© 2017. Published by The Company of Biologists Ltd | Journal of Experimental Biology (2017) 220, 4035-4046 doi:10.1242/jeb.161844

RESEARCH ARTICLE

Mitochondrial capacity, oxidative damage and hypoxia gene expression are associated with age-related division of labor in honey bee (*Apis mellifera* L.) workers

Mário S. Cervoni[1,*], Carlos A. M. Cardoso-Júnior[1], Giovana Craveiro[1], Anderson de O. Souza[2], Luciane C. Alberici[2] and Klaus Hartfelder[1]

ABSTRACT

During adult life, honey bee workers undergo a succession of behavioral states. Nurse bees perform tasks inside the nest, and when they are about 2–3 weeks old they initiate foraging. This switch is associated with alterations in diet, and with the levels of juvenile hormone and vitellogenin circulating in hemolymph. It is not clear whether this behavioral maturation involves major changes at the cellular level, such as mitochondrial activity and the redox environment in the head, thorax and abdomen. Using high-resolution respirometry, biochemical assays and RT-qPCR, we evaluated the association of these parameters with this behavioral change. We found that tissues from the head and abdomen of nurses have a higher oxidative phosphorylation capacity than those of foragers, while for the thorax we found the opposite situation. As higher mitochondrial activity tends to generate more H_2O_2, and H_2O_2 is known to stabilize HIF-1α, this would be expected to stimulate hypoxia signaling. The positive correlation that we observed between mitochondrial activity and *hif-1α* gene expression in abdomen and head tissue of nurses would be in line with this hypothesis. Higher expression of antioxidant enzyme genes was observed in foragers, which could explain their low levels of protein carbonylation. No alterations were seen in nitric oxide (NO) levels, suggesting that NO signaling is unlikely to be involved in behavioral maturation. We conclude that the behavioral change seen in honey bee workers is reflected in differential mitochondrial activities and redox parameters, and we consider that this can provide insights into the underlying aging process.

KEY WORDS: Honey bee, High-resolution respirometry, Redox state, Hypoxia signaling, Nitric oxide

INTRODUCTION

The organization of a colony in the honey bee, *Apis mellifera*, as in all other eusocial insects, involves a structured division of labor. In a typical honey bee colony, the vast majority of individuals are facultative non-reproductive workers. While the queen is responsible for the reproduction of the colony, the workers perform all other social tasks, and in doing so, worker bees undergo a succession of behavioral states during their adult life. While young, they are nurse bees performing tasks inside the hive (e.g. brood care and feeding), and as they age they become forager bees that collect nectar, pollen and water (Seeley, 1982). Thus, associated with their age, workers tend to preferentially perform different kinds of tasks, and this age-related division of labor is also known as temporal or age polyethism (Winston, 1987).

Although worker bees are morphologically all alike, the differential behavioral states (nurse and forager) are contingent on certain physiological alterations, including differences in the levels of juvenile hormone (JH) (Huang and Robinson, 1992; Robinson, 2002; Sullivan et al., 2003) and the yolk precursor vitellogenin circulating in hemolymph (Excels, 1974; Hartfelder and Engels, 1998; Amdam et al., 2004; Guidugli et al., 2005), as well as in the insulin/insulin-like signaling (IIS) pathway (Ament et al., 2008), and in general aspects of gene expression (Ben-Shahar et al., 2002). The age at which a worker initiates foraging is a strong lifespan determinant, owing to both the extrinsic mortality caused by the risks of foraging, as well as intrinsic senescence, especially of the innate immune defence system (Amdam et al., 2004, 2005; Remolina et al., 2007).

Senescence or aging is a natural process affecting all animals and can be defined as a time-dependent deterioration. One of the long proposed and most investigated and accepted mechanisms concerning the molecular basis of aging is the oxidative stress hypothesis, which proposes that reactive oxygen species (ROS) are the main cause of the aging process (Harman, 1956). At low levels, ROS serve as signaling molecules exerting important functions in the regulation of biological and physiological processes (Finkel, 2011). However, when present in high levels and exceeding the normal limit, ROS promote oxidative stress and can induce damage to lipids, proteins and DNA (Cross et al., 1987). ROS are mainly produced by mitochondria during the process of respiration in the electron transport chain (ETC) (Brand, 2010). Leakage of electrons at ETC Complex I and Complex III leads to partial reduction of oxygen to form superoxide ($O_2^{\bullet-}$) radicals. These are quickly dismutated to hydrogen peroxide (H_2O_2) by superoxide dismutase (SOD), and are then further inactivated by catalase (CAT). Both $O_2^{\bullet-}$ and H_2O_2 are considered to be ROS.

Another free radical that plays an important biological role is nitric oxide (NO). NO is a gaseous molecule that is able to diffuse freely across cell membranes and to function as an inter- and intracellular messenger (Moncada, 1993). Another interesting feature of NO is that it can react with $O_2^{\bullet-}$ and can therefore modulate cellular redox signaling, resulting in alterations in biological systems promoted by ROS. In addition, NO can bind to cytochrome c oxidase (complex IV of ETC) where it competes with O_2; this interaction modulates mitochondrial activity, affecting cell

[1]Departamento de Biologia Celular e Molecular, Faculdade de Medicina de Ribeirão Preto, Universidade de São Paulo, Av. Bandeirantes 3900, 14049-900, Ribeirão Preto, São Paulo, Brazil. [2]Departamento de Física e Química, Faculdade de Ciências Farmacêuticas de Ribeirão Preto, Universidade de São Paulo, Av. do Café, s/n, 14040-903, Ribeirão Preto, São Paulo, Brazil.

*Author for correspondence (cervoni@usp.br)

M.S.C., 0000-0003-1178-6144

Received 8 May 2017; Accepted 7 September 2017

Journal of Experimental Biology (2017) 220, 4035-4046 doi:10.1242/jeb.161844

respiration and mitochondrial signaling (Brown, 2001; Cooper, 2002). Because of the competition between NO and O_2, increases in NO concentration can prevent the cytochrome c oxidase from using available oxygen, causing a 'metabolic hypoxia' state (Moncada and Erusalimsky, 2002), and such a situation has profound biological consequences. These include an influence on ROS generation by regulating mitochondrial activities and affecting the molecular function of hypoxia-inducible factors (HIFs), which are major regulators of the cellular adaptation to hypoxia.

HIF-1 is a heterodimeric transcription factor composed of α and β subunits. At normal O_2 concentrations (normoxia), a family of prolyl hydroxylases (PHD) tags the HIF-1α subunit for ubiquitination and subsequent proteasomal degradation. This diminished HIF-1α stability avoids its transfer to the nucleus and dimerization with HIF-1β (Wang et al., 1995). At low O_2 concentrations (1–3%), the enzymatic activity of the oxygen sensor PHD is inhibited, leading to the accumulation of HIF-1α, its transfer to the nucleus and dimerization with HIF-1β, where it forms the functional HIF-1 transcription factor that can then activate the expression of hypoxia-responsive target genes (Liu et al., 2012). Although this is the typical mechanistic framework underlying the response to exogenous hypoxia, HIF-1α stabilization can also occur under normoxic conditions. In such cases it has been shown that the redox state (Pan et al., 2007), ROS (Pagé et al., 2002; Zhou et al., 2007), NO (Mateo et al., 2003; Metzen et al., 2003) and IIS (Treins et al., 2002) can participate in and lead to a non-hypoxic stabilization of HIF-1α.

In insects, the hypoxia response and its molecular underpinnings are best studied in *Drosophila melanogaster*, and primarily so with respect to the formation of the tracheal system during embryonic development (Dekanty et al., 2005; Centanin et al., 2008, 2010). The insect tracheal system is a highly efficient oxygen transport system, so that free-living insects should rarely experience a hypoxia condition. However, this is not the case in holometabolous larvae with a digging lifestyle, such as that of endophytic herbivores (Pincebourde and Casas, 2016). Quite strikingly, in honey bees the question of a hypoxia response has arisen after non-intuitive results on IIS/target of rapamycin (TOR) pathway functions, indicating that the hypoxia response may be a possible regulatory mechanism involved in caste differentiation in the late larval stages, as the three hypoxia core genes were all found to be overexpressed in worker larvae in comparison with queen larvae (Azevedo et al., 2011). As queen and worker larvae are reared next to each another in open brood cells, this was interpreted as representing an endogenous hypoxia response that was recently shown to be related to differences in mitochondrial dynamics and activity (Santos et al., 2016).

With this in mind, we asked whether changes in mitochondrial activity and ROS are associated with this temporal polyethism. Similar to other studies (Corona et al., 2007; Azevedo et al., 2011) we analyzed the three main body compartments (head, thorax and abdomen) separately, because these compartments are related to specific processes, such as cognition learning, flight and heat production, and intermediary metabolism and excretion, respectively. By the separate analysis of these compartments we expected that this would allow us to establish more clearly connections with these processes, which in whole body analyses might remain hidden in the data. We first analyzed the mitochondrial flux characteristics by high resolution respirometry and then investigated ROS (H_2O_2 and NO) levels and genes regulated by ROS, protein carbonylation and lipid peroxidation as indicators of oxidative stress. Furthermore, we measured the transcript levels of the honey bee homologs of *hif-1α* (*Amsima*), *hif1β* (*Amtango*) and *phd* (*Amfatiga*) (Azevedo et al., 2011), as well as of genes coding for enzymes with antioxidant properties such as *CuZnSOD*, *MnSOD*, *catalase* and *Gtpx-1* (Corona et al., 2005). With these data, we expected to shed light on the role of cellular and organismic redox states and on their correlative association with the process of aging and division of labor in honey bees, especially on the transition from nursing to foraging activities.

MATERIALS AND METHODS

Honey bees

Workers of Africanized *Apis mellifera* Linnaeus 1758 were obtained from hives maintained by standard beekeeping practices at the experimental apiary of the Department of Genetics, University of São Paulo, Ribeirão Preto, SP, Brazil. Nurses and foragers were identified according to standard criteria (Huang and Robinson, 1992). Nurses were collected directly from brood frames when seen to perform the nurse-specific behavior of inserting their heads into brood cells to feed larvae, and foragers were collected when returning to the entrance of the colony with either pollen loads in their corbiculae or distended abdomens (nectar or water foragers). Nurses and foragers were collected at the same time and from the same colony and put on ice for 5 min before the start of each experiment. The sting and intestine were removed, the head, thorax and abdomen compartments were separated, placed in phosphate-buffered saline (PBS), and an incision was made to allow penetration of the respective reaction medium into the tissue.

