Journal of Insect Physiology 118 (2019) 103935

Contents lists available at ScienceDirect

Journal of Insect Physiology

journal homepage: www.elsevier.com/locate/jinsphys

Fluctuating thermal regimes prevent chill injury but do not change patterns of oxidative stress in the alfalfa leafcutting bee, *Megachile rotundata*

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ARTICLE INFO

Keywords:
FTR
Megachile rotundata
Oxidative stress
Fluctuating thermal regime

ABSTRACT

In insects, prolonged exposure to unseasonably low temperatures can lead to detrimental physiological effects known as chill injury. Changes to active and passive transport across epithelia during chilling likely drive the collapse of ion gradients, metabolic imbalance and potentially oxidative stress. In the alfalfa leafcutting bee, *Megachile rotundata* transcriptomic evidence provides support for these responses at the level of gene expression, but variable expression profiles between life stages in *M. rotundata* indicate that different mechanisms could be responsible for repairing and protecting against chill injuries across development. Herein, we test the hypotheses that 1) chill injury leads to oxidative stress and damage in insects and 2) exposure to a fluctuating thermal regime (FTR) promotes an increased oxidative stress response leading to a decrease in damage by reactive oxygen species. We measured the expression of transcripts with products known to have antioxidant properties in overwintering prepupae as well as total antioxidant capacity and lipid peroxidation during both extended overwintering in prepupae and low temperature stress during pupal development. We observed differential gene expression for the antioxidant *glutathione peroxidase* and several transcripts with putative antioxidant properties including *vitellogenin*, *apolipoprotein D*, *glutathione S-transferase*, and *nuclear protein 1*. However, the expression of transcripts coding for other enzymatic antioxidants did not change between treatments. Neither life stage varied in their capacity to cope with an induced oxidative stress after FTR exposure and we did not observe evidence of lipid peroxidation in chill injured (STR) prepupae. These results did not support our initial hypotheses and indicate that oxidative-stress-induced damage is neither a causal factor or symptom of chill injury.

1. Introduction

Reactive oxygen species (ROS) are normal byproducts of aerobic metabolism. Reactive oxygen species are mainly produced through the mitochondrial respiratory chain, but ROS levels can also increase disproportionately under environmental stress (Monaghan et al., 2009). Oxidative stress occurs when the production of ROS exceeds the organism's capacity to eliminate excess ROS via their antioxidant system. Excess ROS can cause structural damage to lipids, nucleic acids, and proteins (Monaghan et al., 2009) and lead to the malfunction of key systems critical to normal physiological function. Oxidative stress is a constant challenge for all animals and plants and increased oxidative stress has been associated with decreased longevity and early senescence across taxa (De Block and Stoks, 2008; Haddadi et al., 2014; Reynolds and Phillips, 2013). In insects, oxidative stress is associated with increased senescence in long-lived worker honeybees (Aurori et al., 2014), compensatory growth in the damsel fly, *Lestes viridis* (De Block and Stoks, 2008), age-related memory impairment in *Drosophila melanogaster* (Haddadi et al., 2014), ultraviolet radiation in the Antarctic midge, *Belgica antarctica* (López-Martínez et al., 2008), and gamma radiation and anoxia in the Caribbean fruit fly, *Anastrepha suspense* (López-Martínez and Hahn, 2012). Oxidative stress is also thought to lead to damage in insects exposed to low temperatures (Joanisse and Storey, 1996; Joanisse and Sotrey, 1998; Lalouette et al., 2011) and contribute to chill injury (Rojas and Leopold, 1996).

Low temperature exposure in insects can lead to chill injury – a phenotype characterized by sublethal effects on organismal performance. The biochemical mechanisms that underlie chill injury are likely integrated across multiple levels of biological organization and are thought to be caused by changes to active and passive transport across epithelial tissue (Overgaard and MacMillan, 2017). These temperature-dependent changes to transport likely drive observed

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https://doi.org/10.1016/j.jinsphys.2019.103935
Received 20 February 2019; Received in revised form 22 August 2019; Accepted 22 August 2019
Available online 28 August 2019

disruptions of ion gradients, metabolic imbalance, and potentially oxidative stress (Grumiaux et al., 2019; Koštál et al., 2007; Koštál et al., 2006; Lalouette et al., 2011; Renault et al., 2004; Rojas and Leopold, 1996). Similar perturbations in ion transport are likely to occur in the mitochondria during chilling and could lead to extracellular ROS accumulation. Increased ROS or decreased antioxidant function could lead to structural damage of tissues via lipid peroxidation.

