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ORIGINAL PAPER

Lifetime- and caste-specific changes in flight metabolic rate and muscle biochemistry of honeybees, *Apis mellifera*

Marie-Pierre Schippers · Reuven Dukas · Grant B. McClelland

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Abstract Honeybees, *Apis mellifera*, who show temporal polyethism, begin their adult life performing tasks inside the hive (hive bees) and then switch to foraging when they are about 2–3 weeks old (foragers). Usually hive tasks require little or no flying, whereas foraging involves flying for several hours a day and carrying heavy loads of nectar and pollen. Flight muscles are particularly plastic organs that can respond to use and disuse, and accordingly it would be expected that adjustments in flight muscle metabolism occur throughout a bee's life. We thus investigated changes in lifetime flight metabolic rate and flight muscle biochemistry of differently aged hive bees and of foragers with varying foraging experience. Rapid increases in flight metabolic rates early in life coincided with a switch in troponin T isoforms and increases in flight muscle maximal activities (V_{max}) of the enzymes citrate synthase, cytochrome c oxidase, hexokinase, phosphofructokinase, and pyruvate kinase. However, further increases in flight metabolic rate in experienced foragers occurred without additional changes in the in vitro V_{max} of these flight muscle metabolic enzymes. Estimates of in vivo flux (v) compared to maximum flux of each enzyme in vitro (fractional velocity, v/V_{max}) suggest that most enzymes operate at a higher fraction of V_{max} in mature foragers compared to young hive bees. Our results indicate that honeybees develop most of their flight muscle metabolic machinery early in life. Any further increases in flight metabolism with age or foraging experience are most likely achieved by operating metabolic enzymes closer to their maximal flux capacity.

Keywords Bee · Flight · Insect · Ontogeny · Metabolic enzyme · Metabolic rate · Troponin T

Abbreviations

CS	Citrate synthase
COx	Cytochrome c oxidase
HK	Hexokinase
PFK	Phosphofructokinase
PK	Pyruvate kinase
V_{max}	Enzyme maximum activity
v	In vivo pathway flux
v/V_{max}	Enzyme fractional velocity
V_{CO_2}	Rate of carbon dioxide production

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M.-P. Schippers · G. B. McClelland (✉)
Department of Biology, McMaster University,
1280 Main Street West, Hamilton, ON L8S 4K1, Canada
e-mail: grantm@mcmaster.ca

R. Dukas
Departments of Psychology, Neuroscience and Behaviour,
McMaster University, Hamilton, ON L8S 4K1, Canada

Introduction

Flight muscles are among the most energetically costly organs to power and maintain. They can constitute as much as 65% of body mass and thus represent a substantial energetic investment (Marden 2000). In fact, life history tradeoffs can involve a circumvention of this investment when flight is unnecessary (Zera and Denno 1997; Marden 2000). Indeed, flight muscles are extremely plastic organs that can respond in a flexible manner to environmental factors and to use and disuse. Well-known examples include the considerable increases in flight muscle

metabolic enzyme capacities in migratory birds during migration (Lundgren and Kiessling 1985; Driedzic et al. 1993; Guglielmo et al. 2002) and the rapid muscle generation and degeneration for differential investments in flight or reproduction in flying insects (Zera and Denno 1997; Robertson 1998; Marden 2000).

Muscle plasticity could be particularly important in Honeybees, *Apis mellifera,* who show temporal polyethism (Winston 1987). Honeybees typically spend the first 2–3 weeks of their adult life performing tasks inside the hive (hive bees) and then start foraging for another 1–2 weeks until they die (foragers). Therefore, bees with the same foraging experience can vary in age. Hive bees rarely leave the hive before the onset of foraging, and accordingly, flying activities are minimal. In contrast, foragers can spend over 10 h a day foraging and carrying loads of pollen or nectar nearly as heavy as their own body weight (Winston 1987). Previous research has shown that honeybee foragers increase their foraging performance as they gain foraging experience (Dukas and Visscher 1994; Schippers et al. 2006; Dukas 2008). That is, the rate of nectar and/or pollen brought back to the hive by individual foragers increases with days of foraging experience. One can thus hypothesize that flight muscle machinery is upregulated to enhance glycolytic flux, aerobic capacity, and force output in foragers compared to hive bees, as well as with increasing foraging experience. Our previous study on the physiological basis of this increase in foraging performance revealed increases in a structural component of honeybee flight muscle (Troponin T 10A) in experienced (mature) foragers compared to hive bees. There was also a modest increase in the maximal in vitro capacity (V_{max}) of a mitochondrial enzyme (citrate synthase), but no changes in the V_{max} of key glycolytic enzymes as bees gained foraging experience (Schippers et al. 2006).

In flying insects, increases in ATP turnover rates are accompanied by matching increases in oxygen consumption because ATP supplies involve fully aerobic metabolic pathways (Suarez et al. 1996; Wegener 1996). Honeybees only use carbohydrates (hexoses) to power flight (Rothe and Nachtigall 1989; Blatt and Roces 2001) and any metabolic adaptations affecting flight would act to increase the flux capacity through the glycolytic pathway. Although no changes were previously observed in the activity of glycolytic enzymes in vitro (Schippers et al. 2006), it is possible that in vivo, flux rates through metabolic pathways increase. In fact, honeybee flight muscles are so highly aerobic that they may have reached an upper limit in the amount of metabolic enzymes muscle fibers can contain without affecting myofibrils and therefore power outputs (Pennycuick and Rezende 1984; Suarez et al. 1997, 1999, 2000). Thus, any increases in pathway flux would occur by operating enzymes at a higher fraction of V_{max} in vivo. As a result, an examination of in vivo flux through pathways in relation to the onset of foraging and to foraging experience is warranted.

Little is known regarding changes over the lifetime in flight metabolic rates, especially in relation to foraging experience. Previous research has shown that estimates of whole-animal and mass-specific maximal metabolic rates increase with age (Allen 1959; Harrison 1986; Harrison and Fewell 2002), but it is still unknown whether the same is true with increasing foraging experience which can be independent of a bee's age (Robinson 2002). In addition, little is known about the timing of flight muscle metabolic development in hive bees. Our previous results have shown either small or no increases in V_{max} of mitochondrial and glycolytic enzymes at the onset of foraging and with foraging experience (Schippers et al. 2006), suggesting that most of the muscle metabolic development occurs before the onset of foraging, at some undetermined point in time when bees perform tasks inside the hive.

Flight metabolism is not only a function of the metabolic components of flight muscles but is also a function of its structural machinery. We previously reported an increase in a major structural component in muscle (Troponin T isoform(s) containing alternative exon 10A) in foragers compared to hive bees (Schippers et al. 2006). In insect muscle, troponin T is a major regulatory protein with Ca^{2+} binding properties that generally shows many tissue-specific and developmentally determined isoforms (Domingo et al. 1998; Herranz et al. 2005). The relative abundance of different troponin T transcripts has previously been associated with calcium sensitivity, power output, and rate of energy consumption in flight muscles of other insects (Marden et al. 2001, 2008). In honeybees, the appearance of one troponin T isoform of 46 kDa in bees younger than 5 days old has been associated with the acquisition of flight early in life (Domingo et al. 1998). However, studies on the relative abundance of troponin T isoforms through development in honeybees are scarce (Domingo et al. 1998; Herranz et al. 2005) with knowledge mostly at the mRNA level rather than at the protein level.