Oxygen consumption

Parameters of oxidative phosphorylation (OXPHOS) in body compartments were determined by high-resolution respirometry using the Oxygraph-2k system (Oroboros Instruments, Innsbruck, Austria) as previously described for honey bee larvae (Santos et al., 2016). In brief, the respective compartments (head, thorax or abdomen) were separated from the body and the compartments containing the respective intact tissues (not mitochondrial homogenates) were incubated in 2 ml of MiR05 respiration buffer at 30°C and the reaction chamber was closed. Subsequently, we determined the following mitochondrial respiratory states in sequence: the routine (R) state was determined using a mix containing Complex I-linked substrates (malate and pyruvate, 10 mmol l^{-1}) to evaluate the respiration supported by CI; the phosphorylation (P) state was measured after adding ADP (220 mmol l^{-1}); the leak (L) or non-phosphorylating state was determined by the addition of the ATP synthase inhibitor oligomycin (1 mg ml^{-1}); the electron transport system state (ETS) or uncoupled state was determined via addition of the protonophore carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP) (four additions of 1 µmol l^{-1} each); and Rox (residual) or non-mitochondrial respiration was evaluated after Complex III inhibition by antimycin A (AA, 3 mmol l^{-1}). At the end of the assay, the tissue samples were retrieved and transferred to tubes containing radioimmunoprecipitation assay (RIPA) buffer and stored frozen at −20°C. The tissue samples were then used to determine the citrate synthase (CS) content as a measure to normalize the respirometry data, which are expressed as O_2 flux per mitochondrial content [pmol O_2 (CS units) s^{-1}]. The values of residual respiration (oxygen consumption not related to the mitochondrial electron transport) were subtracted from the all the respiratory states.

Citrate synthase (CS) activity

CS activity was measured as a correlate for the number of mitochondrial functional units in the different tissues of nurse

Journal of Experimental Biology (2017) 220, 4035-4046 doi:10.1242/jeb.161844

bees and foragers. Each tissue sample was homogenized in triethanolamine-HCl buffer (0.1 mol l^{-1}, pH 8.0), centrifuged at 12,000 ***g*** for 10 min at 4°C, and the supernatant was collected. CS activity was measured spectrophotometrically by adding 10 µg of supernatant protein to the reaction mixture containing triethanolamine-HCl buffer (0.1 mol l^{-1}, pH 8.0), 0.3 mmol l^{-1} acetyl-CoA, 0.5 mmol l^{-1} oxaloacetate, 0.25% Triton X-100 and 0.1 mmol l^{-1} 5,5′-dithiobis-2-nitrobenzoic acid (DTNB) (Lemos et al., 2003; Spinazzi et al., 2012; Santos et al., 2016). After 10 min the enzyme activity was measured in a Cary 50MPR Varian spectrophotometer (Varian Ltd, Melbourne, Australia) according to Srere (1969). Each sample was measured in quintuplicate and the absorbance values were expressed per total amount of protein in the tissue homogenates, previously quantified by the Bradford method.

H_2O_2 production

Head, thorax and abdomen samples were incubated in 2 ml of MiR05 respiration buffer at room temperature, in the presence of the same substrates used in the oxygen consumption assay, followed by the addition of 4 µl of horseradish peroxidase (0.1 U ml^{-1}) and 2 µl of the 50 µM Amplex Red Hydrogen Peroxide/Peroxidase Assay Kit (Molecular Probes/ThermoFisher, Eugene, OR, USA). The reaction mix was incubated in a F-4500 Hitachi fluorescence spectrophotometer (Hitachi Ltd, Tokyo, Japan) under excitation/emission of 571/585 nm and constant stirring for 10 min. H_2O_2 levels were determined fluorometrically by measuring the conversion of Amplex Red to resorufin, a highly fluorescent compound. The fluorescence values were expressed per total amount of protein, quantified by the Bradford method. A standard curve was established by sequential addition of H_2O_2 (40 nmol l^{-1}) to the reaction medium, to estimate the amounts of H_2O_2 present in the sample.

Determination of NO levels

Determination of the NO levels in the bees' body compartments was done by the chemiluminescence NO/ozone technique. As NO is very unstable, what is essentially measured are the stable oxidation products of NO metabolism (nitrates). Samples were deproteinized by alcohol precipitation (95% ethanol at 4°C for 30 min and centrifugation for 5 min at 5000 ***g***). Aliquots of the supernatant were injected into a reaction chamber of a Sievers NO analysis apparatus (Sievers 280 NOA, Sievers, Boulder, CO, USA) containing a reducing agent (0.8% vanadium chloride in 1 N HCl at 95°C), which converts nitrate (NO_3^-) to NO in equimolar amounts. NO is then aspirated into the chemiluminescence chamber where its reaction with ozone (O_3) causes the emission of red light. Emitted photons converted into an electrical signal are represented as the area under the curve generated by the electric current corresponding to the sample's nitrate content. The respective concentration was then calculated by regression against a standard curve established from known concentrations (0, 5, 10, 15, 30 and 60 pmol) of sodium nitrate.

Oxidative stress damage

Oxidative stress damage in head, thorax and abdomen tissues was quantified by measuring protein carbonylation and lipid peroxidation. For the protein carbonylation assays, the samples were homogenized in 100 µl ice-cold Tris buffer (0.1 mol l^{-1}, pH 7.4) and centrifuged at 1500 ***g*** for 10 min at 4°C. Supernatants were separated into two aliquots and each was incubated with 10% tricholoracetic acid (TCA) and centrifuged at 5000 ***g*** for 10 min at 4°C. The pellet of one aliquot was resuspended in 1 ml of 10 mmol l^{-1} 2,4-dinitrophenylhydrazine (DNPH) in 2.5 mol l^{-1} HCl, and the other in 1 ml of 2.5 mol l^{-1} HCl only (Reznick and Packer, 1994). After incubation for 1 h at 37°C in the dark, the samples were placed on ice for 10 min before 1 ml of 10% TCA was added and the samples were centrifuged at 5000 ***g*** for 5 min at 4°C. The pellets were washed three times with 1 ml of ethanol/ethyl acetate (1:1) and centrifuged at 5000 ***g*** for 5 min at 4°C. The pellets were dissolved in 6 mol l^{-1} guanidine and stirred for 40 min. Protein carbonyl content was assessed spectrophotometrically at 340 nm in a Cary 50MPR spectrophotometer.

Lipid peroxidation levels were measured using the TBARS assay, which quantifies lipid peroxidation products based on the formation of substances reacting with 0.4% 2-thiobarbituric acid (TBA) in 0.2 mol l^{-1} HCl and 0.2% butylated hydroxytoluene under heating at 90°C. After a 45 min incubation, 1 ml of *n*-butanol was added and the samples were maintained at 10°C for 10 min before centrifugation at 1000 ***g*** for 2 min. Readings were taken at 535 nm in a Cary 50MPR spectrophotometer.

Relative expression analysis of hypoxia response and antioxidant enzyme genes

Total RNA was extracted from the honey bee tissues using TRIzol reagent (Invitrogen) and treated with DNase I (Invitrogen). RNA quality and quantity were assessed in a NanoVue spectrophotometer (GE Healthcare Life Sciences, Pittsburgh, PA, USA), followed by first strand cDNA synthesis using Superscript II (Life Technologies, Carlsbad, CA, USA) enzyme and oligo$(dT)_{12–18}$ primer (Life Technologies) at 42°C for 50 min and 70°C for 15 min. Quantitative RT-PCR (RT-qPCR) analyses were performed with 1.5 µl of 10× diluted cDNA, 7.5 µl of Power SYBR PCR Green Master Mix (Life Technologies), 0.5 µl of each forward and reverse primer (10 pmol $µl^{-1}$) and 5 µl of deionized water (MilliQ, Millipore, Billerica, MA, USA). Reactions were run in a real-time PCR StepOne Plus system (Applied Biosystems, Foster City, CA, USA) under the following conditions: 50°C for 2 min, 95°C for 10 min, 40 cycles of 95°C for 15 s and 60°C for 1 min, followed by melting curve analysis to confirm the specificity of the amplification products. Each biological replicate was analyzed in technical triplicate. For sample normalization, we used the honey bee *rp49* gene (also named *rpl32*), which has previously been established as a suitable endogenous control gene (Lourenço et al., 2008). Relative expression levels were calculated by the comparative threshold

Table 1. Primer sequences of *Apis mellifera* honey bee workers

Gene	Primer sequence	Reference
CuZn SOD	F: GTCGTTCCGTGTAGTCGAGAA R: TCCTTTGACTTCACCCTGAAGA	Corona et al., 2005
Mn SOD	F: GGTGGTGGTCATTTGAATCATTC R: AAGAAGTGCAGCGTCTGGTTTAC	Corona et al., 2005
Catalase	F: TGGAGCAAGTCCTGATAAAATGC R: TGGGCCAAGACGATGTCTATG	Corona et al., 2005
Gtpx-1	F: CGACAACTATAAGGAAGCGAAA R: AGATAGAAAAACGTCTTCGCCT	Corona et al., 2005
sima	F: TGAACGACAGCATGGCCGA R: CGTTCGTTGCTCCTTCTCCG	Azevedo et al., 2011
tango	F: ATGAAACAACACAATCGCCTAT R: TGTCTTCCAATAGCCCATGC	Azevedo et al., 2011
fatiga	F: GTAGTGATCAAATAACGTGGC R: CCTTTGTCCTTCCATTGATTGT	Azevedo et al., 2011
rp49	F: CGTCATATGTTGCCAACTGGT R: TTGAGCACGTTCAACAATGG	Lourenço et al., 2008

Journal of Experimental Biology (2017) 220, 4035-4046 doi:10.1242/jeb.161844

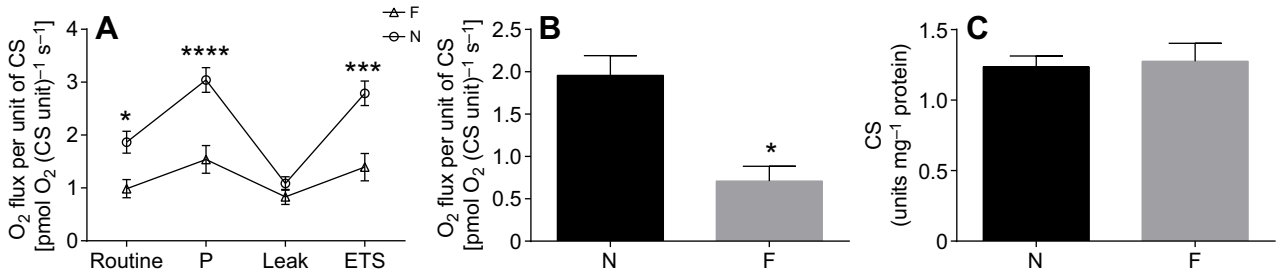

Fig. 1. Mitochondrial capacity and density in heads of *Apis mellifera* honey bee workers. (A) Respiration state. The mitochondrial states routine, phosphorylation (P), leak and electron transport system (ETS) in head tissue of nurse and forager bees, based on oxygen consumption. (B) Oxidative ATP production capacity (P–leak). (C) Citrate synthase (CS) content as an estimate of mitochondrial density. B and C show the phosphorylation state (P) in nurses (N) and foragers (F). Data are expressed as means and s.e.m. of five biological replicates. Statistical analysis by two-way ANOVA (A) and two-tailed unpaired Student's *t*-tests (B,C): **P*<0.05, ****P*<0.001, *****P*<0.0001.

cycle (C_t) method as $2^{-\Delta\Delta Ct}$ (Livak and Schmittgen, 2001). Primer sequences are shown in Table 1.

