




Cold stress induces specific antioxidant responses in honey bee brood

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Abstract – Multiple harmful factors cause chronic sub-lethal stress in managed honey bee colonies. In weak colonies, worker bees may not efficiently maintain the optimum temperature (33–36°C) in peripheral brood development areas. A short period at 25°C induces physiological adjustments and increases the mortality in honey bee brood reared *in vitro*. Molecular damage due to oxidative imbalance can be one of the main causes of mortality when organisms are under stress. Here, we demonstrate that *in vitro* rearing honey bee brood exposed 3 days at sub-optimal temperature (25°C) does not lead to H₂O₂ accumulation and oxidative protein damage. Cold-stressed honey bee brood showed increased levels of both total antioxidant status (TAS) and expression of peroxiredoxins *PRX1* and *PRX4*, but not of catalase activity. Results indicate that specific antioxidant defenses induced in honey bee brood under moderated cold stress are enough to keep H₂O₂ levels under control and avoid major protein damages. It is concluded that the increase of mortality in cold-stressed brood are due to multifactor's, beyond uncontrolled oxidative injuries.

honey bee brood / cold stress / oxidative damage / antioxidant responses

1. INTRODUCTION

Honey bee brood is extremely stenothermic, and it relies on precise regulation of rearing temperature (33–36°C) for proper development (Seeley and Visscher 1985). Under cold stress, thermal regulation in the colony is accomplished by the cooperation of all colony members (Stabentheiner et al. 2010). This includes active (endothermic) heat production by honey bees on the combs and inside cells, changes of bee density within the colony, evaporative cooling with water,

and regulation of internal heat transport via convection (Lindauer 1955; Seeley 1983; Stabentheiner et al. 2010; Schäfer et al. 2011).

Managed honey bee colonies, wild bees, and other pollinators currently experience population losses representing a global concern (Goulson et al. 2015). Habitat loss and fragmentation of natural lands, pesticide application and environmental pollution, the spread of pathogens, and climate change represent serious threats to pollinator populations (Potts et al. 2010). Honey bees react to stressors by accelerating their behavioral development and foraging precociously (Schulz et al. 1998; Woyciechowski and Moroń 2009; Colin et al. 2019). Precocious foragers are less effective than normal-aged foragers to sustain the nutrient demand of the colony; they die soon resulting in a rapid colony population decline and inefficient conditions for brood rearing (Perry

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et al. 2015). As a result, the number of newly emerged bees decreases and, again, heating and nursing processes becomes ineffective, leading to a detrimental cycle that ends with colony collapse (Becher et al. 2009).

In a recent study, we demonstrated that a short period of sub-optimal temperature (25°C) increases approximately 40% the mortality of *in vitro*-reared honey bee brood (Ramirez et al. 2017). Moreover, low temperature induced the expression of genes involved in the antioxidant and heat shock responses in honey bee brood (Ramirez et al. 2017). In *Drosophila melanogaster* adults growing at low temperatures, metabolic pathways associated to processes avoiding or repairing oxidative damages were identified via transcriptomic and metabolomic approaches (MacMillan et al. 2016). Oxidative stress results when the dynamic balance between reactive oxygen species (ROS) generation exceeds the capacity of antioxidant defenses to remove those toxic species. ROS are mostly neutralized by enzymatic and non-enzymatic detoxification mechanisms. Key components of the antioxidant enzyme system have been identified from the honey bee genome (Corona and Robinson 2006). Non-enzymatic antioxidant compounds are less specific than antioxidant enzymes; some examples are albumin, glutathione, ascorbate, tocopherol, carotenoids, among others. The whole suite of these antioxidants present in a certain biological matrix may be estimated measuring total antioxidant status (Costantini 2011).

The transcript levels of *Cytochrome C*, an electron carrier in mitochondria, increase in cold-stressed honey bee brood, suggesting a raise in the metabolic rate (MR) (Ramirez et al. 2017). It was also shown that cold-stressed brood experienced high MR (Ramirez et al. 2020). Higher MR has been linked to greater ROS production and has been suggested to be a physiological cost of compensatory growth occurring after the cold stress period (De Block and Stoks 2008). Thus, keeping MR high may represent an imbalance of ROS levels and an increase of antioxidant responses in cold-stressed organisms (Lalouette et al. 2011). Herein, we challenged the hypothesis that sub-optimal rearing temperature of 25°C leads to cellular damage by ROS in honey bee brood. We

determined brood survival, hydrogen peroxide (H₂O₂) accumulation, protein carbonylation levels, total antioxidant status (TAS), catalase activity, and transcript level of two peroxiredoxin enzymes, during the cold stress and 1 day after returning honey bee brood to standard rearing temperature of 34°C.