In both laboratory and agricultural settings, insects are commonly stored during winter months under constant, low temperatures (static thermal regime, STR) – a treatment that can lead to chill injury. However, exposure to daily, periodic increases in ambient temperatures (fluctuating thermal regime, FTR) during this period can protect against and/or repair chill injury in many species (Bennett et al., 2015; Colinet et al., 2006; Colinet et al., 2015; Koštál et al., 2007; Lalouette et al., 2011; Renault et al., 2004; Rinehart et al., 2013). Decreased levels of oxidative stress as a result of FTR exposure in chill-injured insects have only been directly observed in adult lesser mealworms, *Alphitobius diaperinus* (Lalouette et al., 2011), but transcriptomic evidence in the alfalfa leafcutting bee, *Megachile rotundata*, also provides support for oxidative stress as a factor in chill injury and the FTR response (Melicher et al., 2019; Torson et al., 2015, 2017), suggesting that this response could be conserved across taxa. However, no studies have provided direct evidence of damage cause by ROS in chill-injured insects or a decrease in said damage resulting from FTR exposure.

Observations of damage caused by oxidative stress will be critical to determine if oxidative stress and recovery/protection against it are important in chill injury and the FTR response, respectively. Herein, we test the hypotheses that 1) oxidative stress and its resulting structural damage contributes to chill injury and 2) that FTR exposure provides a protective/reparative effect by decreased oxidative stress. We measured antioxidant gene expression during prepupal overwintering and total antioxidant capacity and lipid peroxidation in both overwintering prepupal and low-temperature-stressed developing pupae exposed to either an FTR or STR rearing protocol. We predicted that FTR exposure would promote increased gene expression of antioxidants and increased antioxidant capacity relative to STR in both overwintering prepupae and developing pupae. Furthermore, we expected evidence of damage caused by oxidative stress to be higher in STR-reared, chill-injured bees and that this damage will increase throughout the duration of exposure.

2. Materials and methods

2.1. Insects

We purchased all leafcutting bees of Canadian origin (*Megachile rotundata;* Fabricius) from JWM Leafcutter, Inc. (Nampa, ID) as loose brood cells. *Megachile rotundata* samples for extended overwintering qPCR where derived from the 2009 field season and arrived after overwintering at a constant temperature (4–6 °C). Bees for both total antioxidant capacity and lipid peroxidation were from the 2014 field season and arrived after similar overwintering conditions as the qPCR samples. Upon arrival, we housed diapausing prepupae in Percival model I-30BLL reach-in incubators at 6 °C ± 0.5 °C under darkness.

2.2. Temperature protocols

2.2.1. Extended overwintering

We maintained post-diapause quiescent *M. rotundata* prepupae at 6 °C until the end of their typical overwintering period (mid-April) and then placed them into either a Static Thermal Regime (STR) or a Fluctuating Thermal Regime (FTR; Fig. 1). The STR protocol consisted of continued exposure at 6 °C under darkness. For the FTR protocol, we exposed the bees to a constant 6 °C with a daily, one-hour pulse of 20 °C with a one-hour ramp-up and ramp-down time (0.23 °C/min).

2.2.2. Interrupted development

We maintained post-diapause quiescent prepupae at 6 °C prior to pupal development. After their overwintering period, we transferred prepupae to 29 °C to initiate development and maintained them at this temperature until they reached a “red eye” stage in development characterized by melanization of the eyes. We assessed their developmental progression visually using guide plates. The guide plates used prepupae that were not destined for experimental treatment and had been removed from their brood cells. We placed each 24-well plate within a chamber containing a NaCl solution to maintain 75% humidity (Winston and Bates, 1960). Once ~50% of the guide-plate bees had reached the “red eye” stage, we placed experimental prepupae into their respective low temperature stress treatments (STR, FTR) for seven days (Fig. 1). Both STR and FTR protocols for this life stage are consistent with those that we used during extended overwintering.

2.3. Gene expression

2.3.1. RNA collection and sample preparation

We exposed post-diapause quiescent prepupae to either an FTR or STR extended overwintering rearing protocol. Samples were harvested starting at 20 weeks of exposure (September 15; Fig. 1) and were sampled every two weeks until there were no viable STR-reared individuals. We extracted total RNA for qPCR from whole prepupae exposed to either the FTR or STR treatment during extended overwintering (3 biological replicates), using the TRI Reagent (Molecular Research Center, Inc., Cincinnati, OH USA). We stored the RNA precipitates under ethanol at −80 °C until needed. We then diluted the RNA samples in water to equal concentrations and removed genomic DNA contamination using Invitrogen DNase I (Carlsbad, CA, USA). We used Invitrogen Super Script III first strand synthesis system for RT-PCR (Carlsbad, CA, USA) for first strand synthesis, using 1 µg RNA for each biological replicate. Prior to experimental runs, we used a pooled sample of all biological replicates to determine optimal dilution of our cDNA samples.