Statistical analysis

Data were analyzed using the software GraphPrad Prism version 6.0 (GraphPad Software, La Jolla, CA, USA). Values were $\log_{10}$ transformed to normalize variation before analyses by two-tailed unpaired Student's *t*-test. Values of $P<0.05$ were considered statistically significant. An *F*-test was used to compare variances. The data regarding mitochondrial states modulation were analyzed by two-way ANOVA.

RESULTS

Here we report the results for tissue mitochondrial respiratory capacity and density, the cellular redox state, and hypoxia signaling for each of the three body compartments.

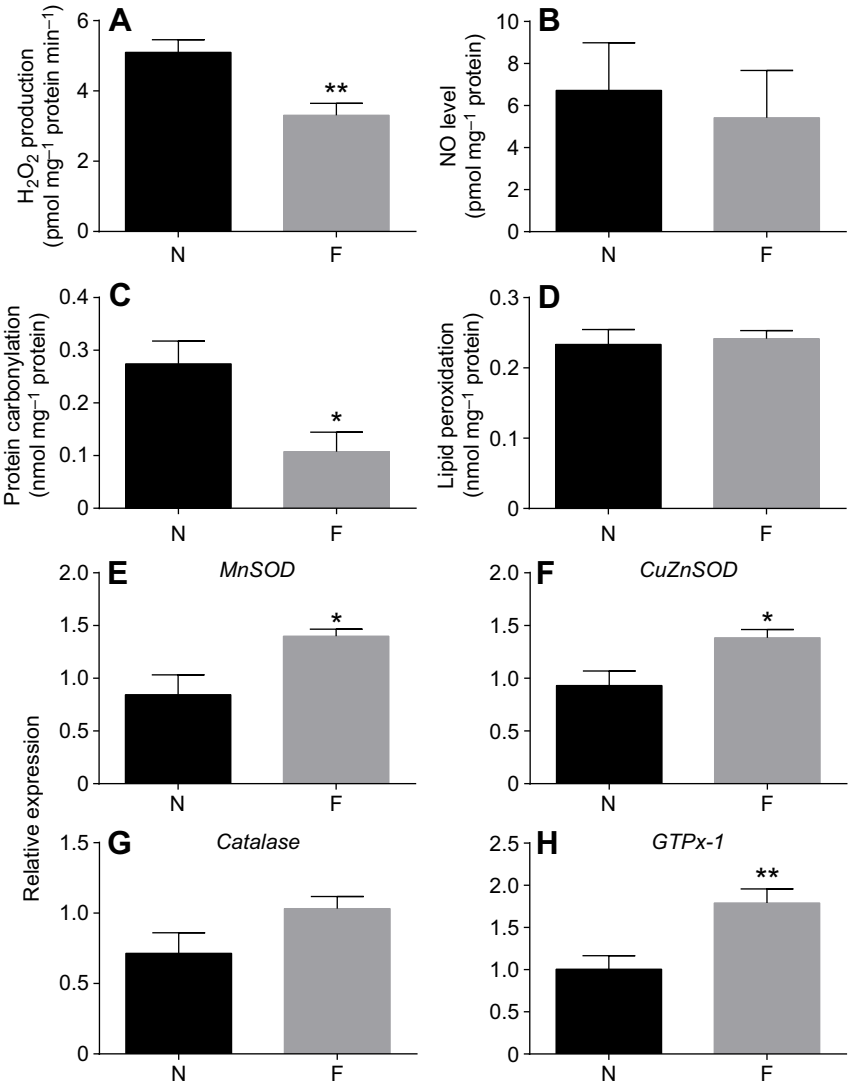

Fig. 2. Cellular redox environment in heads of honey bee workers. (A) H_2O_2 production in heads of nurse and forager bees was quantified by the Amplex Red assay. (B) NO quantification was done by chemiluminescence quantification in a Sievers NO analysis system. (C) Protein carbonylation was measured spectrophotometrically by reading specific carbonyl absorbance at 340 nm after DNPH reaction. (D) Lipid peroxidation was measured by TBARS absorbance at 535 nm. (E–H) Relative RT-qPCR quantification of *MnSOD*, *CuZnSOD*, *catalase* and *GTPx-1* transcript levels in head compartment. Relative expression levels were calculated as $2^{-\Delta\Delta Ct}$, with *rp49* serving as endogenous control. (A–H) N, nurse (black column); F, forager (gray column). Data are expressed as means and s.e.m. of five biological replicates. Statistical analysis by two-tailed unpaired Student's *t*-tests: **P*<0.05, ***P*<0.01.

Journal of Experimental Biology (2017) 220, 4035-4046 doi:10.1242/jeb.161844

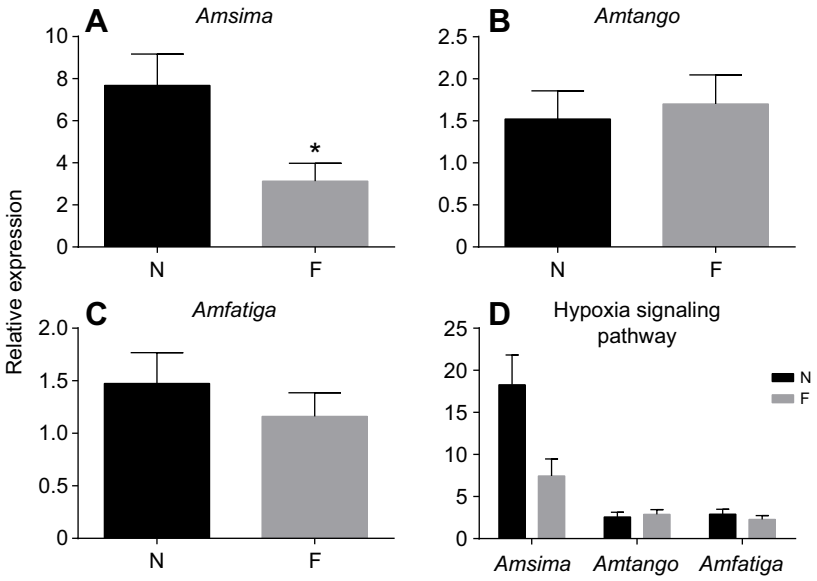

Fig. 3. Relative expression of hypoxia signaling pathway genes in heads of honey bee workers. (A–C) Relative quantification of *Amsima* (*HIF-1α*), *Amtango* (*HIF-1β*) and *Amfatiga* (*PHD*) transcript levels in nurse and forager heads were calculated as $2^{-\Delta\Delta Ct}$ with *rp49* serving as endogenous control, followed by calibration against a reference sample for each case (A–C); in D they are calibrated against the same reference sample. N, nurse; F, forager. Data are expressed as means and s.e.m. of five biological replicates. Statistical analysis by two-tailed unpaired Student's *t*-tests: **P*<0.05.

Head mitochondrial activity, redox state and hypoxia gene expression in honey bee workers

Mitochondrial respiratory capacity was evaluated using high-resolution respirometry. After determining the basal O_2 consumption, the respiratory states of the routine (R), phosphorylation (P), leak (L) and electron transport system (ETS) states were determined by sequential addition of compounds modulating mitochondrial activity. The respirometry results (Fig. 1A) were analyzed by two-way ANOVA (mitochondrial state $F_{(3,32)}$=17.12, P<0.0001; caste $F_{(1,32)}$=46.23, P<0.0001; interaction $F_{(3,32)}$=3.71, P<0.005). *Post-hoc* tests carried out as Sidak's multiple comparisons revealed a significant difference between the castes for states R (P<0.05), P (P<0.0001) and ETS (P<0.001). The oxidative ATP production capacity (P minus L) in the head turned out to be higher in nurses than in foragers. Thus the elevated respiratory rates in the R and P states (Fig. 1A), and the oxidative ATP production capacity (P minus L) (Fig. 1B) indicates that nurse bees have significantly higher OXPHOS capacity than foragers, or in other words, mitochondria in the head of nurses are capable of producing higher amounts of ATP per unit of time.

The numbers of mitochondrial functional units inferred from CS activity are shown in Fig. 1C. We observed no differences in CS activity between nurses and foragers, indicating similar mitochondrial densities. Taken together, these results suggest that, although the bees have similar numbers of mitochondria in their head tissues, the OXPHOS system in the head of nurses is more efficient, allowing them to respond to a higher ATP demand in a faster mode.

Next, we measured the levels of H_2O_2 and NO and damages from oxidative stress. The analyses showed that nurses have significantly higher rates of H_2O_2 production (Fig. 2A), while the amounts of NO were similar between the two life cycle stages (Fig. 2B). Damages caused by oxidative stress were inferred from the levels of carbonylated protein and peroxidized lipids. Nurse bees exhibited higher levels of protein carbonylation in their heads (Fig. 2C), which indicates that the respective tissues suffered more oxidative damage in comparison with foragers. No difference was seen, however, for lipid peroxidation (Fig. 2D). As the observed oxidative damage to proteins in nurse heads could be also due to differences in antioxidant system functions, we addressed this by analysing the transcript levels of four antioxidant enzymes. Of the four antioxidant genes analyzed, three exhibited significantly higher expression in the head of foragers (Fig. 2E–H), which is in accordance with the lower levels of oxidative damage seen in this compartment (Fig. 2C). These data indicate notable differences in the cellular redox state in head tissue of nurses and foragers and provide evidence for oxidative stress signals, characterized by protein carbonylation and high levels of H_2O_2 in nurses. The high H_2O_2 levels may be associated with a reduced expression of antioxidant enzymes, contributing to the oxidative stress observed.