2. MATERIALS AND METHODS

2.1. *In vitro* rearing of honey bee brood

Larvae of *Apis mellifera* (*A. mellifera ligustica* - *A. mellifera mellifera*) of some hours from hatching were collected from hives belonging to “Centro de Investigación en Abejas Sociales” (CIAS) located in Santa Paula, route 226, Km 10, Mar del Plata, Argentina. Larvae were transferred to rearing plates and were incubated under standardized conditions at the laboratory: 34°C and 96% humidity during the larval stage (the first 6 days since larva hatching) and 34°C and 70% humidity during the prepupal stage (days 7 and 8 since larva hatching) (Crailsheim et al. 2013; Medrzycki et al. 2010; Ramirez et al. 2017). The amount and composition of the diet of honeybee larvae were as detailed in Ramirez et al. (2017). For the sub-optimal temperature treatment, a group of 4-day-old larvae was transferred into an incubator at 25°C for 3 days (cold-stressed larvae) and then returned to 34°C for 1 day. Figure 1 shows the experimental design used to investigate the oxidative stress in honey bee brood exposed to cold stress.

2.2. Survival determination

Survival was measured as the percentage of brood alive from an initial number of brood (100%) at the start point of cold stress treatment. Dead brood was detected by both lack of mobility and growth, and their dark appearance; they were counted daily and removed from brood plates.

2.3. Quantification of hydrogen peroxide in hemolymph

Honey bee larvae were individually washed with 1× phosphate-buffered saline (PBS; 0.43-

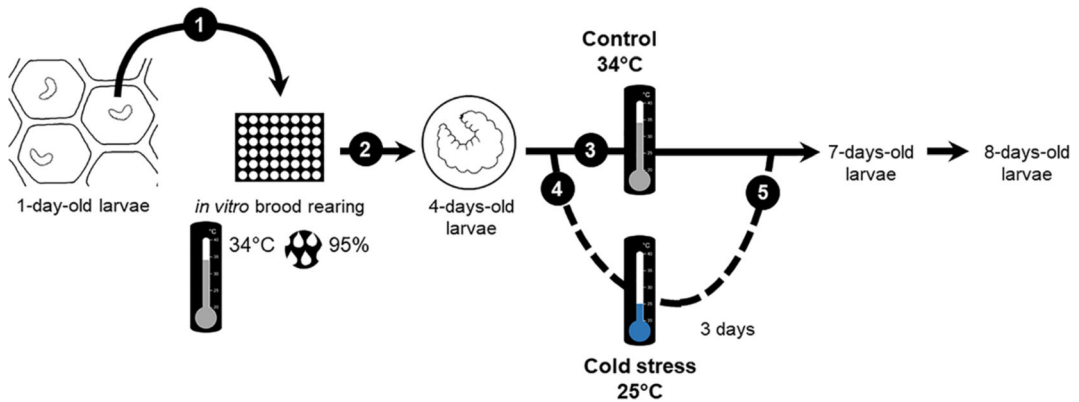


Fig 1. Experimental design used for *in vitro* rearing studies of honey bee brood and cold stress exposure. Brood comb with larvae less than 1-day-old was chosen from a hive and the larvae were transferred to 48-well culture plate which was placed into a desiccator and maintained at a relative humidity of 96% in a 34°C incubator (1). The *in vitro* rearing method was adjusted accordingly to Aupinel et al. (2005), Crailsheim et al. (2013), and Ramirez et al. (2017). Brood was kept in the standardized rearing conditions for 4 days (2) and then the larvae were separated into two groups, one was continuously reared at control temperature (3) and the other group was cold-stressed at 25°C for 3 days (4). After the cold stress, brood at 25°C was returned to control temperature (34°C) for 1 day (5).

