scientific reports

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Prepupal diapause reduces adult lifespan in the solitary alfalfa leafcutter bee

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Diapause is an adaptation that allows many insects to survive adverse environmental conditions. While diapause-associated trade-offs with reproduction are well-documented, its potential impact on lifespan and long-term adult physiology remains poorly understood. In this study, we used *Megachile rotundata*, a bivoltine solitary bee species with facultative diapause, to investigate the effects of prepupal diapause on adult lifespan and physiology. We compared individuals that underwent diapause to those that did not. Our findings reveal a trade-off between prepupal diapause and adult lifespan, with diapause females exhibiting shorter adult lifespans. Newly-emerged females showed no differences in immune response capacity but females that emerged from diapause had higher levels of some measures of oxidative stress and slightly elevated *prothoracicotropic hormone* (*PTTH*) expression. These results highlight the physiological consequences of alternative developmental pathways and provide new insights into how diapause strategies shape life-history traits in insects.

Keywords Prothoracicotropic hormone, Oxidative stress, Developmental theory of aging, Life history tradeoffs, Encapsulation response, Immunity

Lifespan varies significantly among species and even among individuals within the same species[1]. Evolutionary theories of aging explain how differences in lifespan are shaped by both genetic and environmental factors[2,3]. One such factor is diapause, a state of dormancy that allows many insects to survive unfavorable environmental conditions. Diapause can be obligatory, where developmental or reproductive arrest occurs regardless of environmental cues, or facultative, where individuals alternate between direct development and diapause in response to specific stimuli[4]. Facultative diapause, in particular, results in remarkable differences in total lifespan among individuals of the same species, driven by the extended time spent in diapause at a specific life stage. However, diapause may also come with costs, leading to life history tradeoffs.

A well-documented trade-off in many species is between diapause and reproduction. Decreased fecundity and egg-laying are commonly observed in insect species that diapause in the prepupal or pupal stage[5]. In contrast, this trade-off is more variable in species that diapause as adults, with some showing no reproductive costs[6] and others producing more offspring than non-diapause females[7]. Another potential, but understudied trade-off, is between prepupal diapause and adult lifespan. While diapause itself extends lifespan during dormancy, adult diapause is often associated with adaptations that slow senescence and extend adult lifespan[8,9]. However, in holometabolous insects, prepupal diapause is followed by the drastic changes of metamorphosis, including renewal of somatic tissues, potentially reducing selection on delayed senescence among adults[9]. It is therefore unclear whether the physiological adaptations required for survival during prepupal diapause have long-term consequences on adult fitness beyond reproductive costs.

Adaptations necessary for diapause survival may contribute to trade-offs. For example, in the butterfly *Araschnia levana*, pupae in diapause invest more in their immune system than pupae that undergo direct development, as evidenced by higher phenoloxidase (PO) activity and improved survival to a bacterial infection challenge[10,11]. However, activating the immune system is energetically costly[12]. The activation of phenoloxidase generates free radicals that, if unbalanced, might cause oxidative stress and tissue damage, which could lead to trade-offs with longevity[13,14]. Oxidative stress has been linked to aging, as reactive oxygen species (ROS) can damage proteins, lipids, and DNA over time. If this damage accumulates, it can interfere with cellular function and accelerate senescence[15,16]. However, elevated oxidative stress and an increased immune response are not universal consequences of diapause. In some species, these responses are only triggered when diapause individuals encounter environmental challenges or pathogens[17].

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Another potential source of trade-offs between diapause and lifespan is the expression of the *prothoracicotropic hormone* (*PTTH*) gene. PTTH regulates the release of ecdysone by activating the tyrosine kinase receptor TORSO, promoting molting or metamorphosis in insects[18]. Reduced PTTH levels delay the larva-pupa transition in *Drosophila*[19] and PTTH is a major regulator of larval and pupal diapause across insects[17]. Interestingly, *PTTH* loss-of-function mutants in *Drosophila* have extended adult lifespan, increased oxidative stress resistance, and enhanced female fecundity, but only if the knockdown occurs during development[20]. This suggests that the expression of *PTTH* during development might have detrimental effects on the adult lifespan. If so, then we would expect reduced expression among individuals with longer lifespan. In bees, *PTTH* and its receptor *TORSO* genes are lost in species that diapause as adults or do not diapause[21], most of which are social bee species[22]. In contrast, species that diapause during the prepupal stage retain both *PTTH* and *torso* genes. This makes *PTTH* a particularly interesting candidate for exploring trade-offs between diapause and adult lifespan.

In bees, diapause may be facultative or obligatory, depending on the species and geographic distribution[23–26]. In most species, diapause occurs at the prepupal stage[27] and involves profound physiological changes that can affect survival, reproduction, and other life-history traits[5]. Despite being a common life-history strategy, the long-term consequences of undergoing diapause versus direct development on adult physiology remain poorly understood. Few studies have investigated the relationship between immune response and oxidative stress during diapause in bees, and the findings are highly variable among species. Worker honeybees that overwinter live longer than summer worker bees, possibly due to upregulation of antioxidant enzymes and antimicrobial peptides in aged winter bees[28]. In contrast, bumble bee (*Bombus terrestris*) queens do not consistently exhibit increased immune response during diapause. Antimicrobial peptides can be elevated after mating and persist during diapause[29], but genes like *defensin* (antimicrobial response) and *phenoloxidase subunit A3-like* are down-regulated during diapause[24]. Among solitary species that diapause as prepupa, immune-related genes are up-regulated during diapause in *Tetrapedia divesipes*[30], and in post-diapause quiescent prepupae of *Megachile rotundata*[31]. These findings suggest that bees may exhibit trade-offs as a result of the diapause program.

