

## RESEARCH ARTICLE

# Larval starvation improves metabolic response to adult starvation in honey bees (*Apis mellifera* L.)

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## ABSTRACT

Environmental changes during development have long-term effects on adult phenotypes in diverse organisms. Some of the effects play important roles in helping organisms adapt to different environments, such as insect polymorphism. Others, especially those resulting from an adverse developmental environment, have a negative effect on adult health and fitness. However, recent studies have shown that those phenotypes influenced by early environmental adversity have adaptive value under certain (anticipatory) conditions that are similar to the developmental environment, though evidence is mostly from morphological and behavioral observations and it is still rare at physiological and molecular levels. In the companion study, we applied a short-term starvation treatment to fifth instar honey bee larvae and measured changes in adult morphology, starvation resistance, hormonal and metabolic physiology and gene expression. Our results suggest that honey bees can adaptively respond to the predicted nutritional stress. In the present study, we further hypothesized that developmental starvation specifically improves the metabolic response of adult bees to starvation instead of globally affecting metabolism under well-fed conditions. Here, we produced adult honey bees that had experienced a short-term larval starvation, then we starved them for 12 h and monitored metabolic rate, blood sugar concentrations and metabolic reserves. We found that the bees that experienced larval starvation were able to shift to other fuels faster and better maintain stable blood sugar levels during starvation. However, developmental nutritional stress did not change metabolic rates or blood sugar levels in adult bees under normal conditions. Overall, our study provides further evidence that early larval starvation specifically improves the metabolic responses to adult starvation in honey bees.

**KEY WORDS:** Nutrition, Eusocial species, Environmental stress, Stress response, Anticipatory mechanism, Adaptive response, Metabolic rate, Respiratory quotient, Glycogen, Triglycerides, Glucose, Trehalose

## INTRODUCTION

Organisms are constantly faced with environmental alterations and stresses, and environmental changes during animal development are as important as genetic factors in causing phenotypic variation. Across taxa, phenotypic variation and plasticity play a critical role in helping organisms cope with specific environmental conditions.

The textbook examples of phenotypic variation and plasticity are well-known polyphenisms in insects (Whitman et al., 2007), sex determination in reptiles (Janzen and Phillips, 2006) and phenotypically plastic responses such as acclimation, immune response adaptation and learning (Gilbert and Epel, 2009). For example, whether embryos of desert locusts (*Schistocerca gregaria*) develop into gregarious or solitary forms is determined based on their population density (Applebaum and Heifetz, 1999), and these two adult forms are advantageous in high- and low-density populations, respectively (Simpson et al., 1999, 2001).

Developmental environment, especially adverse conditions, can have profound and negative effects on adult health and life, and produce phenotypic deficits in adults. For example, prenatal or postnatal nutritional perturbation increases susceptibility to adult metabolic disorders such as type II diabetes, obesity and heart disease in humans. Also, it is common in various species that developmental nutritional stress decreases body size (Searcy et al., 2004), reduces immune function (Butler and McGraw, 2011), impairs cognition (Fisher et al., 2006) and suppresses reproduction (Lindström, 1999). However, recent studies have suggested that these phenotypes induced by developmental stress have adaptive value under specific environmental conditions. The phenotype of diabetes II resulting from poor prenatal nutrition (insulin resistance, a predisposition for central fat and smaller muscle mass) is adaptive in an energy-insufficient environment (Gluckman et al., 2007). Similarly, *Bicyclus anynana* butterflies and zebra finches (*Taeniopygia guttata*) become smaller if they experience developmental food restriction, but both of them perform better under stressful conditions: butterflies starved during development can better cope with forced flights by increasing allocation of energy resources to the thorax (Saastamoinen et al., 2010; van den Heuvel et al., 2013) and developmental starvation of female zebra finches accelerates exploratory and foraging behavior of offspring (Krause et al., 2009). Therefore, this phenomenon leads to a fundamental question: are those ‘negative’ adult phenotypes affected by developmental stress a part of an adaptive mechanism shaped by evolution?

It has been suggested that developmental organisms have a mechanism to anticipate future conditions based on the preceding environment, which sets the developmental trajectory to adapt to the predicted condition (Gluckman et al., 2005a,b, 2007). In complex multicellular eukaryotes, the anticipatory capability as shown by classical Pavlovian conditioning is a fundamental biological process to capture connections between events in their environment (Pavlov, 1927). Microorganisms can also make adaptive predictions of the environment (Mitchell et al., 2009). Diverse organisms such as birds (Krause et al., 2009), fish (Villeneuve et al., 2013), insects (van den Heuvel et al., 2013) and microorganisms (Mitchell et al., 2009) are able to adaptively respond to specific conditions that have similarities to developmental environments (Mitchell et al., 2009; Crino and Breuner, 2015). Although studies

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have suggested this adaptive mechanism may be conserved across species, evidence is still limited at the morphological and behavioral levels and is lacking at the physiological level.

Our previous studies on honey bees suggested that a developmental adaptive response exists in eusocial insects. We subjected fifth (last) instar worker-destined larvae to a short period of starvation and found that the adult workers were smaller and had fewer ovarioles (Wang et al., 2014), but were more resistant to starvation (Wang et al., 2016) (Fig. S1). The changes that these bees showed in metabolic physiology, endocrinology and behavior suggest a potential mechanism underlying the improved resistance to starvation in the adults (Janković-Hladni, 1991; Gruntenko et al., 1996; Suarez et al., 1996; Arrese and Soulages, 2010). We also performed an RNAseq study (Y.W., R.E.P. and G.V.A., unpublished data) and gene expression analysis on metabolic pathways [insulin/insulin-like growth factor signaling pathway (IIS) and adipokinetic hormone (AKH) signaling; Wang et al., 2016] in those bees that experienced short-term larval starvation, but were kept in a normal nutritional environment. Interestingly, our data show that larval starvation had minimal effects on gene expression in the adults. These results led us to ask whether that early starvation specifically targets the capability to cope with nutritional stress instead of having global effects on adult physiology and metabolism. Such a specific effect would make sense if this is an adaptive mechanism shaped by evolution to increase fitness in a predicted event. Here, we tested the hypothesis that honey bees which experienced larval starvation have adaptive metabolic responses to starvation during adulthood.