mM Na_2HPO_4 , 0.14-mM KH_2PO_4 , 13.7-mM NaCl , 0.27-mM KCl) and dried on filter paper to remove food debris. Larvae were punctured in the ventral side of the cuticle with a sterile G30 needle and hemolymph was collected using a micropipette. Then, it was transferred to a sterile tube and kept on ice until use. Accumulation of H_2O_2 was determined using a colorimetric method based on the commercial kit Bioxytech® H_2O_2 -560 (Oxis International). The working reagent for H_2O_2 detection was prepared mixing one part of reactive 1 (25-mM $\text{SO}_4(\text{NH}_4)_2 + 2.5\text{-M H}_2\text{SO}_4$; R1) with 100 parts of reactive 2 (100-mM Sorbitol + 125-mM Xylenol Orange in water; R2). Ten microliters of hemolymph was mixed with 90 μL of working reagent (R1+R2) in a 96-well plate. A control without $\text{SO}_4(\text{NH}_4)_2$ was prepared for each sample. Reactions were incubated at room temperature for 30 min. Then, absorbance was measured in a 96-well spectrophotometer at 560 nm (EL \times 800, BioTek). H_2O_2 was quantified by interpolating the absorbance values in a standard curve made with commercial H_2O_2 .

2.4. Quantification of protein carbonylation

Protein carbonylation was measured as a proxy for oxidative stress, following the method

described by Wehr and Levine (2013). Whole body homogenates were used for each determination. Each sample was weighed, frozen in liquid nitrogen, and ground to a fine powder with a mortar and pestle. One hundred milligrams of powder was homogenized with 200 μL of 100-mM sodium phosphate buffer (pH 7.4) and centrifuged at 10,000 $\times g$ for 10 min at 4°C. Supernatants were separated and protein concentration measured using the Bradford method (Bradford 1967). Proteins were separated into two aliquots of 500 μg , one aliquot was incubated in 0.5 mL of 10-mM 2,4-dinitrophenylhydrazine (DNPH) and 2-M HCl, and the other in 0.5 mL of 2-M HCl only. After incubation for 30 min at 37°C in the dark at room temperature, mixing occasionally, a volume of 20% trichloroacetic acid (TCA) was added and the samples were centrifuged at 1000 $\times g$ for 2 min. The pellets were washed three times with 1 mL of ethanol/ethyl acetate (1:1) and centrifuged at 1000 $\times g$ for 2 min. The pellets were redissolved in 500 μL of guanidine solution (6-M guanidine, 500-mM KCl, pH 2.5). The carbonyl concentration in the sample was determined by reading the absorbance at 370 nm in a spectrophotometer (Gene-Quant 1300, GE Healthcare Life Sciences) and dividing the absorbance by the molar extinction coefficient of hydrazone

(22,000) as detailed in Wehr and Levine (2013). The results were expressed as nmol of carbonyl per mg of protein.

2.5. Determination of total antioxidant status

TAS is a measure of complete antioxidant status due to the presence of different types of molecules preventing the oxidation of a biological system. The whole body of honey bee larvae was used for the determination of TAS. Each sample was weighed, frozen in liquid nitrogen, and ground to a fine powder with a mortar and pestle. One hundred milligrams of powder was then extracted with 600 μL of 100% ethanol and was incubated overnight at 4°C, in dark with agitation. Resulting crude homogenates were centrifuged at 10,000 $\times g$ for 10 min at 4°C. Subsequently, 150 μL of supernatant was mixed with 2.85 mL of 0.004% (w/v) DPPH (1,1-diphenyl-2-picryl hydrazyl) in 100% ethanol, and incubated overnight at 4°C in dark with agitation. The blank contained all the reaction reagents except the sample. A Trolox (6-hydroxy-2, 3, 7, 8-tetramethyl chroman-2-carboxylic acid) standard curve was prepared for each assay using a range of 0–50 μg . Absorbance was read at 514 nm using a spectrophotometer (Gene-Quant 1300, GE Healthcare Life Sciences). The results were expressed in mg Trolox equivalents per g of fresh weight.

2.6. RNA isolation, cDNA synthesis, and transcript level quantification by real-time PCR

Total RNA from each individual was isolated using TRIzol (Invitrogen, Thermo Fisher Scientific) according to the manufacturer's protocol. RNA concentration and purity was measured using a nanospectrophotometer (ThermoFisher), while the integrity of RNA was verified by electrophoresis on a 1% (w/v) agarose gel. RNA was treated with DNase I (Thermo Scientific) for 30 min at 37°C to remove any contaminating genomic DNA. Complementary DNA (cDNA) was synthesized using M-MLV Reverse Transcriptase (Invitrogen) following the

manufacturer's protocol, using 1 μg of total RNA. Negative controls for reverse transcription reactions were performed without adding reverse transcriptase enzyme.