We next investigated if these differences seen in nurse and forager heads could be associated with the expression of the honey bee

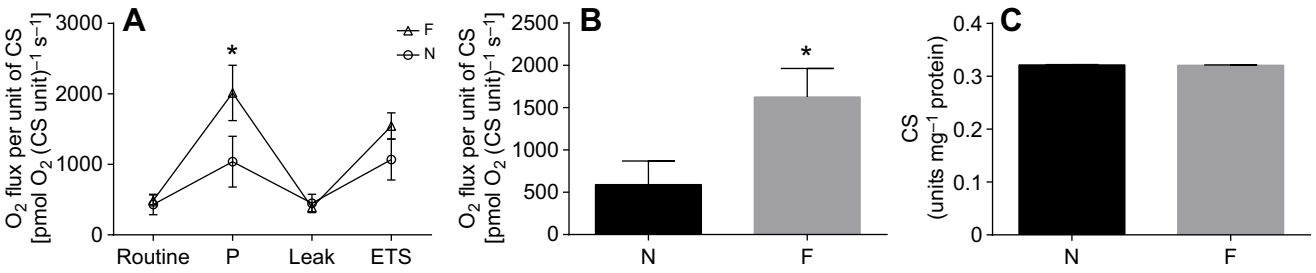

Fig. 4. Mitochondrial capacity and density in the thorax of honey bee workers. (A) Respiration state. The mitochondrial states routine, phosphorylation (P), leak and electron transport system (ETS) in thorax tissue of nurse and forager bees, based on oxygen consumption. (B) Oxidative ATP production capacity (P–leak). (C) CS content as an estimate of mitochondrial density. B and C show the phosphorylation (P) state in nurses (N) and foragers (F). Data are expressed as means and s.e.m. of five biological replicates. Statistical analysis by two-way ANOVA (A) and two-tail unpaired Student's *t*-tests (B,C): **P*<0.05.

Journal of Experimental Biology (2017) 220, 4035-4046 doi:10.1242/jeb.161844

hypoxia response core genes, the *hif-1α*, *hifF-1β* and *PHD* homologs *Amsima*, *Amtango* and *Amfatiga*. As shown in Fig. 3A, the heads of nurses had significantly higher transcription levels of *Amsima*, but no differences were seen for *Amtango* and *Amfatiga* (Fig. 3B,C). To directly compare the levels of the three hypoxia response genes, we calibrated their relative transcript levels against the same reference sample (Fig. 3D). This showed that *Amsima* expression is clearly elevated in nurse bees compared with foragers (Fig. 3A,D) and the other hypoxia response genes, and hence the honey bee HIFα protein, could represent an important factor in the physiological biochemistry of the brain in young, brood-rearing worker bees.

Thorax

The respirometry results analyzed by two-way ANOVA revealed significant differences for mitochondrial state ($F_{3,32}$=11.58, P<0.0001) and caste ($F_{1,32}$=4.62, P<0.005) but not for the interaction term. *Post hoc* tests carried out as Sidak's multiple comparisons revealed a significant difference (P<0.05) between the castes for the P state (Fig. 4A). Oxidative ATP production capacity (P minus L) was significantly increased in foragers (Fig. 4B). CS activity did not differ between nurses and foragers (Fig. 4C), indicating that they are similar with respect to mitochondrial functional units. These data indicate that the thorax tissue of foragers has a more efficient OXPHOS system. This higher mitochondrial capacity is probably related to the functional indirect flight musculature that enables foragers to achieve long-distance flights.

Regarding the two ROS signaling moieties, we did not observe any differences between the two groups (Fig. 5A,B). Upon investigating the indicators of oxidative stress, we found statistically significant lower levels for protein carbonylation in foragers compared with nurse bees (Fig. 5C), but again, no difference in lipid peroxidation levels (Fig. 5D). The expression levels for the antioxidant enzyme genes *MnSOD* and *catalase* were significantly higher in foragers (Fig. 5E,G), while *CuZnSOD* and *GTPx-1* did not differ (Fig. 5F,H). Taken together, these data suggest that the high levels of oxidative damage to protein seen in nurse bees could be related to a less efficient antioxidant system.

With respect to the expression levels of the three hypoxia signaling genes, we did not see statistically significant differences (Fig. 6A–C), even though the mean expression level for *Amsima* tended to be higher in nurses. However, when comparing the transcript levels for the three genes together, *Amsima* again showed a higher degree of expression compared with the other two genes.

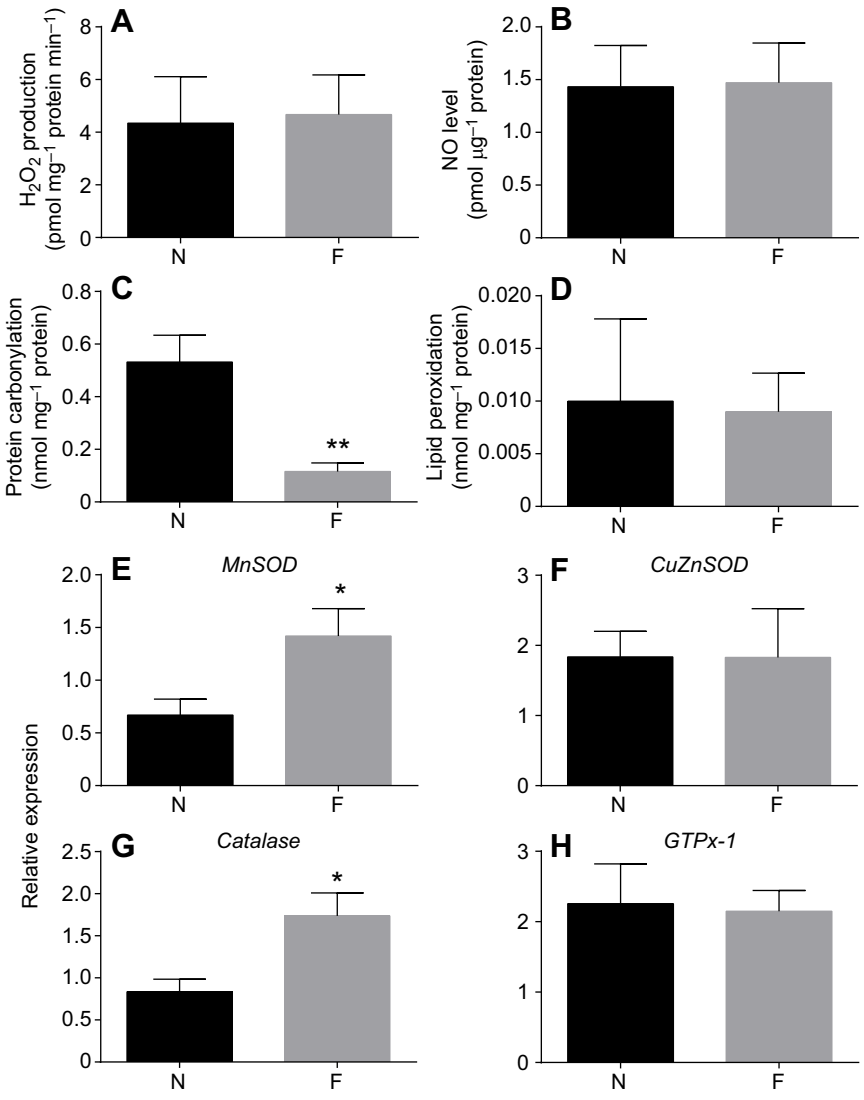

Fig. 5. Cellular redox environment in the thorax of honey bee workers. (A) H_2O_2 production in thorax of nurse and forager bees was quantified by the Amplex Red assay. (B) NO quantification was done by chemiluminescence measurement in a Sievers NO analysis system. (C) Protein carbonylation was measured photospectrometrically by reading specific carbonyl absorbance at 340 nm after DNPH reaction. (D) Lipid peroxidation was measured by TBARS absorbance at 535 nm. (E–H) RT-qPCR quantification of *Mn SOD*, *CuZn SOD*, *catalase* and *GTPx-1* transcript levels. Relative expression levels were calculated as $2^{-\Delta\Delta Ct}$, with *rp49* serving as endogenous control. (A–H) N, nurse (black column); F, forager (gray column). Data are expressed as means and s.e.m. of five biological replicates. Statistical analysis was done by two-tailed unpaired Student's *t*-tests: *P<0.05, **P<0.01.

Journal of Experimental Biology (2017) 220, 4035-4046 doi:10.1242/jeb.161844

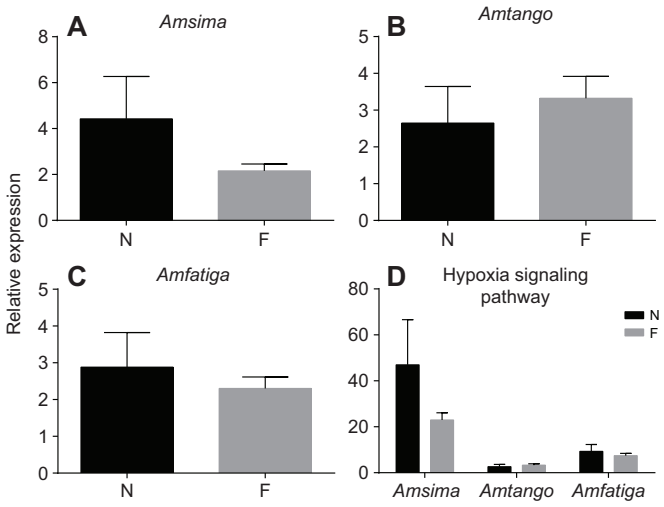

Fig. 6. Relative expression of hypoxia signaling pathway genes in the thorax of honey bee workers. (A–C) Relative quantification of *Amsima* (*HIF-1α*), *Amtango* (*HIF-1β*) and *Amfatiga* (*PHD*) transcript levels in nurse and forager thorax compartments, calculated as $2^{-\Delta\Delta Ct}$ with *rp49* serving as endogenous control, followed by calibration against a reference sample for each case (A–C), or (D) calibrated against the same reference sample. (A–D) N, nurse (black column); F, forager (gray column). Data are expressed as means and s.e.m. of five biological replicates.

Abdomen

The respirometry results analyzed by two-way ANOVA showed significant differences for mitochondrial state of the abdomen ($F_{3,32}$=11.37, P<0.0001), caste ($F_{1,32}$=67.98, P<0.0001) and also for the interaction term ($F_{3,32}$=3.13, P<0.005). *Post hoc* tests carried out as Sidak's multiple comparisons revealed a significant difference between the castes for the R (P<0.05), P (P<0.0001) and ETS states (P<0.0001) (Fig. 7A). The P minus L value was higher in nurses (Fig. 7B). These data for the abdominal compartment indicate that nurse bees have a more efficient OXPHOS capacity (Fig. 7A,B), a pattern similar to that found in the head (Fig. 1A,B). However, distinct from the other two body segments, the two life cycle stages differed in terms of their numbers of mitochondrial functional units, which were higher in foragers (Fig. 7C). These data imply that, even though they have fewer mitochondria in their abdominal tissues, nurse bees have a higher phosphorylating capacity per mitochondrial functional unit.

With respect to ROS levels and the cellular redox state, we found significant difference in abdominal H_2O_2 levels, which were elevated in foragers (Fig. 8A). These higher ROS levels could be associated with the higher mitochondrial density, as evidenced from the CS activity measurement (Fig. 7C), once mitochondria are an important source for ROS production (Muller, 2000; Turrens, 2003). Again, no differences in NO levels were found (Fig. 8B). The levels of oxidative damage to proteins and lipids also did not differ significantly between the two groups (Fig. 8C,D). The expression analyses for antioxidant enzyme genes (Fig. 8E–H) revealed significantly higher transcript levels for *catalase* in foragers. This suggests that the elevated ROS production seen in the abdomen of foragers may be counteracted by a more efficient antioxidant system, especially catalase activity.