A greater capacity to survive starvation, as has been observed for bees starved during the larval stage, could be due to a number of different physiological changes. Larval starvation could reduce adult metabolic rate, particularly during starvation (Dulloo and Jacquet, 1998). It could increase the content of energy stores, including glycogen, triglycerides (Dulloo and Jacquet, 1998), glucose or trehalose (Zauner et al., 2000) in adults. Finally, while honey bees are well known for primarily utilizing carbohydrate for fuel, conceivably, larval starvation might influence shifts in adults toward use of other fuels such as lipids (Zauner et al., 2000). Here, we starved fifth instar larval bees using the previously established protocol (Wang et al., 2014; Wang et al., 2016). We measured metabolic rates, hemolymph sugar concentration and metabolic reserves in the brain, thorax and abdomen of adults before and during starvation. Overall, we found few differences in metabolism or nutrient levels in well-fed adult controls and bees starved as larvae. The early-starved bees did not have lower metabolic rates or higher initial nutrient levels under normal nutritional condition. However, during starvation, adult bees that had experienced larval starvation showed a great reduction in metabolic rate, were able to quickly shift away from carbohydrate metabolism and maintained stable hemolymph sugar concentrations, potentially explaining their improved capacity to survive starvation.

## MATERIALS AND METHODS

### Bee preparation

Bees were maintained at the Honey Bee Research Laboratory at the Arizona State University Polytechnic Campus (Mesa, AZ, USA). Three wild-type colonies were used as donors for the experiments. Each queen was caged on a wax comb and allowed to lay eggs for 24 h, after which the comb was placed in a separate foster colony. A pre-starvation procedure (Wang et al., 2014; Wang et al., 2016) was performed at 180 h after oviposition when the larvae entered the last feeding sub-phase of fifth instar (Michelette and Soares, 1993). Half

the resulting larvae were isolated with two layers of pushing cages for 10 h until the larvae entered the spinning phase (and feeding ceased) (Rembold et al., 1980; Michelette and Soares, 1993). When the cages were removed, the larval cells were sealed by nurse bees. Just prior to adult emergence (20 days after egg laying), the combs were collected and placed in an incubator, set at 34°C and 80% relative humidity, where the bees emerged. Exclusion cages were used to prevent starved and untreated bees from intermingling as they emerged.

Twenty newly emerged bees were collected for measuring body mass. The remaining bees were marked on their thoraces and placed in the foster colony. Six days later, the marked bees were collected from the colony and taken to the lab for monitoring physiological parameters during adult starvation as described below.

### Validation of early-starvation effects on body mass and ovariole number

Twenty 7 day old bees from each group (control and starvation group) were chilled to immobility then weighed on a digital scale (VWR, Randnor, PA, USA). Individuals were dissected under a microscope to count the number of ovarioles from both ovaries. Because ovariole number does not change during adult maturation (Atkins et al., 1975), we only monitored ovariole number in 7 day old bees.

### Respirometry

Metabolic rate for individual honey bees was determined by measuring oxygen consumption ( $\dot{V}_{O_2}$ ) and carbon dioxide emission ( $\dot{V}_{CO_2}$ ) using closed-system respirometry (Lighton, 2008; Cease et al., 2010). Respiratory chambers consisted of 60 ml syringe barrels with three-way stopcocks mounted on the tip. Compressed ambient air was scrubbed of water vapor and CO<sub>2</sub> using a Balston purge-gas generator (Haverville, MA, USA) and scrubbed secondarily with columns of Drierite<sup>®</sup> (W. Hammond Drierite Company, Xenia, OH, USA) and Ascarite<sup>®</sup> (Arthur H. Thomas Company, Swedesboro, NJ, USA). Air was pushed through mass-flow control valves that regulated flow at 200 ml min<sup>-1</sup> standard temperature and pressure (STP) and directed to the reference cell of the LI-COR 6252 infrared CO<sub>2</sub> analyzer. The CO<sub>2</sub> reference outlet was pumped through the sample cell and then to the Oxzilla Dual Paramagnetic Oxygen analyzer (Sable Systems International, Las Vegas, NV, USA). A small injection port was placed at the entrance of the CO<sub>2</sub> analyzer sample cell to allow for a bolus injection of air from the respirometry chambers. Air was scrubbed of CO<sub>2</sub> and water vapor between the CO<sub>2</sub> analyzer and the O<sub>2</sub> analyzer to simplify calculations of oxygen consumption rate. A data acquisition system (model UI-2, Sable Systems International) digitized analog output from the gas analyzers, and was interfaced to the ExpeData software (Sable Systems International).

Our companion study (Wang et al., 2016) showed that after 12 h of starvation at 35°C, almost 80% of 7 day old bees that had experienced larval starvation were still alive, while only 50% of control bees were alive (Fig. S1). Therefore, we chose to focus on metabolic rates during the first 12 h of starvation. Eighty bees were collected from the foster colony, and mounted individually on small straw holders to limit activity and for easy handling during respirometry measurements. These bees were starved during the experiment at room temperature (25°C), and the time of the starvation was determined by the order of measurements. Control bees and those starved as fifth instar larvae were chosen in pairs at random and each individual bee was placed into a 60 ml syringe barrel at 10 min intervals. Then, the syringe was flushed with dry, CO<sub>2</sub>-free air and sealed for 60 min. After 60 min, a subsample of air

was injected through the injection port at a slow constant rate into the airstream directed through the CO<sub>2</sub> and O<sub>2</sub> analyzers. The molar rates of CO<sub>2</sub> and O<sub>2</sub> transport in the airstream were calculated and recorded by ExpeData from the output of the gas analyzers and mass flow meter. Each injection was preceded by 5 min of baseline recording; molar quantities of CO<sub>2</sub> and O<sub>2</sub> were calculated by integrating the peak created by the bolus versus time, subtracting the baseline. Measurements of  $\dot{V}_{O_2}$  and  $\dot{V}_{CO_2}$  were calculated as described using the manual bolus integration and equation 4.23 from Lighton (2008), where  $\dot{V}_{O_2}$  is oxygen consumption rate ( $\mu\text{l O}_2 \text{ h}^{-1}$ ) and  $\dot{V}_{CO_2}$  is carbon dioxide emission rate ( $\mu\text{l CO}_2 \text{ h}^{-1}$ ).  $\dot{V}_{O_2}$  was converted to metabolic rate (MR) using:

$$\text{MR} = (15.97 + (5.164 \times \text{RQ})) \times \dot{V}_{O_2}, \quad (1)$$

according to Lighton (1991), where MR is in watts (W), respiratory quotient (RQ) =  $\dot{V}_{CO_2}/\dot{V}_{O_2}$ , and  $\dot{V}_{CO_2}$  and  $\dot{V}_{O_2}$  are expressed in  $\text{ml s}^{-1}$ . Because there was a significant difference in body mass between control and starved bees, and fuel stores were measured as concentrations, we report mass-specific metabolic rate ( $\text{W kg}^{-1}$ ); with this approach, a decrease in metabolic rate with constant initial fuel stores would clearly indicate a reduced rate of use of fuel.

We also calculated RQ for both control bees and those starved as larvae during the 12 h adult starvation. RQ is an indicator of substrate oxidation and is the ratio of CO<sub>2</sub> produced to O<sub>2</sub> consumed ( $\dot{V}_{CO_2}/\dot{V}_{O_2}$ ) while the fuel is used. Usually, RQ is between 0.7 and 1, where an RQ of 1.00 indicates pure carbohydrate catabolism, and an RQ of 0.71 indicates a pure lipid substrate for energy metabolism (Kleiber, 1961).

### Metabolic assays

Bees were collected before adult starvation and during the 12 h of starvation. A 1  $\mu\text{l}$  sample of hemolymph was collected between the third and fourth abdominal tergites. The head and thorax were quickly cut into pieces and the abdomen was collected by removing the digestive tract and sting apparatus. All samples were quickly flash-frozen in liquid nitrogen. Glucose and trehalose were measured in the hemolymph, and glycogen and triglycerides were measured in the brain, thorax and abdominal fat body.

### Concentrations of glucose and trehalose in the hemolymph

Glucose concentration in the hemolymph was measured by adding 400  $\mu\text{l}$  glucose reagent (Sigma-Aldrich, including hexokinase and glucose 6-phosphate dehydrogenase) into 1  $\mu\text{l}$  hemolymph. The mix was incubated for 15 min at 37°C. Using a spectrophotometer (Bio-Rad xMARK Microplate spectrophotometer), absorbance at 340 nm ( $A_{340}$ ) was measured, and glucose concentration was determined from a standard curve for standard solutions treated identically to the samples. Three replicates were tested for each sample. After measuring glucose concentration, the same samples were used to measure trehalose (Broughton et al., 2005) by adding the enzyme trehalase (Sigma-Aldrich) to a final concentration of 0.05 U  $\text{ml}^{-1}$ . The resulting solution was incubated at 37°C overnight followed by a second  $A_{340}$  reading. The amount of glucose produced from trehalose was calculated by subtracting the amount in the first reading from that in the final reading. The amount of trehalose was estimated using the equation: trehalose ( $\mu\text{g}$ ) = glucose ( $\mu\text{g}$ )  $\times$  342.3 / (180.2  $\times$  2) (Hartfelder et al., 2013; Wang et al., 2013).

### Glycogen and lipid reserves in the brain, thorax and fat body

The results from our metabolic measurements suggested that a comparison between samples collected before the starvation and

toward the end of the 12 h adult starvation period might be the most informative, so we measured fuel reserves for samples collected before adult starvation and after 9 h of starvation (9–12 h), using modified versions of previously described methods (Arrese and Wells, 1997; Iijima et al., 2009; Palanker et al., 2009).

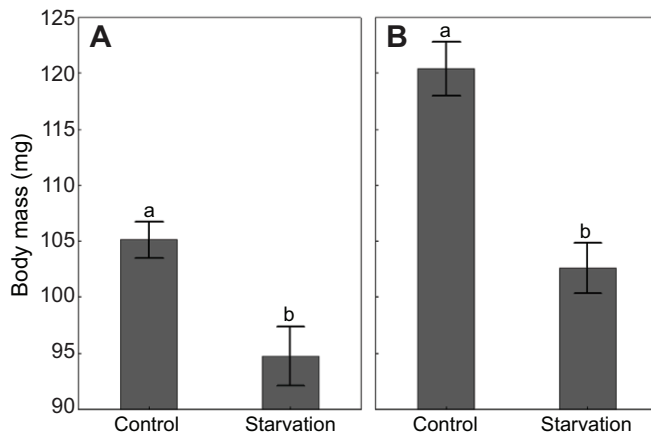
Each tissue sample was homogenized on ice in 200  $\mu\text{l}$  of a glycogen assay buffer containing 10  $\text{mmol l}^{-1}$  KH<sub>2</sub>PO<sub>4</sub> and 1  $\text{mmol l}^{-1}$  EDTA (pH 7.4). The homogenates were then heated at 75°C for 5 min to degrade general enzymes before being centrifuged at 2000 rpm at 4°C for 10 min. The supernatants were then transferred into another set of tubes. Total protein level was measured using the Quick-Start Bradford Reagent (Sigma-Aldrich). The  $A_{540}$  was read using a spectrophotometer (Bio-Rad), and protein concentrations calculated from a standard curve for BSA (Bio-Rad).

For measuring glycogen, we adopted and slightly revised a widely used method in *Drosophila* (Iijima et al., 2009). Two sets of 5  $\mu\text{l}$  of the tissue homogenates were used: one was incubated with 400  $\mu\text{l}$  of glucose reagents (Sigma-Aldrich) at 37°C and the other was incubated with 1 U amyloglucosidase and 400  $\mu\text{l}$  of glucose reagents at 37°C. After 30 min of incubation, we measured the  $A_{540}$ , and glucose concentration was calculated as described above. For each individual, concentrations were averaged for three replicates, and normalized relative to protein concentration. The amount of glycogen was calculated as the additional glucose in the homogenate containing amyloglucosidase.

