~48 for Ax 3, and ~16 min for Ax1 bees to fully recover and resume locomotory activity. Ax1 bees were more active following full recovery from anoxia (Fig. 3B; $F_{3,26} = 11.88$, $p < 0.0001$). There was no difference between the activity levels of Ax1 and Ax3 bees which concurs with previous work in beetles showing that 1 and 3 h of anoxia are the in hormetic portion of the dose response curve (De La Torre and López-Martínez unpub).

3.4. Longevity

The longevity of bees treated with anoxia as pupae shows there was an effect of treatment and sex (Fig. 4A, B; $p_{treatment} = 0.0002$; $p_{sex} < 0.0001$). For females, Ax6 treated bees lived the longest and were not different from the controls (Ax0; $p = 0.4$), but Ax1 and Ax3 bees had shorter lifespans meaning that hormesis did not occur at either dose ($p = 0.015$). In prepupal males there was no difference between Ax0 and Ax1 ($p = 0.19$), but Ax3 males had the shortest lifespan (Fig. 4B; $X^2 = 10.11$, $p = 0.018$). Ax6 male longevity was not different from controls (Ax0; $p = 0.46$), but it did differ from Ax1 ($p = 0.0495$) and Ax3 ($p = 0.004$). For treated adults, anoxia treatment results in decreased survivorship (Fig. 4C; $X^2 = 17.46$, $p = 0.0006$). Ax3 and Ax6 females had the most dramatic decrease in lifespan, living about half as long as the controls (Ax0). The Ax1 bees lived as long as the Ax0 ones but had a sharper decline in survivorship early in life ($p = 0.017$). Ax6 males had a strong decline in survivorship in the first week following treatment (Fig. 4D; $X^2 = 29.14$, $p < 0.0001$). Ax1 and Ax3 male longevity was not different from the controls ($p = 0.36$). Female bees lived longer than males (Fig. 4C, D; $X^2 = 235.88$, $p < 0.0001$). Adult longevity data represents a different response to anoxia than that seen for treated immature pupal bees. Immature females were only negatively impacted by the highest dose, while all three doses affected adult females. Immature males lived longer when treated in anoxia for 6 h, the same treatment that dramatically decreased adult male longevity.

3.5. Energy biochemistry

There were no weight differences between the prepupae that were randomly selected and treated with anoxia ($X^2 = 0.44$, $p = 0.93$). Sugars (mono- and disaccharides), were higher in prepupae (Fig. 5A; $X^2 = 38.6$, $p < 0.0001$) but not different between adults (Fig. 5A; $p = 0.68$). Glycogen content was also higher in the prepupae (Fig. 5B; $X^2 = 57.12$, $p < 0.0001$). Glycogen content did not vary by treatment in emerged adults (Fig. 5B; $p_{treatment} = 0.77$) but was higher in males