2.3.2. Primer design and reference gene selection

We designed the primers for all experimental targets and reference gene candidates (Table S1) using Integrated DNA Technologies' IDR program (Coralville, IA, USA). We used existing RNA-seq data from *M. rotundata* reared under the same conditions (Torson et al., 2015) to select 13 candidate reference genes. We then measured the expression of each candidate reference gene using qPCR and used geNorm (Vandesompele et al., 2002) to select the most stably-expressed candidates. Two transcripts, ubiquitin-fold-modifier-conjugating enzyme 1-like and peptidyl-prolyl cis–trans isomerase-like 2-like (Table S1), were selected as reference genes by geNorm for the normalization of all experimental targets.

2.3.3. qPCR

We conducted all qPCR runs on a Roche LightCycler 480 II (Indianapolis, ID, USA) using Roche FastStart Universal SYBR Green I Master Mix with ROX (Indianapolis, IN, USA). To correct for inter-plate variation, we included three calibrator reactions consisting of a pooled template sample and different three verified primer sets on each plate. These reactions also served as positive controls. Additionally, negative, no template, controls consisting of all enzymatic components and lacking template were present for each experimental primer set assayed on each plate. We used Biogazelle qbase+ (Ghent, Belgium) for plate annotation and normalization against reference genes. We $\log_{10}$ transformed the calibrated normalized relative quantities (CNRQ) of each transcript to allow for parametric statistics and conducted the statistical analysis and graphical representation using GraphPad Prism 8 (La Jolla, CA USA). We assessed the effects of thermal regime and time on the expression of each transcript using a two-way ANOVA with Sidak's correction for multiple comparisons.

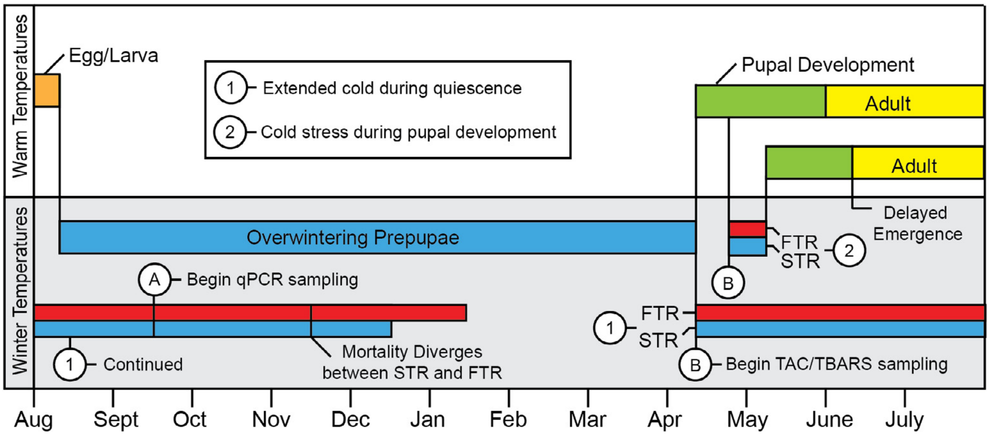

Fig. 1. Timing of experimental treatments. *M. rotundata* were exposed to an STR (blue bar) or FTR (red bar) protocol during either extended exposure to overwintering conditions (1) or pupal development (2). STR (blue bar) was a constant 6 °C, and FTR (red bar) was 6 °C with a 1 h pulse of 20 °C and 1 h ramp times. Prepupal samples (1) for antioxidant capacity and lipid peroxidation were collected from 2 to 20 weeks in treatment (A). Pupal samples (2) for antioxidant capacity and lipid peroxidation were collected before the treatment, each of the seven days during treatment, and after treatment (A). Prepupal samples for qPCR (1) were collect from 20 to 30 weeks in treatment (B). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2.4. *Total antioxidant capacity*

We assayed total antioxidant capacity (TAC) using the Cayman Chemical Antioxidant Assay Kit (Ann Arbor, MI USA) in accordance with the manufacturer protocol. We harvested samples starting two weeks after initial exposure to their respective treatments (Fig. 1). Samples were immediately frozen on dry ice and stored at −80 °C until needed. We homogenized the samples in 1 ml PBS (pH 7.4) using a Bullet Blender Blue (Next Advance; Averill Park, NY, USA) with a mixture of 0.5 mm and 1.0 mm Zirconium oxide beads for three minutes. Interrupted development and extended overwintering samples were diluted 1:30 and 1:40, respectively. Absorbance was read at 405 nm. The results are expressed in mM Trolox equivalents per mg of protein. We measured total protein content using a modified Bradford Assay (Bio-Rad, Berkeley, CA, USA) read spectrophotometrically at 595 nm against a 0.05–0.5 mg/ml bovine serum albumin (BSA) standard. We analyzed the effect of thermal regime and time on TAC using two-way ANOVA with Sidak's correction for multiple comparisons to assess differences between treatments at individual time points.