For measuring triglycerides, we adopted and slightly revised another well-established method from *Drosophila* (Palanker et al., 2009). Two sets of 10  $\mu\text{l}$  homogenates from each tissue were used: one homogenate was incubated with 25  $\mu\text{l}$  PBS for 30 min at 37°C and the other homogenate was incubated for 30 min at 37°C with 25  $\mu\text{l}$  triglyceride reagent, which contains lipase to produce glycerol from triglycerides (Sigma-Aldrich). Then, each sample was incubated with 200  $\mu\text{l}$  Free Glycerol Reagent (Sigma-Aldrich) for 5 min at 37°C, the  $A_{540}$  was taken, and the glycerol concentration was calculated from a standard curve. The triglyceride level was calculated as the additional glycerol in the sample containing the triglyceride reagent, and normalized relative to tissue protein content.

### Statistics

Results are expressed as means  $\pm$  s.e. Student's *t*-test was used to evaluate treatment effect of the larval starvation on adult body mass, ovariole number and baseline levels of glucose and trehalose concentrations in the hive bees. The change of metabolic rate, RQ, hemolymph glucose and trehalose during the adult starvation were analyzed by using factorial ANOVA to detect interaction effects and main effects of treatment (control versus larval starvation) and adult starvation (1–12 h). In the analysis, larval starvation was a categorical factor and adult starvation time was used as a continuous factor. For the *post hoc* exploration, we were more interested in the changes between the early stage of adult starvation and the end of the starvation in both bees starved during the fifth larval instar and control bees. Therefore, we pooled data from 0–4 h and 9–12 h of the adult starvation as the early stage and late stage periods of starvation, and applied a Fisher LSD test to test the difference between groups in the factorial design. The changes in glycogen and triglycerides were analyzed by using factorial ANOVA and followed by a Fisher LSD test. The treatment (control versus larval starvation) and adult starvation time (before and 9–12 h after the start of starvation) were used as independent categorical variables and the measurements of glycogen and triglycerides were used as dependent variables.  $P < 0.05$  was considered



**Fig. 1. Effect of larval starvation on the body mass of honey bees.**

(A) Newly emerged bees. The 7 day old bees starved during the fifth instar had a lower body mass than control bees. (B) 7 day old bees. The 7 day old bees had a higher body mass than newly emerged bees in both treatment groups. But the bees starved as larvae still had a lower body mass than controls. Different letters indicate a significant difference between groups.

statistically significant, with a significant interaction effect (larval starvation treatment × time of adult starvation), indicating that the treatment groups responded differently to starvation.

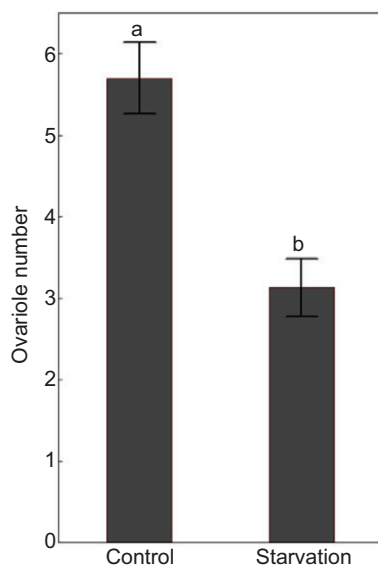
## RESULTS

### Treatment validation

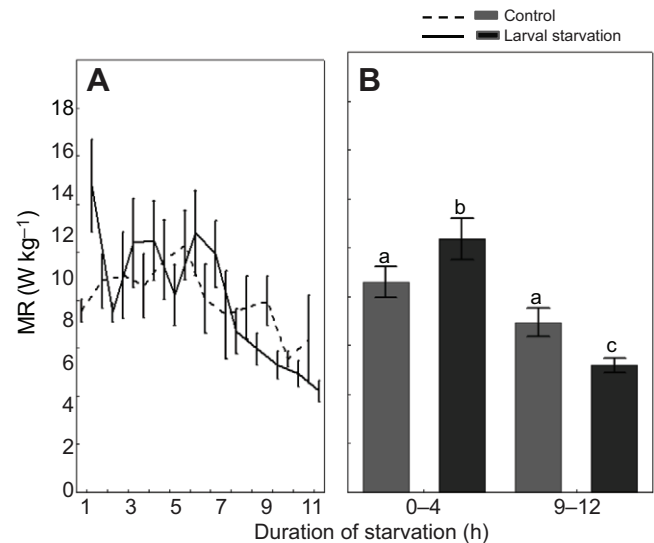
Larval starvation significantly reduced body mass (Student's *t*-test,  $t=5.38$ ,  $N=60$ ,  $P<0.0001$ ) and ovariole number (Student's *t*-test,  $t=3.87$ ,  $N=122$ ,  $P<0.0001$ ; Figs 1 and 2) in both newly emerged and 7 day old bees. These results confirmed starvation treatment effects previously described (Wang et al., 2014; Wang et al., 2016).

### Metabolic rate

The response of metabolic rate to 12 h of adult starvation changed depending on larval starvation treatment (factorial ANOVA,



**Fig. 2. Effect of larval starvation on ovariole number in honey bees.** The bees starved during the fifth larval stage had fewer ovarioles than control bees. Different letters indicate a significant difference between groups.



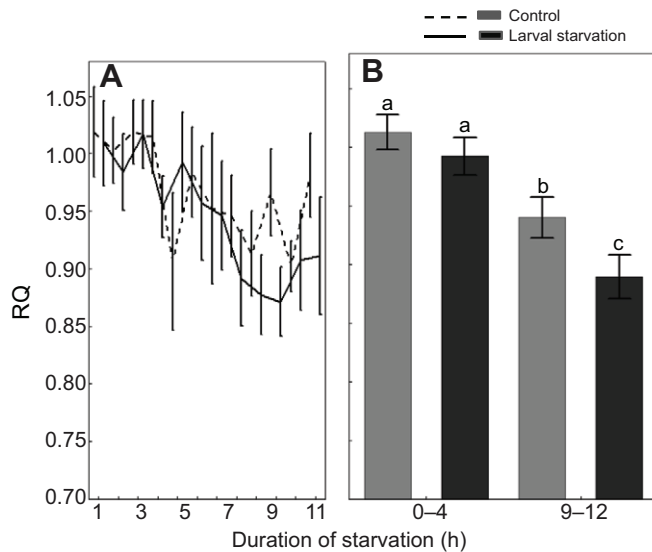
**Fig. 3. Effect of larval starvation and 12 h adult starvation on metabolic rate of 7 day old bees in a factorial design.**