Real-time PCR reactions were performed in StepOne real-time PCR machine (Applied Biosystems). Reaction mixtures consisted of 2 μL of 1:5 diluted cDNA, 1 \times Fast Universal SYBR Green Master Rox (Roche), 0.48 μM of each primer, and RNase free water until a final volume of 10 μL . PCR program consisted of an initial step at 95°C for 10 min and 40 cycles of 95°C for 15 s and 60°C for 1 min. Specificity of the reaction was checked by analysis of the melting curve of the final amplified product. Primers used for quantitative real-time PCR of honeybee transcripts were designed using the Primer-BLAST tool from NCBI and are summarized in Table I. Amplification results were expressed as the starting concentrations (expressed in arbitrary fluorescence units) in RNA samples for each transcript according to the method described by Ruijter et al. (2009), employing the transcript level ratio between each target gene and the reference gene (actin related protein).

2.7. Catalase activity

Catalase assays were performed as previously described by Weirich et al. (2002), with minor modifications. Whole body homogenates from honeybee larvae were used for each determination. Homogenates were performed in liquid nitrogen with a mortar and pestle and 100 mg of powder was dissolved in 200 μL of 50-mM potassium phosphate buffer (pH 7.4) with 1 μL of protease inhibitor cocktail (Sigma-Aldrich). The homogenates were centrifuged at 10,000 $\times g$ for 10 min and total protein concentration was quantified in the supernatants using the Bradford method (Bradford 1967). Assay mixtures contained 40 μg of total proteins and 10-mM H_2O_2 in a final volume of 1 mL completed with 50-mM potassium phosphate buffer (pH 7.4). A blank was prepared with 10-mM H_2O_2 in 1 mL of 50-mM potassium phosphate buffer (pH 7.4). The decomposition of H_2O_2 was recorded at 240 nm using a spectrophotometer (Gene-Quant 1300, GE Healthcare Life Sciences). The catalase activity

Table I. Sequences of primers used for real-time RT-PCR

Gene name	Gen ID	Forward primer (5'-3')	Reverse primer (5'-3')	Reference
<i>Peroxiredoxin 1</i>	409954	CAGCATCTACTGATTCTCAC TTC	AGACCACGAAATGG AACTCC	
<i>Peroxiredoxin 4</i>	551975	ATCCAACATCCACTGGACGC AATGT	TTTCACCTGGCACC CAGTTAGCAG	
<i>Actin related protein</i>	406122	TGCCAACACTGTCTTTCTG	AGAATTGACCCACC AATCCA	(Mutti et al. 2011)

was calculated using the molar extinction coefficient of H_2O_2 at 240nm ($43.6 \text{ M}^{-1} \text{ cm}^{-1}$) and was expressed as nanomoles of H_2O_2 degraded per minute per mg of total protein.

2.8. Statistics

All data are presented as mean \pm SE. Generalized linear mixed model (GLMM) with a binomial error structure and “logit” link function was used to model survival. Lognormal or gamma (“inverse” link function) error structures were used to model H_2O_2 accumulation, protein carbonylation, TAS, relative gene expression, and catalase activity as responses in relation to the explanatory variable (rearing temperature, 34 and 25°C). ANOVA was used to test for significance of the explanatory variable in each response assessed. All statistical tests used $\alpha=0.05$ to establish significance. The “rearing temperature” was included in the model as fixed effect and “the independent experiment” was included as random effects. All analyses were performed with R (R Core Team 2015) using the packages lme4 and nlme.

3. RESULTS

3.1. Survival, H_2O_2 accumulation, and oxidative damage to proteins in cold-stressed honey bee brood

The survival rate of honey bee brood exposed to cold stress (25°C) from day 4 to day 7 decreased progressively compared to brood reared continuously at control temperature (34°C), but this was not statically significant (5-day-old: $t_{12}=1.24$, $P=0.2358$; 6-day-old: $t_{12}=1.43$, $P=0.1763$; 7-day-old: $t_{12}=1.98$, $P=0.0706$;

Figure 2). When cold-stressed brood was returned to 34°C (8-day-old brood), the survival rate significantly decreased by approximately 20% compared to brood continuously reared at control temperature ($t_{12}=2.82$; $P<0.05$; Figure 2). Previously, we demonstrated that cold-stressed brood has a high MR (Ramirez et al. 2017), and this could lead to a greater accumulation of ROS and, consequently, be a factor causing the brood mortality. Thus, we assessed H_2O_2 accumulation and protein carbonylation levels in cold-stressed brood. No differences were found in either H_2O_2 accumulation (5-day-old: $t_{30}=-0.01$, $P=0.9914$; 7-day-old: $t_{38}=0.3$, $P=0.7616$; Figure 3a) or protein carbonylation levels (5-day-old: $t_{15}=-0.8$, $P=0.4115$; 7-day-old: $t_{18}=-0.9$, $P=0.3509$, Figure 3b) in cold-stressed honey bee brood compared to brood reared continuously at 34°C. Several samples required for the evaluation of H_2O_2 and protein carbonylation in 8-day-old honey bee brood could not be considered in the analysis because they presented contamination with fat body and other substances that interfere with such determinations