2.5. *Lipid peroxidation*

We measured the concentrations of thiobarbituric acid reactive substances (TBARS), a common proxy for oxidative stress, for both pupae exposed to the interrupted development protocol and prepupae exposed to extended overwintering conditions using a TBARS (TCA method) Assay Kit (Cayman Chemical; Ann Arbor, MI USA). We harvested and stored samples for both life stages in a similar fashion to the TAC assay. We homogenized samples at 4 °C using a Bullet Blender Blue (Next Advance, Averill Park, NY, USA) in 250 µl RIPA buffer (250 mM Tris-HCl, pH 7.6, containing 750 mM NaCl, 5% NP-40, 2.5% sodium deoxycholate, and 0.5% SDS) with 0.5 mm and 1.0 mm zirconium oxide beads. We treated the RIPA buffer with Roche cOmplete™ protease inhibitor cocktail tablets (Roche, Indianapolis, IN USA). We diluted each sample 1:10 to ensure TBARS concentrations fell within the standard curve. Reactions were run in duplicate and sample TBARS concentrations (nmol/ml) were calculated from a standard curve (0–50 nmol/ml) of malondialdehyde (MDA). TBARS measurements were corrected for total protein using a Bradford assay (Bio-Rad, Berkeley, CA USA) and results are represented as µM MDA/mg protein. We analyzed the effects of thermal regime and time on TBARS concentrations using two-way ANOVA followed by Sidak's correction.

3. Results

3.1. *Gene expression*

We measured antioxidant gene expression in *M. rotundata* prepupae exposed to either an STR or FTR rearing protocol every two weeks from 20 to 30 weeks of extended overwintering (Figs. 2, 3). These transcripts had been previously identified as differentially expressed under these conditions (Fig. 2; Torson et al., 2015), or their products are known to mitigate oxidative stress in other contexts (Fig. 3; Imlay, 2003). We predicted that these transcripts would show increased expression in FTR-exposed *M. rotundata*.

We observed a significant treatment effect on the expression of *eater* ($F_{1,4} = 333.3$, $P < 0.0001$), explaining 85.49% of the variation in gene expression. The differential expression of eater was consistent across the sampling period ($F_{6,24} = 2.09535$, $P = 0.1559$), with FTR-exposed individuals having, on average, 95.5 times higher expression of *eater* than those exposed to STR throughout the sampling period (Fig. 2A). Expression of *apolipoprotein D* (*ApoD*; Fig. 2B) was also significantly influenced by treatment ($F_{1,4} = 75.31$, $P = 0.0010$) and treatment × time interaction ($F_{6,24} = 4.083$, $P = 0.0058$). All time points except 20 weeks were differentially expressed between FTR and STR. A linear regression indicated that the slopes of the FTR and STR expression profiles varied significantly ($F_{1,38} = 14.61$, $P = 0.0005$); suggesting that expression may continue to diverge with increased exposure time. We observed a similar trend in *short-chain dehydrogenase reductase* expression (Fig. 2C) with treatment explaining 80.41% of the variation in expression ($F_{1,4} = 1000$, $P < 0.0001$), with expression in both STR ($F_{1,19} = 0.3597$, $P = 0.5558$) and FTR ($F_{1,19} = 0.3503$, $P = 0.5609$) consistent over time. Expression of the egg yolk protein precursor *vitellogenin* (*Vg*; Fig. 2D) had a significant treatment effect ($F_{1,4} = 63.61$, $P = 0.0013$). Temperature treatment significantly affected expression of *glutathione S-transferase* (*GST*; $F_{1,4} = 16.99$, $P = 0.0292$) with three time points being differentially expressed between treatments. Furthermore, *GST* expression significantly increased in STR over time (Fig. 2E; $F_{1,19} = 6.139$, $P = 0.0228$). *Nuclear protein 1* (*NUPR1*; Fig. 2F) was differentially expressed at 20, 24, and 30 weeks of exposure with treatment explaining 62.71% of variation in expression ($F_{1,4} = 118.0$, $P = 0.0004$). Expression of *NUPR1* in either STR or FTR did not change over time ($F_{1,38} = 0.666368$, $P = 0.4194$), again showing that the initial deviation in gene expression occurred prior to sampling.

Enzymatic antioxidant expression varied little between STR and FTR treatments (Fig. 3A). Expression of superoxide dismutase (SOD) had significant time ($F_{6,24} = 3.127$, $P = 0.0349$) and time × treatment effects ($F_{6,24} = 4.035$, $P = 0.0062$), but only one time point (28 weeks)