In this study, we focused on the lifetime- and caste-specific changes in flight metabolic rate, flight muscle metabolic components, and changes in a structural component of flight muscles. Specifically, the objectives of this study were (1) to estimate the lifetime changes in maximum in vivo fluxes through pathways by measuring whole-animal hovering metabolism, (2) to elucidate the timing of muscle biochemical development prior to the onset of foraging and with foraging experience by measuring in vitro flux capacities (V_{max}) of metabolic enzymes, (3) to estimate lifetime changes in the fractional velocity of enzymes, and (4) to assess lifetime changes in the relative

abundance of flight muscle troponin T isoforms. Overall, our results provide detailed information on lifetime changes in flight metabolism of honeybees in relation to both age and foraging experience.

Materials and methods

Experiments were carried out in two different years in Southern Ontario, Canada from mid-June to mid-July in 2005 and 2006. The 2005 experiment was carried out at the University of Guelph and the 2006 experiment was performed at McMaster University. Both sites offered plenty of nearby flower patches during the time of the experiments. In 2005, we individually tagged 1,000 newly eclosed bees using differently colored number tags and introduced them all at once into a four-frame nucleus containing approximately 8,000 bees. Ten days after introduction, hive entries and exits of individually marked bees were recorded for at least 2 h a day, every day, between 12:00 and 17:00 hours to determine individual bees' foraging experience. Bees that performed trips longer than 5 min and/or carried pollen were considered foragers. We omitted all shorter trips assuming they were orientation trips by bees about to initiate foraging (Dukas and Visscher 1994; Capaldi et al. 2000).

In 2006, we tagged 300 newly eclosed bees and introduced them all at once into a two-frame observation hive containing about 2,000 bees. Twenty-three days after introduction, we monitored the entries and exits of individually marked bees for 1 day from 10:00 to 12:00 hours and from 14:00 to 16:00 hours to identify foraging bees as described above. Bees identified as foragers were collected 3 days later. That is, in the 2006 study, we distinguished between foragers and hive bees but did not record foraging experience.

Flight metabolic rate

Since honeybee flight is exclusively fuelled by carbohydrates (respiratory exchange ratio is 1.0, $V_{CO_2} = V_{O_2}$) (Rothe and Nachtigall 1989), we measured V_{CO_2} as an index of metabolic rate. In 2005, hive bees and foragers of different ages and foraging experiences were collected from the hive and immediately brought to the lab located 10 m from the hive for V_{CO_2} analysis. Hive bees were collected directly from the frames, whereas foragers were captured after returning from a foraging trip. All bees were individually contained in a transparent cylindrical vial and placed in the dark until the V_{CO_2} analysis. Hive bees and foragers were analyzed within 60 and 30 min, respectively, following removal from the hive. All measurements were performed between 9:30 and 13:00 hours.

Individual bees were placed in a 500 ml flow-through respirometry chamber. Rates of CO_2 production were measured using a respirometry system (Sable Systems International Inc., Las Vegas, NV, USA). Ambient air was scrubbed free of CO_2 and water before entering the respirometry chamber at a rate of 350–500 ml/min set by a calibrated Subsampler/Pump/Mass flow meter unit. Carbon dioxide concentrations were determined using a CO_2 analyzer, a data acquisition interface, and Expedata software. Bees were stimulated to fly by placing the respirometry chamber next to an open window and by agitating the chamber to induce flight when bees landed. We only used data from bees that exhibited sustained flight (voluntarily or agitated) for at least 1 min. Since a limited number of bees were selected, we combined bees into the following life stage categories: hive bees: 1 day old, 2 days old, 3–4 days old, 8–12 days old; foragers: 1 day of foraging experience, 4–5 days of foraging experience, 8–10 days of foraging experience. Body weights (mean ± SEM) fluctuated across life stages, although not significantly (ANOVA; $F_{6,89} = 2.0$, $P = 0.07$): 1 day old: 115 ± 6 mg, 2 days old: 119 ± 4 mg, 3–4 days old: 142 ± 6 mg, 8–12 days old: 128 ± 4 mg, 1 day of foraging experience: 120 ± 6 mg, 4–5 days of foraging experience: 112 ± 6 mg, 8–10 days of foraging experience: 126 ± 6 mg. The average daily outdoor temperature (± SEM) at 11:00 on days that we performed the V_{CO_2} analyses was 25.4 ± 1.2°C, and mean flight metabolic rates for each day were not significantly correlated with daily temperatures (ANOVA regression; $F_{1,5} = 2.6$, $P = 0.17$).

Flight metabolic rates were also measured in 2006 in bees of different ages; however, a technical problem with the V_{CO_2} analyzer throughout the 2006 experiment prevented us from considering the data as reliable. We thus do not report these data even though a trend consistent with the 2005 data was observed.

Flight muscle enzyme activity

In both experiments (2005 and 2006), hive bees and foragers of different ages and foraging experiences collected from the hive were anesthetized on ice. We then removed their thoraxes and immediately placed them in liquid nitrogen. Subsequently, all thoraxes were stored at −80°C and later homogenized as described previously (Schippers et al. 2006). Briefly, whole thoraxes were powdered using a liquid N_2-cooled mortar and pestle and homogenized on ice using a glass on glass homogenizer for 1 min in 20 volumes of extraction buffer consisting of 75 mM potassium phosphate (pH 7.3) and 10 mg/ml Lubrol® (Suarez et al. 1996). All enzymes were measured at 37°C in a Spectromax Plus 384, 96-well microplate reader (Molecular Devices, Sunnyvale, CA, USA). Assays were performed in

triplicate and control rates without substrate were determined for each assay.

Enzyme activities of cytochrome c oxidase (COx), phosphofructokinase (PFK), and hexokinase (HK) were measured on fresh homogenates. Enzyme activities of pyruvate kinase (PK) and citrate synthase (CS) were measured after having been frozen and thawed once and three times, respectively. Nine or ten thoraxes were used for each life stage. In 2005, we assessed flight muscle enzyme activity in the following life stages (mean ± SEM of thorax weights in mg are in parentheses, $N = 9$–10 per life stage): hive bees: 1 day old (27.5 ± 0.3), 2 days old (27.4 ± 0.3), 3 days old (30.3 ± 0.5), 10 days old (28.6 ± 0.6); foragers: 1 day of foraging experience (28.2 ± 0.4), 4–5 days of foraging experience (28.8 ± 0.6) and 8–10 days of foraging experience (30.3 ± 0.9). In 2006, we focused primarily on hive bees and assessed flight muscle enzyme activity in the following life stages (mean ± SEM of thorax weights in parentheses, $N = 10$ per life stage): hive bees: 1 day old (29.9 ± 0.5), 2 days old (29.4 ± 0.5), 3 days old (31.3 ± 0.7), 8 days old (31.3 ± 0.5), 12 days old (32.3 ± 0.3); foragers (unknown foraging experience): 26 days old (30.8 ± 0.3). Assay conditions were as described previously (Schippers et al. 2006). Briefly, COx 50 mmol l^{-1} potassium phosphate (pH 7.5), 50 μmol l^{-1} cytochrome c, PFK 10 mmol l^{-1} fructose-6-phosphate (F6P) (omitted in control), 1 mmol l^{-1} ATP, 0.15 mmol l^{-1} NADH, 2 mmol l^{-1} AMP, 10 mmol l^{-1} $MgCl_2$, 100 mmol l^{-1} KCl, 5 mmol l^{-1} dithiothreitol (DTT), 1 U aldolase, 5 U triose phosphate isomerase and 5 U α-glycerophosphate dehydrogenase, in 50 mmol l^{-1} imidazole (pH 7.4); HK 5 mmol l^{-1} D-glucose (omitted in control), 4 mmol l^{-1} ATP, 10 mmol l^{-1} $MgCl_2$, 100 mmol l^{-1} KCl, 0.5 mmol l^{-1} NADP, 5 mmol l^{-1} DTT, 1 U glucose-6-phosphate dehydrogenase, in 50 mmol l^{-1} HEPES (pH 7.0); PK 5 mmol l^{-1} phosphoenol pyruvate (omitted in control), 5 mmol l^{-1} ADP, 2.5 mmol l^{-1} $MgCl_2$, 0.15 mmol l^{-1} NADH, 10 mmol l^{-1} fructose-1,6-phosphate, 100 mmol l^{-1} KCl, 9.25 U lactate dehydrogenase, in 50 mmol l^{-1} imidazole (pH 7.4); CS 0.5 mmol l^{-1} oxaloacetate (omitted in control), 0.09 mmol l^{-1} acetyl-CoA, and 0.1 mmol l^{-1} dithiobisnitrobenzoic acid in 20 mmol l^{-1} Tris (pH 8.0).

