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Lifetime performance in foraging honeybees: behaviour and physiology

M.-P. Schippers[1], R. Dukas[2], R. W. Smith[1], J. Wang[3], K. Smolen[1,4] and G. B. McClelland[1,*]

Departments of [1]Biology, [2]Psychology, Neuroscience & Behaviour and [3]Regional Centre for Mass Spectrometry, Department of Chemistry, McMaster University, Hamilton, ON, L8S 4K1, Canada and [4]University of Waterloo, ON, N2L 3G1, Canada

*Author for correspondence (e-mail: grantm@mcmaster.ca)

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Summary

Honeybees, *Apis mellifera*, gradually increase their rate of forage uptake as they gain foraging experience. This increase in foraging performance has been proposed to occur as a result of learning; however, factors affecting flight ability such as changes in physiological components of flight metabolism could also contribute to this pattern.

Thus, the purpose of this study was to assess the contribution of physiological changes to the increase in honeybee foraging performance. We investigated aspects of honeybee flight muscle biochemistry throughout the adult life, from non-foraging hive bees, through young and mature foragers, to old foragers near the end of their lifespan. Two-dimensional gel proteomic analysis on honeybee thorax muscle revealed an increase in several proteins from hive bees to mature foragers including troponin T 10a, aldolase and superoxide dismutase. By contrast, the activities (V_{max}) of enzymes involved in aerobic performance, phosphofructokinase, hexokinase, pyruvate kinase and cytochrome *c* oxidase, did not increase in the flight muscles of hive bees, young foragers, mature foragers and old foragers. However, citrate synthase activity was found to increase with foraging experience. Hence, our results suggest plasticity in both structural and metabolic components of flight muscles with foraging experience.

Key words: insect, muscle, flight, performance, honeybee, *Apis mellifera*, troponin T, superoxide dismutase, metabolic enzyme, foraging.

Introduction

Research on age-specific performance in several taxa including humans has documented a strikingly similar lifetime performance curve consisting of low initial performance rising to a much higher peak followed by decline late in life (Clutton-Brock, 1991; Newton, 1988; Stephan and Levin, 1992; Wooler et al., 1990). In forager honeybees (*Apis mellifera*), Dukas and Visscher (Dukas and Visscher, 1994) documented a similar pattern of more than twofold increase in the rate of food delivery by individually marked foragers from the time they commenced foraging until their peak several days later, followed by decreased performance of old foragers. Whereas this increase in performance has been proposed to occur as a result of learning, other factors such as physiological components of flight muscle and metabolism, aerodynamic mechanisms and motivational state could also shape this pattern. To the authors' knowledge, no research has explicitly evaluated the contribution of any of these factors to increases in foraging performance. In this study, we thus investigated aspects of flight muscle biochemistry that could be associated with improved foraging performance in honeybees.

In flying insects, ATP turnover rates increase several hundredfold from resting rates. This dramatic increase is accompanied by matching increases in oxygen consumption because ATP supplies involve fully aerobic metabolic pathways (Suarez et al., 1996; Wegener, 1996). Honeybees rely exclusively on carbohydrates (hexoses) to power flight (Blatt and Roces, 2001; Rothe and Nachtigall, 1989) and any metabolic adaptations affecting flight would act to increase the flux capacity through the glycolytic pathway. As well, there may be increases in oxidative stress associated with the large increase in mitochondrial oxygen consumption during flight. To date, few studies have measured the components (i.e. enzyme activities, V_{max}) of glycolysis, Krebs cycle, electron transport chain or antioxidant defence systems in honeybees (Harrison, 1986; Seehuus et al., 2006; Suarez, 2000; Suarez et al., 1996). Moreover, there is currently no information available on the changes in these components in relation to directly quantified foraging performance.

Similarly, little research has focused on differences in structural components of flight muscles and their effects on flight performance in insects (Domingo et al., 1998; Marden et al., 2001; Marden et al., 1998; Marden et al., 1999; Strohm and Daniels, 2003). Specifically, the calcium sensitivity of the troponin-tropomyosin complex can affect cross-bridge recruitment and muscle force generation necessary for

sustained flight (Marden et al., 1999). Insect troponin T isoforms have Ca^{2+}-binding capabilities and generally show more developmental and tissue-specific diversity than other isoforms of troponins I and C (Reiser et al., 1992; Sabry and Dhoot, 1991; Schiaffino and Reggiani, 1996). For example, Marden et al. (Marden et al., 1999) found that the relative abundance of different troponin T transcripts affects muscle calcium sensitivity and the power output in the flight muscles of dragonflies (*Libellula pulchella*). Thus structural components of honeybee flight muscles may also contribute to increased flight capacity and, in the same manner, increased foraging performance.

Honeybees show age polyethism (Winston, 1987). They spend the first 1–3 weeks of their adult life performing tasks inside the hive and then switch to foraging, which becomes their primary task until they die, typically within 1 to 2 weeks. Some of the physiological changes associated with the onset of foraging include a decrease in body mass, an increase in thorax glycogen stores and an increase in metabolic rate (Harrison, 1986). The timing of the transition from hive bee to forager is extremely variable (Robinson, 2002). Hence physiological changes after the onset of foraging must be related to foraging experience rather than age.