3.2. Antioxidant responses

To further study oxidative stress in cold-stressed honey bee brood, we evaluated the induction of some antioxidant responses. We measured the levels of TAS as the ability of non-enzymatic components in the organism to prevent ROS damage (Ghiselli et al. 2001). TAS was unchanged in 5-day-old brood exposed one day at 25°C ($t_{12}=1.1$, $P=0.2829$; Figure 4). In brood exposed for 3 day at 25°C (measured on day 7) and brood exposed to cold stress and transferred back to 34°C for 1 day (measured on day 8), we determined a significant increase in TAS compared to

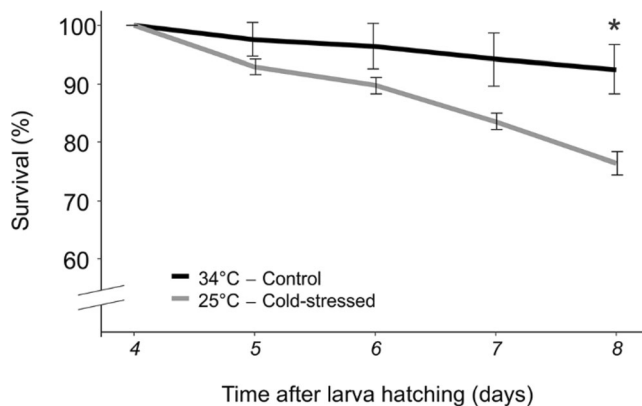


Fig 2. Survival rate of honey bee brood reared at sub-optimal temperature of 25°C. Four-day-old honey bee larvae were reared at control temperature (34°C, black line) or cold-stressed (25°C, grey line) for 3 days, from day 4 to day 7, and then were returned to 34°C for 1 day. Survival rate was expressed as a percentage of living brood in each day taking as 100% the number of individuals at the onset of the experiment (day 4). Data are shown as mean \pm SE of at least three independent experiments. Asterisk indicates significant differences between values at $\alpha = 0.05$.

brood reared continuously at 34°C (7-day-old larvae: $t_8 = -3.9$, $P < 0.05$; 8-day-old brood: $t_{13} = -6.8$, $P < 0.0001$; Figure 4).

The transcript levels of peroxidases *PRX1* and *PRX4* did not change in 5-day-old brood exposed at 25°C for 1 day compared with the brood continuously reared at 34°C (*PRX1*: $t_{23} = -1.2$, $P = 0.2498$; *PRX4*: $t_{23} = 0.7$, $P = 0.4993$; Figure 5). Figure 5 shows that *PRX1* and *PRX4* expression increased ≈ 3 and ≈ 18 times, respectively, in brood

exposed 3 days at 25°C compared with the control group (7-day-old brood, *PRX1*: $t_{23} = -7.1$, $P < 0.0001$; *PRX4*: $t_{28} = -11.1$, $P < 0.0001$; Figure 5) and these differences were maintained in 8-day-old brood (*PRX1*: $t_{25} = -11.4$, $P < 0.0001$; *PRX4*: $t_{11} = -9.3$, $P < 0.0001$; Figure 5).

We evaluated the effect of cold stress on the catalase activity in honey bee brood. No significant differences were found on catalase activity between brood reared at control