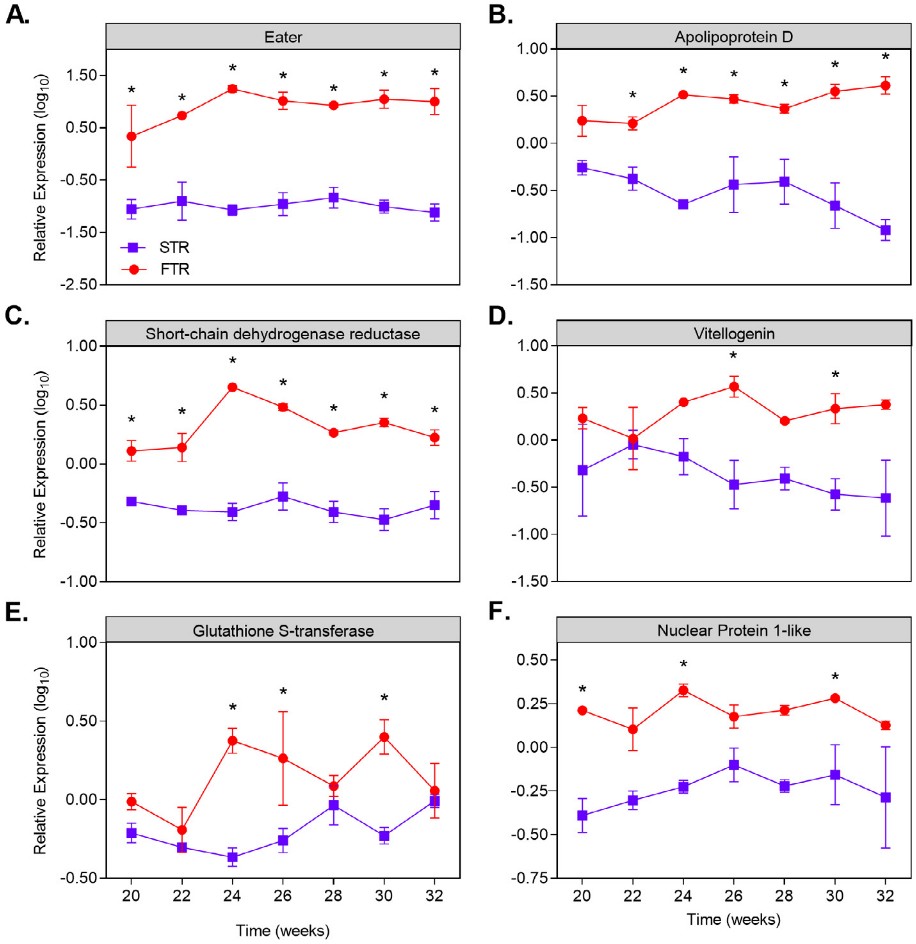

Fig. 2. Expression profiles of putative antioxidant-functioning transcripts. Gene expression from 20 to 32 weeks of extended prepupal overwintering. Relative expression is represented as Mean ± S.E. of Log_{10} transformed calibrated normalized relative quantities (CNRQ). Asterisks indicate statistical significance between STR (Blue) and FTR (Red). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

was differentially expressed between STR and FTR treatments. However, glutathione peroxidase (*GPx*) was enriched in STR from 24 to 32 weeks of exposure (Fig. 3B). *GPx* expression also decreased significantly over time in FTR ($F_{1,\ 19} = 14.04$, $P = 0.0014$). The expression of *catalase* (*Cat*; Fig. 3C) did not differ between STR and FTR at any individual time point, but FTR-exposed individuals also showed a

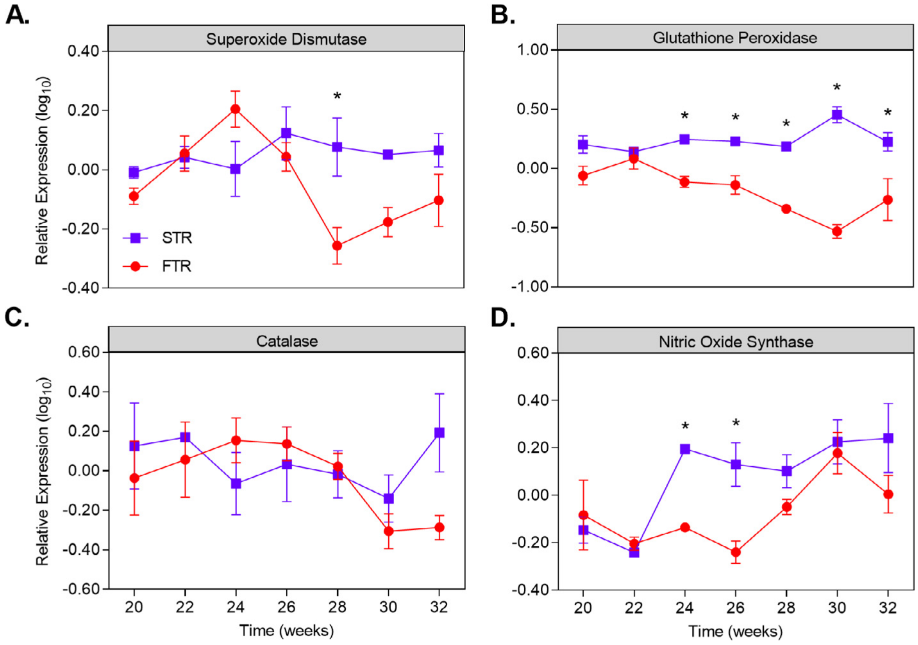

Fig. 3. Expression profiles of enzymatic antioxidants. Relative gene expression during extended prepupal overwintering. Data are represented as Mean ± S.E. of Log_{10} transformed calibrated normalized relative quantities (CNRQ). Asterisks indicate statistical significance between STR (Blue) and FTR (Red). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