In this study, we used *Megachile rotundata* as a model to investigate how prepupal diapause affects newly-emerged adults compared to individuals that developed directly without diapause. *M. rotundata* is a solitary bee species with a bivoltine life cycle and facultative diapause. Individuals emerging in the spring undergo direct development, while those from late summer arrest development at the prepupal stage and overwinter until the following spring[26]. Diapause trade-offs with provision size and reproduction have been reported for this *Megachile* species. Females that undergo diapause provide their offspring with smaller provisions[32–34], which, in turn, influences whether the offspring will enter diapause[32,33]. Additionally, females which develop directly build significantly more brood cells than the diapause females after emergence under certain environmental conditions[32]. Understanding trade-offs between prepupa diapause and adult lifespan will advance our knowledge about diapause physiology and may help to improve rearing methods in this economically important pollinator.

We first evaluated whether a trade-off exists between prepupal diapause and adult lifespan. Next, we examined three key physiological traits in newly-emerged adults: immune response, oxidative stress, and *PTTH* transcript abundance to identify potential mechanisms underlying lifespan variation. We found that there is a trade-off between prepupal diapause and adult lifespan. Females that overwintered in diapause had shorter lifespans than individuals with direct development. Newly-emerged adults did not show differences in their capacity to respond to an immune challenge, but females that overwintered had lower levels of peroxidized lipids, higher levels of carbonylated proteins, and higher transcript abundance of *PTTH*. These findings provide insights into the physiological consequences of alternative developmental pathways and contribute to a broader understanding of how diapause strategies shape insect life histories.

Methods

Experimental setup

M. rotundata bees were offspring collected from a commercial alfalfa farm in Malad, ID in the summer of 2022 (diapause) and 2023 (non-diapause). Parents of these offspring were purchased from a bee supplier for commercial pollination (Watts Solitary Bees, USA). Nests were brought to the laboratory to determine diapause fate using x-ray imagery (Faxitron MX-20 with a computed radiography high-resolution system; Faxitron X-Ray LLC, Linconshire, IL). Bees were classified as diapause if they spent October to June overwintering at 4 °C, and as non-diapause if they had direct development. Bees remained as prepupa until the start of the experiment, at which time cocoons (both diapause and non-diapause) were transferred to 29 °C to complete development. Diapause and non-diapause bees completed development and emerged as adults within the same 1-week period (August 10 to August 17) in summer 2023. Cocoons were checked daily for new emergence. Most bees were entered into the lifespan study, but a subset was collected upon emergence by submerging them in liquid nitrogen with subsequent storage at – 80 °C. These bees were used to assay oxidative stress and *PTTH* transcript abundance. Another subset was collected live on the day of emergence for immunity assays.

Lifespan

Newly-emerged diapause and non-diapause females were placed into individual cages (9 × 7.5 cm) upon emergence. Each individual was provided with artificial nectar *ad libitum*. We prepared and combined cane syrup and invert syrup in a 1:1 ratio following Rowe et al.[35]. We included preservatives (citric acid and sorbic acid), but did not include any additional supplements (e.g., amino acids, feeding stimulants). The artificial nectar was stored at 4 °C and replaced in the cages every 2–3 weeks during the experiment. Bees were kept in a Percival incubator chamber set at 29 °C, 10–35% relative humidity, and a constant 0:24 L:D cycle. Mortality was monitored daily, and dead bees were frozen individually in labelled tubes at – 20 °C. Body size was measured as thorax intertegular distance (ITD) using a Leica M80 microscope and Leica Application Suite software.

Immune assays

Newly emerged diapause and non-diapause females were individually placed in 15 mL conical tubes and immobilized by chilling on ice packs. Under a stereo microscope, immobilized bees were constrained using two entomological pins crossed above the junction between the thorax and abdomen. The ventral part of the abdomen was exposed and cleaned using a cotton swab embedded in distilled water. A disposable 32G needle (Knixxo, USA) was used to puncture a hole under the third abdominal sternite, through which a 3 mm long nylon filament (0.06 mm in diameter) was inserted. The filament was pre-roughened with sandpaper and stored in 70% ethanol until use. Before insertion, the filament was dried with sterilized tissue paper and then immersed in a lipopolysaccharide (LPS) solution (0.5 µg/µL) from *Escherichia coli* (Sigma-Aldrich, USA). Approximately one-third of the filament's length was left protruding from the bee for easier removal using fine forceps. The bees with the filaments inserted were kept in an incubator for four hours at a controlled temperature of 28 °C. By the end of this time, if the bee was still alive and active, the nylon filament was removed and pictures were taken for subsequent measurement of the immune response.

The filament was placed on a small piece of a white plastic (cut from disposable weighing trays) for imaging. Three pictures were taken from different angles using a Leica stereo microscope equipped with an embedded camera connected to a laptop. A new, unused filament was placed to the left of each melanized filament as a reference for analysis. The microscope magnification varied, but a 1 mm scale was included in each image. Both top and back lights were set to maximum for each of the images. The filaments were preserved in glycerol, and the bees were killed in liquid nitrogen before being stored in a – 80 °C freezer.