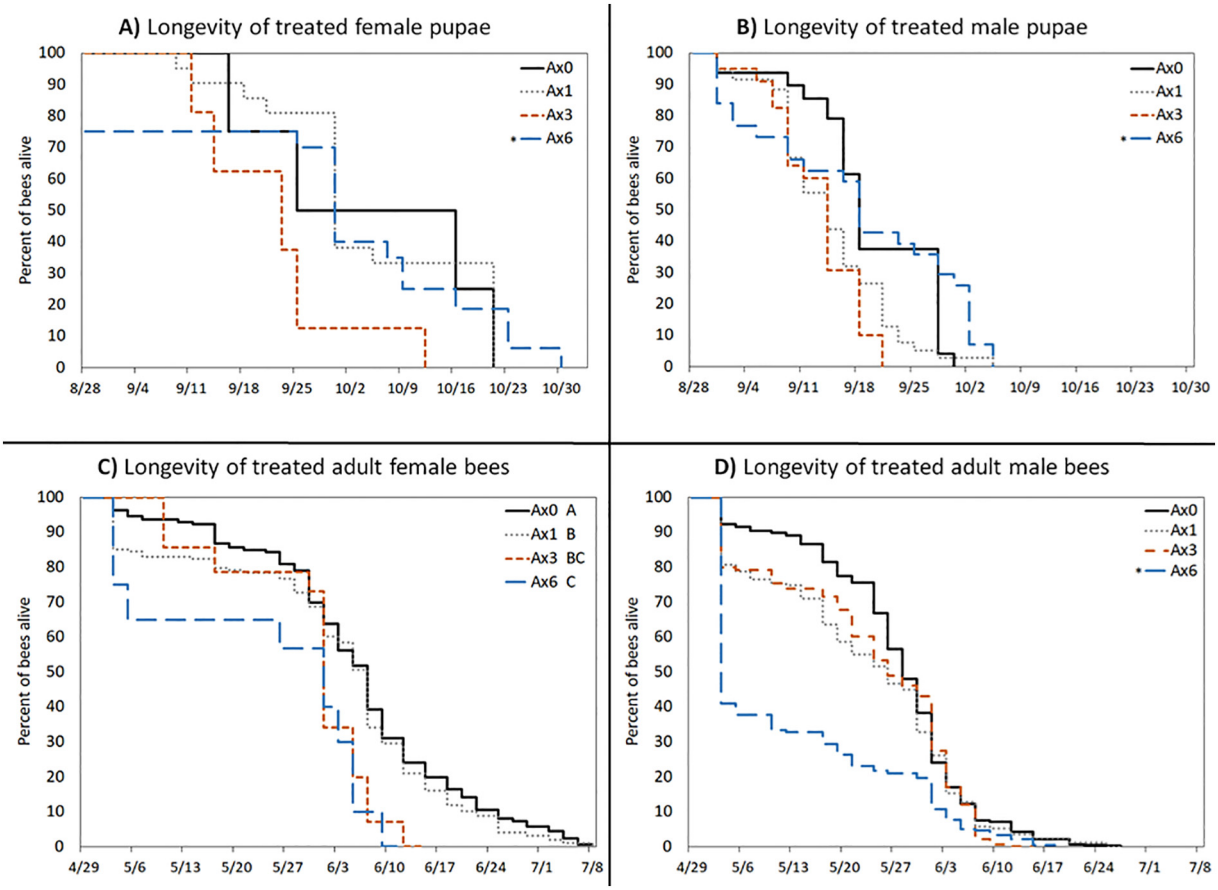

Fig. 4. A) Out of our four anoxia treatments (0, 1, 3, or 6 h), the longest-lived females were those treated with the harshest dose, Ax6 ($p = 0.015$), but the other treatments (1 and 3 h) lived as long as to the controls (0 h). B) Male longevity was not different between Ax0 and Ax1, but Ax3 males had the shortest lifespan ($p = 0.018$). Ax6 male longevity was not different from controls (Ax0), but it did differ from Ax 1 and Ax3. For treated adults, anoxia hormesis had no effect on longevity, but females (C) lived longer than males (D; $p < 0.0001$). Asterisks and letters denote differences.

in all treatments ($p_{sex} = 0.0008$). Total lipid content did not vary with treatment (Fig. 5C; $p_{treatment} = 0.32$) but varied with age ($p_{age} < 0.0001$). There was a treatment by age interaction ($p = 0.03$) in lipid content. Within the treatments, males and females had lower lipid levels at 0, 1, 3, and 6 h of anoxia ($p < 0.0005$). For the 6-h group, week 1 pupae had the highest levels of lipids ($p = 0.0008$). Total protein content changed with age and adults had the lowest levels of protein (Fig. 5D; $p_{age} < 0.0001$), while prepupae had the highest. Even though we found no treatment effects on protein content ($p = 0.16$), we found an interaction of treatment and age ($p_{treatment*age} = 0.005$) where Ax3 had the highest protein levels in males ($X^2 = 8.86$, $p = 0.03$) and Ax1 in females ($X^2 = 10.3$, $p = 0.016$).