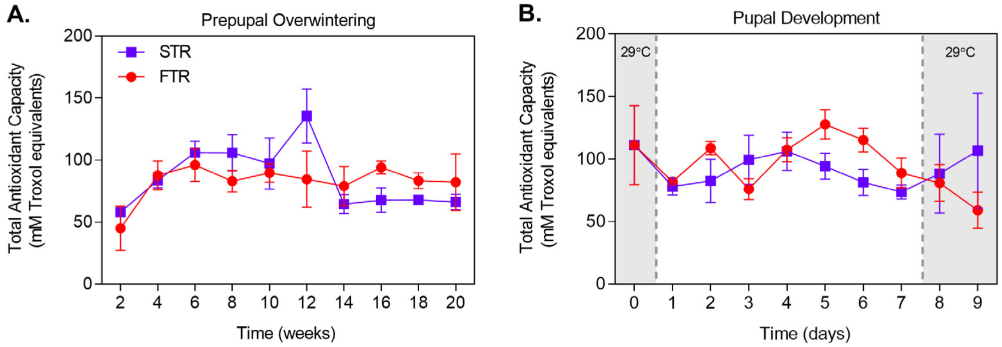

Fig. 4. Total antioxidant capacity. A) Overwintering prepupae and B) interrupted development pupae. Grey boxes indicate exposure to normal developmental temperatures. Data are expressed as mM Trolox equivalents/mg protein (mean ± S.D.).

significant decrease in *Cat* expression over time ($F_{1,19} = 5.233$, $P = 0.0338$). *Nitric oxide synthase* (*NOS*; Fig. 3D) expression was significantly influence by both treatment ($F_{1,4} = 10.45$, $P = 0.0319$), time ($F_{6,24} = 6.902$, $P = 0.0002$) and their interaction ($F_{6,24} = 2.521$, $P = 0.0491$), but only varied significantly between individual time points at 24 and 26 weeks.

3.2. *Total antioxidant capacity*

The total antioxidant capacity (TAC) during prepupal extended overwintering (leading up to the gene expression time series, 20 weeks) did not vary between treatments (ANOVA, $F_{1,4} = 0.6461 P = 0.4665$). Time explained 34.76% of the variation in TAC ($F_{9,36} = 2.963$, $P = 0.00097$). Antioxidant capacity in weeks 14–20 decreased significantly from week 12 in STR (Fig. 4A), but no global increase was observed using linear regression ($F_{1,29} = 1.019$, $P = 0.3214$). We observed similar trends in TAC during interrupted pupal development. There was no treatment effect between STR and FTR ($F_{1,4} = 0.7891$, $P = 0.4246$) and at no point were TAC measurements significantly different between STR and FTR (Fig. 4B).

3.3. *Lipid peroxidation*

Lipid peroxidation, reported as μM MDA/mg protein, did not vary between treatments before, during, or after exposure to low temperature stress in the pupal life stage (Fig. 5A). In prepupal extended overwintering, lipid peroxidation between the two treatments was stable for the first 14 weeks of treatment but increased in both treatments from 16 to 20 weeks of exposure (Fig. 5B). There were no statistical differences between treatments (Two-way ANOVA, $F_{1,4} = 0.8683$, $P = 0.4042$), but time explained 40.16% of the variation in lipid peroxidation ($F_{5,20} = 3.922$, $P = 0.0122$).

4. Discussion

Oxidative stress is hypothesized to be a symptom of chill injury and its decrease associated with the protective effect of FTR (Hayward et al., 2014; Lee, 2010). However, few studies have provided evidence of increased oxidative stress in chill-injured or overwintering insects (Joanisse and Storey, 1996; Lalouette et al., 2011; Rojas and Leopold, 1996) and only one has given evidence of decreased levels after FTR exposure (Lalouette et al., 2011). Importantly, no evidence exists to suggest that ROS is accumulated in high enough levels to lead to damage during chill injury. We predicted that chill-injured (STR) *M. rotundata* would accumulate damage from increased ROS production and those exposed to FTR would have higher antioxidant capacity and lower levels of damage. Exposure to FTR changed gene expression of antioxidants, but did not influence total antioxidant capacity or diminish lipid peroxidation levels.