Immune challenges, such as the injection of live bacteria or the insertion of foreign objects like nylon filaments, are frequently used to elicit immune responses in insects[36]. The filament coated with LPS acts as a foreign object, triggering the encapsulation immune response, which involves the activation of the PO enzyme and the melanization process[37]. The deposition of melanin on the nylon filament serves as a proxy for the intensity of the immune response in each individual bee.

The immune response was inferred through the grey value measurements of the nylon filament pictures analyzed using ImageJ Fiji[38]. The images were first uploaded and converted to 16 bit. Rectangles were drawn around both the sections of the melanized filament which was inside the bee and a blank, unmelanized filament which was used as a reference in the same image. The rectangles were drawn as close to the perimeter of the filaments as possible. The average grey value of each box was then measured and recorded. To account for variability in photograph lighting, the grey value of the blank filament was subtracted from the grey value of the melanized filament. The difference of the grey values was then averaged across the three photographs taken for each filament. Higher grey values indicate more melanization and thus a stronger immune response.

Oxidative stress assays – protein carbonylation

Protein oxidation leads to the formation of stable carbonyl groups, which serve as markers of oxidative damage. To assess oxidative stress in the female bees' thoraxes, we used the Pierce BCA Protein Assay Kit with Dilution-Free BSA Protein Standards (Thermo Fisher Scientific, Catalog #s A55864, A55865) to quantify total protein concentration and the Protein Carbonyl Content Assay Kit (Sigma-Aldrich, Catalog # MAK094) to quantify carbonyl content.

Prior to both assays, the frozen thorax was crushed to a paste consistency using a plastic pestle in 1.5 mL centrifuge tubes and then diluted with 280 µL of 1% PBS to solubilize as much tissue as possible. The samples were kept on ice when not manipulated. Each sample was vortexed and centrifuged in the Eppendorf 5430R refrigerated centrifuge at 4 °C and 13,000 rpm for 5 min to separate tissue and cuticle debris from the homogenized cellular suspension. The supernatant was collected for use in both the BCA and Carbonyl Content assays. For the BCA assay, 10 µL of the supernatant was diluted with 90 µL of 1% PBS to achieve a 1:10 dilution. The assay was performed following the manufacturer's protocol, and sample absorbance was measured at a wavelength of 562 nm using the Agilent BioTek Epoch Microplate Spectrophotometer and Gen 5 microplate reader (version 3.10.06). Each sample was run in triplicate.

For the Protein Carbonyl Content assay, 20 µL of the supernatant was diluted with 80 µL of purified water. Following protocol, 10 µL of 10% streptozocin solution was added to prevent interference from nucleic acids. After adding 30 µL of 87% TCA solution and a 5-min incubation on ice, samples were centrifuged in a refrigerated centrifuge at 13,000 rpm and 5 °C for 2 min. This process was repeated following the addition of ice-cold acetone and 5-min incubation at – 20 °C. Subsequently, 6 M guanidine solution was added to the samples, and most proteins re-solubilized without further vortexing or incubation. The absorbance of the samples, including negative controls, was measured at 375 nm. Each sample was run in duplicate.

Oxidative stress assays – lipid peroxidation

We measured lipid peroxidation by quantifying thiobarbituric acid reactive substances (TBARS) in the abdomens from the same set of 24 females used to measure protein carbonylation. We used the Pierce BCA Protein Assay Kit with Dilution-Free BSA Protein Standards (Thermo Fisher Scientific, 205 Catalog # A55865 Lot YG379902) to quantify total protein concentration and the Cayman Chemical TBARS Assay kit (Item 10009055, Batch 0803381) to quantify the concentration of malondialdehyde (MDA). Samples were suspended in RIPA Buffer (Pierce, Ref 89901, Lot AB405689). Both kits were used according to the manufacturer's recommendations. The BCA assay was run in duplicate and the TBARS assay was run in triplicate.

The frozen abdomens were manually homogenized in 250 µl of RIPA buffer using a plastic pestle on dry ice. The homogenate was then centrifuged for 10 min at 10,000 rpm at 4 °C to precipitate pieces of tissue and cuticle. The supernatant was collected for use in both the BCA and TBARS assays. For the BCA assay, 30 µL of the supernatant was diluted with 70 µL of RIPA buffer to achieve either a 3:10 or 2:10 dilution. Bovine Serum albumin (BSA) standard dilutions from (0–1500 µg/ml) served as a standard curve. Sample absorbance was

measured at a wavelength of 562 nm using the Agilent BioTek Epoch Microplate Spectrophotometer and Gen 5 microplate reader (version 3.10.06). For the TBARS assay, 100 µL of the supernatant was added to the 800 µL color reagent and 100 µL TBA SDS solution. Following protocol, the colorimetric standards were prepared with RIPA buffer. The absorbance of the samples, including negative controls, was measured at 535 nm. MDA concentration was normalized to protein concentration.

Transcript abundance

RNA was extracted for n = 12 diapause and non-diapause newly-emerged female bees using the whole head. The RNeasy Mini Kit (Qiagen) was used following the manufacturer's protocol, including the DNase treatment step. RNA was quantified using the Qubit 2.0 Fluorometer (Thermo Scientific). For the RT-qPCR analysis, forward and reverse primers were designed for the prothoracicotropic hormone (*PTTH*) gene (forward: 5'-CGTGCT CGGACGTAAACTTCAT-3'; reverse: 5'-GTCACGTTCGCATCGGTACA-3') using Primer-BLAST, and their specificity was checked against the *M. rotundata* core nucleotide database. The primers were designed to span an exon-exon junction, including at least one intron between them. The genes RPS18 and RPL8, which are uniformly expressed across all life stages, including diapause and non-diapause, were used as controls[39].