Distinct from the other two body compartments, we found significant differences in the abdomen for the transcript levels of two of the three hypoxia response genes, with elevated expression of *Amsima* and *Amfatiga* in foragers, suggesting a negative feedback mechanism between the HIFα factor and its negative regulator PHD. Although PHD acts as a negative regulator of HIFα, the activation of HIF signaling can, in turn, promote the upregulation of PHD activity, and hence this upregulation can also be interpreted as indicative of HIF signaling activation (Li et al., 2013). In the joint comparison of the three genes (Fig. 9D), we found a strong overexpression of *Amsima*, as observed in the other two body compartments, but with a different pattern concerning nurses and foragers. Thus although the negative regulator, *Amfatiga*, was seen as highly transcribed in foragers in comparison with nurses, the pathway was clearly dominated by the overexpression of *Amsima* in the abdomen of foragers, indicating a strong abdominal hypoxia response in this life cycle stage.

DISCUSSION

We show that the oxidative parameters and the differential expression of genes related to these processes exhibit characteristic alterations for the two main life cycle stages of honey bee workers and also with respect to body compartment. The differences seen among the body compartments for each behavioral state reflect, of course, nothing other than the different functions for the main tissues in these body parts (brain, flight muscle and fat body, respectively). Although apparently trivial, this is frequently overlooked in whole body biochemical and gene expression studies.

Head mitochondrial activity, redox state and hypoxia gene expression in honey bee workers

With respect to task switching in adult honey bee workers, most studies focus on brain functions, especially so when high-throughput transcriptomic approaches became feasible (Whitfield et al., 2003, 2006; Garcia et al., 2009; Khamis et al., 2015). These revealed that genes upregulated in the brain of nurse bees are

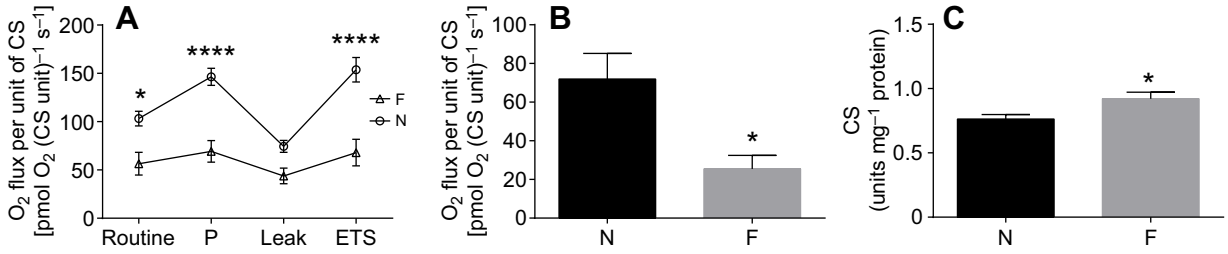

Fig. 7. Mitochondrial capacity and density in the abdomen of honey bee workers. (A) Respiration state. The mitochondrial states routine, phosphorylation (P), leak and electron transport system (ETS) in abdomen tissue of nurse and forager bees, based on oxygen consumption. (B) Oxidative ATP production capacity (P–leak). (C) CS content as an estimate of mitochondrial density. B and C show the phosphorylation state (P) in nurses (N) and foragers (F). Data are expressed as means and s.e.m. of five biological replicates. Statistical analysis by two-way ANOVA (A) and two-tailed unpaired Student's *t*-tests (B,C): **P*<0.05, *****P*<0.0001.

Journal of Experimental Biology (2017) 220, 4035-4046 doi:10.1242/jeb.161844

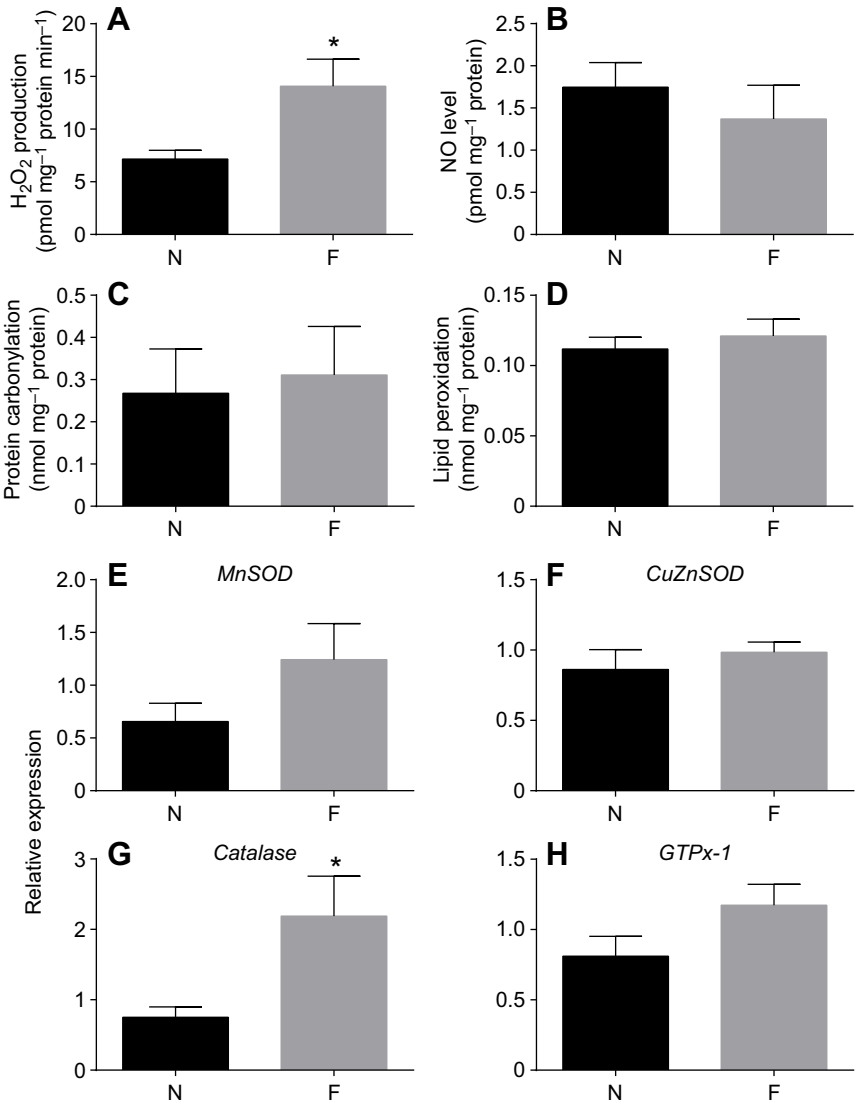

Fig. 8. Cellular redox environment in the abdomen of honey bee workers. (A) H_2O_2 production in abdomen tissue of nurse and forager bees was quantified by the Amplex Red assay. (B) NO quantification was done by chemiluminescence measurement in a Sievers NO analysis system. (C) Protein carbonylation was measured photospectrometrically by reading specific carbonyl absorbance at 340 nm after DNPH reaction. (D) Lipid peroxidation was measured by TBARS absorbance at 535 nm. (E–H) RT-qPCR quantification of *Mn SOD*, *CuZn SOD*, *catalase* and *GTPx-1* transcript levels. Relative expression levels were calculated as $2^{-\Delta\Delta Ct}$, with *rp49* serving as endogenous control. N, nurse; F, forager. Data are expressed as means and s.e.m. of five biological replicates. Statistical analysis was done by two-tailed unpaired Student's *t*-tests: *P<0.05.

enriched in gene ontology (GO) categories related to oxidoreductase activity (Whitfield et al., 2006; Ament et al., 2012), glycolysis (Ament et al., 2012) and mitochondrial and ribosomal components (Naeger et al., 2011). In contrast, in the brain of foragers, the enriched GO categories were linked to nervous system development (Sinha et al., 2006; Ament et al., 2012) synaptic/neurotransmission (Chandrasekaran et al., 2011), receptor signaling pathways (Whitfield et al., 2006), protein kinase activity (Whitfield et al., 2006; Lutz et al., 2012), G-protein-coupled receptor signaling (Grozinger et al., 2003; Lutz et al., 2012), insulin receptor signaling (Naeger et al., 2011), protein folding (Whitfield et al., 2006; Ament et al., 2012; Lutz et al., 2012) and response to heat (Ament et al., 2012; Lutz et al., 2012).

Our analyses of mitochondrial capacity in the head compartment of nurse and forager bees now provide evidence that nurses have higher OXPHOS capacity compared with foragers (Fig. 1A,B). This result is in agreement with the mitochondrial component GO categories enriched in the nurse bee brain transcriptomes (Naeger et al., 2011) and suggests a strong correlation between biochemical mitochondrial function and tissue transcriptome.

The higher amounts of H_2O_2 found in the head of nurses (Fig. 2A) and the elevated oxidative stress damage in nurse brains, evidenced by their higher protein carbonylation levels (Fig. 2C), could be caused by this elevated ROS production, together with a less efficient antioxidant system, as evidenced by the lower expression of antioxidant genes in this group of bees (Fig. 2E–H). The enriched GO categories linked to protein folding (Whitfield et al., 2006; Ament et al., 2012; Lutz et al., 2012) and response to heat (Ament et al., 2012; Lutz et al., 2012) in brains of foragers had already suggested an elevated potential to control a cellular stress environment, and this was confirmed by the oxidative damage analyses shown here. In contrast, Williams et al. (2008), comparing nurses and forager head tissue, did not find differences, neither regarding the total antioxidant capacity nor in protein carbonylation levels.

Strikingly, we did not find differences with respect to mitochondrial densities (Fig. 1C) or lipid peroxidation in head tissue of the two worker task groups (Fig. 2D), indicating that not all the oxidative parameters follow the same tendency. We also did not encounter a significant variation for NO levels in worker heads (Fig. 2B), although previous studies had indicated that this signaling molecule is likely to play important roles in olfactory learning and discrimination (Müller, 1996; Müller and Hildebrandt, 1995, 2002), as well as in various brain functions of worker bees (Watanabe et al., 2007).