Fractional velocity of enzymes

Fractional velocity of enzymes was estimated only in the 2005 experiment in bees from which both hovering flight metabolic rates and flight muscle enzyme activities were measured. Life stages are as follows: hive bees: 3–4 days old; foragers: 1 day of foraging experience, 4–6 days of foraging experience and 8–10 days of foraging experience. Briefly, fractional velocity of enzymes were calculated by dividing the maximal enzyme activity (V_{max}) measured in vitro by the in vivo flux rate of metabolic pathways, multiplied by 100 (Suarez et al. 1996). The in vivo flux rates of pathways were determined using CO_2 production rates converted into μmol min^{-1} g^{-1} of glycolytic flux rate, Krebs cycle rate, and electron transport chain rate as described in Suarez et al. (1996).

Protein identification and abundance of protein bands

Frozen thoraces from the 2005 experiment were homogenized using a motorized homogenizer and myofibrillar isolation was performed as described previously (Churcott et al. 1994) with the following modifications: (1) a protease inhibitor cocktail (Complete Mini, Roche Diagnostics, Indianapolis, IN, USA) was added to all solutions instead of PMSF, (2) thoraces were homogenized in 500 μl of solution A (25 mmol l^{-1} imidazole, 25 mmol l^{-1} KCl, 5 mmol l^{-1} EDTA, 1 mmol l^{-1} DTT, protease inhibitor cocktail) and (3) samples were incubated for 10 min on ice in solution B (50 mmol l^{-1} imidazole, 25 mmol l^{-1} KCl, 1 mmol l^{-1} EGTA, 22 μmol l^{-1} $CaCl_2$, 0.5% Triton X-100, 1 mmol l^{-1} DTT, protease inhibitor cocktail). Protein concentration was determined with the bicinchoninic acid assay (Thermo Scientific, Rockford, IL, USA) and samples were diluted to 5 μg of protein in 20 μl of solution D (50 mmol l^{-1} imidazole, 100 mmol l^{-1} KCl, protease inhibitor cocktail) and 5 μl of loading buffer (48 mmol l^{-1} Tris–HCl pH 6.8, 4% glycerol, 3.2% SDS, 600 mmol l^{-1} 2-mercaptoethanol, 1.6% bromophenol blue). Samples were then denatured by boiling for 5 min, loaded onto a 12% SDS-polyacrylamide gel (Bio-Rad), and electrophoresed for ~ 5 h and 30 min at 85 V. Gels were then fixed in 40% methanol and 10% acetic acid for 15 min, stained with SYPRO-ruby stain (Invitrogen, Eugene, OR, USA) overnight, and washed twice in 10% methanol, 7% acetic acid for 1 h. Two protein bands of apparent molecular mass within the range of Troponin T isoforms (~ 50 kDa) and one protein band within the range of actin (~ 42 kDa) were manually cut from one gel and subjected to in-gel tryptic digestion and nano electrospray quadropole time of flight mass spectroscopy analysis (nanoES Q-TOF) as in Schippers et al. (2006). Imaging of gels was carried out using the Perkin Elmer Pro-Express gel imaging system and quantification of protein bands were determined with Phoretix 2DTM software (Nonlinear Dynamics), which combines both pixel density and area of band to quantify band volume. Volume values of each band were normalized to actin to control for any loading differences.

Statistical analyses

Changes in hovering flight metabolic rates and V_{max} of each enzyme were determined by performing a one-way

analysis of variance (ANOVA; SPSS version 12.0, SPSS Inc., Chicago, IL, USA) followed by post hoc analyses using either Hochberg's GT2 test when homogeneity of variance was assumed (Levene's test, $P > 0.07$) or Games–Howell test when variances were expected to be unequal (Levene's test, $P < 0.07$). Changes in fractional velocity of each enzyme were also analyzed using a one-way ANOVA and post hoc analyses were performed using either the Tukey test (equal variances) or the Dunnet T3 test (unequal variances). Troponin T isoforms abundance was analyzed with the non-parametric Kruskal–Wallis test and post hoc Mann–Whitney *U* tests with Bonferroni correction.

Results

Flight metabolic rate

Generally, whole-body and mass-specific flight metabolic rates showed similar trends (Fig. 1). Flight metabolic rates first increased relatively early in life, and were then further enhanced when bees became foragers. A significant 68% increase in whole-body metabolic rates occurred by 3–4 days of age (Games–Howell; $P = 0.001$). Subsequently, little changes were observed in hive bees as there were no significant differences between 3 to 4-day-old and 8 to 12-day-old hive bees. The onset of foraging was associated with enhanced flight metabolic rates but no significant changes were found between 3 to 4-day-old hive bees and first-day foragers (Games–Howell; $P > 0.9$). On the other hand, foragers generally had higher flight metabolic rates than hive bees. Foragers with 4–5 days of foraging experience (mature foragers) achieved significantly higher mass-specific metabolic rates than hive bees of any age (Games–Howell; $P \leq 0.041$). However, only non-significant increases were observed in whole-body metabolic rates between mature foragers and 3 to 4-day-old hive bees (Games–Howell; $P = 0.4$). In addition, no significant changes were found among foragers of differing foraging experience even though hovering metabolic rates were enhanced in the first 5 days of foraging.