For each individual, 60 ng of RNA was converted to cDNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems), following the manufacturer's instructions. For the qPCR reaction, 1 µl of cDNA (3 ng) was mixed with 0.2 µl of each forward and reverse primer (10 µmol), 4.6 µl of water, and 5 µl of iQ SYBR Green Supermix (Bio-Rad). The reactions were run in a CFX Opus 384 machine (Bio-Rad) and PCR conditions followed the 2-step real-time PCR protocol suggested in the iQ SYBR Green Supermix manual (95 –180 s; 40 x: 95 °C–15s, 60 °C–60 s; melt curve: 55–95 °C in 0.5 °C increments – 30 s). Two negative controls were included: a reaction using a mix of all cDNA samples without the reverse transcriptase enzyme and water in place of the RNA sample, which was included every time a new cDNA reaction was prepared. PCR product quality and specificity were verified using melt curve analysis in the BR.io Cloud Platform (Bio-Rad). Samples were run in triplicate and averaged for use in the statistical analysis. Transcript abundance levels of the candidate gene were normalized to the geometric mean of the two control genes using the 2-ΔΔCt method.

Statistical analysis

All statistical analyses were completed in R (v4.3.2 or v4.4.2[40]) using data manipulation functions from tidyverse v2.0.0[41]. Survival rate was compared for diapause and non-diapause females with the Survival package v3.7-0[42] and plotted with Survminer v0.4.9[43]. Cox hazard models were fit and plotted with diapause and body size using the *coxph* and *ggforest* functions in the ggfortify package v0.4.16[44]. Body size of females used for lifespan was fit to a linear model with diapause status as predictor. Immunity (average grey values) and oxidative stress (carbonylation/total protein or MDA/total protein) data were visually inspected for outliers and fit to a normal distribution using qq plots and histograms. The assumptions for fit to a linear model were tested with an Anderson-Darling test (nortest v1.0-4[45]), Levene's test of homogeneity (car v3.1-2[46]), and a Shapiro-Wilk test (stats v4.4.2[40]). Immunity was fit to a linear model with diapause status and body size as predictor variables (lme4 v1.1-35.1[47]). Protein carbonylation was fit to a mixed effects linear model with diapause status and body size as fixed effects, and assay batch as a random effect (lme4 v1.1-35.1[47]). Lipid peroxidation was fit to a mixed effects linear model with diapause status as the only fixed effect (because the AIC value was lower without inclusion of body size) and assay batch as a random effect (lme4 v1.1-35.1[47]). The relationship between lipid peroxidation and protein carbonylation was tested with cor.test (stats v4.4.2[40]). *PTTH* transcript abundance differences were assessed by comparing ΔCt values between diapause and non-diapause groups. First, an F-test was conducted to verify the assumption of equal variance between the two groups (stats v4.3.2[40]). Subsequently, a one-tailed and two-tailed independent two-sample t-test were performed to determine whether there was a significant difference in *PTTH* transcript abundance (stats v4.3.2[40]).

Results

Lifespan

Diapause had a significant impact on the female adult lifespan. Females that went through diapause had a median lifespan that was 9 days (17.3%) shorter than those who developed directly (43 ± 2.19 SE vs. 52 ± 2.71 SE). Diapause females had a lower rate of survival in a Kaplan-Meier analysis ($p = 0.02$; Fig. 1A). Diapause and body size were significant predictors in a Cox Proportional Hazards model ($p = 0.009$; Fig. 1B). Diapause increased the probability of mortality compared to non-diapause (HR = 2.72, $p = 0.003$). Increased body size decreased the probability of mortality (HR = 0.14, $p = 0.04$). Diapause females were 9.9% larger (D: mean = 2.59 mm ± 0.03 SE; ND: mean = 2.36 ± 0.03 SE), on average, than non-diapause females (lm; $p < 0.001$; Fig. 2A).

Immunity and oxidative stress

The difference in adult survival was not likely caused by differences in immunity. There were no significant differences in immune response, measured as the encapsulation/melanization response, between newly emerged individuals that diapaused or developed directly ($F_{1,21} = 0.02$, $p = 0.88$; Fig. 2B). However, there were significant differences in oxidative stress between diapausing and non-diapausing females (Fig. 3). Diapausing females had 26% higher levels of protein carbonylation in the thorax than those that developed directly. Diapause ($X^2 = 12.45$, $p = 0.0004$) and increased body size ($X^2 = 5.68$, $p = 0.02$) significantly increased carbonylated proteins (overall model: $X^2 = 22.69$, $p = 4.69e\text{-}5$, $n = 12$ per condition). Conversely, non-diapausing females had 34% higher levels of lipid peroxidation in the abdomen than diapausing females ($X^2 = 4.50$, $p = 0.03$, $n = 12$ per condition). There was not a significant correlation between thorax protein carbonylation and abdominal lipid peroxidation within females ($r = 0.14$, $p = 0.52$).