With respect to the hypoxia response, we found that the transcription factor *Amsima* is highly expressed in heads of

Journal of Experimental Biology

Journal of Experimental Biology (2017) 220, 4035-4046 doi:10.1242/jeb.161844

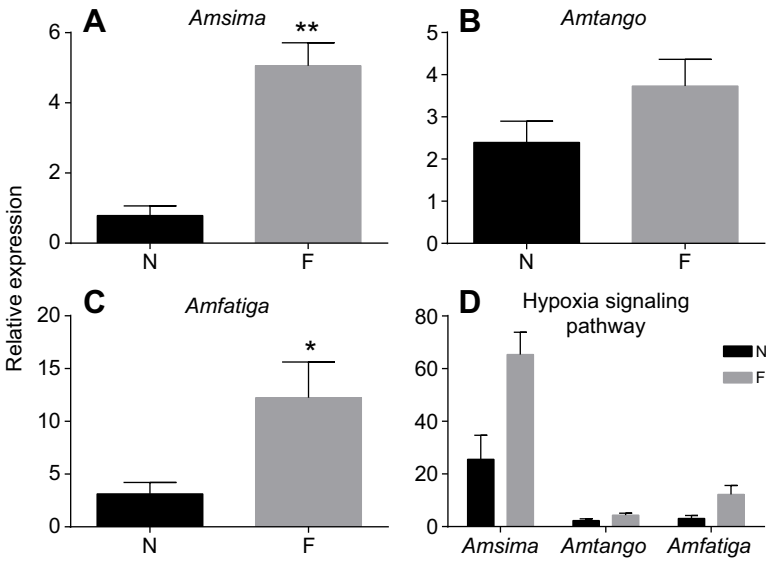

Fig. 9. Relative expression of hypoxia signaling pathway genes in the abdomen of honey bee workers. (A–C) Relative quantification of *Amsima* (*HIF-1α*), *Amtango* (*HIF-1β*) and *Amfatiga* (*PHD*) transcript levels in abdomens of nurses and foragers, calculated as $2^{-\Delta\Delta Ct}$ with *rp49* serving as endogenous control, followed by calibration against a reference sample for each case (A–C), or (D) calibrated against the same reference sample. N, nurse; F, forager. Data are expressed as means and s.e.m. of five biological replicates. Statistical analysis by two-tailed unpaired Student's *t*-tests: **P*<0.05, **P<0.01.

nurses, while *Amtango* and *Amfatiga* showed similar transcript levels (Fig. 3A–C). These higher *Amsima* transcript levels correlate with the higher H_2O_2 levels which, knowingly, could contribute to the stability of HIF-1α protein under normoxic conditions. As the activation of the hypoxia response pathway is mainly characterized by the negative regulation of HIF-1α by PHD, the higher amounts of *Amsima* transcripts (Fig. 3D) are indicative that this signaling cascade is activated in the head of nurse bees.

Thoracic mitochondrial activity, redox state and hypoxia gene expression in honey bee workers

The mitochondria of the thoracic flight musculature are responsible for supplying the energy demand necessary to perform long-distance flights. In insects, this energy demand is guaranteed aerobically via a highly efficient tracheal system network (Kammer and Heinrich, 1978; Beenakkers et al., 1984). The flight metabolism of honey bees utilizes primarily carbohydrate substrates that are oxidatively catabolized via glycolysis and the Krebs cycle (Beenakkers, 1969; Beenakkers et al., 1984).

Using high-resolution respirometry we found evidence that the mitochondria of the thorax of foragers possess a more efficient OXPHOS system and that they are able to produce higher amounts of ATP (Fig. 4A,B). These results are in accordance with previous findings, showing that maximal V_{O_2} for the thorax of foragers is significantly greater than in nurses (Harrison, 1986). This observation was expected because foragers perform long flights, up to 2 km from the hive and can carry loads equivalent to their body mass (Roberts and Harrison, 1999). Schippers et al. (2010) suggested that honey bees develop their flight musculature early in life, and the enhancement in flight metabolism in mature foragers is achieved by metabolic enzymes that operate close to their maximal capacities. According to our results (Fig. 4C) and also those of Harrison (1986), these higher mitochondrial capacities are not due to higher mitochondrial density, as we did not detect significant differences in citrate synthase activity between the two worker life cycle stages. It is important to note that Schippers et al. (2010) detected elevated levels of citrate synthase activity in foragers, but this was only observed when comparing foragers with young nurses. Nurses that were older than 5 days exhibited citrate synthase activity levels similar to foragers (Schippers et al., 2010). It is worth mentioning here that citrate synthase is not always a good marker of mitochondrial volume, as citrate synthase levels may vary depending on tissue source (Jackman and Willis, 1996). Hence changes in fat body tissue composition and mitochondrial type (and possibly volumes) between nurses and foragers may have contributed in part to the observed patterns.

In our analyses of oxidative damage, we found that nurses have higher levels of carbonylated protein (Fig. 5C), a result that differs from that of Williams et al. (2008) who did not find differences concerning this aspect. The levels of lipid peroxidation were the same between the two worker task types (Fig. 5D). Regarding the expression of antioxidant genes, foragers had higher *MnSOD* and *catalase* transcript levels in their thorax tissue (Fig. 5E,G). This is in agreement with earlier findings (Schippers et al., 2006; Williams et al., 2008), showing that the levels of catalase and CuZnSOD, as well as the total antioxidant capacity are elevated in foragers. Previous work also demonstrated that foragers have higher *hsp70* expression in the thorax than nurse bees (Roberts and Elekonich, 2005; Williams et al., 2008). This molecular chaperone participates in oxidative protection, maturation, maintenance and degradation of diverse proteins in stressed cells (Feder and Hofmann, 1999; Kaszubowska et al., 2017). Together with our results, these data strongly suggest that foragers have a higher potential to avoid oxidative stress conditions. Finally, no differences were encountered in hypoxia signaling genes, although *Amsima* expression tended to be slightly higher in nurses (Fig. 6A,D). Taken together, these data clearly show that, with respect to mitochondrial activity, oxidative damage and antioxidant system activity, the thoracic compartment differs considerably from the two other body compartments in relation to the age-related division of labor in honey bee workers, thus deserving closer attention in future studies.

Abdominal mitochondrial activity, redox state and hypoxia gene expression in honey bee workers

The abdomen of honey bees contains very different tissue types, including the reproductive tract, the sting apparatus, the dorsal heart vessel, the intestinal tract and associated Malphigian tubules, the epidermis and its glands underlying the cuticle and the parietal fat body. As we had removed the intestinal tract and sting apparatus during tissue preparation and due to the fact that the reproductive tract of workers contributes very little to the abdominal tissue mass, we consider that our data primarily reflect the mitochondrial activity

Journal of Experimental Biology (2017) 220, 4035-4046 doi:10.1242/jeb.161844

and gene expression of the parietal fat body. The insect fat body functions in detoxification and in the storage and utilization of energetic moieties (Arrese and Soulages, 2010), making it analogous to the vertebrate liver and adipose tissue (Law and Wells, 1989; Liu et al., 2009). During the transition from in-hive to foraging activities, the fat body of honey bee workers is extensively remodeled (Chan et al., 2011), with nurse bees having a more developed fat body (Ament et al., 2011), followed by a decrease in abdominal lipid content that precedes the onset of foraging (Toth et al., 2005).

Our respirometry data revealed that the abdominal tissue of nurses has an elevated mitochondrial capacity (Fig. 7A), as seen in head tissue (Fig. 1A,B). Hsu and Chuang (2014) had previously shown that: (i) the content of energy-regulated molecules in fat body cells differs between young and old workers, (ii) young workers have higher levels of ATP and ADP concentrations, and (iii) young workers have higher cAMP concentrations, which may reflect higher levels of signal transduction and a more active cellular metabolism. Their results are in accordance with the higher OXPHOS capacity that we find in nurses. However, the citrate synthase assay showed that foragers have an elevated mitochondrial density in abdominal tissues (Fig. 7C), which was not the case in head tissue (Fig. 1C). This higher number of mitochondria could be a possible explanation for the higher levels of H_2O_2 production detected in foragers compared with nurses (Fig. 8A). We also found that foragers have significantly higher transcript levels of *catalase* (Fig. 8G), which could be a response to counteract the elevated H_2O_2 production.

The other oxidative parameters (NO levels and oxidative damage to proteins and lipids) did not show differences between nurses and foragers (Fig. 8B–D), suggesting that in the abdomen the differences in mitochondrial system function and H_2O_2 production do not seem to promote major modifications in the cellular redox state. Previous work has demonstrated, however, that old workers possess higher levels of lipid peroxidation and protein oxidation (Hsieh and Hsu, 2011) and that the levels of ROS decrease with aging in the fat body cells of worker bees (Hsu and Hsieh, 2014). These discrepant results could be due to differences in the sample selection. While we selected our samples according to specific behaviors, nursing or foraging, Hsu and Hsieh selected the individuals by age (1 day for younger and 50 days for older bees). Even though the behavioral tasks are age related, they are not age fixed, as can be clearly seen in bees of the same age performing different tasks and showing different gene expression profiles in single age cohort colonies (Whitfield et al., 2003).

The relative expression of hypoxia signaling genes demonstrated higher expression of *Amsima* and of its negative regulator *Amfatiga* in foragers (Fig. 9A,C). Even though the negative regulator is also elevated, *Amsima* expression is much higher than that of *Amfatiga* (Fig. 9D), suggesting an activated hypoxia response pathway in foragers. Similarly, the IIS/TOR pathways also seem to be activated in foragers, as indicated by higher transcript levels of IIS genes in the abdomen foragers (Ament et al., 2008) and the finding that inhibition of the TOR pathway delays the onset of foraging. As a connection between the IIS/TOR axis and the hypoxia response via HIF-1α stabilization previously demonstrated in *Drosophila melanogaster* (Dekanty et al., 2005), we believe that the changes seen here in abdominal mitochondrial activity and the associated balance between ROS production and antioxidant system activity are probably closely related to the dietary switch (Haydak, 1970; Toth et al., 2005) and nutrient-sensing (IIS/TOR) pathway activity during the nurse-to-forager transition.

Conclusions

Our results on mitochondrial physiology, biochemistry and gene expression of signaling pathway genes revealed that worker bees show differential mitochondrial capacities as they perform different tasks during their adult life cycle. These differences involve alterations in the cellular redox state and in the differential expression of genes likely to be regulated by ROS, including the core genes of the hypoxia response. In other animal models, such changes were shown to be strongly associated with NO signaling (Mateo et al., 2003; Metzen et al., 2003), but this does not seem to be the case in honey bee workers. Rather unexpectedly, we found signatures of higher oxidative stress damage in nurse bees compared with foragers. In the thorax, for example, foragers have high levels of oxidative phosphorylation needed to provide ATP moieties for sustained indirect flight muscle function, but at the same time, this is balanced by higher expression levels of enzymes with antioxidant function.

With the current data, it is now possible to gain insights into functional differences among the three elementary body compartments of honey bee workers in terms of mitochondrial energy production, ROS production and oxidative damage, and activation of the hypoxia response pathway. As the relationships among these parameters differed between the three compartments, this implies that care must be taken when interpreting whole body data on biochemistry, physiology and transcriptomics of honey bees. Our data, with their focus on mitochondrial function dynamics and cellular redox state, also open a new field in the context of aging research in honey bees. These are becoming an emergent model invertebrate system in this respect (Flatt et al., 2013), with the interesting twist that the transition from in-nest activities to forager tasks represents a lifespan-limiting behavioral/physiological step in the life of an adult worker bee.