The expression profiles of transcripts coding for enzymatic antioxidants (Fig. 3) were, albeit inconsistently, upregulated in STR-exposed prepupae and not in FTR as we had initially predicted. The STR profiles are consistent with previous observations of enzyme activity in overwintering insects. *SOD* was consistently expressed during STR and was higher than FTR late in the time series (Fig. 3). This STR profile is consistent with what Joanisse and Storey observed in SOD enzyme activity throughout overwintering in the freeze avoidant moth *Epiblema scudderiana* (1996). Superoxide dismutase converts the superoxide ion to hydrogen peroxide (H_2O_2) and singlet oxygen – both of which are still capable of causing damage to cells. The resulting H_2O_2 is scavenged by Cat in more localized responses and GPx, which is more broadly expressed in the organism (Monaghan et al., 2009). *GPx* expression was elevated in STR-exposed individuals from 24 to 32 weeks of treatment; suggesting that *M. rotundata* could be combatting an influx of H_2O_2 produced by SOD. This is correlated with an increased expression of *SOD* in STR in later time points, relative to FTR. Because *Cat* was not differentially expressed (Fig. 3B), it is likely that its more localized

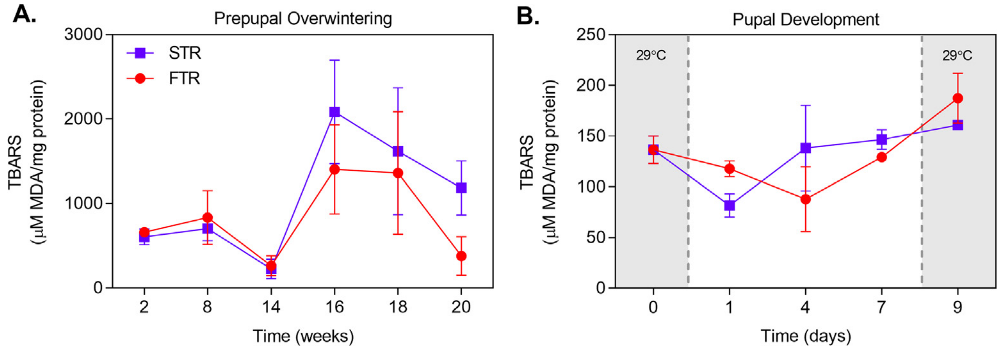

Fig. 5. Lipid peroxidation. TBARS measurements between STR and FTR in A) extended overwintering prepupae and B) low- temperature-exposed pupae. Grey indicates maintenance at normal temperatures for pupal development (29 °C). Measurements are recorded as mean ± S.D.

effect is not contributing differentially to this stress response or that its effect has been masked by our whole-organism sampling. Increased expression of these transcripts in STR indicates that either 1) STR-exposed prepupae are actively upregulating these transcripts in response to increased generation of free radicals, or 2) FTR-exposed prepupae are downregulating expression in response to diminished oxidative stress. It is also possible that, even with changes in antioxidant gene expression, the warm temperature exposure during FTR could modulate enzyme activity independently of gene expression.

We also predicted that FTR exposure would promote an increase in antioxidant capacity and that would correlate with lower levels of damage throughout treatment. However, we did not observe differences in total antioxidant capacity (Fig. 4) or lipid peroxidation between treatments for either life stage (Fig. 5). This result is perhaps not surprising considering we observed only marginal differences in the gene expression of enzymatic antioxidants (Fig. 3). We appreciate that an increase in gene expression does not always yield a proportionate increase in protein abundance (Maier et al., 2009) and that enzyme activity can be modulated independent of enzyme abundance (Hochachka et al., 1998). With this, and given constant TAC in both treatments, it is possible that compensatory mechanisms (independent of gene expression) are sufficient to keep oxidative damage stable in both STR and FTR environments. Nonetheless, these results, in combination with only marginal differences in the expression of enzymatic antioxidants do not support our initial hypotheses that chill injured insects are succumbing to an accumulation of oxidative-stress-induced damage and the FTR exposure reduces oxidative stress.

With little evidence of a difference in oxidative stress response between treatments, it is possible that the marked differential expression of transcripts with putative antioxidant function (Fig. 2) might be serving other protective or reparative functions in FTR. For example, in addition to its antioxidant properties, Vitellogenin (Vg) is involved in anti-inflammatory responses (Corona et al., 2007; Salmela et al., 2016; Sun and Zhang, 2015) and its increased expression is associated with longevity in honeybees (Aurori et al., 2014; Ihle et al., 2015). In honeybees, Vg has a high affinity to bind to damaged cell membranes and can also bind to healthy cells, increasing oxidative stress tolerance (Havukainen et al., 2013). This association with damaged cells could indicate a reparative mechanism in *M. rotundata* prepupae. Bayley et. al. recently provided a convincing putative mechanism for the initiation of cellular apoptosis in chill-injury *Locusta migratoria* via Ca^{2+}-induced cellular depolarization (2018). If FTR does in fact provide a reparative effect, removal of dead and damaged tissue would be an essential component of the response.