Flight muscle enzyme activity

In both 2005 and 2006, the V_{max} of all enzymes (COX, CS, PK, PFK and HK) increased early in life (Figs. 2, 3). However, little changes were observed either during the transition from hive bees to foragers or with increased foraging experience (Fig. 2). In 2005, the V_{max} of all enzymes except PK significantly increased between 1-day-old and 3-day-old hive bees (Hochberg's GT2 post hoc test; $P \leq 0.004$). More specifically, 58, 55, 91, and 43% of the total lifetime increases in COx, CS, PFK, and HK V_{max},

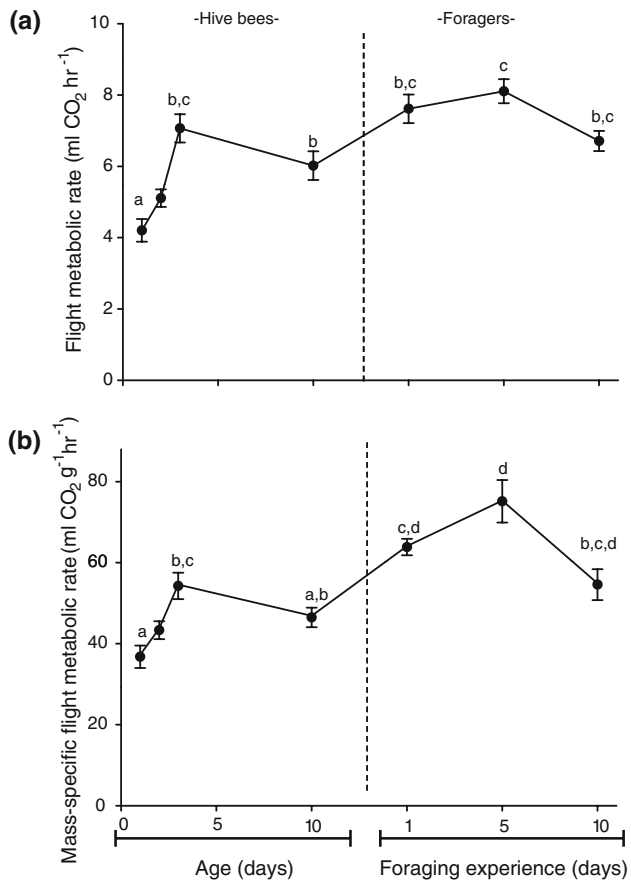

Fig. 1 Hovering metabolic rates across lifespan and foraging experience (mean ± SEM) expressed as **a** whole-body CO_2 production rates and **b** mass-specific CO_2 production rates in honeybees collected in 2005. $N = 9$–25 bees for each life stage. *Different letters* show significant pair-wise differences (Hochberg's GT2 post hoc test; $P < 0.05$)

respectively, occurred by the age of 3 days. As well, most of the increase in PK V_{max} (66% of total increase) occurred by 10 days of age (Hochberg's GT2; $P = 0.001$). Thus, for all enzymes measured in 2005, most of the increase in enzyme flux capacity occurred early in life, in hive bees younger than 10 days old, and similar results were found for all enzymes measured in our 2006 experiment. In 2006, the V_{max} of PFK and HK also significantly increased between day 1 and day 3 (Games–Howell and Hochberg's GT2, respectively; $P < 0.001$) and all other enzymes significantly increased by day 8 (between day 1 and day 8; Hochberg's GT2; $P \leq 0.007$). In 2005, the increases in PK and HK V_{max} occurred gradually between 3-day-old hive bees and first-day foragers (Hochberg's GT2; $P = 0.002$ and $P = 0.004$, respectively). Conversely, COx V_{max} significantly decreased between 10 day-old hive bees and first-day foragers (Hochberg's GT2; $P = 0.015$). No significant changes in V_{max} in any of the other enzymes (CS, PK, PFK, HK) were observed during the transition from hive bees to foragers (between 10 days old bees and

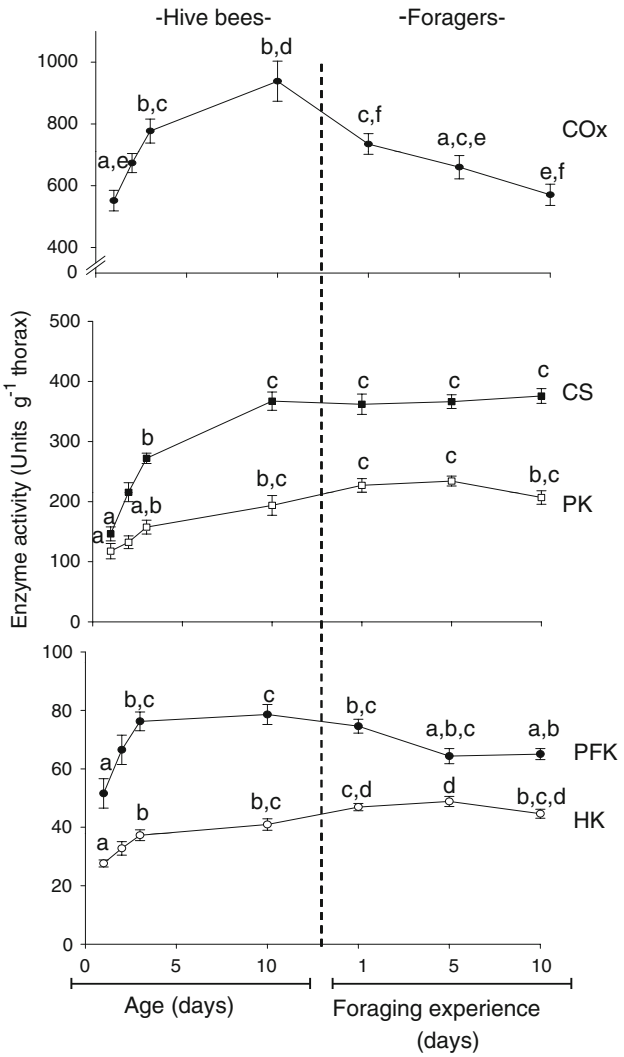

Fig. 2 Activities of metabolic enzymes ($V_{max} \pm$ SEM) across life span and foraging experience in honeybee thoraxes collected in 2005. *COx* cytochrome c oxidase, *CS* citrate synthase, *PK* pyruvate kinase, *HK* hexokinase, *PFK* phosphofructokinase. $N = 9$–10 thoraxes for each life stage. *Different letters* show significant pair-wise differences (Hochberg's GT2 or Games–Howell post hoc test; $P < 0.05$)