To understand the usage of macronutrients during development, we looked at changes (loss or gain). There were no changes in sugar content during development by sex nor treatment (Fig. 5E; $p = 0.67$). But glycogen consumption during development was higher for females (Fig. 5F; $p = 0.0002$). Lipid consumption was unaffected by treatment during week 1 of development (Fig. 6A; $X^2 = 1.64$, $p = 0.65$). There was a strong treatment effect in lipid usage during week 2 where the 6-h bees spent the largest proportion of lipids (Fig. 6B; $X^2 = 16.31$, $p = 0.001$). At the end of the third week adult bees emerged and bees in the 6-h group gained the least lipids, regardless of sex (Fig. 6C, D; $p < 0.0001$).

Anoxia treatment had no effect on protein content during the first week (Fig. 7A; $X^2 = 3.6$, $p = 0.31$) or the second week of development (Fig. 7B; $X^2 = 5.9$, $p = 0.12$). A linear contrast analysis of week 2 protein changes shows that Ax1 bees gained more protein than Ax 6 bees ($X^2 = 4.004$, $p = 0.045$). By the time adults emerged there were changes in protein content due to treatment (Fig. 7C, D; $p = 0.0348$), where Ax3 bees lost the least amount of protein content during the final stages of adult development. This effect was found in males (Fig. 7C; $p = 0.003$), but not females ($p = 0.31$).

4. Discussion

We previously determined that the range of anoxia hormesis for multiple species of insects ranged between 1 and 3 h of exposure to an oxygen-free environment (López-Martínez and Hahn, 2012, López-Martínez et al., 2014, López-Martínez et al., 2016b, De La Torre and López-Martínez unpub). In those insects where anoxia hormesis triggered a stimulatory response, performance improvements ranged from no negative effects in treatment survival and emergence to more individuals flying, mating, and living longer. For alfalfa leafcutting bees we found that 1 h provides the highest level of protection associated with improvements in performance. This dose leads to no negative effects in emergence, starvation, or longevity; unlike the other anoxia doses. The bees also recover faster from 1 h of anoxia, and they are more active following the exposure. Additionally, we see that unlike 3 and 6 h exposures, energy reserves are not depleted by 1 h exposures. Thus, there is potential for the 1 h dose to improvement other performance metrics in this bee that we have not yet measured; such as flight and stress challenges later in life. Three hours did not improve performance as robustly as one, but it was mildly hormetic and both treatments are energetically neutral in the cost of mounting the hormetic response. Six hours of anoxia exceeds the no observed adverse effect level (NOAEL; Calabrese and Baldwin, 2001), and bee performance is negatively impacted likely due to the large reallocation of resources necessary for recovery. We saw that the week following anoxia had