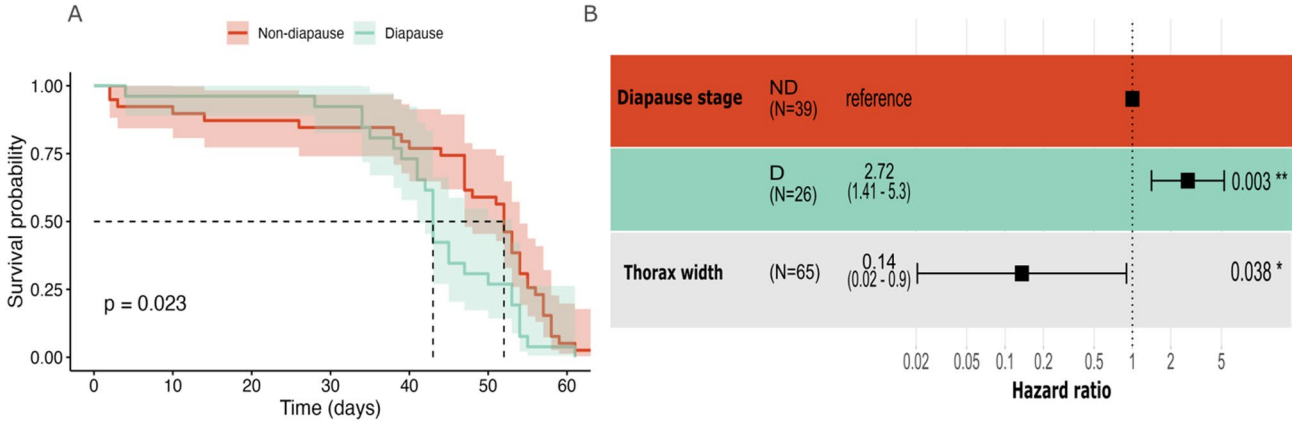

Fig. 1. Lifespan is significantly reduced by diapause. (**A**) Kaplan-Meier survival curves for diapause (D, green) and non-diapause (ND, red) bees with 95% confidence intervals (shaded). Dotted lines indicate median survival for each group. A log-rank test shows a significant difference between groups ($p = 0.023$). (**B**) Cox proportional hazards analysis assessing the effect of diapause and body size (thorax width) on mortality risk. Hazard ratios (HR) are shown with 95% confidence intervals in parentheses. Diapause bees (D) had a 2.72-fold higher mortality risk than non-diapause bees (ND) (HR = 2.72, 95% CI: 1.41–5.3, $p = 0.003$**). In contrast, larger body size was associated with a significantly lower mortality risk (HR = 0.14, 95% CI: 0.02–0.9, $p = 0.038$*). The black squares represent hazard ratio estimates, and horizontal error bars indicate the 95% confidence intervals (CI). The dotted vertical line at HR = 1 is the reference value used as a comparison point and represents no effect.

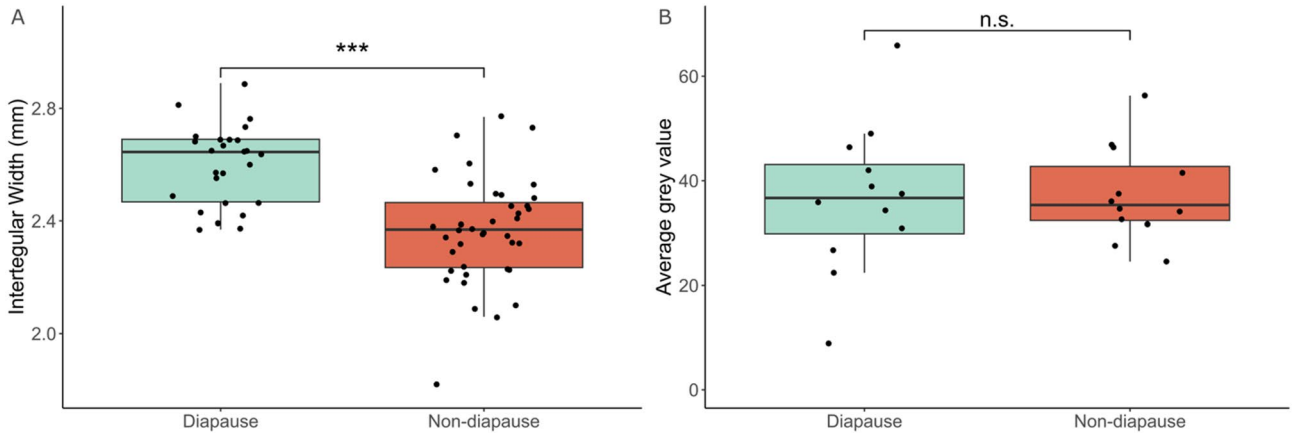

Fig. 2. Body size and immunity in newly emerged females. (**A**) Body size, measured by intertegular width (mm), of *Megachile rotundata* females used in the lifespan assay. Diapause females are significantly larger than non-diapause females (***: $p < 0.001$). (**B**) Diapause did not significantly influence immune response (measured as grey value in an encapsulation assay) at emergence. n.s. - not significant. Boxes represent the interquartile range (IQR), with the horizontal line indicating the median. Whiskers extend to show the range of most data points, excluding outliers. The minimum/maximum whisker values are calculated as the first quartile (Q1) and the third quartile (Q3) –/+ 1.5 * IQR. Dots represent individual data points.