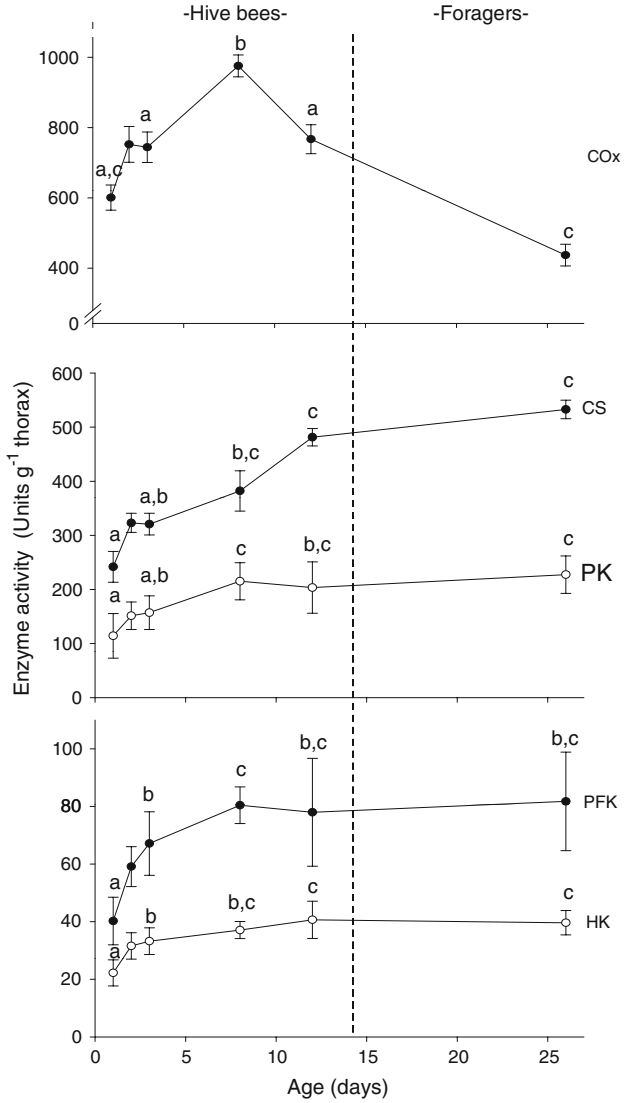

Fig. 3 Activities of metabolic enzymes ($V_{max} \pm$ SEM) across life span and foraging experience in honeybee thoraxes collected in 2006. *COx* cytochrome c oxidase, *CS* citrate synthase, *PK* pyruvate kinase, *HK* hexokinase, *PFK* phosphofructokinase. $N = 10$ thoraxes for each life stage. *Different letters* show significant pair-wise differences (Hochberg's GT2 or Games–Howell post hoc tests; $P < 0.05$)

first-day foragers; Hochberg's GT2; $P \geq 0.29$), and no significant changes in V_{max} of any enzyme were observed with increased foraging experience (between 1 day of foraging experience and 4–5 days of foraging experience; Hochberg's GT2; $P \geq 0.6$). In the 2006 experiment, no significant changes in V_{max} of all enzymes except COx were found after 8 days of age. COx V_{max} significantly decreased between 8 and 12 days of age (Hochberg's GT2; $P = 0.007$) and then further decreased in 25-day-old foraging bees (Hochberg's GT2; $P < 0.001$).

Fractional velocity of enzymes

Generally, the fractional velocity of most enzymes slightly increased in life until reaching a peak in foragers of 4–6 days of foraging experience, and then decreased in more experienced foragers (8–10 days of foraging experience) (Fig. 4). Fractional velocity of COx and PFK significantly increased by 43 and 32%, respectively, between 3 to 4-day-old hive bees and foragers of 4–6 days of foraging experience (Tukey; $P < 0.02$). Conversely, the fractional velocity of PK significantly decreased between 3 to 4-day-old hive bees and first-day foragers (Dunnet T3; $P = 0.046$). Significant decreases in fractional velocity of PFK and CS were also observed at the end of a bee's life, in foragers with 8–10 days of foraging experience compared to foragers with 4–6 days of foraging experience (Tukey; $P \leq 0.03$). On the other hand, no changes in the

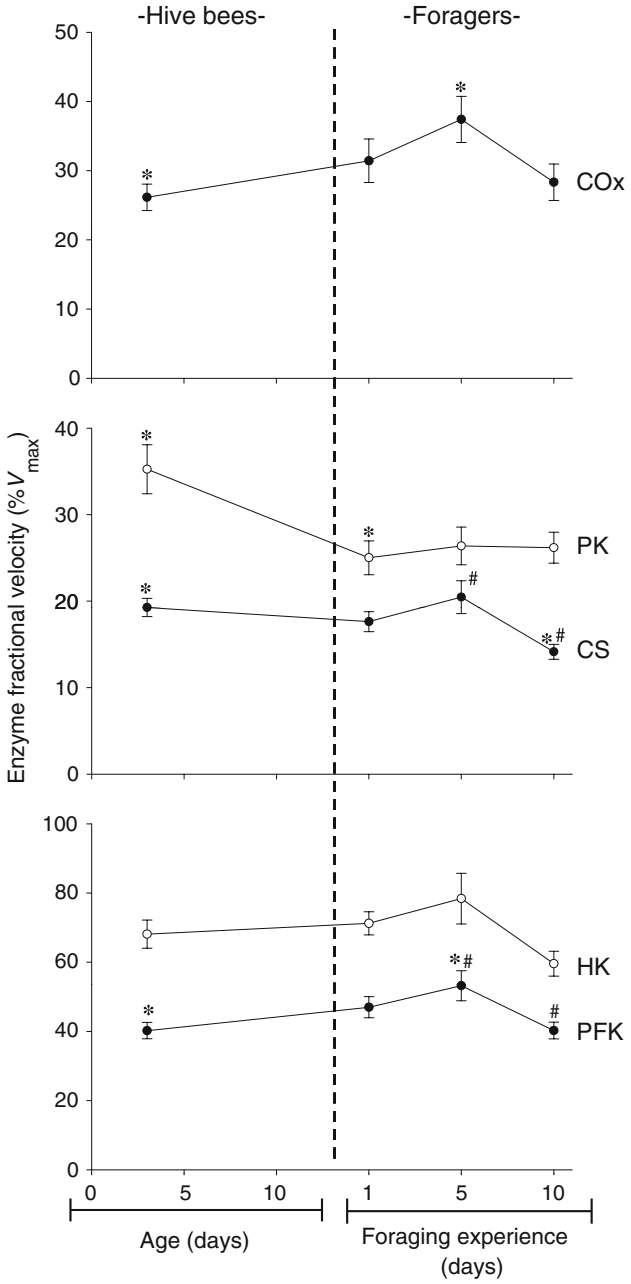

Fig. 4 Fractional velocities of enzymes in honeybee thoraxes across lifespan and foraging experience. *COx* cytochrome c oxidase, *CS* citrate synthase, *PK* pyruvate kinase, *HK* hexokinase, *PFK* phosphofructokinase. $N = 8$–13 bees per life stage. *Asterisks* or *octothorpes* indicate significant pair-wise differences (Tukey or Dunnet T3 post hoc tests; $P < 0.05$)

lifetime fractional velocity of HK were found (ANOVA; $F_{3,35} = 2.4$, $P = 0.08$).

Troponin T isoform abundance

Typical 1-D gels showing the myofibrillar fraction of thorax samples from different lifestages are presented in

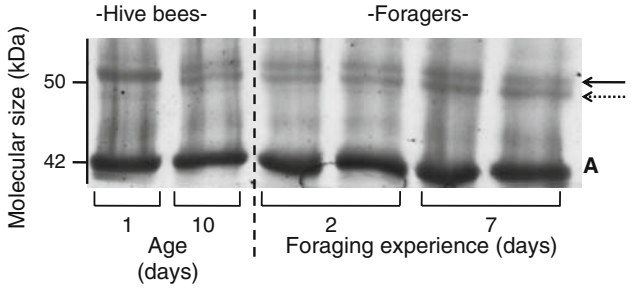

Fig. 5 Representative 1D electrophoresis protein gel from the myofibrillar fraction of honeybee thoraces. The *dotted arrow* indicates the 48-kDa Troponin T bands and the *solid arrow* indicates the 52-kDa Troponin T bands. Actin *bands* are indicated by the letter "*A*." Molecular sizes shown on gel are approximate

Fig. 5. NanoES Q-TOF mass spectroscopy identified one protein band (apparent molecular weight of 42 kDa) as actin and two protein bands (apparent molecular weights of 48 and 52 kDa) as troponin T (isoform identity could not be determined). Detailed results of the identification analysis are presented in Table 1. Both the 48 and 52 kDa protein bands were contaminated with actin: NCBI accession no. gil4220623, MOWSE scores = 105 (50) and 37 (34), respectively (minimum scores for significant homology shown in parentheses, $P < 0.05$); and there were some evidence for the 52-kDa protein band to be contaminated with ATP synthase subunit alpha, mitochondrial (NCBI accession no. gil5921205), but the MOWSE score was barely significant: MOWSE score = 40 (39).