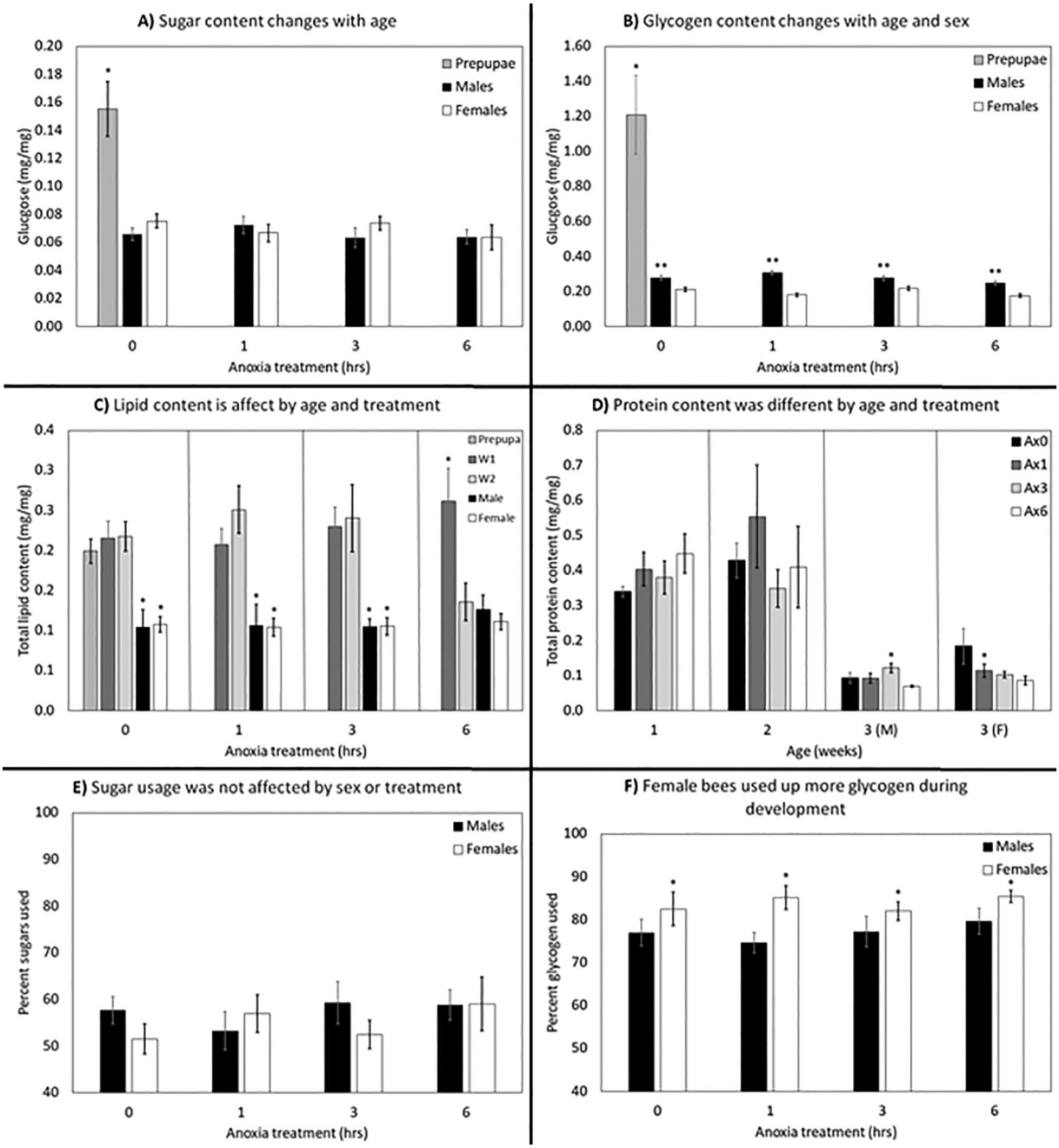

Fig. 5. Prepupae bees prior to development have higher macronutrient concentrations [A) sugar $p < 0.0001$, B) glycogen $p < 0.0001$, and C) lipids $p < 0.0001$] than adults. D) Protein concentration varies during development with immature stages having a higher concentration of protein ($p < 0.0001$). E) Sugar usage was not affected by treatment or sex ($p = 0.4$), but F) male bees had higher glycogen stores because females used a higher proportion of their glycogen during development ($p = 0.0008$).