We also observed increased expression of *ApoD* in FTR-reared *M. rotundata.* Similarly to Vitellogenin, ApoD, a lipid transport molecule, is involved in oxidative stress response and longevity in *Drosophila melanogaster* (Walker et al., 2006) and oxidative stress and bacterial challenges in *Bombyx mori* (Chen et al., 2016). The absence of the ApoD homolog (Glial Lazarillo; GLaz) in *D. melanogaster* is associated with reduced longevity and accelerated neurological degradation (Sanchez et al., 2006), indicating a protective effect. Chill-injured *M. rotundata* pupae exposed to STR exhibit increased neuromuscular dysfunction relative to those exposed to FTR (Bennett et al., 2015). The increase in ApoD in *M. rotundata* exposed to the protective effects of FTR could be associated with the protein's role in neuromuscular repair and not related to anti-oxidant function.

The upregulation of *NUPR1*, also known as *p8* (Goruppi and Iovanna, 2010), in FTR individuals also suggests that FTR exposure is allowing for a response to stress. *NUPR1* is not as well studied as the transcripts that we have discussed previously, at least in insects, but it serves as a transcriptional regulator to cellular stress pathways in mammalian systems and knockdowns have been shown to lead to increased sensitivity to oxidative stress (Jin et al., 2009). NUPR1 appears to also have a regulatory function associated with DNA repair mechanisms (Gironella et al., 2009). NUPR1 could provide repair of this damage (perhaps caused by oxidative stress) during FTR exposure. Although these observations have not been made in insects, the high sequence conservation suggests their stress response roles may also be conserved.

Eater was the most differentially expressed transcript in this study (Fig. 2A). Although it has antioxidant properties, its function may not be directly associated with oxidative stress, but rather in the identification of damaged cells. Eater is a transmembrane protein located within hemocyte membranes that recognizes and binds pathogens in the initial steps of phagocytosis during an immune response (Kocks et al., 2005). There are several immune response genes upregulated in FTR-exposed *M. rotundata* prepupae (Torson et al., 2015), supporting an immune-response-centric function of Eater. However, extracellular domains of Eater show similarity to proteins functioning in the removal of apoptotic cells during metamorphosis in the flesh fly, *S. crassipalpis* (Hori et al., 2000) and *C. elegans* (Zhou et al., 2001). During flesh fly metamorphosis, Eater's homolog is present in hemocyte membranes that function to clear apoptotic cells during tissue remodeling. Given Eater's function during an immune response, the protein may be binding to dead or dying cells, but this apoptotic-function in chill injured insects needs additional study (Bayley et al., 2018; Kocks et al., 2005).

Increased expression of Vg, ApoD, NUPR1 and Eater during FTR do not correlate with increased antioxidant capacity (Fig. 4) or lipid peroxidation (Fig. 5), indicating that their products are likely not serving an antioxidant function in this context. It is possible that these proteins, especially Vg, ApoD, NUPR1, and Eater could be acting in a reparative fashion that is independent of oxidative stress in FTR-reared individuals. Vg and ApoD's association with longevity still suggests that these could be contributing to the beneficial effects of FTR. For example, Vg is thought to have antioxidant properties, but studies in honeybees support the hypothesis that longer-lived drones are not actually increasing response to oxidative stress or repairing damage but instead an increased tolerance to induced oxidative stress (Li-Byarlay et al., 2016). An increased stress tolerance could be assayed in *M. rotundata* by, for example, paraquat injection or knockdowns of candidate genes, such as those presented in Fig. 2.

Overall, the results of this study do not support our hypotheses that 1) chill injury leads to damage caused by oxidative stress and 2) that FTR provides a protective effect via increased antioxidant function and reduced oxidative damage. We did not observe evidence of differential lipid peroxidation between treatments, but damage to DNA and protein could still occur and warrant measurement. Tissue-level measurements could also increase the resolution of the results we have presented in this study – identifying smaller-scale damage that could have been masked by whole-organism sampling. With little evidence of oxidative stress, functional genomic techniques could test the hypothesized apoptotic and autophagic functions of the upregulated genes we have measured during FTR and how manipulated expression influences the chill injury phenotype.

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

Acknowledgements

We would like to thank Marnie Larson of the USDA for her help on the RNA extraction for the extended overwintering study. We also thank Giancarlo López-Martínez and two anonymous reviewers for their comments on earlier drafts of the manuscript. This work was supported by the United States Department of Agriculture funding to JPR and GDY and NSF EPSCoR to JHB.

Funding

This research was funded by the United States Department of Agriculture and NSF EPSCoR (1826834).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jinsphys.2019.103935.

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