In the present study, we did not dissociate worker task performance and age, as we sampled bees exclusively based on the tasks that they performed in a natural colony setting. So here we can draw inferences only with respect to task, not with respect to aging or senescence. This is important because the nurse-to-forager transition is not age fixed, as seen in single cohort colonies (Whitfield et al., 2003). It can even be reverted dependent on colony condition, and such task reversion is associated with improved immunosenescence parameters, especially so via vitellogenin production in the fat body (Amdam et al., 2005). Our results should therefore stimulate functional studies that address questions on whether and how age and especially the reversion of foragers to nurse tasks is also reflected in mitochondrial functional dynamics, cellular redox state and hypoxia response, or in other terms, what is the coupling strength between age and behavior in relation to mitochondrial function?

Acknowledgements

We thank L. R. Aguiar for assistance in beekeeping and sampling of bees; we are also grateful for the assistance provided by Milene M. Lopes and Professor José A. Rodrigues during nitric oxide measurements.

Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: M.S.C., C.A.C., G.C., A.d.S., L.C.A., K.H.; Methodology: M.S.C., C.A.C., G.C., A.d.S., L.C.A.; Validation: M.S.C.; Formal analysis: M.S.C., C.A.C., G.C., A.d.S.; Investigation: M.S.C., C.A.C., G.C., A.d.S., L.C.A., K.H.; Resources: K.H.; Data curation: M.S.C., C.A.C., G.C., A.d.S., L.C.A., K.H.; Writing - original draft: M.S.C., L.C.A., K.H.; Writing - review & editing: M.S.C., L.C.A., K.H.; Visualization: M.S.C., C.A.C., G.C., A.d.S., L.C.A., K.H.; Supervision: L.C.A., K.H.; Funding acquisition: L.C.A., K.H.

Funding
The study received financial support from the Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP grants 2011/03171-5 and 2016/23509-4) and a PhD fellowship from Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) to M.S.C.

References

Amdam, G. V., Aase, A. L. T. O., Seehuus, S.-C., Kim Fondrk, M., Norberg, K. and Hartfelder, K. (2005). Social reversal of immunosenescence in honey bee workers. *Exp. Gerontol.* **40**, 939-947.
Amdam, G. V., Simões, Z. L. P., Hagen, A., Norberg, K., Schrøder, K., Mikkelsen, Ø., Kirkwood, T. B. L. and Omholt, S. W. (2004). Hormonal control of the yolk precursor vitellogenin regulates immune function and longevity in honeybees. *Exp. Gerontol.* **39**, 767-773.
Ament, S. A., Blatti, C. A., Alaux, C., Wheeler, M. M., Toth, A. L., Le Conte, Y., Hunt, G. J., Guzmán-Novoa, E., Degrandi-Hoffman, G., Uribe-Rubio, J. L. et al. (2012). New meta-analysis tools reveal common transcriptional regulatory basis for multiple determinants of behavior. *Proc. Natl. Acad. Sci. USA* **109**, E1801-E1810.
Ament, S. A., Chan, Q. W., Wheeler, M. M., Nixon, S. E., Johnson, S. P., Rodriguez-Zas, S. L., Foster, L. J. and Robinson, G. E. (2011). Mechanisms of stable lipid loss in a social insect. *J. Exp. Biol.* **214**, 3808-3821.
Ament, S. A., Corona, M., Pollock, H. S. and Robinson, G. E. (2008). Insulin signaling is involved in the regulation of worker division of labor in honey bee colonies. *Proc. Natl. Acad. Sci. USA* **105**, 4226-4231.
Arrese, E. L. and Soulages, J. L. (2010). Insect fat body: energy, metabolism, and regulation. *Annu. Rev. Entomol.* **55**, 207-225.
Azevedo, S. V., Caranton, O. A. M., de Oliveira, T. L. and Hartfelder, K. (2011). Differential expression of hypoxia pathway genes in honey bee (*Apis mellifera* L.) caste development. *J. Insect Physiol.* **57**, 38-45.
Beenakkers, A. M. T. (1969). Carbohydrate and fat as a fuel for insect flight. A comparative study. *J. Insect Physiol.* **15**, 353-361.
Beenakkers, A. M. T., Van der Horst, D. J. and Van marrewijk, W. J. A. (1984). Insect flight muscle metabolism. *Insect Biochem.* **14**, 243-260.
Ben-Shahar, Y., Robichon, A., Sokolowski, M. B. and Robinson, G. E. (2002). Influence of gene action across different time scales on behavior. *Science* **296**, 741-744.
Brand, M. D. (2010). The sites and topology of mitochondrial superoxide production. *Exp. Gerontol.* **45**, 466-472.
Brown, G. C. (2001). Regulation of mitochondrial respiration by nitric oxide inhibition of cytochrome c oxidase. *Biochim. Biophys. Acta* **1504**, 46-57.
Centanin, L., Dekanty, A., Romero, N., Irisarri, M., Gorr, T. A. and Wappner, P. (2008). Cell autonomy of HIF effects in *Drosophila*: tracheal cells sense hypoxia and induce terminal branch sprouting. *Dev. Cell* **14**, 547-558.
Centanin, L., Gorr, T. A. and Wappner, P. (2010). Tracheal remodelling in response to hypoxia. *J. Insect Physiol.* **56**, 447-454.
Chan, Q. W. T., Mutti, N. S., Foster, L. J., Kocher, S. D., Amdam, G. V. and Wolschin, F. (2011). The worker honeybee fat body proteome is extensively remodeled preceding a major life-history transition. *PLoS ONE* **6**, e24794.
Chandrasekaran, S., Ament, S. A., Eddy, J. A., Rodriguez-Zas, S. L., Schatz, B. R., Price, N. D. and Robinson, G. E. (2011). Behavior-specific changes in transcriptional modules lead to distinct and predictable neurogenomic states. *Proc. Natl. Acad. Sci. USA* **108**, 18020-18025.
Cooper, C. E. (2002). Nitric oxide and cytochrome oxidase: substrate, inhibitor or effector?. *Trends Biochem. Sci.* **27**, 33-39.
Corona, M., Hughes, K. A., Weaver, D. B. and Robinson, G. E. (2005). Gene expression patterns associated with queen honey bee longevity. *Mech. Ageing Dev.* **126**, 1230-1238.
Corona, M., Velarde, R. A., Remolina, S., Moran-Lauter, A., Wang, Y., Hughes, K. A. and Robinson, G. E. (2007). Vitellogenin, juvenile hormone, insulin signaling, and queen honey bee longevity. *Proc. Natl. Acad. Sci. USA* **104**, 7128-7133.
Cross, C. E., Halliwell, B., Borish, E. T., Pryor, W. A., Ames, B. N., Saul, R. L., McCord, J. M. and Harman, D. (1987). Oxygen radicals and human disease. *Ann. Intern. Med.* **107**, 526-545.
Dekanty, A., Lavista-Llanos, S., Irisarri, M., Oldham, S. and Wappner, P. (2005). The insulin-PI3K/TOR pathway induces a HIF-dependent transcriptional response in *Drosophila* by promoting nuclear localization of HIF-α/Sima. *J. Cell Sci.* **118**, 5431-5441.
Excels, W. (1974). Occurrence and significance of vitellogenins in female castes of social Hymenoptera. *Am. Zool.* **14**, 1229-1237.
Feder, M. E. and Hofmann, G. E. (1999). Heat-shock proteins, molecular chaperones, and the stress response: evolutionary and ecological physiology. *Annu. Rev. Physiol.* **61**, 243-282.
Finkel, T. (2011). Signal transduction by reactive oxygen species. *J. Cell Biol.* **194**, 7-15.
Flatt, T., Amdam, G. V., Kirkwood, T. B. L. and Omholt, S. W. (2013). Life-history evolution and the polyphenic regulation of somatic maintenance and survival. *Q. Rev. Biol.* **88**, 185-218.
Garcia, L., Saraiva Garcia, C. H., Calábria, L. K., Costa Nunes da Cruz, G., Sánchez Puentes, A., Báo, S. N., Fontes, W., Ricart, C. A. O., Salmen Espindola, F. and Valle de Sousa, M. (2009). Proteomic analysis of honey bee brain upon ontogenetic and behavioral development. *J. Proteome Res.* **8**, 1464-1473.
Grozinger, C. M., Sharabash, N. M., Whitfield, C. W. and Robinson, G. E. (2003). Pheromone-mediated gene expression in the honey bee brain. *Proc. Natl. Acad. Sci. USA* **100** Suppl. 2, 14519-14525.
Guidugli, K. R., Nascimento, A. M., Amdam, G. V., Barchuk, A. R., Omholt, S., Simões, Z. L. P. and Hartfelder, K. (2005). Vitellogenin regulates hormonal dynamics in the worker caste of a eusocial insect. *FEBS Lett.* **579**, 4961-4965.
Harman, D. (1956). Aging: a theory based on free radical and radiation chemistry. *J. Gerontol.* **11**, 298-300.
Harrison, J. M. (1986). Caste-specific changes in honeybee flight capacity. *Physiol. Zool.* **59**, 175-187.
Hartfelder, K. and Engels, W. (1998). Social insect polymorphism: hormonal regulation of plasticity in development and reproduction in the honeybee. *Curr. Top. Dev. Biol.* **40**, 45-77.
Haydak, M. H. (1970). Honey bee nutrition. *Annu. Rev. Entomol.* **15**, 143-156.
Hsieh, Y.-S. and Hsu, C.-Y. (2011). Honeybee trophocytes and fat cells as target cells for cellular senescence studies. *Exp. Gerontol.* **46**, 233-240.
Hsu, C.-Y. and Chuang, Y.-L. (2014). Changes in energy-regulated molecules in the trophocytes and fat cells of young and old worker honeybees (*Apis mellifera*). *J. Gerontol. A Biol. Sci. Med. Sci.* **69**, 955-964.
Hsu, C.-Y. and Hsieh, Y.-S. (2014). Oxidative stress decreases in the trophocytes and fat cells of worker honeybees during aging. *Biogerontology* **15**, 129-137.
Huang, Z. Y. and Robinson, G. E. (1992). Honeybee colony integration: worker-worker interactions mediate hormonally regulated plasticity in division of labor. *Proc. Natl. Acad. Sci. USA* **89**, 11726-11729.
Jackman, M. R. and Willis, W. T. (1996). Characteristics of mitochondria isolated from type I and type IIb skeletal muscle. *Am. J. Physiol.* **270**, C673-C678.
Kammer, A. E. and Heinrich, B. (1978). Insect flight metabolism. *Adv. Insect Physiol.* **13**, 133-228.
Kaszubowska, L., Foerster, J., Kaczor, J. J., Schetz, D., Ślebioda, T. J. and Kmieć, Z. (2017). Expression of cellular protective proteins SIRT1, HSP70 and SOD2 correlates with age and is significantly higher in NK cells of the oldest seniors. *Immun. Ageing* **14**, 3.
Khamis, A. M., Hamilton, A. R., Medvedeva, Y. A., Alam, T., Alam, I., Essack, M., Umylny, B., Jankovic, B. R., Naeger, N. L., Suzuki, M. et al. (2015). Insights into the transcriptional architecture of behavioral plasticity in the honey bee *Apis mellifera*. *Sci. Rep.* **5**, 11136.
Law, J. H. and Wells, M. A. (1989). Insects as biochemical models. *J. Biol. Chem.* **264**, 16335-16338.
Lemos, D., Salomon, M., Gomes, V., Phan, V. N. and Buchholz, F. (2003). Citrate synthase and pyruvate kinase activities during early life stages of the shrimp *Farfantepenaeus paulensis* (Crustacea, Decapoda, Penaeidae): effects of development and temperature. *Comp. Biochem. Physiol. B Biochem. Mol. Biol.* **135**, 707-719.
Li, Y., Padmanabha, D., Gentile, L. B., Dumur, C. I., Beckstead, R. B. and Baker, K. D. (2013). HIF- and non-HIF-regulated hypoxic responses require the estrogen-related receptor in *Drosophila melanogaster*. *PLoS Genet.* **9**, e1003230.
Liu, Y., Liu, H., Liu, S., Wang, S., Jiang, R.-J. and Li, S. (2009). Hormonal and nutritional regulation of insect fat body development and function. *Arch. Insect Biochem. Physiol.* **71**, 16-30.
Liu, W., Shen, S. M., Zhao, X. Y. and Chen, G. Q. (2012). Targeted genes and interacting proteins of hypoxia inducible factor-1. *Int. J. Biochem. Mol. Biol.* **3**, 165-178.
Livak, K. J. and Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta Ct}$ method. *Methods* **25**, 402-408.
Lourenço, A. P., Mackert, A., dos Santos Cristino, A. and Simões, Z. L. P. (2008). Validation of reference genes for gene expression studies in the honey bee, *Apis mellifera*, by quantitative real-time RT-PCR. *Apidologie* **39**, 372-385.
Lutz, C. C., Rodriguez-Zas, S. L., Fahrbach, S. E. and Robinson, G. E. (2012). Transcriptional response to foraging experience in the honey bee mushroom bodies. *Dev. Neurobiol.* **72**, 153-166.
Mateo, J., García-Lecea, M., Cadenas, S., Hernández, C. and Moncada, S. (2003). Regulation of hypoxia-inducible factor-1α by nitric oxide through mitochondria-dependent and -independent pathways. *Biochem. J.* **376**, 537-544.
Metzen, E., Zhou, J., Jelkmann, W., Fandrey, J. and Brüne, B. (2003). Nitric oxide impairs normoxic degradation of HIF-1α a by inhibition of prolyl hydroxylases. *Mol. Biol. Cell* **14**, 3470-3481.
Moncada, S. (1993). The L-arginine: nitric oxide pathway, cellular transduction and immunological roles. *Adv. Second Messenger Phosphoprotein Res.* **28**, 97-99.
Moncada, S. and Erusalimsky, J. D. (2002). Does nitric oxide modulate mitochondrial energy generation and apoptosis? *Nat. Rev. Mol. Cell Biol.* **3**, 214-220.
Müller, U. (1996). Inhibition of nitric oxide synthase impairs a distinct form of long-term memory in the honeybee, *Apis mellifera*. *Neuron* **16**, 541-549.
Muller, F. (2000). The nature and mechanism of superoxide production by the electron transport chain: its relevance to aging. *J. Am. Aging Assoc.* **23**, 227-253.