no effect on bee energy stores, but by week two dramatic lipid usage took place in the 6-h bees. The following week the lipid imbalance was still present, and without the ability to replenish those lipids (bees are actively developing inside their overwintering cocoons), the emerging adults have very poor starvation resistance; one of the few performance metrics for which both females and males were equally negatively impacted. The change in lipid stores during development indicates that adult emergence is a priority and likely the reason we see no treatment effects on adult emergence. The energy usage of the 6-h bees is starkly different than any other treatment yet their emergence rates are between 80 and 85%, similarly to the controls (85 to 90%); which seems to indicate that as a species spring time emergence is very important. When treated as adults, 6-h bees take longer to recover and their overall activity is the lowest of all the treatments. Adult longevity experiments revealed that about 50% of the male bees treated with 6 h of anoxia die in the first week post treatment. This indicates there is a strong connection between available lipids and surviving a longer exposure of anoxia. This may be due to the fact that ischemia-related hypoxia/anoxia inhibits beta oxidation (Eaton et al., 1996) and lipid use becomes an inviable way to replenish glucose and/or ATP. These changes in lipid content were not seen in the other treatments, suggesting that anoxia may lead to efficient use of energy reserves in those shorter treatments and/or supplementation via lipid metabolism was not required.

However, the dose is not the only crucial component to a hormetic response. A second important factor is age at treatment. The application of a hormetic treatment at the wrong age can be the difference between improvement and harm (Berry and López-Martínez, 2020). In essence,