In this study, we (1) assessed honeybee foraging performance by monitoring foraging experience and rate of food delivery to the hive; (2) identified flight muscle proteins that showed changes between hive bees and peak (mature) foragers by means of a proteomic analysis; and concurrently, (3) determined the activities of flight muscle metabolic enzymes (V_{max}) in bees of varying foraging experience. Besides suggesting the contribution of physiology to the increase in foraging performance, our results provide a template of potential components of flight muscles that could affect flight performance in insects.

Materials and methods

Foraging performance

The research was carried out in southern Ontario, Canada from early June to early July 2004. The average (± s.e.m.) daily high temperature was 23.2±0.65°C. Forage during this period was abundant. The empty honeycomb we placed in the observation hive at the start of the experiment was 100% full 29 days later. Assuming a full frame mass of 4.5 kg (Sammataro, 1998), this corresponds to an average daily increase in frame mass of 155 g. We marked newly eclosed bees (*Apis mellifera* L.) with individually numbered tags and introduced them into a two-frame observation hive containing approximately 2000 bees. We made four introductions of 80 bees 3 days apart in order to have bees commencing foraging over several days.

Two weeks after introducing the first bee cohort, we removed a few bees that had already initiated foraging and began data recording. All bees departing and entering the hive travelled through a transparent Plexiglas tunnel. The marked bees were diverted into a side tunnel, caged and weighed on an analytical balance with precision of 0.1 mg. The balance reported the bee weight and time of day to the computer, and we added the bee identity, her travel direction and whether she carried pollen. We recorded data from the start of bee activity in the morning until 18:00 h on a total of 20 successive days, skipping a single rainy day with no foraging activity. At the end of the experiment, we edited the data set to include only trips longer than 5 min. We omitted all shorter trips assuming they were orientation trips by bees about to initiate foraging (Capaldi et al., 2000; Dukas and Visscher, 1994; Ribbands, 1953). For each foraging trip, we calculated the trip duration in min, the mass of forage in mg and the food delivery rate, defined as the mass of forage over trip duration.

Concurrently, entries and exits were monitored on a separate set of marked honeybees, which were used for proteomic and enzymatic analyses. These bees were collected at four different life stages: hive bees (11–15 days old), young foragers (2 days of foraging experience), mature foragers (4–11 days of foraging experience) and old foragers (≥12 days of foraging experience). Upon collection, bees were placed on ice and dissected so that only their thoraxes were kept. Thoraxes were immediately frozen in liquid nitrogen and stored at –80°C for future analyses.

It was essential to compare the behaviour of the same individual bees throughout their life to control for the possibility of a positive correlation between foraging performance and lifespan. Hence, following published methods (Dukas and Visscher, 1994), the main statistical analysis involved repeated measures ANOVA on the data set of food delivery rates over the first 7 days by the 24 bees that foraged for at least 7 days. Only four of these 24 bees collected pollen on at least half their foraging trips, precluding a detailed analysis of pollen foragers. To evaluate the effect of senescence, we conducted a second analysis of the performance of the 14 bees that foraged for at least 12 days. Sample sizes were insufficient for analyses beyond 12 days of foraging experience.

Two-dimensional electrophoresis and proteome analysis

Similar sized thoracic sections from nine hive bees and nine mature foragers [mass=27.0±0.6 mg and 26.3±0.1 mg (mean ± s.e.m.), respectively] were individually homogenised in 500 μl of ice cold two-dimensional gel lysis buffer [as detailed elsewhere (Smith et al., 2005)] using a motorised dounce homogeniser. The homogenate was clarified by centrifugation (18 000 ***g***, for 5 min at 4°C) and the supernatant desalted using commercially available protein desalting columns (Pierce, Rockford, IL, USA).

From each homogenate 200 μg total protein was resolved by two-dimensional (2D) gel electrophoresis. All 2D electrophoresis was carried out using the Investigator™ electrophoresis system (Genomic Solutions, Ann Arbor, MI, USA) according to the manufacturer's instructions and using the pre-made rehydration/solubilisation, equilibration and running buffers. Briefly, the first dimension was resolved on pHIash (pH 3–10) immobilised pH gradient (IPG) strips, using

the pHaser isoelectric focussing apparatus pre-programmed ramped voltage regimen, for a total of 100 000 volt-hours, and the second dimension was resolved on trycine chemistry/10% duracryl slab gels run on the Investigator™ (Ann Arbor, MI, USA) 2D casting and running apparatus, again using the pre-programmed voltage regimen. After electrophoresis the gels were fixed with water, methanol and acetic acid (in accordance with the instructions provided with the gel stain) and then stained with SYPRO-ruby stain (Genomic Solutions). Imaging of the stained gels was carried out using the Perkin Elmer Pro-Express gel imaging system. The gel images were then analysed using Phoretix 2D™ analytical software version v2004 (Nonlinear Dynamics).