Müller, U. and Hildebrandt, H. (1995). The nitric oxide/cGMP system in the antennal lobe of *Apis mellifera* is implicated in integrative processing of chemosensory stimuli. *Eur. J. Neurosci.* **7**, 2240-2248.
Müller, U. and Hildebrandt, H. (2002). Nitric oxide/cGMP-mediated protein kinase A activation in the antennal lobes plays an important role in appetitive reflex habituation in the honeybee. *J. Neurosci.* **22**, 8739-8747.
Naeger, N. L., Van Nest, B. N., Johnson, J. N., Boyd, S. D., Southey, B. R., Rodriguez-Zas, S. L., Moore, D. and Robinson, G. E. (2011). Neurogenomic signatures of spatiotemporal memories in time-trained forager honey bees. *J. Exp. Biol.* **214**, 979-987.
Pagé, E. L., Robitaille, G. A., Pouysségur, J. and Richard, D. E. (2002). Induction of hypoxia-inducible factor-1alpha by transcriptional and translational mechanisms. *J. Biol. Chem.* **277**, 48403-48409.
Pan, Y., Mansfield, K. D., Bertozzi, C. C., Rudenko, V., Chan, D. A., Giaccia, A. J. and Simon, M. C. (2007). Multiple factors affecting cellular redox status and energy metabolism modulate hypoxia-inducible factor prolyl hydroxylase activity *in vivo* and *in vitro*. *Mol. Cell Biol.* **27**, 912-925.
Pincebourde, S. and Casas, J. (2016). Hypoxia and hypercarbia in endophagous insects: larval position in the plant gas exchange network is key. *J. Insect Physiol.* **84**, 137-153.
Remolina, S. C., Hafez, D. M., Robinson, G. E. and Hughes, K. A. (2007). Senescence in the worker honey bee *Apis mellifera*. *J. Insect Physiol.* **53**, 1027-1033.
Reznick, A. Z. and Packer, L. (1994). Oxidative damage to proteins: spectrophotometric method for carbonyl assay. *Methods Enzymol.* **233**, 357-363.
Roberts, S. P. and Elekonich, M. M. (2005). Muscle biochemistry and the ontogeny of flight capacity during behavioral development in the honey bee, *Apis mellifera*. *J. Exp. Biol.* **208**, 4193-4198.
Roberts, S. P. and Harrison, J. F. (1999). Mechanisms of thermal stability during flight in the honeybee *Apis mellifera*. *J. Exp. Biol.* **202**, 1523-1533.
Robinson, G. E. (2002). Genomics and integrative analyses of division of labor in honeybee colonies. *Am. Nat.* **160**, S160-S172.
Santos, D. E., Alberici, L. C. and Hartfelder, K. (2016). Mitochondrial structure and dynamics as critical factors in honey bee (*Apis mellifera* L.) caste development. *Insect Biochem. Mol. Biol.* **73**, 1-11.
Schippers, M.-P., Dukas, R., Smith, R. W., Wang, J., Smolen, K. and McClelland, G. B. (2006). Lifetime performance in foraging honeybees: behaviour and physiology. *J. Exp. Biol.* **209**, 3828-3836.
Schippers, M.-P., Dukas, R. and McClelland, G. B. (2010). Lifetime- and caste-specific changes in flight metabolic rate and muscle biochemistry of honeybees, *Apis mellifera*. *J. Comp. Physiol. B* **180**, 45-55.
Seeley, T. D. (1982). Adaptive significance of the age polyethism schedule in honeybee colonies. *Behav. Ecol. Sociobiol.* **11**, 287-293.
Sinha, S., Ling, X., Whitfield, C. W., Zhai, C. and Robinson, G. E. (2006). Genome scan for *cis*-regulatory DNA motifs associated with social behavior in honey bees. *Proc. Natl. Acad. Sci. USA* **103**, 16352-16357.
Spinazzi, M., Casarin, A., Pertegato, V., Salviati, L. and Angelini, C. (2012). Assessment of mitochondrial respiratory chain enzymatic activities on tissues and cultured cells. *Nat. Protoc.* **7**, 1235-1246.
Srere, P. A. (1969). Citrate synthase. *Method Enzymol.* **13**, 3-11.
Sullivan, J. P., Fahrbach, S. E., Harrison, J. F., Capaldi, E. A., Fewell, J. H. and Robinson, G. E. (2003). Juvenile hormone and division of labor in honey bee colonies: effects of allatectomy on flight behavior and metabolism. *J. Exp. Biol.* **206**, 2287-2296.
Toth, A. L., Kantarovich, S., Meisel, A. F. and Robinson, G. E. (2005). Nutritional status influences socially regulated foraging ontogeny in honey bees. *J. Exp. Biol.* **208**, 4641-4649.
Treins, C., Giorgetti-Peraldi, S., Murdaca, J., Semenza, G. L. and Van Obberghen, E. (2002). Insulin stimulates hypoxia-inducible factor 1 through a phosphatidylinositol 3-kinase/target of rapamycin-dependent signaling pathway. *J. Biol. Chem.* **277**, 27975-27981.
Turrens, J. F. (2003). Mitochondrial formation of reactive oxygen species. *J. Physiol.* **552**, 335-344.
Wang, G. L., Jiang, B. H., Rue, E. A. and Semenza, G. L. (1995). Hypoxia-inducible factor 1 is a basic-helix-loop-helix-PAS heterodimer regulated by cellular O_2 tension. *Proc. Natl. Acad. Sci. USA* **92**, 5510-5514.
Watanabe, T., Kikuchi, M., Hatakeyama, D., Shiga, T., Yamamoto, T., Aonuma, H., Takahata, M., Suzuki, N. and Ito, E. (2007). Gaseous neuromodulator-related genes expressed in the brain of honeybee *Apis mellifera*. *Dev. Neurobiol.* **67**, 456-473.
Whitfield, C. W., Cziko, A. M. and Robinson, G. E. (2003). Gene expression profiles in the brain predict behavior in individual honey bees. *Science* **302**, 296-299.
Whitfield, C. W., Ben-Shahar, Y., Brillet, C., Leoncini, I., Crauser, D., Leconte, Y., Rodriguez-Zas, S. and Robinson, G. E. (2006). Genomic dissection of behavioral maturation in the honey bee. *Proc. Natl. Acad. Sci. USA* **103**, 16068-16075.
Williams, J. B., Roberts, S. P. and Elekonich, M. M. (2008). Age and natural metabolically-intensive behavior affect oxidative stress and antioxidant mechanisms. *Exp. Gerontol.* **43**, 538-549.
Winston, M. L. (1987). *The Biology of the Honey Bee*. Cambridge, MA: Harvard University Press.
Zhou, Q., Liu, L.-Z., Fu, B., Hu, X., Shi, X., Fang, J. and Jiang, B.-H. (2007). Reactive oxygen species regulate insulin-induced VEGF and HIF-1α expression through the activation of p70S6K1 in human prostate cancer cells. *Carcinogenesis* **28**, 28-37.