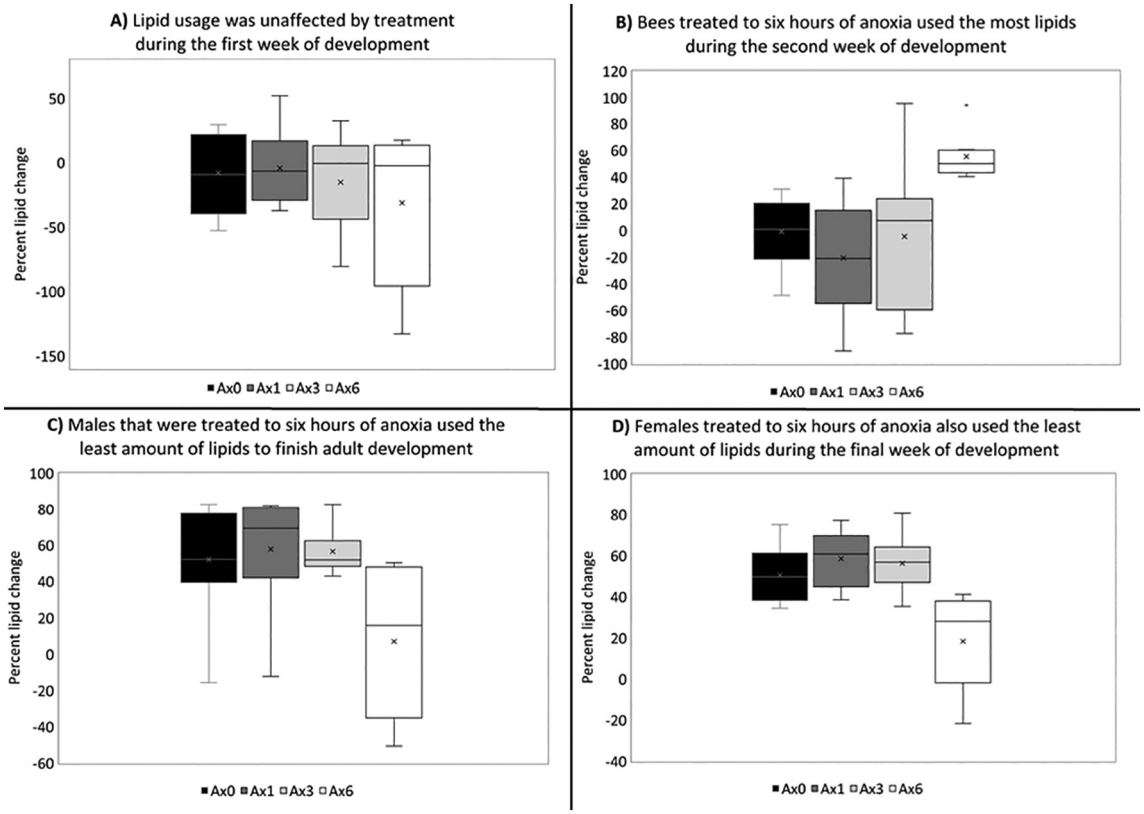

Fig. 6. Lipid usage did not vary during the 1st week of development ($p = 0.65$), but it did during weeks 2 and 3. B) During the 2nd week of development most bees used lipids as did control bees but 6-h bees had a dramatic gain in total lipid content (p 0.001). By week 3, both males (A) and females (B) used large proportions of lipids while 6-h bees were depleted of lipids ($p < 0.0001$).

the NOAEL could shift with age, and we found different responses to anoxia hormesis depending on the time of application. Males were the most affected as their patterns of emergence were dramatically different if treated as prepupae or 2.5 weeks later as pupae. Early in development exposure to anoxia causes a bigger disruption as the bees struggle to finish development and their patterns of emergence are shifted. The biological imperative for this species is to complete development and emerge with the goal of reproduction. Our data indicates that bees continue development, and the higher emergence rates that we see for treated pupae likely come at a cost to them later in life. The adult emergence of treated older developing pupal bees was not affected by treatment and more of them emerged. This could be due to being further along in their development and therefore being able of tolerating the stress better. This pattern of differential treatment response in immature bees was also seen in treated adults. If we compare the longevity (Fig. 4) of the best performing treatment (1-h), treated pupae have a median lifespan of 18 days for males and 36 for females. Treated adults have a median lifespan of 26 days for males and 37 for females. Median lifespan in this species is about 28 days for males and 37 for females. Thus, the stimulatory effect of hormesis present in treated adults is likely absent in bees treated just a few days prior to emergence during a time of high metabolic flux and rapid development (Visser et al., 2018). The race towards adult emergence may cost these bees more than energy resources and this additional metabolic "help" might be difficult to pay back. Upon emergence, food is unlimited, and we expected that longevity would not be impacted once resources are plentiful. Like we have previously recorded in flies (López-Martínez and Hahn, 2012, 2014), hormesis applied during development can have dramatic lifelong consequences where we suspect a shift in metabolic efficiency or activity happens which allows performance to remain high into advanced age (López-Martínez 2014, De La Torre and López-Martínez unpub). This also seems to be the case for 6-h treated bees, of both sexes, that have low levels of activity, but live just as long as untreated bees do.

The responses seen here between males and females highlight the fact that stress responses are multifarious and inter-sex differences in performance occur. Even when treated during their immature stages where sexual characteristics have not yet developed, males are more vulnerable to stress than females. These sex-specific responses might be related to energetics and/or connected to female egg protection. Males consistently have high levels of glycogen stores and treatment has no effect on this. The lack of plasticity on their glycogen stores might be related to the strong requirement that flight puts on available glycogen. Even during the last week of development, male bees used more lipid than females in the 6-h treatment while maintaining their muscle glycogen stores. A possible explanation is that if males fall below some theoretically threshold, it could impact flight as adults. This does not explain why females used more of their glycogen reserves during development but ongoing investigations into flight differences between males and females might provide additional insight.

Our expectation that unlimited resources would aid in treatment recovery was proven to be incorrect. Immediately following recovery from anoxia, the bees had unlimited access to food. If the harmful effects of anoxia exposure were solely related to availability of macronutrients, we would have seen positive changes in bee activity and longevity. It is very likely that the accumulation of free radical damage that occurs as a consequence of prolonged exposure to anoxia (López-Martínez and Hahn, 2012) and the protective mechanism related to the preparation for oxidative stress hypothesis (Hermes-Lima et al., 1998) has its limits. While there seems to be overlap between the mechanisms of hormesis and the POS hypothesis, where the protection conferred by POS overlaps with low dose stimulatory effects of hormesis (Oliveira et al., 2018; Berry and López-Martínez, 2020), there must be a threshold where exposure is too high, and damage accumulates.