Proteome changes thought to be associated with the transition from hive activity to foraging were selected according to similar criteria to those used by Smith et al. (Smith et al., 2005). The protein spot was present on all hive bee and all foraging bee gels (i.e. the protein was consistently resolved) yet there was a significant change in mean normalized spot volume, a parameter offered by the Phoretix analytical software which combines spot area and intensity to give an overall index of expression, between hive and foraging gels.

Selected protein spots (see Results) were cut from the gel using the Perkin Elmer Pro-Pick robotic work station and the gel plugs preserved in 2% glycerol at 4°C until they were subjected to in-gel trypsin digestion and peptide analysis.

In-gel tryptic digestion and nano electrospray quadropole time of flight mass spectroscopy analysis

The gel plugs, containing the protein spots of interest (see above), were destained with 50 mmol l^{-1} ammonium bicarbonate, containing 50% acetonitrile and air dried. The proteins were then reduced by adding 30 μl of 10 mmol l^{-1} dithiotreitol (DTT) in 25 mmol l^{-1} ammonium bicarbonate to each gel plug and incubating for 1 h at 56°C. After cooling to room temperature, the DTT solution was removed and the gel plugs treated with the same volume of 100 mmol l^{-1} iodoacetamide in 50 mmol l^{-1} ammonium bicarbonate. After 60 min incubation at ambient temperature, in the dark, the gel plugs were washed with 30 μl of 25 mmol l^{-1} ammonium bicarbonate for 15 min and then dehydrated with 100% acetonitrile. After 10 min the liquid phase was removed, and the gel plugs were completely dried in air. The proteins were then subjected to in-gel digestion: 0.015 μg trypsin in 30 μl of 50 mmol l^{-1} ammonium bicarbonate solution containing 10% acetonitrile was added to each gel plug and these were incubated at 37°C overnight. The digested proteins were desalted and concentrated using a Millipore C18 ZipTip prior to MS analysis and the peptides were finally eluted in 8 μl of 50% aqueous acetonitrile containing 0.2% formic acid. All protein digests were analyzed by a Q-TOF Global Ultima (Micromass Waters, Manchester, UK) with a nanoES source. Capillary voltage was typically 1.2–1.6 kV, cone voltage was 50–100 V and the voltage was 100 V. Mass spectra in time of flight mass spectroscopy (TOF MS) and MSMS mode were in a mass range 50–1800 m/e with a resolution of 8000 full width at half maximum height (FWHM). Argon was used as collision gas.

Normalized spot volumes from hive bees and mature foragers 2D gels were compared by Student's *t*-test (Statistix analytical software).

Enzyme activities

Thoraxes from each life stage (hive bees, young foragers, mature foragers, old foragers; mean ± s.e.m. thorax mass= 27.8±0.8 mg, 27.1±0.5 mg, 26.5±0.3 mg and 26.6±0.6 mg, respectively) were powdered using a liquid N_2-cooled mortar and pestle. Thorax weight did not differ among life stages (ANOVA; $F_{3,39}$=1.0, P=0.41). Whole thoraxes were then homogenized on ice using a glass on glass homogenizer for 1 min in 20 volumes of extraction buffer consisting of 75 mmol l^{-1} potassium phosphate (pH 7.3) and 10 mg ml^{-1} 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 activity of cytochrome *c* oxidase (COx), phosphofructokinase (PFK) and hexokinase (HK) were measured on fresh thorax homogenates. Enzyme activity of pyruvate kinase (PK) and citrate synthase (CS) were measured after having been frozen and thawed once and twice, respectively. Nine to eleven thoraxes were used for each life stage. Assays condition were, 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} 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, 50 mmol l^{-1} Hepes (pH 7.4); PK: 5 mmol l^{-1} phosphoenol pyruvate (PEP; omitted in control), 50 mmol l^{-1} imidazole (pH 7.4), 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 and 9.25 U lactate dehydrogenase (LDH); 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 (DTNB) in 20 mmol l^{-1} Tris (pH 8.0).

For each enzyme, we tested whether enzyme activity increased from hive bees to young foragers, mature foragers and old foragers by performing an analysis of variance linear contrast (ANOVA linear contrast; SPSS version 12.0, SPSS Inc.). *Post hoc* analysis was performed using the Dunn–Sidak test.

Results

Foraging performance

Bee experience was significantly associated with bees' rate of food delivery (repeated measures ANOVA, $F_{6,12}$=6.6,

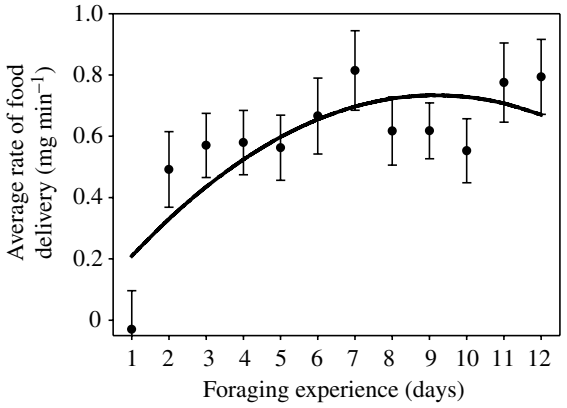

Fig. 1. The average (± s.e.m.) rate of food delivery as a function of foraging experience of honeybees. The line is a quadratic regression fit. The averages are based on individual averages of all bees (N=24) active on each day.