Transcript abundance

The level of transcript abundance of the *PTTH* gene was 1.3 times higher in the diapause group compared to the non-diapause group (Fig. 4) Although this difference is significant in a one-tailed t-test (t = – 1.91, df = 22, $p = 0.03$), that is not maintained in a two-tailed test ($p = 0.07$).

Discussion

In the current study, we tested the hypothesis that prepupal diapause influences adult lifespan of *M. rotundata* by comparing diapausing and non-diapausing females. We also compared immune response, oxidative stress levels, and *PTTH* transcript abundance upon emergence as potential explanations for differences in lifespan. Our findings show evidence for a trade-off between diapause and adult lifespan in females. Bees that went through diapause had reduced adult lifespans compared to those that developed directly, despite having a significant body size advantage which promotes longevity. Thus, the lifespan shortening effects of diapause are strong

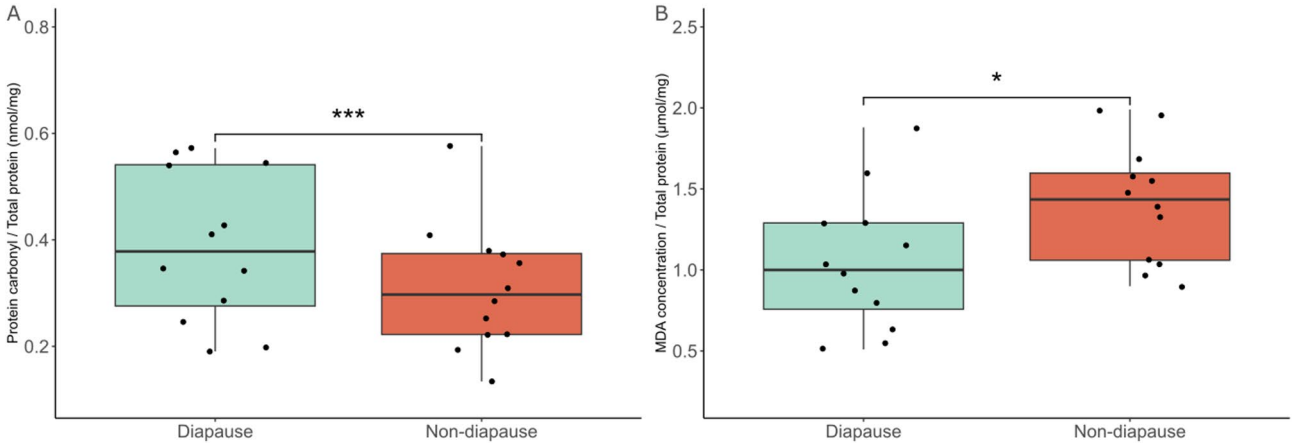

Fig. 3. Oxidative stress in newly emerged females. (**A**) Females who went through diapause had significantly higher levels of protein carbonylation at emergence. ***$p < 0.001$. (**B**) Females who did not diapause had significantly higher levels of lipid peroxidation at emergence. *$p < 0.05$. Boxes represent the interquartile range (IQR), with the horizontal line indicating the median. Whiskers extend to show the range of most data points, excluding outliers. The minimum/maximum whisker values are calculated as the first quartile (Q1) and the third quartile (Q3) –/+ 1.5 * IQR. Dots represent individual data points.