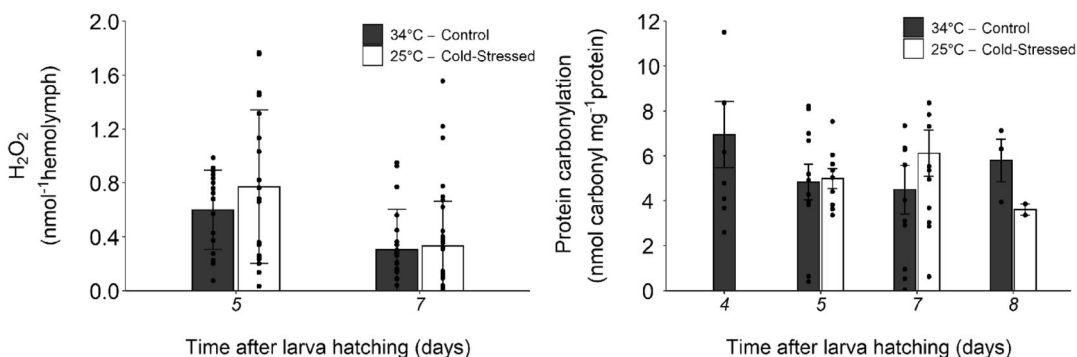


Fig 3. H₂O₂ and protein carbonylation levels in honey bee brood reared at 34°C and sub-optimal temperature of 25°C. Four-day-old honey bee larvae were reared at control temperature (34°C, grey bar) or cold-stressed (25°C, white bar) for 3 days, from day 4 to day 7, and then were returned to 34°C for 1 day. (A) H₂O₂ accumulation in hemolymph and (B) protein carbonylation in full body were measured as described in “Materials and methods.” Data are shown as mean \pm SE of at least three independent experiments of each measurement, except for protein carbonylation at day 8 (one experiment).

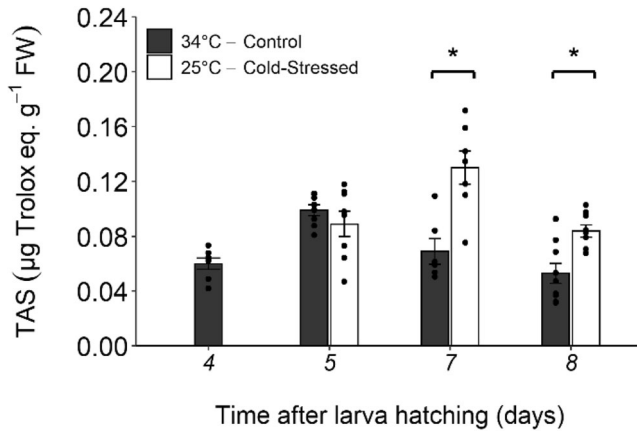


Fig 4. Total antioxidant status (TAS) in honey bee brood reared at 34°C and sub-optimal temperature of 25°C. Four-day-old individuals were reared at control temperature (34°C, grey bars) or cold-stressed at 25°C (white bars) for 3 days and then were returned to 34°C for 1 day. TAS was determined as described in “Materials and methods.” FW: fresh weight. Data are shown as mean \pm SE of at least three independent experiments. Asterisks indicate significant differences between values at $\alpha = 0.05$.

temperature and cold-stressed brood (5-day-old: $t_{14} = -0.54$, $P = 0.5936$; 7-day-old: $t_{26} = -0.9$, $P = 0.3418$; 8-day-old: $t_{23} = -1.5$, $P = 0.1567$; Figure 6).

4. DISCUSSION

In several studies, oxidative stress has been linked to stressors influencing honey bee health and colony productivity. The movement of managed honey bee colonies across different agricultural landscapes and the location of colonies inside greenhouses impact on the levels of oxidative stress (Morimoto et al. 2011; Simone-Finstrom et al. 2016). Moreover, flight activity, invasive predator species, and pathogens in hives as well as pesticides used for pest control in numerous crops disturb the honey bee oxidative homeostasis (Baleira et al. 2018; Gregorc et al. 2018; Margotta et al. 2018; Leza et al. 2019; Li et al. 2020). Oxidative stress has been suggested to lead to damage in insects exposed to low temperatures (Lalouette et al. 2011; Kojić et al. 2019; Tawfik et al. 2020). Here, we studied the impact of sub-optimal rearing temperature on the oxidative status and the antioxidant responses induced in honey bee brood. We report that sub-optimal rearing temperature of 25°C increases brood mortality

from day 4 to day 8, becoming statically significant in 8-day-old individuals exposed 3 days to cold stress and transferred back for 1 day to standard rearing temperature of 34°C (Figure 2). We originally had hypothesized that higher metabolic rate (MR) in cold-stressed honey bee brood (Ramirez et al. 2020) leads to increased ROS formation, and consequently results in elevated cell injury and mortality. However, contrary to expectations, we did not find differences in the levels of H₂O₂ or oxidative damage in proteins in cold-stressed brood compared to brood reared at control temperature (Figure 3). This unexpected result could be linked to the activation and the increased levels of antioxidant mechanisms in brood reared at sub-optimal temperature. Accordingly, we determined high levels of TAS in honey bee brood reared under cold stress (Figure 4), suggesting a role for non-enzymatic antioxidant compounds in neutralizing ROS. Other works have demonstrated the relevant role of TAS controlling ROS levels. For instance, in honey bees fed syrup containing ascorbic acid, the prevalence and intensity of *Varroa destructor* infestation were lower and this was associated with elevated levels of TAS (Farjan et al. 2014). TAS in the hemolymph of 30-day-old honey bees was greater than for 1-day-old adult bees. This increased