(A) Bees starved as larvae had a higher metabolic rate than controls, and both groups of bees exhibited a reduction in metabolic rate (MR) during a 12 h starvation, with those subjected to larval starvation showing a more significant decline. (B) *Post hoc* analysis showed that bees starved as larvae had a higher metabolic rate than controls in the early stages (0–4 h) of adult starvation but a lower metabolic rate at 9–12 h. There was no change between 0–4 h and 9–12 h of adult starvation in controls, but bees starved at the fifth instar showed a significant decrease after 9–12 h of adult starvation. Different letters indicate a significant difference between groups.

interaction effect,  $F_{1,181}=6.237$ ,  $N=183$ ,  $P=0.01$ ; Fig. 3A). To compare metabolic rates between the beginning and end of the 12 h adult starvation period, groups were pooled from 0 to 4 h and 9 to 12 h for each treatment group. *Post hoc* tests showed that bees starved as larvae had a higher metabolic rate than controls during the first 4 h of adult starvation and had a lower metabolic rate in the last 4 h (control versus starvation: 0–4 h,  $P=0.05$ ; 9–12 h,  $P=0.045$ ). Metabolic rates did not significantly change between the beginning and end of starvation for the control group (0–4 h versus 9–12 h: control,  $P=0.06$ ); however, the group starved during the fifth instar showed a significant decrease in metabolic rate (0–4 h versus 9–12 h: starvation,  $P<0.0001$ ; Fig. 3B). These results indicate that bees that had been starved during the larval stage (but not control bees) were able to reduce metabolic rate when starved as adults.

### RQ

RQ in both control bees and those starved during the fifth larval instar responded similarly to 12 h adult starvation (factorial ANOVA, interaction effect,  $F_{1,176}=1.14$ ,  $N=183$ ,  $P=0.197$ ; Fig. 4A). RQ decreased in both control and treated bees during the 12 h starvation (main effect of adult starvation,  $F_{1,176}=15.49$ ,  $P<0.001$ ). The progressive decrease of RQ in bees starved as larvae was greater than in control bees (main effect of larval starvation, one-tailed,  $F_{1,176}=4.33$ ,  $P=0.03$ ; Fig. 4A). During the early stages (0–4 h) of adult starvation, the RQ did not differ between controls and bees starved as larvae (*post hoc* analysis, control versus starvation:  $P=0.403$ ; Fig. 4B); however, RQ in bees starved during the fifth instar was significantly lower than that in controls after 9–12 h of adult starvation (control versus starvation:  $P=0.033$ ). Between 0–4 h and 9–12 h of adult starvation, the RQ was reduced in both groups of bees (*post hoc* analysis, 0–4 h versus 9–12 h: control,  $P=0.003$ ; starvation,  $P<0.001$ ; Fig. 4B). These results

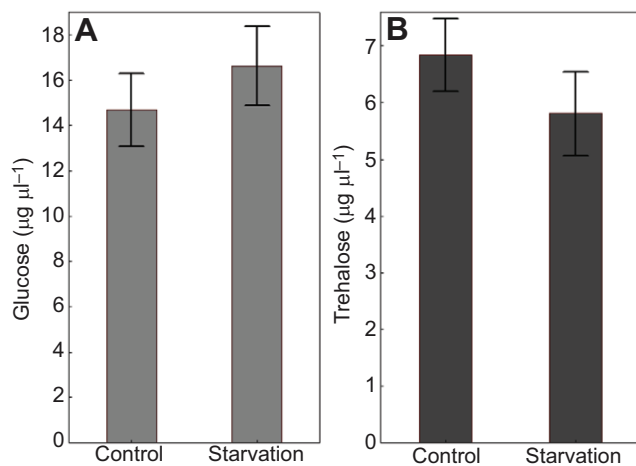


**Fig. 4. Effects of larval starvation and 12 h adult starvation on respiration quotient (RQ) of 7 day old bees in a factorial design.** (A) During a 12 h adult starvation, RQ was slowly decreased in both bees subjected to larval starvation and control bees, but the reduction in the former group was greater. (B) *Post hoc* analysis showed that during the early stages (0–4 h) of adult starvation, there was no difference in RQ between bees starved as larvae and controls, but at 9–12 h of starvation, the former group had a lower RQ than controls. Additionally, both controls and bees starved as larvae showed a reduction in RQ from 0–4 h to 9–12 h, with the reduction in the early-starved bees being more significant than in controls. Different letters indicate a significant difference between groups.

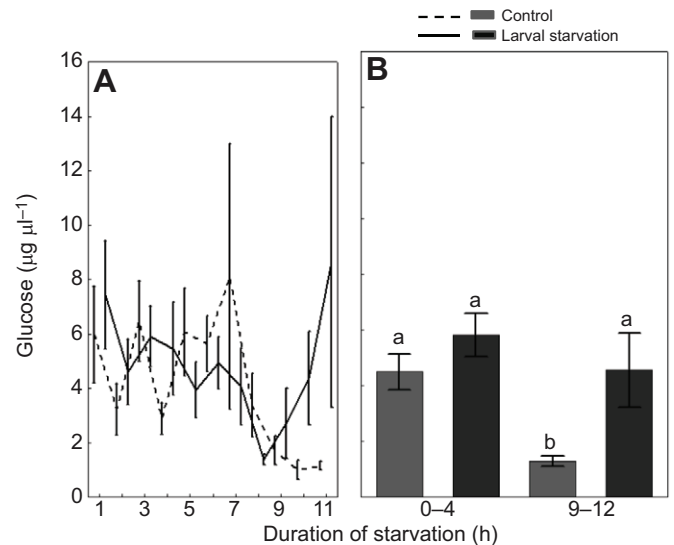
suggest that bees that were starved as larvae were able to more quickly shift from carbohydrate to mixed energy resources (protein or fat) during adult starvation.