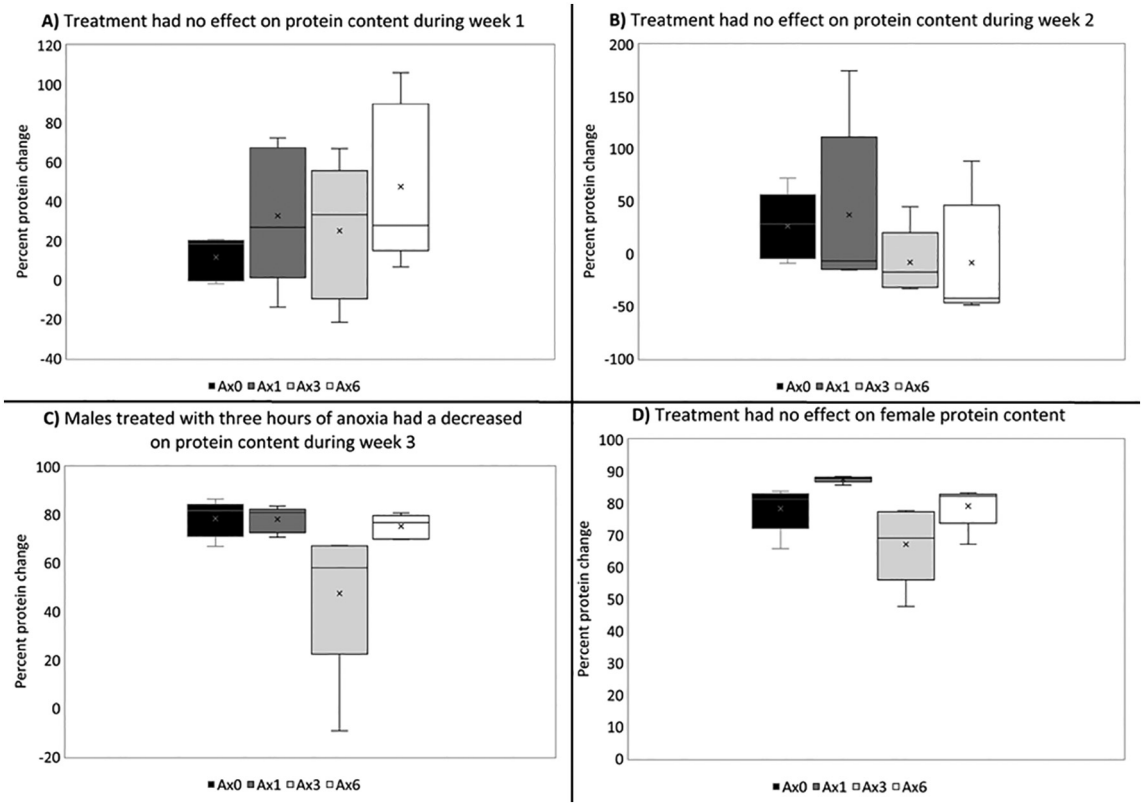

Fig. 7. There were no treatment effects on total protein content during the first two weeks of development following anoxia treatment (A: $p = 0.31$, B: $p = 0.12$), but Ax1 bees gained more protein than 6-h bees by the end of the second week ($p = 0.045$). C) 3-h male bees had a decrease in protein content upon emergence ($p = 0.0007$), but we recorded no difference for female bees (D).