Bee thoraces of all lifestages contained both troponin T bands except for 1-day-old bees, which lacked the 48-kDa troponin T band. This lower molecular weight troponin T band appeared at 3 days of age; however, no significant changes in intensity were observed between lifestages (Mann–Whitney *U* with Bonferroni correction: $P \geq 0.07$). Likewise, no significant changes in intensity between lifestages were found in the 52-kDa troponin T band (Mann–Whitney *U* with Bonferroni correction: $P \geq 0.1$; Table 2).

Discussion

This study attempted to elucidate ontogenic changes in flight metabolism and muscle biochemistry of honeybees, taking into account both caste transition (hive worker to forager) and days of foraging experience. Honeybees' flight metabolic rates were rapidly enhanced early in life (by 3 days of age). This increase could be explained by the concurrent increases in flight muscle in vitro metabolic flux capacities, as most of the increases in enzymatic capacity of several aerobic and regulatory glycolytic enzymes also occurred by 3 days of age. In addition, changes in structural components of flight muscles were also observed at

Table 1 Identification results of protein bands by nanoES Q-TOF mass spectroscopy

Protein band apparent M_r	Protein description	NCBI accession no.	Peptide sequence	M_r (exp.)	M_r (calc.)	MOWSE score[a]
42 kDa	Actin (*Apis mellifera*)	gil4220623	SYELPDGQVITIGNER	1789.79	1789.88	79 (43)
		gil66509789	GYSFTTTAER	1131.39	1131.52	49 (46)
		gil66509789	QEYDESGPGIVHR	1485.58	1485.68	62 (44)
		gil66509789	VAPEEHPVLLTEAPLNPK	1952.99	1953.06	44 (37)
48 kDa	Troponin T (*Apis mellifera*)	gil94400893	DLAGNLTSAQLER	1386.68	1386.71	78 (51)
52 kDa	Tropornin T (*Apis mellifera*)	gil94400893	DLAGNLTSAQLER	1386.59	1386.71	87 (51)

M_r refers to apparent molecular mass on gel, see Fig. 5, M_r *(exp.)* experimentally determined molecular mass and M_r *(calc.)* calculated molecular mass for the peptide sequence

[a] Values in parentheses indicate MOWSE score required for significant homology ($P < 0.05$)

Table 2 Mean ± SEM of troponin T bands intensities expressed as % of actin intensity of honeybee thoraces at different ages and foraging experience

Troponin T bands	Age (days)			Foraging experience (days)	
	1	3	10	2	7–9
48 kDa band intensities (% actin)	n.d.	16.0 ± 2.2	11.4 ± 2.7	6.8 ± 1.4	9.2 ± 1.4
52 kDa band intensities (% actin)	15.8 ± 3.0	2.9 ± 0.6	3.8 ± 1.2	4.6 ± 1.6	4.8 ± 1.9

n.d. Non-detectable

the same time. A troponin T isoform, not present in 1-day-old bees first appeared by the age of 3 days. Hive bees' flight metabolic rates, and thus estimates of flux through glycolysis and oxidative phosphorylation, remained unchanged thereafter up until the onset of foraging, where it gradually increased as bees gained foraging experience until reaching a peak in mature foragers (4–5 days of foraging experience). These increases could not be adequately explained by changes in flight muscle metabolic capacity, as the V_{max} of all enzymes measured either remained unchanged or decreased (COx) with foraging experience. In fact, we found that the fractional velocity (v/V_{max}) of most enzymes was higher in mature foragers compared to hive bees and significantly so for COx and PFK. Thus, foragers seem to operate most of their metabolic enzymes at a higher fraction of their enzymatic capacity (V_{max}) to support elevated flight metabolic rates. Moreover, since our measurements of in vivo flux represent underestimates of true maximum rates, we assume that foragers in the field who undertake forward flight and carry heavy loads of nectar or pollen operate at a much higher fraction of their V_{max} than reported here and thus in vivo flux rates approach in vitro enzymatic capacities.

Flight metabolic rates

Knowledge on maximum metabolic rates in hive bees younger than 4 days of age is scarce (Harrison and Fewell 2002; Stabentheiner et al. 2003) probably because, at this age, bees are not able to sustain flight for longer than a few seconds. It is important to note that to get a measure of flight metabolic rate, we forced bees to sustain flight by agitating the metabolic chamber every time they landed. In bees younger than 4 days, the metabolic chamber was almost constantly agitated because young bees were not able to hover for longer than a few seconds. Previous research (Suarez et al. 1999) has shown that metabolic rates achieved during agitated flight are significantly higher than those reached while hovering. We thus believe that our values of agitated flight for these bees represent reasonable estimates of flight metabolic rate at this age. On the other hand, hovering flight metabolic rates in bees older than 4 days of age are surely underestimates of maximal metabolic rates. For instance, heavy loads of forage are known to increase flight metabolic rates (Wolf et al. 1989; Feuerbacher et al. 2003). In fact, bees carrying extra loads during free directional forward flight in a wind tunnel yielded up to 40% higher metabolic rates than unladen bees (Wolf et al. 1989). True maximal metabolic rates are difficult to determine in insects and have not been determined previously for honeybees. However, measurements of hovering flight metabolic rate of carpenter bees in low air densities (mixture of O_2, N_2 and He) were found to be about 30% higher than in normal air (Roberts et al. 2004). In the present study, bees were partially laden (20–40% heavier than unladen state) and mass-specific V_{CO_2} values

of mature foragers were approximately 6 and 15–25% lower than those reported in previous studies for partially laden and unladen foragers, respectively (Harrison 1986; Coelho and Mitton 1988; Wolf et al. 1989; Suarez et al. 1996; Roberts and Harrison 1999; Feuerbacher et al. 2003; Woods et al. 2005). Thus, flight metabolic rates reported here are evidently lower than the true maximum metabolic rate that can be achieved by honeybee foragers. Yet, significantly higher metabolic rates were still observed in foragers compared to hive bees. If we assume, as previous studies have (Suarez et al. 1996, 1997), that maximal metabolic rates in foragers are at least 30% higher than the ones measured, we would expect even greater differences in flight V_{CO_2}, and hence in the calculated in vivo flux, between hive bees and foragers.