#### Glucose and trehalose concentrations

Larval starvation did not affect glucose and trehalose concentrations in the hemolymph of fed bees collected directly from the hive (Student's *t*-test, glucose,  $t=-0.7816$ ,  $N=43-44$ ,  $P=0.4366$ ; trehalose,  $t=1.0634$ ,  $N=43-44$ ,  $P=0.2906$ ; Fig. 5).



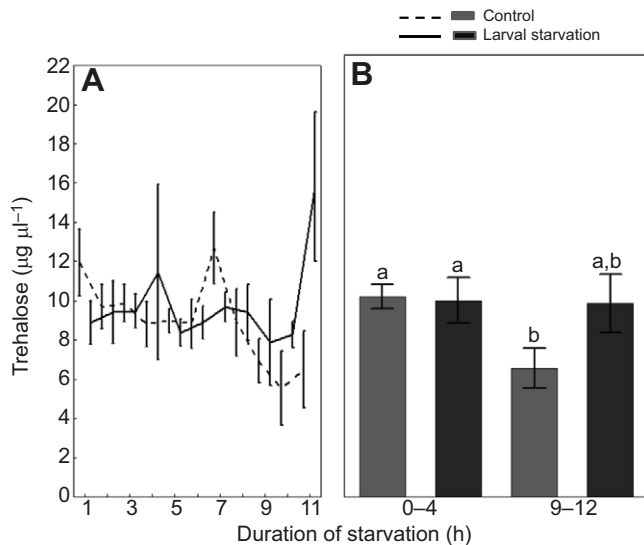
**Fig. 5. Effect of larval starvation on glucose and trehalose concentrations of honey bees.** Data are for 7 day old bees. (A) Hemolymph glucose titer in 7 day old bees. The bees subjected to larval starvation did not differ in glucose titer from control bees under normal condition. (B) Hemolymph trehalose titer in 7 day old bees. The bees subjected to larval starvation did not differ in trehalose titer from control bees under normal condition.



**Fig. 6. Effect of larval starvation and 12 h adult starvation on glucose concentration of 7 day old bees in a factorial design.** (A) During a 12 h adult starvation, the glucose level was significantly reduced in both bees starved as larvae and control bees, but there was no significant difference in glucose level between the two treatment groups. (B) *Post hoc* analysis showed that during the early stages (0–4 h) of adult starvation, there was no difference in glucose concentration between bees starved as larvae and controls, but at 9–12 h of starvation, the early-starved bees had a higher glucose level than controls. Comparing the two stages (0–4 and 9–12 h) of starvation, the controls had a significant reduction in glucose, but the bees starved as larvae did not. Different letters indicate a significant difference between groups.

Bees starved during the fifth larval instar were better able to conserve hemolymph glucose concentrations during adult starvation. Hemolymph glucose concentrations in control bees and those starved as larvae responded similarly to 12 h of adult starvation (factorial ANOVA, interaction effect,  $F_{1,136}=0.003$ ,  $N=138$ ,  $P=0.96$ ; Fig. 6A). During the 12 h adult food restriction, a significant reduction in glucose concentration was observed (main effect of adult starvation,  $F_{1,136}=6.53$ ,  $N=138$ ,  $P=0.01$ ), but larval starvation did not significantly affect glucose levels ( $F_{1,136}=0.03$ ,  $N=138$ ,  $P=0.86$ ; Fig. 6A). *Post hoc* analysis showed that during the early stages (0–4 h) of adult starvation, the concentration of glucose did not differ between controls and bees starved as larvae (control versus starvation:  $P=0.16$ ; Fig. 6B), but at 9–12 h, the bees starved during the last larval stage had a significantly higher glucose concentration than controls (control versus starvation:  $P=0.04$ ). From 0–4 h to 9–12 h of adult starvation, glucose was significantly reduced in controls, but not in bees starved as larvae (0–4 h versus 9–12 h: control,  $P=0.01$ ; starvation,  $P=0.36$ ; Fig. 6B).

There was mixed evidence in support of the hypothesis that the bees which experienced larval starvation kept more stable trehalose levels in the blood than control bees during adult starvation. Hemolymph trehalose concentration in control bees and bees starved as larvae responded similarly to 12 h of adult starvation (factorial ANOVA, interaction effect,  $F_{1,136}=3.17$ ,  $N=138$ ,  $P=0.07$ ; Fig. 7A). There was neither a main effect of adult starvation nor a main effect of larval starvation on hemolymph trehalose (main effect of adult starvation,  $F_{1,136}=1.82$ ,  $N=138$ ,  $P=0.18$ ; main effect of larval starvation  $F_{1,136}=2.06$ ,  $N=138$ ,  $P=0.15$ ; Fig. 7A). *Post hoc* analysis showed that during the early stages (0–4 h) of starvation, the concentration of trehalose did not differ between controls and treated bees (control versus starvation:  $P=0.87$ ; Fig. 7B). At 9–12 h



**Fig. 7. Effects of larval starvation and 12 h adult starvation on trehalose concentration of 7 day old bees in a factorial design.** (A) During a 12 h adult starvation, the bees in both treatment groups did not change trehalose level and did not differ in trehalose level. (B) *Post hoc* analysis showed that during the early stages (0–4 h) of adult starvation, there was no difference in trehalose concentration between bees starved as larvae and controls, but at 9–12 h of starvation, the early-starved bees showed a trend for a higher level of trehalose relative to controls. Comparing the two stages (0–4 and 9–12 h) of starvation, the controls had a significant reduction in trehalose, but the bees starved as larvae did not. Different letters indicate a significant difference between groups.

of starvation, the bees starved as larvae had a trend of an increase in trehalose concentration compared with controls, but it was not significant (one-tailed test, control versus starvation:  $P=0.06$ ). From 0–4 h to 9–12 h of starvation, the trehalose level in controls was