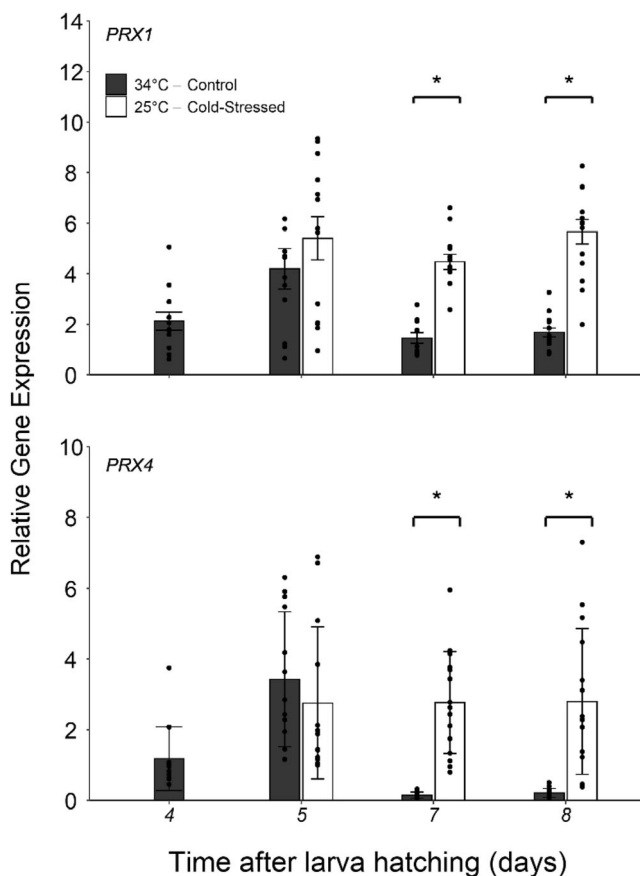


Figure 5. Peroxiredoxin 1 (PRX1) and 4 (PRX4) transcription levels measured in honey bee brood reared at 34°C and sub-optimal temperature of 25°C. Four-day-old honey bee larvae were reared at control temperature (34°C, grey bar) or cold-stressed (25°C, white bar) for 3 days, from day 4 to day 7, and then were returned to 34°C for 1 day. The relative gene expression of *PRX1* and *PRX4* was determined by RT-qPCR using actin related protein as a reference gene for normalization. Data are shown as mean \pm SE of at least three independent experiments. Asterisks indicate significant differences between values at $\alpha = 0.05$.

protection could be associated with ageing and transition of worker bees to foraging (Słowińska et al. 2016).

ROS include a diverse variety of chemical species like superoxide (O_2^-), hydroxyl radicals (OH^-), and H_2O_2 . Previously, we reported that transcript levels of superoxide dismutase (SOD) were increased in cold-stressed honey bee brood (Ramirez et al. 2017), suggesting an increased conversion of O_2^- to H_2O_2 and, consequently, H_2O_2 should accumulate in cold-stressed brood. However, as we described above, H_2O_2 does not accumulate in cold-stressed brood. Several antioxidant enzymes contribute to scavenge the

accumulation of H_2O_2 produced by SOD, thus reducing the levels of oxidative damage to proteins, lipids, and nucleic acids (Monaghan et al. 2009). Catalase converts H_2O_2 to water and oxygen while PRXs also reduces H_2O_2 when coupled to a sulfhydryl-reducing system such as thioredoxin or glutathione (Tasaki et al. 2017). Here, we determined an increase in *PRX1* and *PRX4* transcription levels (Figure 5) in cold-stressed brood, but no changes in catalase activity were detected (Figure 6). These results suggest that PRXs are probably involved in H_2O_2 decomposition preventing its accumulation and, consequently, cell damage in honey bee brood. Termite