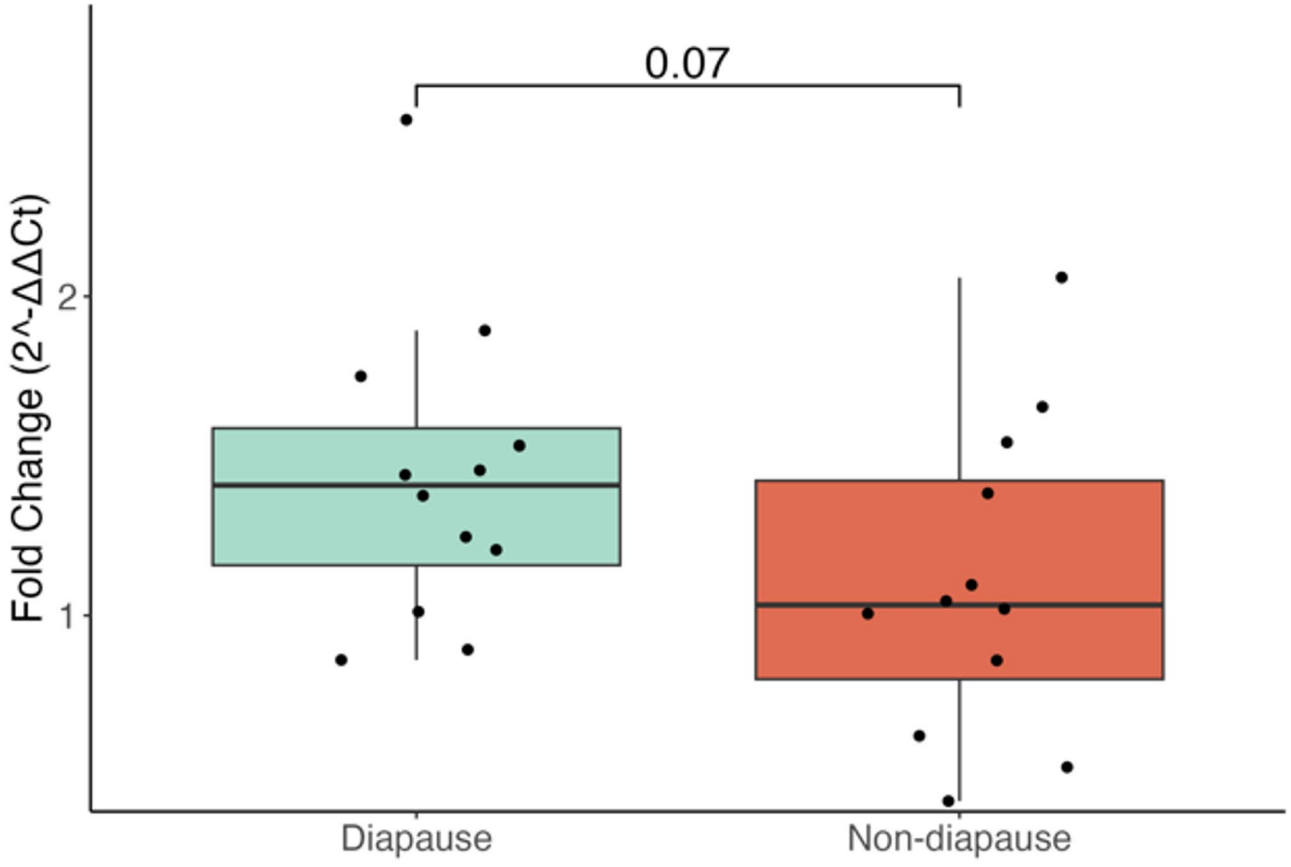

Fig. 4. *PTTH* transcript abundance in newly emerged females. *PTTH* is slightly more abundant in females that went through diapause compared to females with direct development (non-diapause) ($p = 0.07$). Transcript abundance is represented by the fold change values. Boxes represent the interquartile range (IQR), with the horizontal line indicating the median. Whiskers extend to show the range of most data points, excluding outliers. The minimum/maximum whisker values are calculated as the first quartile (Q1) and the third quartile (Q3) –/+ 1.5 * IQR. Dots represent individual data points.

enough to overcome the longevity-promoting effects of larger body size. However, our results are in contrast to those observed in a distantly related parasitoid wasp (*Nasonia vitripennis*), in which diapause extended adult lifespan[48]. This suggests that the effects of diapause on adult lifespan are varied across species, and that further research is needed to understand how developmental delays, such as diapause, affect the physiological mechanisms underpinning adult longevity. Our physiological results provide some preliminary insights.

We found differences in diapause were associated with differences in oxidative stress, but the nature of this association is mixed. Upon emergence, bees that experienced diapause had higher protein carbonylation in the thorax, but lower lipid peroxidation in the abdomen, than those that developed directly. Similarly, lipid peroxidation in the head was different from protein carbonylation in the thorax among honey bee drones treated with paraquat[49] and tissue-specific differences in oxidative stress are common in bees[50,51]. It is tempting to speculate that differences in oxidative stress at emergence may be responsible for lifespan differences between diapausing and non-diapausing females. However, the relationship between oxidative stress and aging in bees is far from clear. In honey bees, oxidative carbonylation damage to proteins in the brain is more strongly associated with social role than age[52]. Foragers showed increase in reactive oxygen species (ROS) production after extensive flying[50,53], coupled with reduced antioxidant capacity with age, suggesting that declining antioxidant capacity contributes to senescence[50]. Concordantly, both summer and long-lived winter honey bee workers show reduced antioxidant capacity with age[54]. In another study, however, oxidative stress was associated with higher survival in both honey bee drones[49] and workers[51], suggesting that at least some measures may indicate tolerance or buffering to oxidative damage.

In adult *M. rotundata*, previous research has shown that oxidative stress measured as protein damage increases over time without a corresponding decline in antioxidant capacity, suggesting no direct relationship between oxidative stress and aging[55]. Further, lipid peroxidation was found to be lower in adults than prepupae who had completed diapause[56], suggesting that some oxidative damage can reverse with age. Interestingly, antioxidant activity increased with the length of time *M. rotundata* spent in diapause, without a corresponding increase in lipid peroxidation[56]. This could suggest that increased risk of lipid damage during diapause is mitigated by an upregulation of antioxidant enzymes. This explanation is consistent with our results that females who experienced diapause emerged from development with lower levels of lipid peroxidation than those that did not diapause. Our study did not measure antioxidant activity, which would be necessary to understand whether our observations are due to increased compensatory activity in females who experienced diapause. Moreover, further studies are needed to understand the long-term effects of higher oxidative stress at emergence in diapause bees. A useful approach would be to compare oxidative stress and antioxidant activity in diapause and non-diapause bees throughout their adult lifespan to determine whether the differences observed at emergence persist throughout the adult lifespan.

We also observed slightly higher transcript abundance of the *PTTH* gene in newly-emerged diapause bees. This is partially consistent with the role of PTTH in *Drosophila*, where *PTTH* knockout during development prevents pro-inflammatory immune responses, extending adult lifespan[20]. However, *PTTH* expression is low in *Drosophila* adults compared to prepupal and pupal stages, and global knockdown of *PTTH* in adults reduces, rather than extends, lifespan[20]. Likewise, in the brown planthopper, *Nilaparvata lugens*, the absence of PTTH throughout the entire lifespan shortens adult lifespan[57]. In contrast, reduced levels of *PTTH* transcripts at emergence among *M. rotundata* females are not associated with an apparent cost to longevity. However, it is unclear whether *PTTH* transcript abundance remained elevated throughout the lifespan in bees that experienced diapause. The role of *PTTH* during development, diapause, and emergence in bees is not yet fully understood. Knockdown studies during development and adulthood could clarify whether the observed higher *PTTH* expression affects lifespan or other physiological traits in bees.