Flight muscle metabolic enzymes

Since honeybee flight is obligatory aerobic and is fueled entirely by carbohydrates (hexoses), we measured three enzymes (PK, PFK, HK) important for determining the overall flux of glycolysis (Hochachka and Somero 2002), an aerobic enzyme from the Krebs cycle (CS) and from the electron transport chain (COx). Although V_{max} represents the maximum flux that can be achieved at a particular step of a biochemical pathway in vitro, it can also provide insights into flux capacity in vivo (Newsholme and Crabtree 1986; Suarez et al. 1996, 1997). V_{max} is a function of enzyme concentration and catalytic efficiency (k_{cat}), and since k_{cat} is generally constant (Suarez et al. 2000), V_{max} corresponds to the functional concentration of enzymes. Most of the increase in activity of all enzymes occurred early in life and then either remained unchanged or gradually increased up until reaching a peak before or at the onset of foraging. Honeybees thus acquire most of their flight muscle metabolic machinery well before undertaking foraging activities. A previous study reported a rapid increase in the V_{max} of PK and CS in the first 4 days and no further increases beyond 5 days of age (Harrison 1986). Similarly, our prior study (Schippers et al. 2006) showed either modest (CS) or no significant changes (HK, PFK, PK, COx) in V_{max} between 11- and 15-day-old hive bees and foragers (various ages). The rapid increases in glycolytic flux and oxidative capacity of flight muscles early in life coincided with the increases in metabolic rates and the appearance of flight ability, which occurs around 2–4 days of age (Harrison and Fewell 2002; Roberts and Elekonich 2005). However, when bees start foraging and gain foraging experience, they elevate fluxes through glycolytic (as indexed by hovering metabolic rate) and aerobic pathways without increasing the V_{max} of enzymes. Flying honeybees are known to attain one of the highest mass-specific metabolic rates ever measured in the animal kingdom and these high in vivo flux rates are thought to be achieved not only by increasing enzyme content but also by operating enzymes at higher fractional velocities than other animals (Suarez et al. 2000). Despite the fact that our measure of in vivo flux is an underestimate of true maximal flux rates achievable, we can still conclude that COx, PFK, and HK operate at a higher percentage of V_{max} in mature foragers compared to hive bees (although not significantly so for HK). Yet, these fractional velocity values agree with the ones previously reported in honeybee workers (Suarez et al. 1996). We can expect that honeybees carrying extra loads (pollen or nectar), which lead to up to 40% higher metabolic rates (Wolf et al. 1989), operate their enzymes even closer to capacity (for example, HK might be expected to operate at almost 100% of V_{max}). It is possible that honeybees have reached close to an upper limit in the biochemical capacity of flight muscles (Suarez 2000; Suarez et al. 1996). Honeybee flight muscles are highly aerobic: mitochondria account for approximately 43% of fiber volume whereas myofibrils occupy roughly 54% (Suarez et al. 2000). Further increases in aerobic enzyme content to accommodate high flux rates would require greater mitochondrial densities, which would come at the detriment of myofibrils and consequently power output (Suarez 2000; Suarez et al. 1997, 2000). Moreover, since myofibrils and mitochondria account for almost all of the cytosolic space, glycolytic enzymes and glycolytic intermediates are packed in the same extramitochondrial space as myofibrils, which may lead to specific interactions that affect the regulation of glycolysis (Suarez 2000).

While higher in vivo flux rates in foragers can be explained by either elevated or stable enzymatic capacities (with high fractional velocities), a decrease in V_{max}, on the other hand, seems counterintuitive. Unlike the other enzymes investigated in this study, COx V_{max} dramatically decreased in foragers in both the 2005 and 2006 experiments. Moreover, our previous study conducted in 2004 showed similar results (Schippers et al. 2006). Highly oxidative muscles must deal with an elevated production of reactive oxygen species (ROS), usually by increasing antioxidant defense to prevent oxidative damage (Powers et al. 1999). In fact, earlier proteomic analyses revealed that honeybee foragers have increased antioxidant defenses compared to hive bees (Schippers et al. 2006; Wolschin and Amdam 2007). However, this increase may not be sufficient to cope with the high production of ROS during foraging flights. It is possible that the extremely high aerobic demands of flight and the associated spatial constraints to further increase antioxidant enzymes may prevent adequate antioxidant defense in honeybee foragers. This supports the notion that senescence and ROS damage may not correlate with chronological age in honeybees (Münch et al. 2008). The decrease in COx V_{max} could thus

be due to oxidative damage. Several studies have documented the vulnerability of complex IV/COx to oxidative stress (Benzi et al. 1991; Schwarze et al. 1998; Ferguson et al. 2005) and experimental increases of H_2O_2 in *Drosophila melanogaster* have been found to decrease COx activity (Schwarze et al. 1998). Moreover, age-related declines in COx activities have been found in the absence of changes in COx I protein abundance, suggesting that the decrease in COx activity is not the result of protein abundance but of oxidative inactivation (Ferguson et al. 2005).

Flight muscle structural components

Honeybees contain several troponin T isoforms, some of which are tissue specific and/or developmentally determined (Domingo et al. 1998; Herranz et al. 2005). Previous findings indicate several troponin T transcripts in honeybee thorax, with one predominantly found in indirect flight muscles: exon-10A containing transcript lacking all 5′ region alternatively spliced exons (Herranz et al. 2005). We previously found an increase in troponin T isoform(s) containing exon 10A (troponin T 10A) in mature foragers compared to 11–15 days old hive bees (Schippers et al. 2006). In the present study, we could not adequately quantify specific isoforms. However, we report here a shift in the relative abundance of troponin T isoforms by 3 days of age. This shift is consistent with the findings of a previous study (Domingo et al. 1998) and occurs concurrently with the metabolic development of muscles and flight capability observed here and elsewhere (Harrison 1986; Harrison and Fewell 2002; Schippers et al. 2006). In dragonflies, variations in the relative abundance of troponin T transcripts have been correlated with calcium sensitivity and maximum specific power output of muscles (Marden et al. 2001). Hence these data suggest a possible role for the differential abundance of troponin T isoforms in affecting honeybee flight. Further studies on age-related variation in troponin T isoforms and its functional role in flight muscles are needed.

Conclusion

This study demonstrates lifetime changes in honeybee flight metabolism and flight muscle biochemistry that are both segregated by age and foraging experience. Our results suggest that most of the flight muscle metabolic development occurs within a few days after eclosion, which coincides with the appearance of sustained flight capability. No further increases in metabolic enzyme capacities were observed at the onset of foraging or with increased foraging experience even though mature foragers showed significantly higher in vivo metabolic flux rates. These findings reinforce current concepts on the upper limits of biochemical capacities of hymenopteran flight muscles and suggest that honeybee foragers sustain high flight metabolism by operating enzymes in vivo closer to capacity (V_{max}).

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References

Allen MD (1959) Respiration rates of worker honeybees of different ages and at different temperatures. J Exp Biol 36:92–101

Benzi G, Curti D, Pastoris O, Marzatico F, Villa RF, Dagani F (1991) Sequential damage in mitochondrial complexes by peroxidative stress. Neurochem Res 16:1295–1302

Blatt J, Roces F (2001) Haemolymph sugar levels in foraging honeybees (*Apis mellifera carnica*): dependence on metabolic rate and in vivo measurement of maximal rates of trehalose synthesis. J Exp Biol 204:2709–2716

Capaldi EA, Smith AD, Osborne JL, Fahrbach SE, Farris SM, Reynolds DR, Edwards AS, Martin A, Robinson GE, Poppy GM, Riley JR (2000) Ontogeny of orientation flight in the honeybee revealed by harmonic radar. Nature 403:537–540

Churcott CS, Moyes CD, Bressler BH, Baldwin KM, Tibbits GF (1994) Temperature and Ph effects on Ca2 + sensitivity of cardiac myofibrils—a comparison of trout with mammals. Am J Physiol 267:R62–R70