P<0.01; Fig. 1). On average, the bees showed little success on their first day of foraging but performed much better on day 2. From then on, the bees increased their foraging success, peaking on day 7 at about 80% higher food collection rate than on day 2. The change in food delivery rate was attributed to an increase in the average weight of food collected per trip from 6.9±3.3 mg (mean ± s.e.m.) on day 1 and 23.9±3.2 mg on day 2 to 31.8±2.7 mg on day 7. Trip duration remained approximately constant, 43.6±3.6 min on day 1, 45±3.3 min on day 2, and 41.1±3.2 min on day 7. The date at which the various bees commenced foraging did not significantly affect the rate of food delivery ($F_{4,2}$=8.8, P=0.1). Bees that foraged for at least 12 days did not exhibit a decrease in food collection rate between day 7 and day 12 (repeated measures ANOVA, $F_{5,10}$=0.8, P=0.57).

Overall, we recorded the behaviour of 38 bees. These bees commenced foraging at an average age of 14±0.3 days. The average foraging life span of the 27 bees that died before the end of the experiment was 9.7±0.9 days, and the median foraging span was 8 days.

Two-dimensional electrophoresis, proteome analysis and protein identification

The minimum and maximum number of proteins resolved from hive and foraging bees were 194 and 259, and 197 and 276, respectively. Fig. 2 depicts a typical 2D gel from a foraging bee and also indicates the five spots that fulfilled the selection criteria given above (see Materials and methods, 2D electrophoresis and proteome analysis) and were selected for identification (Table 1). There were no completely consistent inductions or deletions in the proteome; i.e. there were no examples of protein spots that were absent from all hive bees but induced in all foragers, nor any which were present in all hive bees and deleted from all foragers. Quantitative proteome changes were selected according to the criteria used by Smith et al. (Smith et al., 2005). Specifically, the protein spot was present on all hive bee and all foraging bee gels (i.e. the protein

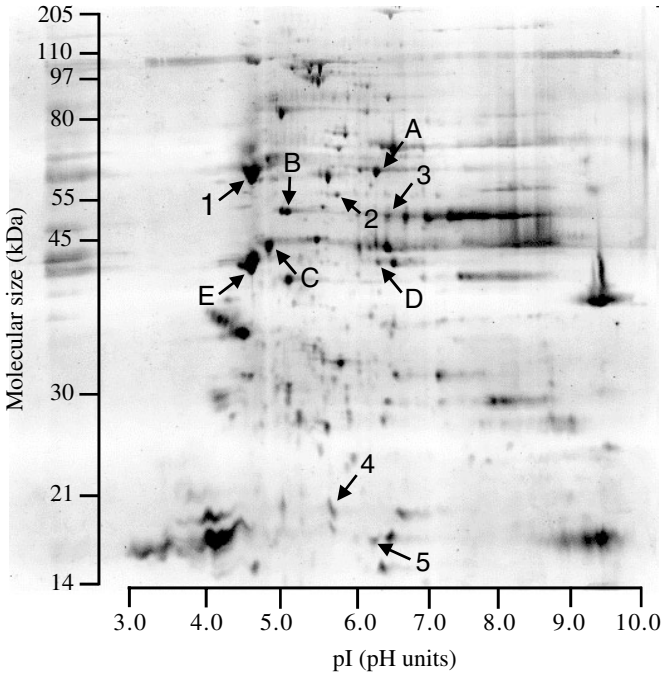

Fig. 2. Representative 2D electrophoresis protein gel from the thoracic section of a mature forager honeybee. Proteins that either increased or decreased in foragers, compared to hive bees, are indicated by numbers: 1, troponin T 10°: 2, translation elongation factor-1 γ; 3, fructose 1,6-biphosphate aldolase; 4, unidentified protein; 5, Cu/Zn superoxide dismutase (for further details see Table 1, and the extent of protein abundance change is illustrated in Fig. 3). Additional proteins, which were identified but did not show any change in expression, are indicated by capital letters: A, enolase; B, actin; C, tropomyosin 2; D, CG6084-PA; E, tropomyosin (*Dermatophagoides farinae*) (for further details see Table 2).

was consistently resolved) yet there was a significant change in mean normalized spot volume, a parameter offered by the Phoretix analytical software (see Materials and methods) which combines spot area and intensity to give an overall index of protein expression.

The increase or decrease in abundance of the differentially expressed proteins is given in Fig. 3 and results of the identification analysis of these proteins are given in Table 1. Of these, troponin T 10A, fructose-1,6-biphosphate aldolase (aldolase) and Cu/Zn superoxide dismutase increased in foraging bees, whilst translation elongation factor-1 gamma and spot 4 (no database matches) decreased.