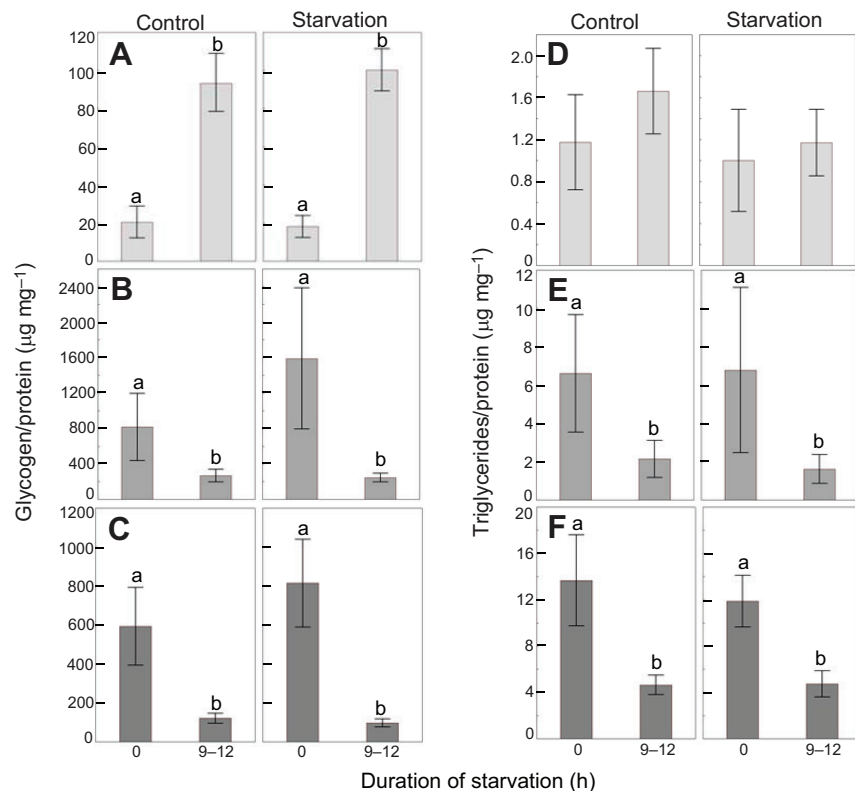
significantly reduced, but not in treated bees (0–4 h versus 9–12 h: control,  $P=0.02$ ; starvation,  $P=0.94$ ; Fig. 7B).

### Glycogen, glycerol and triglyceride levels

Glycogen and triglyceride levels in control bees and those starved at the fifth larval instar responded similarly to 12 h of adult starvation (no significant interaction effects in the factorial ANOVA,  $P>0.05$ ; Fig. 8). Also, there was no main effect of treatment on the glycogen and triglyceride stores in the brain, thoraces and fat body (all main effects of treatment,  $P>0.05$ ; Fig. 8). There were significant effects of adult starvation duration. Glycogen increased after 12 h of adult starvation in the brain ( $F_{1,94}=19.61$ ,  $N=98$ ,  $P<0.0001$ ; Fig. 8A). However, glycogen decreased in the thorax ( $F_{1,94}=9.34$ ,  $N=98$ ,  $P=0.003$ ; Fig. 8B) and fat body ( $F_{1,94}=31.14$ ,  $N=98$ ,  $P<0.0001$ ; Fig. 8C). Triglycerides did not change in the brain after 12 h of adult starvation ( $F_{1,94}=0.57$ ,  $N=98$ ,  $P=0.45$ ; Fig. 8D) and fat body ( $F_{1,94}=18.74$ ,  $N=98$ ,  $P<0.0001$ ; Fig. 8F).

### DISCUSSION

Previously, we found that adult honey bees that experienced short-term starvation during larval development survived better when they encountered food deprivation as adults (Wang et al., 2016). However, our gene expression analysis suggested that early nutritional stress had minor effects on metabolic pathways under normal nutritional conditions. The goal of this study was to test whether nutritional stress during larval development specifically affected metabolic responses of adult bees to nutritional challenges. Our results support this hypothesis. Metabolic reserves of adults sampled from the hive, measured as blood glucose and trehalose levels, or as brain, thorax or fat body glycogen and triglyceride levels, were not affected by larval starvation. However, bees that were starved as larvae were better able to maintain their blood



**Fig. 8. Effect of 12 h adult starvation on glycogen and triglyceride stores in the brain, thorax and abdominal fat body in both control bees and bees subjected to larval starvation.** (A,D) Brain; (B,E) thorax; (C,F) fat body. (A–C) Glycogen levels in multiple tissues. After 9–12 h of starvation, brain glycogen levels were increased in both controls and bees starved as larvae, but glycogen levels in the thorax and abdominal fat body were significantly reduced in all bees. (D–F) Triglycerides in multiple tissues. After 9–12 h of starvation, triglycerides in the brain were not affected in either controls or bees subjected to larval starvation. Triglycerides in the thorax and fat body were significantly reduced in all bees. Different letters indicate a significant difference between groups.

glucose levels during starvation. This preservation of blood sugar levels is at least partially explained by the better capacity of bees starved during the fifth larval instar to decrease their metabolic rates and shift away from carbohydrate use during starvation. Adaptive responses to starvation such as a decrease in glucose oxidation and an increase in mobilization of fatty acids are of major importance to survival in diverse organisms (Rion and Kawecki, 2007; Aggarwal, 2014). Our results further support the hypothesis that the early developmental program influenced by nutritional stress specifically targets metabolic responses to nutritional stress in adults.

The improved capacity of bees starved as larvae to survive adult starvation and conserve blood glucose levels could be partially explained by lower metabolic rates during the later stages of the starvation period. Bees starved as larvae had higher mass-specific metabolic rates during the first 4 h of adult starvation (Fig. 3), likely due to their reduced body size (Enquist et al., 2003; Brown et al., 2004). However, as starvation progressed, these bees showed a much stronger suppression of metabolic rate, decreasing energy use by about 50% relative to initial conditions (Fig. 3). This decrease in metabolic rate cannot be explained by lack of fuel, as our data showed that fuel levels in the hemolymph were well maintained. The decrease in metabolic rate could also not be explained by mortality; over 95% of bees survived the 12 h adult starvation in the current study. The metabolic rates of these starved honey bees ( $\sim 9 \text{ W kg}^{-1}$ ) were quite low compared with those of flying honey bees ( $\sim 500 \text{ W kg}^{-1}$ ; Harrison and Fewell, 2002) or whole hives ( $\sim 60 \text{ W kg}^{-1}$ ; Kronenberg and Heller, 1982), and just higher than those measured for sleeping honey bees ( $\sim 5 \text{ W kg}^{-1}$ ; Schmolz et al., 2002), indicating that the bees were quite quiescent and were likely only rarely activating their flight muscles.