Coelho JR, Mitton JB (1988) Oxygen consumption during hovering is associated with genetic variation of enzymes in honey-bees. Funct Ecol 2:141–146

Domingo A, Gonzalez-Jurado J, Maroto M, Diaz C, Vinos J, Carrasco C, Cervera M, Marco R (1998) Troponin-T is a calcium-binding protein in insect muscle: in vivo phosphorylation, muscle-specific isoforms and developmental profile in *Drosophila melanogaster*. J Muscle Res Cell Motil 19:393–403

Driedzic WR, Crowe HL, Hicklin PW, Sephton DH (1993) Adaptations in pectoralis-muscle, heart mass, and energy-metabolism during premigratory fattening in semipalmated sandpipers (*Calidris pusilla*). Can J Zool 71:1602–1608

Dukas R (2008) Life history of learning: performance curves of honeybees in the wild. Ethology 114:1195–1200

Dukas R, Visscher PK (1994) Lifetime learning by foraging honeybees. Anim Behav 48:1007–1012

Ferguson M, Mockett RJ, Shen Y, Orr WC, Sohal RS (2005) Age-associated decline in mitochondrial respiration and electron transport in *Drosophila melanogaster*. Biochem J 390:501–511

Feuerbacher E, Fewell JH, Roberts SP, Smith EF, Harrison JF (2003) Effects of load type (pollen or nectar) and load mass on hovering metabolic rate and mechanical power output in the honey bee *Apis mellifera*. J Exp Biol 206:1855–1865

Guglielmo CG, Haunerland NH, Hochachka PW, Williams TD (2002) Seasonal dynamics of flight muscle fatty acid binding protein

and catabolic enzymes in a migratory shorebird. Am J Physiol Regul Integr Comp Physiol 282:R1405–R1413

Harrison JM (1986) Caste-specific changes in honeybee flight capacity. Physiol Zool 59:175–187

Harrison JF, Fewell JH (2002) Environmental and genetic influences on flight metabolic rate in the honey bee, *Apis mellifera*. Comp Biochem Physiol A Mol Integr Physiol 133:323–333

Herranz R, Mateos J, Mas JA, Garcia-Zaragoza E, Cervera M, Marco R (2005) The coevolution of insect muscle TpnT and TpnI gene isoforms. Mol Biol Evol 22:2231–2242

Hochachka PW, Somero GN (2002) Biochemical adaptation: mechanism and process in physiological evolution. Oxford University Press, New York

Lundgren BO, Kiessling KH (1985) Seasonal-variation in catabolic enzyme-activities in breast muscle of some migratory birds. Oecologia 66:468–471

Marden JH (2000) Variability in the size, composition, and function of insect flight muscles. Annu Rev Physiol 62:157–178

Marden JH, Fitzhugh GH, Girgenrath M, Wolf MR, Girgenrath S (2001) Alternative splicing, muscle contraction and intraspecific variation: associations between troponin T transcripts, Ca2 + sensitivity and the force and power output of dragonfly flight muscles during oscillatory contraction. J Exp Biol 204:3457–3470

Marden JH, Fescemyer HW, Saastamoinen M, MacFarland SP, Vera JC, Frilander MJ, Hanski I (2008) Weight and nutrition affect pre-mRNA splicing of a muscle gene associated with performance, energetics and life history. J Exp Biol 211:3653–3660

Münch D, Amdam GV, Wolschin F (2008) Ageing in a eusocial insect: molecular and physiological characteristics of life span plasticity in the honey bee. Funct Ecol 22:407–421

Newsholme EA, Crabtree B (1986) Maximum catalytic activity of some key enzymes in provision of physiologically useful Information about metabolic fluxes. J Exp Zool 239:159–167

Pennycuick CJ, Rezende MA (1984) The specific power output of aerobic muscle, related to the power-density of mitochondria. J Exp Biol 108:377–392

Powers SK, Ji LL, Leeuwenburgh C (1999) Exercise training-induced alterations in skeletal muscle antioxidant capacity: a brief review. Med Sci Sports Exerc 31:987–997

Roberts SP, Elekonich MA (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 SP, Harrison JF (1999) Mechanisms of thermal stability during flight in the honeybee *Apis mellifera*. J Exp Biol 202:1523–1533

Roberts SP, Harrison JF, Dudley R (2004) Allometry of kinematics and energetics in carpenter bees (*Xylocopa varipuncta*) hovering in variable-density gases. J Exp Biol 207:993–1004

Robertson IC (1998) Flight muscle changes in male pine engraver beetles during reproduction: the effects of body size, mating status and breeding failure. Physiol Entomol 23:75–80

Robinson GE (2002) Genomics and integrative analyses of division of labor in honeybee colonies. Am Nat 160:S160–S172

Rothe U, Nachtigall W (1989) Flight of the honey bee.4. Respiratory quotients and metabolic rates during sitting, walking and flying. J Comp Physiol B Biochem Syst Environ Physiol 158:739–749

Schippers MP, Dukas R, Smith RW, Wang J, Smolen K, McClelland GB (2006) Lifetime performance in foraging honeybees: behaviour and physiology. J Exp Biol 209:3828–3836

Schwarze SR, Weindruch R, Aiken JM (1998) Oxidative stress and aging reduce COX I RNA and cytochrome oxidase activity in *Drosophila*. Free Radic Biol Med 25:740–747

Stabentheiner A, Vollmann J, Kovac H, Crailsheim K (2003) Oxygen consumption and body temperature of active and resting honeybees. J Insect Physiol 49:881–889

Suarez RK (2000) Energy metabolism during insect flight: biochemical design and physiological performance. Physiol Biochem Zool 73:765–771

Suarez RK, Lighton JRB, Joos B, Roberts SP, Harrison JF (1996) Energy metabolism, enzymatic flux capacities, and metabolic flux rates in flying honeybees. Proc Natl Acad Sci USA 93:12616–12620

Suarez RK, Staples JF, Lighton JRB, West TG (1997) Relationships between enzymatic flux capacities and metabolic flux rates: nonequilibrium reactions in muscle glycolysis. Proc Natl Acad Sci USA 94:7065–7069

Suarez RK, Staples JF, Lighton JRB (1999) Turnover rates of mitochondrial respiratory chain enzymes in flying honeybees (*Apis mellifera*). J Exp Zool 284:1–6

Suarez RK, Staples JF, Lighton JRB, Mathieu-Costello O (2000) Mitochondrial function in flying honeybees (*Apis mellifera*): respiratory chain enzymes and electron flow from complex III to oxygen. J Exp Biol 203:905–911

Wegener G (1996) Flying insects: model systems in exercise physiology. Experientia 52:404

Winston ML (1987) The biology of the honey bee. Harvard University Press, Cambridge

Wolf TJ, Schmidhempel P, Ellington CP, Stevenson RD (1989) Physiological correlates of foraging efforts in honeybees—oxygen-consumption and nectar load. Funct Ecol 3:417–424

Wolschin F, Amdam GV (2007) Comparative proteomics reveal characteristics of life-history transitions in a social insect. Proteome Sci 5:10

Woods WA, Heinrich B, Stevenson RD (2005) Honeybee flight metabolic rate: does it depend upon air temperature? J Exp Biol 208:1161–1173

Zera AJ, Denno RF (1997) Physiology and ecology of dispersal polymorphism in insects. Annu Rev Entomol 42:207–230