Fig. 2 also indicates four further proteins that were identified (using the same method as previously described) but did not show differential expression between hive and foraging bees: tropomyosin, tropomyosin 2, actin and enolase (Table 2). The mean ± s.e.m. normalized spot volumes of these proteins in hive and foraging bees are; 8.82±1.45 and 8.38±1.41, 3.29±0.66 and 2.88±0.15, 2.62±0.62 and 2.28±0.33, and 2.93±0.58 and 3.71±0.35, respectively. These proteins were selected for identification primarily because, being some of the more abundant, they could be used to improve the digestion

Table 1. *Proteins significantly increased or decreased in abundance, as determined by changes to mean normalised protein spot volume, between hive workers and mature foragers*

Spot no.*	Protein description	Database	Accession no.	Peptide sequence†	M_r (exp.)	M_r (calc.)	MOWSE score‡
1	Troponin T 10A (*Apis mellifera*)	MSDB	Q6IFYO APIME	23–KGPNFTITR–31	1032.52	1032.57	37 (32)
		MSDB	Q6IFYO APIME	33–DLAGNLTSAQLER–45	1386.65	1386.71	70 (33)
		MSDB	Q6IFYO APIME	64–IKPLEIDGFSIEK–76	1487.82	1487.82	37 (27)
		MSDB	Q6IFYO APIME	163–LFEGGYDTLLAEINEK–178	1810.79	1810.90	84 (36)
2	Translation elongation factor-1 γ (*Apis mellifera*)	NCBInr	gi|66519222	31–IADDFVFGETNK–42	1354.62	1354.64	35 (25)
		NCBInr	gi|66519225	225–ETIEDLDPADAALAAEPK–242	1867.88	1867.91	29 (23)
3	Fructose 1,6-biphosphate aldolase (*Apis mellifera*)	NCBInr	gi|66499111	111–VDTGVVTLFGTEEETTTQGLDNLQAR–136	2793.30	2793.37	41 (22)
		NCBInr	gi|66499111	246 –ASPQEIAAATVTALLR–261	1610.89	1610.90	22 (20)
4	Unidentified protein§						
5	Cu/Zn superoxide dismutase (*Apis mellifera*)	NCBInr	gi|33089104	14–GTIFFEQPESTNSVK–28	1682.71	1682.82	28 (22)

*See Fig. 2.

†Figures at beginning and end indicate location of peptide in overall protein amino acid sequence; the complete sequence being available in the databases, under the accession numbers given.

‡Figures in parentheses indicate MOWSE score required for significant homology (P<0.05). The MOWSE score provides an index of the probability that the match between the experimental data and the database amino acid sequence is a random event. For more information refer to Pappin et al. (Pappin et al., 1993).

§Amino acid sequence unavailable due to inadequate spectra from digested protein.

M_r (exp.) is the experimentally determined molecular mass and M_r (calc.) is the calculated molecular mass for the peptide sequence.

and analysis procedure of the proteins that did exhibit expression changes (refer to Table 1). As a result we were able to optimise the amount of trypsin to ensure adequate digestion but avoid interference from an excessive mass spectrometry trypsin signature. Overall, this proved invaluable in obtaining accurate identification; particularly of the lower abundance proteins listed in Table 1 and Fig. 3.

Although these proteins did not show significant changes in expression they are included: (a) to increase the knowledge of the bee proteome and (b), in the case of tropomyosin and tropomyosin 2, to reinforce the very precise nature of the change in the major structural proteins.

Flight muscle enzyme activity

Of the five metabolic enzymes measured, only CS activity significantly increased with foraging experience (Fig. 4; ANOVA linear contrast; $F_{1,36}$=6.2, P=0.018). Activities of glycolytic enzymes PK, PFK and HK, as well as electron transport chain enzyme COx, did not increase with foraging experience. In fact, contrary to our prediction, there was a decreasing trend in PFK and COx activities, while PK and HK activities did not show any pattern with foraging experience. Pairwise comparisons also showed no significant differences between any life stages (Dunn–Sidak *post hoc* test; $P \geqslant 0.16$).

Discussion

This study attempted to explain *in vivo* performance changes (foraging success) in honeybees by examining flight muscle proteomic and biochemical changes both between casts (hive workers and foragers) and among foragers with increasing levels of foraging experience. We found that bees increased their foraging performance (rate of forage delivery) as they gained foraging experience. This result replicates an earlier detailed behavioural study (Dukas and Visscher, 1994). Compared to hive bees, foragers at peak foraging performance (mature foragers) showed an increase in an isoform of an important regulatory muscle structural component, Troponin T 10A. As well, we expected *a priori* that foragers would show increases in the activities of several metabolic enzymes important in aerobic capacity. Results from our *in vitro* analysis of enzymatic flux capacity followed our prediction for CS, but