We found no differences in immune response between diapause and non-diapause bees. Immune activity was measured through melanization levels produced during encapsulation and activation of the phenoloxidase cascade. Enhanced encapsulation responses or increased phenoloxidase activity have been observed in many species during, or as a result of, diapause. For example, adult butterflies (*Pieris napi*) that went through diapause exhibit enhanced encapsulation responses[58]. Winter honey bee workers show higher levels of proPOX and POX enzyme activity upon emergence, which are key enzymes in the melanization process[54], and antibacterial effector genes are actively expressed[59]. In *M. rotundata* prepupae that have completed diapause, immune-related transcripts are elevated and further respond to temperature changes[31]. While immune activity appears to be increased in bee diapause, it is unclear whether these differences persist into adulthood. Our results suggest they do not, as diapause and non-diapause bees showed no differences in immune response intensity in our assays. Additional measures of immunity, such as antimicrobial activity, are needed to provide a more complete picture of how prepupal diapause influences adult immune function.

There are alternative mechanisms by which prepupal diapause may influence adult lifespan that we did not measure. For example, telomere shortening has been linked to aging in many organisms, but recent work with *M. rotundata* suggest that telomeres lengthen following diapause and then remain stable throughout adulthood[56,60]. It is unknown how telomere length is influenced by development in bees that do not experience diapause, but these findings suggest there may be unique aspects to telomere function during development in bees.

One limitation to our study is that we cannot account for differences in mortality that diapause and non-diapause bees could have experienced during development. A 2013 survey of *M. rotundata* was conducted across the western United States and found that less than 50% of offspring survive to emerge the following season[61], indicating that during the summer development period these bees are at high risk of mortality. Primary mortality was found during development, but not during diapause. On average, only 15% of mortality is attributed to overwintering death in managed populations[62]. Additionally, there is no evidence that size is a driving factor in overwintering mortality for *M. rotundata*[63].

An additional limitation is that our results are representative of a single year of data collection for each treatment, with diapause and non-diapause bees experiencing egg and larval phases in two different years. It is thus possible that climate patterns in the field may have differentially influenced the pre-emergence survival of diapausing and non-diapausing bees. However, there were no noticeable climatic variations between the two years. The average field temperatures were 20.0 °C and 19.6 °C for the years the diapause and non-diapause eggs hatched, respectively (L. McCabe, unpublished data). Additionally, the proportion of bees entering diapause and developing directly was similar to previous years[33] (L. McCabe, personal observation).

Related to yearly variation, female bees in our study lived longer on average than previously reported for *M. rotundata* under laboratory conditions. The combined average adult lifespan of diapause and non-diapause females in our study was 44.6 days ± 1.84 SE, compared to an average of 17.4 days for diapause bees reported by Pithan et al.[55] and less than 20 days reported by Bennett et al.[64]. This discrepancy may be due to differences in conditions during hibernation and post-emergence. For example, bees in Pithan et al. were allowed to mate, and the bees in our study had minimal activity, as they were kept in the dark and did not fly[55]. High metabolic activity, including flight, is known to increase oxidative damage and reduce lifespan[53]. Our results show that bees who diapause during development begin their adult life with higher levels of damaged proteins than those who do not diapause. We would thus expect this disparity to be further exacerbated in free-flying bees accumulating oxidative damage in their flight muscles.

Overall, our findings demonstrate a trade-off between prepupal diapause and adult lifespan in *M. rotundata*. The observed differences in lifespan of *M. rotundata* may be linked to higher levels of oxidative stress and elevated *PTTH* expression in bees that underwent diapause. However, further research is needed to uncover the mechanisms underlying this trade-off and to establish causation for the correlations observed.

Data availability

Data and code for this analysis can be found at https://github.com/kapheimlab/MROT_diapause_lifespan.

Received: 26 June 2025; Accepted: 19 September 2025
Published online: 23 October 2025

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Acknowledgements

We are grateful to members of the Kapheim Lab and anonymous reviewers for helpful feedback on earlier versions of this manuscript. Alex Torson and Hongmei Li-Byarlay provided helpful advice about the oxidative stress assays.

Author contributions

PKFS - formal analysis, investigation, writing—original draft. AL - investigation, CS - investigation, LMM - resources, AMG-Z - investigation, RAW - investigation, DLC-F - resources, KMK - conceptualization, investigation, formal analysis, funding acquisition, project administration, resources, supervision. All authors participated in the Writing – review and editing process.

Funding

This work was supported by a grant from the National Science Foundation (NSF-IOS #2142778 to K.M.K.). The

Utah Agricultural Experiment Station, Utah State University (Project #1588), and approved as journal paper #9853. Partially supported by the U.S. Department of Agriculture, Agricultural Research Service. Mention of trade names or commercial products in this publication is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture.

Declarations

Competing interests
The authors declare no competing interests.

Declaration of generative AI and AI-assisted technologies in the writing process
During the preparation of this work PKFS used OpenAI-ChatGPT to review grammar and improve clarity of the text. After using this tool/service, the authors reviewed and edited the content as needed and take full responsibility for the content of the published article.

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