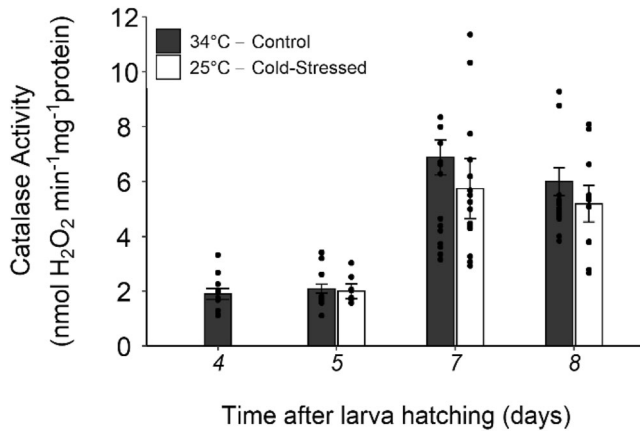


Fig 6. Catalase activity in honey bee brood reared at 34°C and sub-optimal temperature of 25°C. Four-day-old honey bee larvae were reared at control temperature (34°C, grey bar) or cold-stressed (25°C, white bar) for 3 days, from day 4 to day 7, and then were returned to 34°C for 1 day. Catalase activity was determined as described in “Materials and methods.” Data are shown as mean ± SE of at least three independent experiments.

queens have an extended lifespan and they suffer lower levels of oxidative damage than non-reproductive workers. An efficient antioxidant system consisting of several antioxidant enzymes, especially high catalase activity and PRX gene expression levels, seems to play an important role in the oxidative stress resistance of termite queen (Tasaki et al. 2017). In honey bees, brain plasticity is positively associated with the levels of cellular maintenance proteins such as Heat Shock protein 8 (HSP8) and PRX (Baker et al. 2012). Other proteins such as HSP70, HSP90 and vitellogenin, involved in oxidative stress resistance, have been linked to cold stress tolerance in honey bee brood (Ramirez et al. 2017). These proteins have been reported to be involved in the protection of cellular components, principally other proteins, from oxidative damage (Seehuus et al. 2006; King and MacRae 2015).

The results of our study do not support our hypothesis that rearing honey bee brood at sub-optimal temperature leads to protein damage caused by H₂O₂ accumulation. ROS accumulation occurs when their production exceed the organism’s antioxidant defense mechanism (Monaghan et al. 2009). Our results suggest that antioxidant defenses induced in cold-stressed honey bee brood are enough to keep H₂O₂ levels under control and that the increased lethality of stressed brood could be due to multifactors.

However, we cannot ignore the oxidative damage generated by other ROS such as superoxide or hydroxyl radicals, which were not measured in this work. We did not find evidence of differential protein carbonylation, but damage to lipids and DNA could still occur that were not measured here. In situ and tissue-level measurements could also contribute to the resolution of oxidative damage as was suggested (Torson et al. 2019).

Overall, our results show that oxidative damage would not be the cause of the higher mortality determined in the cold-stressed brood. It has been proposed that a cold-induced membrane phase transition is the direct cause of cellular chill injury. Temperature-induced perturbations in the biological membrane organization have a profound impact on its structure and function, and this represents a serious challenge for the maintenance of physiological function in animals (Hazel 1995). Additionally, apoptotic mechanisms are involved in insect chill injury (Yi et al. 1976). It has been proposed that hypothermia induces injury indirectly through the resulting depolarization by inducing Ca²⁺ influx promoting apoptosis/necrosis (Bayley et al. 2018). The energetic cost to keep antioxidant responses elevated could be high. In insects, energy mobilization plays a very important role in stress responses (Even et al. 2012). In spite of increasing interests, the multicausality of cold mortality in honey bee reared at sub-optimal

temperature is yet intriguing and deserves further efforts.

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AUTHOR CONTRIBUTIONS

LR and LL conceived the research and designed experiments; CAM, LR, and RSG performed the experiments and analysis; CAM, LR, and LL interpreted the data and wrote the paper.

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DATA AND MATERIALS AVAILABILITY

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

CODE AVAILABILITY

Not applicable.

DECLARATIONS

Ethics approval Not applicable.

Competing interests The authors declare no competing interests.

Le stress dû au froid induit des réponses antioxydantes spécifiques dans le couvain d'abeilles.

Couvain d'abeilles domestiques / stress dû au froid / stress oxydatif / dommages oxydatifs / réponses antioxydantes.

Kältstress induziert die Antwort spezifischer Antioxidantien in der Brut der Honigbiene.

Honigbienenbrut / Kältstress / oxidativer Stress / oxidative Schäden / Antioxidanzantwort.

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