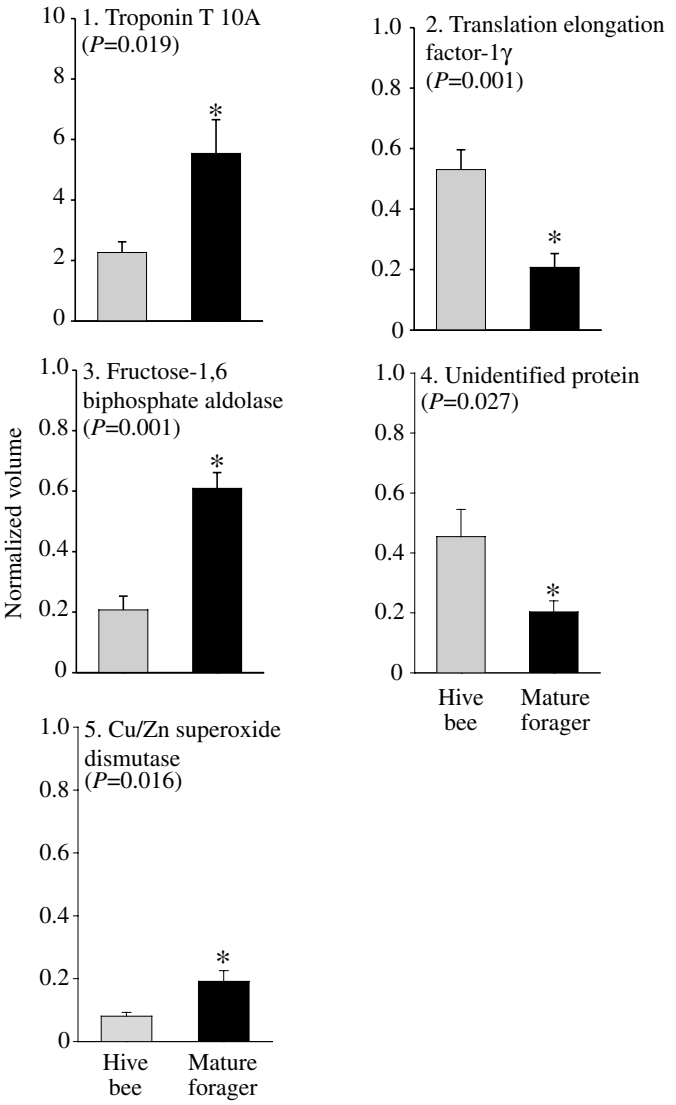

Fig. 3. Changes in normalized spot volume of proteins that were either increased or decreased in mature forager compared to hive bees. Values are means ± s.e.m.; *significant difference from hive bees (*P* values are given in parentheses). *N*=9 forager and 9 hive bees. The numbers next to the protein names refer to the numbers in Fig. 2.

not for COx or any of the glycolytic enzymes measured, which are classically defined as regulatory for overall glycolytic flux. In contrast, proteomic analysis showed that the glycolytic enzyme aldolase increased in mature foragers compared to hive bees. Thus, although the contribution of glycolytic enzymes for flight performance is currently unclear, our results suggest plasticity in both structural and metabolic components of flight muscles with foraging experience.

Foraging performance

Honeybee foragers increased their rate of forage uptake as they gained foraging experience. Dukas and Visscher, who performed a similar experiment (Dukas and Visscher, 1994), found a comparable, but more gradual increase in the rate of forage uptake. We believe that because our study site offered abundant food sources, honeybee foragers had an easier task finding and collecting food, which resulted in a steep increase in the rate of forage uptake with experience. Besides changes affecting flight metabolism and perhaps muscle efficiency associated with aerodynamic mechanisms (Feuerbacher et al., 2003), learning could also play an important role in the observed increase in foraging performance with experience. Bees are known to learn tasks such as flower handling and navigational skills, which could strongly affect the rate of forage uptake (Dukas and Visscher, 1994). Research in progress is focussing on the relative contribution of learning and decision to the observed increase in foraging performance (R.D., unpublished data).

Flight muscle structural components

Insect indirect flight muscle contraction is controlled by a supramolecular complex. As in vertebrates, troponin is a major regulatory protein of this structure. Moreover, insect muscle troponin T is a calcium-binding regulatory protein with many alternatively spliced isoforms (Domingo et al., 1998; Herranz et al., 2005). In honeybees, there was a significant increase in the troponin T 10A isoform (mutually excluded exon 10A) in mature foragers compared to hive bees whereas other components of this supramolecular complex, tropomyosin and tropomyosin 2 did not change between these two casts. Muscle activation results from Ca^{2+} binding to troponin C, which leads to a conformational change in troponin I and tropomyosin to allow cross-bridge formations (Tobacman, 1996). Troponin T joins troponin C and I to tropomyosin, and is thought to affect calcium sensitivity by forming interactions among neighbouring troponin complexes (Marden et al., 1998). In dragonflies, an age-related change in the troponin T isoform was found to affect calcium sensitivity of muscle activation (Fitzhugh and Marden, 1997), and the relative abundance of troponin transcripts explains much of the variation in aerodynamic power output during free flight (Marden et al., 1999). Although dragonfly flight muscle is synchronous and honeybee flight muscle is asynchronous, it is possible that increases in troponin T 10A in honeybees affect calcium sensitivity in a way similar to dragonflies. In addition, because the troponin T 10A isoform is associated with the acquisition of flight in honeybees (Domingo et al., 1998), it most likely affects flight performance in the same manner as that seen in dragonflies. So, although this troponin isoform first appears as part of a developmental-specific program early in life [observed in 5-day old hive bees (Domingo et al., 1998)], we show that the level of expression of this contractile protein further increases as bees become foragers. Thus, our results suggest that increases in troponin T 10A could ultimately affect foraging performance. Further research on the functional role of this protein is warranted.