Outside of understanding organismal responses to anoxia hormesis and the dynamic that development plays in these responses, the use of hormesis in alfalfa leafcutting bees has potential to improve bee pollination services. Hormesis is already known to improvement performance in honeybees where mild doses of caffeine and nicotine improve memory and learning (Cutler and Rix, 2015). Even cupric salts used for *Varroa* mite treatment increase lifespan in worker bees (Bounias et al., 1995). Taken together these improvements to bee performance are likely to increase pollination efforts through prolonged bee activity, as we expect is the case in our bees. *Megachile rotundata* is one of the most managed bee pollinators on the planet (Pitts-Singer and Cane, 2011). Every year millions of bees are used for the pollination of alfalfa and their success depends on how well they are able to survive the overwintering storage of 7 to 10 months at 4 to 5 °C and emerge as "healthy" bees in the spring. The bulk of this time is spent in that metabolic suppressed state we described earlier, diapause. The bees are able to save their energy stores in this state but changes in wintering conditions will lead to changes in energy usage (Marshall et al., 2020). We saw here that exposure to 6 h of anoxia can dramatically alter the use of lipid reserves that shorten survival upon adult emergence. Hence the vulnerability of the bees during their overwintering period extends beyond temperature and likely include a number of additional abiotic factors. Using 1 h of anoxia during the developmental period that follows overwintering has the potential to not only preserve energy reserves but improve post-winter adult performance. Anoxia 1 h bees were most active and had starvation resistance and longevity, as least for males, that was not different from untreated bees. Our preliminary data shows that anoxia hormesis also improves multiple metrics of flight in this species (Pithan and López-Martínez unpub). It is important to understand that as a biological mechanism, hormesis is subjected to life history trade-offs associated with limited resources. And while we can see improvements in multiple traits, the cost for these hormetic responses might be significant. Anoxia hormesis improves performance in green peach aphids (Ayyanath et al., 2013), cabbage loopers moths (López-Martínez et al., 2016b), and *Tenebrio* beetles (De La Torre and López-Martínez unpub), but the cost is a significant decrease in reproductive output. For moths and beetles, we see significant decreases in fecundity (eggs laid) and fertility (eggs hatched). Thus, perhaps with additional work and understanding of anoxia hormesis in this species, it would be possible to design and carry out hormetic treatments for overwintering bees that could result in higher pollination services in the spring.

We conclude that there is potential for anoxia hormesis in this species and anoxia is a good candidate for interventions aimed at improving bee performance following long-term storage or overwintering. We postulate that studies looking into the effects of hormesis, not just anoxia hormesis, would further allow us to understand the dynamics of abiotic dose responses and may uncover potential new ways of improving performance. The use of temperature hormesis is currently applied as fluctuating temperature regimes (FTR) that improve overwintering and storage survival in bees (Yocum et al., 2021) and flies (Melicher et al., 2021). Studies that focus on multiple metrics of performance (treatment survival/emergence, starvation resistance, flight/activity, longevity, etc.) are needed to fully understand the broad scale of responses triggered by hormesis. We know that these types of hormetic interventions can have transgenerational effects in insects (Ayyanath et al., 2013; López-Martínez et al., 2014) and recognize that additional work is needed to fully understand the response. The differences that we saw between males and females add to the growing number of studies showing that there are sex-specific responses to stress. Understanding these sex effects might allow for the design of treatments that maximize performance for the entire bee population, while keeping in mind that the age of application can be as crucial a component to stress survival as the dose itself.

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CRediT authorship contribution statement

Lidia Cervantes and Giancarlo López-Martínez: Conceptualization and design of experiments. Giancarlo López-Martínez: obtained the funding. Lidia Cervantes and Giancarlo López-Martínez: carried out the experiments, data analysis, and wrote the manuscript. Giancarlo López-Martínez: revisions and editing.

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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LC and GLM conceived the idea and designed the experiments. GLM obtained the funding. LC and GLM carried out the experiments, the data analysis, and wrote the manuscript. Research reported in this publication was supported by National Science Foundation Office of Integrative Actives RII Track-2 #1826834 and USDA NACA 58-3060-9-025 to GLM. The authors wish to thank Pollination Nation, where LC was a participant, and the Fargo ICE network for their assistance in the early stage of these experiments. The authors wish to thank multiple anonymous reviewers for making meaningful contributions to the thesis of our manuscript.

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