An interesting difference with our previous paper was that most bees survived 12 h of adult starvation in the present study, while 50% of control bees died in our companion study (Wang et al., 2016). This may be explained by the fact that we used a lower environmental temperature (25°C) in the present study compared with our previous study (34°C). For quiescent bees, metabolic rates increase with temperature (Schmolz et al., 2002), with a  $Q_{10}$  of about 3. Thus, it is likely that the 9°C lower temperatures used here reduced metabolic rate and extended survival time approximately 3-fold.

Our RQ results suggest that during 12 h of adult starvation, all bees increased fat/protein utilization, but bees that were starved as larvae were able to switch more rapidly to non-carbohydrate fuels (Fig. 4). Studies in various organisms have demonstrated that the use of alternative fuel sources such as fatty acids or proteins is a critical metabolic adaptive strategy to starvation, which results in a reduction of the RQ and a conservation of carbohydrate (Nakaya et al., 2002).

Blood sugar homeostasis is critical for fundamental life functions in both vertebrates and invertebrates (Lee and Park, 2004). For regular activities, honey bees are able to keep constant blood (hemolymph) glucose and trehalose concentrations (Blatt and Roces, 2001). However, how hemolymph sugar concentrations change during starvation in honey bees has rarely been studied. Here, we found that larval starvation did not influence the levels of blood glucose and trehalose in adult bees sampled from the hive (Fig. 5); however, 12 h adult starvation significantly reduced glucose and trehalose concentrations in controls but not in bees starved as larvae (Figs 6 and 7). These results demonstrate that the bees that were starved during larval stages can better maintain blood sugar concentrations during nutritional deprivation.

Glycogen in honey bees is mostly stored in the thorax and abdominal fat bodies (present study and Neukirch, 1982; Panzenböck and Crailsheim, 1997). Here, we show that, like other insects that have been studied (McCue, 2010; Sinclair et al., 2011), honey bees can shift away from pure carbohydrate use during food starvation. We also show that after 9–12 h of starvation, glycogen and triglycerides in the fat body and thorax were greatly reduced, suggesting the abdominal fat body and flight muscles are the major energy reservoirs used during starvation in honey bees, and beyond 12 h these stores are nearly completely consumed (Fig. 8).

Our study also suggests that honey bees have very small lipid (triglycerides) stores compared with their glycogen stores, which is consistent with the findings of other studies (Hepburn et al., 1979; Leta et al., 1996). Nonetheless, these triglycerides are used during starvation, and this may be partly why the RQ declined with time. In addition, it is likely that starved bees utilized protein as fuel, contributing to the decline in RQ.

In contrast to the starvation-induced decreasing glycogen stores in the abdominal fat body and thorax, honey bees increase the amount of glycogen in their brains during starvation (Fig. 8). A similar result was obtained in monkeys in which brain glycogen significantly increased during the first 4 days of starvation while liver glycogen was rapidly depleted (Rivera and Jesus, 1974). These results suggest that animals respond to starvation by shifting carbohydrate from peripheral organs to the central brain, which obligatorily depends on carbohydrate fuel. Like other species, the honey bee brain is highly specialized for using carbohydrates (Tsacopoulos and Poiry, 1995). Plausibly, the increase of brain glycogen during starvation is important for conserving brain function during starvation stress, with other tissues being able to shift to using protein or lipid.

Overall, we found that, compared with control bees, the bees that were starved as larvae were able to reduce their metabolic rate, more quickly shift away from carbohydrate use and keep blood sugar concentrations more stable during adult starvation. We conclude that short-term starvation during larval stages alters the metabolic responses of adult bees to starvation, increasing the probability of their survival under starvation. We are the first to test the anticipatory-adaptive mechanism in a eusocial organism and demonstrate its specificity under a condition similar to that experienced during development. Furthermore, it has been suggested that environmental anticipation is an adaptive trait that was selected for during evolution and can be conserved in diverse organisms (Mitchell et al., 2009). The ecological and evolutionary success of eusocial insects is largely built on their division of labor (Hölldobler and Wilson, 2008), in which environmental nutrition plays a critical role (Page, 2013). Honey bee queen and worker phenotypes are determined by differential nutrition (nutrient rich versus nutrient restricted) during the larval stage, then the queens and workers stay in two nutritionally divergent environments (nutrient rich versus nutrient poor), and perform different social tasks (within colony versus outside); the variation of worker ovariole number is induced by developmental nutrition (Wang et al., 2014), and worker division of labor, which is a key component for the colony to adaptively respond to internal and external environmental changes, is regulated by ovariole number and involves nutritional-stress responses (Wang et al., 2016; Page, 2013). Therefore, one speculation that needs to be tested is that an anticipatory-adaptive mechanism was selected during social evolution and was incorporated into the social regulatory network to increase adaptive responses to environmental changes (Wang et al., 2016). Recently, anticipatory mechanisms, referred to as

predictive adaptive responses (PARs), have been used to explain the correlation between prenatal poor nutrition and higher susceptibility to adult metabolic diseases in humans (van den Heuvel et al., 2013): adult phenotypes that were preset by nutritional restriction during development mismatch the modern carbohydrate/lipid-rich diets, resulting in maladaptation and metabolic disorders (Gluckman et al., 2005a,b). Honey bees exhibit a rich repertoire for phenotypic plasticity and responses in morphology, physiology and behavior to environmental nutrition (Page, 2013). Future investigations need to be carried out to elucidate how both developmental and adult environments (nutrition) interactively affect honey bee biology and to test whether these results in honey bees can be generalized in other organisms including humans.

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#### Competing interests

The authors declare no competing or financial interests.

#### Author contributions

Y.W. developed the general concept and approaches in this study and G.V.A., R.E.P. and J.F.H. provided helpful comments. O.K. prepared honey bee colonies and performed the starvation treatment on larvae. Y.W. and J.B.C. performed respiratory assays and Y.W. performed metabolic assays. Y.W. analyzed data and prepared the paper which J.B.C., R.E.P., G.V.A. and J.F.H. edited prior to submission.

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#### Supplementary information

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