Flight muscle metabolic enzymes

Because foraging flight in honeybees is fuelled entirely by the oxidation of hexose sugars, we examined key enzymes in glycolysis (HK, PFK, PK), a Krebs cycle enzyme (CS) and an

Table 2. *Identified proteins that did not increase or decrease in abundance, as defined by changes in normalized spot volume*

Spot no.*	Protein description	Database	Accession no.	Peptide sequence†	M_r (exp.)	M_r (calc.)	MOWSE score‡
A	Enolase [2-phosphoglycerate dehyratase] (*Apis mellifera*)	NCBInr	gi|66558835	264–EALNLIIDSIK–274	1227.68	1227.71	36 (31)
		NCBInr	gi|66558835	308–SDPSTYLDSDSLK–320	1426.63	1426.65	22 (18)
B	Actin (*Culex pipiens pipiens*)	MSDB	Q4JQ54 CULPP	198–GYSFTTTAER–207	1131.52	1131.52	29 (20)
		MSDB	Q4JQ54 CULPP	317–EITALAPSTIK–327	1142.63	1142.65	36 (31)
C	Tropomyosin 2 (*Apis mellifera*)	NCBInr	gi|48129583	92–VQEIEATLTK–101	1130.66	1130.62	43 (37)
		NCBInr	gi|48129583	169–LALVEEELEGAEER–182	1585.88	1585.78	56 (41)
		NCBInr	gi|48129583	194–EDELFIVQNIVK–205	1445.86	1445.78	52 (45)
D	CG6084-PA (*Apis mellifera*)	NCBInr	gi|66525576	272–NIGISNFNSEQIER–173	1619.75	1619.79	50 (25)
		NCBInr	gi|66525576	272–IAENYEVFDFK–282	1373.59	1373.65	34 (25)
E	Tropomyosin (*Dermatophagoides farinae*)	MSDB	TPM DERFA	190–IVELEEELR–198	1128.59	1128.60	47 (43)

*See Fig. 2.

†Figures at beginning and end indicate location of peptide in overall protein amino acid sequence; the complete sequence being available in the databases, under the accession numbers given.

‡Figures in parentheses indicate MOWSE score required for significant homology (P<0.05). The MOWSE score provides an index of the probability that the match between the experimental data and the database amino acid sequence. For more information (see Pappin et al., 1993).

M_r (exp.) is the experimentally determined molecular mass and M_r (calc.) is the calculated molecular mass for the peptide sequence.

electron transport chain component (COx). Of these, only CS showed increases in activity as bees gained foraging experience. Enzyme activities, or, more specifically, maximal enzymatic flux capacities (V_{max}), represent the upper limits of flux at a particular step of a biochemical pathway *in vitro*, but can provide insights on flux *in vivo* (Newsholme and Crabtree, 1986; Suarez et al., 1996; Suarez et al., 1997). V_{max} is a function of enzyme concentration and catalytic efficiency (k_{cat}). Since k_{cat} is constant (Suarez et al., 2000), the differences in V_{max} are necessarily due to differences in enzyme concentrations.

HK, PFK, and PK activities in flight muscles did not change with increasing foraging experience. Since these three enzymes are thought to be major regulatory steps of glycolysis (Hochachka and Sommero, 2002), we assume that they exert most of the control of the overall glycolytic flux. Based on our enzyme activity measurements, the overall flux capacity of glycolysis does not seem to be upregulated with foraging experience. It seems that most of the metabolic capacity of the flight muscles is already present relatively early in the bee life, before the onset of foraging. However, proteomic analysis revealed an increase in aldolase abundance in flight muscles of mature foragers compared to hive bees. The aldolase step in glycolysis is generally thought to be a near-equilibrium reaction and thus its contribution in determining overall flux through pathways is somewhat controversial (Brooks, 1996; Fell, 1998; Pierce and Crawford, 1997). However, there is some evidence for near-equilibrium steps of pathways affecting flight metabolism (Coelho and Mitton, 1988; Harrison et al., 1996). On the other hand, since equilibrium constants differ from *in vitro* values (Connett, 1985), it is not clear how aldolase functions *in vivo*. Moreover, changes in protein abundance may not reflect *in vivo* changes of enzyme flux capacity. For example, recent research on *Drosophila* flight muscle suggests that co-localization of some glycolytic enzymes, including aldolase, along the sarcomeres is required to sustain flight (Sullivan et al., 2003; Wojtas et al., 1997). Hence, not only enzymes need to be present but co-localization is also required for proper muscle function. One more aspect of near-equilibrium enzymes is that they must maintain higher forward flux capacities to maintain a given pathway flux rate relative to enzymes that are far from equilibrium (Staples and Suarez, 1997). Thus, changes in V_{max} of near-equilibrium enzymes must be greater than changes in V_{max} of allosteric enzymes. So it is possible that small changes in V_{max} of HK, PFK and PK were not detected in this study because these changes were smaller than the error associated with enzyme measurements. Possibly honeybees have already reached an upper limit in the amount of enzymes that can be packed within a muscle cell without affecting contractile components, and, therefore, the enzymes operate at a greater fractional velocity v/V_{max} *in vivo* instead of increasing V_{max} itself (Suarez et al., 1996). If this is the case, the overall flux of glycolysis could be upregulated

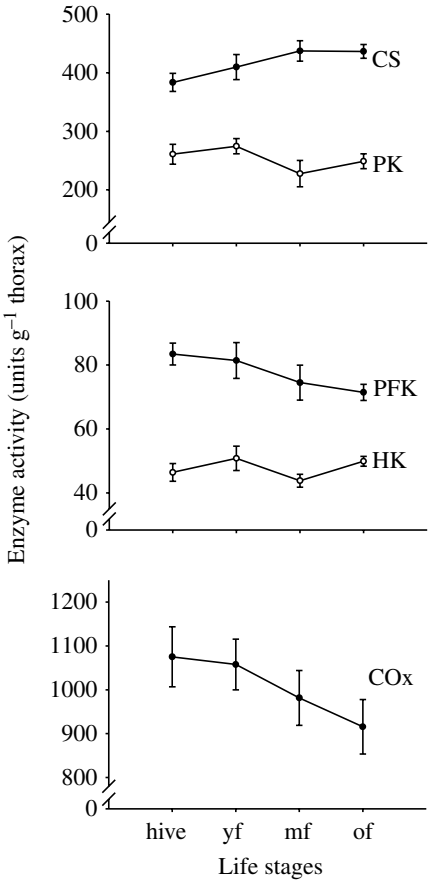

Fig. 4. Mean enzyme activities (V_{max}) of phosphofructokinase (PFK), hexokinase (HK), citrate synthase (CS), pyruvate kinase (PK) and cytochrome *c* oxidase (COx) at different life stages. Bars indicate ± s.e.m.; *N*=9–11 thoraxes per life stage; yf, young forager; mf, mature forager; of, old forager.

even though no changes in enzyme activity (V_{max}) were observed. Moreover, Suarez et al. (Suarez et al., 2005) have proposed for body size variation in metabolism in orchid bees (Apidea: Euglossini), that variation in flux at any step in metabolism may be under hierarchical control (changes in enzyme concentration [E]) or metabolic control (variation in product, substrate or modulator concentration) or both. So, even though we did not see any changes in V_{max} *in vitro*, *in vivo* small changes in [product]/[substrate] or [modulators] may increase total flux rate through glycolysis in forager *versus* hive honeybees.

We measured CS and COx, mitochondrial enzymes involved in the Krebs cycle and in the electron transport chain, respectively, because they are important determinants of aerobic capacity. No increases in COx activities were observed but CS showed a 14% increase in activity as bees gained foraging experience. Comparative studies on insects and mammals have shown that mitochondrial enzyme densities are constant per unit cristae membrane surface area (Suarez et al., 2000). Hence, the modest increase in CS activity observed suggests that honeybees slightly augment flight muscle mitochondrial density as they gain foraging experience, which could enhance aerobic capacity and flight muscle ATP turnover rates.

Enhanced antioxidant defence in foragers

Our proteomic analysis revealed that Cu/Zn superoxide dismutase (Cu/ZnSOD, cytosolic) was increased in mature foragers compared to hive bees. Cu/ZnSOD is involved in antioxidant defence in cells by catalyzing the reaction that converts superoxide radicals into hydrogen peroxide (Fridovich, 1995). Cu/ZnSOD activity is increased in highly oxidative muscles (Powers et al., 1999) offering an enhanced antioxidant defence. It is thought that prolonged muscular exercise increases Cu/ZnSOD activity in mammalian muscle cells to cope with increases in reactive oxygen species (Powers et al., 1999). Recently, the over expression of the Cu/ZnSOD gene has been linked to decreased oxidative damage and increased longevity in *Drosophila* (Parkes et al., 1998; Spencer et al., 2003). It is thus possible that the higher levels of Cu/ZnSOD in mature foragers compared to hive bees allow them to cope with the increased production of free radicals associated with the high aerobic demands of flight.

Conclusion

Our results suggest a physiological basis for increased foraging ability in mature foragers compared to hive bees. Increases in troponin T 10A isoform in mature foragers and increases in CS activity with foraging experience may contribute to enhanced flight performance, but causation between these proteins and foraging success needs to be established. *In vitro* glycolytic enzyme flux capacities (V_{max}) could not explain the observed increase in foraging performance but proteomic analysis revealed that aldolase protein content was increased in mature foragers. Thus, physiological flux rates through glycolysis need to be assessed in relation to foraging experience to further elucidate the physiological determinants of lifetime performance in honeybees. In addition, further experiments are required to assess the functional role of troponin T 10A and its contribution to the foraging performance of honeybees.

List of abbreviations

CS	citrate synthase
COx	cytochrome *c* oxidase
HK	hexokinase
PFK	phosphofructokinase
PK	pyruvate kinase
SOD	superoxide dismutase
V_{max}	enzyme maximum activity

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