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Behavioural, morphological, and metabolic maturation of newly emerged adult workers of the bumblebee, *Bombus impatiens*

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ABSTRACT

Newly emerged adult holometabolous insects must still complete considerable morphological, metabolic, and neural maturation. Despite this, adults have frequently been documented to fly prior to finishing maturation and attaining peak physiological capacity. In some species, flight is limited by the unfurling of the wing, while in other species it may be limited by biochemical capacity. We charted maturation trajectories of adult bumblebee workers (*Bombus impatiens*) for both morphological and flight muscle metabolic capacities, and compared these to the first age at flight. Workers began regular flights as soon as two days after emergence. The unfurling of the wings was completed well before first flights and before any other studied factor, suggesting this did not initially limit flight. Wing beat frequencies, measured as a struggling response to grasping the hindlegs, were about 90% mature by two days old, and did not significantly change after three days. Conversely, by the initiation of flight, the mean enzyme maturation was only 63% completed relative to adult enzyme capacity, though specific enzyme profiles ranged from 42% to 73%. Maximum ADP-stimulated mitochondrial respiratory activity on pyruvate and proline matured along a similar time frame to glycolytic capacity, reaching its maximum three days after emergence. Bumblebees, as other adult insects, thus begin flights prior to fully maturing.

1. Introduction

In order to prepare for energetically intensive flight, metamorphosing insects histolyse the weak larval muscles and replace them with new and more powerful ones (Fernandes et al., 1991). Maturation of the new flight muscles usually continues even after emergence of the new adults, resulting in an overlap between physiological maturation and the onset of flight behaviour. Due to this overlap, early flight competency may be reliant on ongoing changes in muscle properties such as enzyme content (Beenakkers et al., 1975), mitochondrial volume (Herold, 1965), and muscle elasticity (Fielding et al., 1980).

In some cases, the flight of newly emerged adults is dependent only on the unfurling and hardening of the wings, such as in butterfly species of the genus *Pieris* (Petersen et al., 1956). In other species, the initiation of flight may be delayed by the maturation of muscle tissue metabolic capacities. Among six insect species, initial α-glycerol-3-phosphate dehydrogenase (αGPDH) activities in new adults ranged from 2.5% to 68% of the eventual adult maximum, and higher initial activities predicted earlier ages of first flights (Campbell and Birt, 1972). Though Campbell and Birt (1972) suggested that αGPDH alone predicted the start of flight, which enzymes mature, and so which may be initially limiting, is dependent on the flight fuel source of the adults (Beenakkers et al., 1975).

Intriguingly, many insects are capable of flight prior to achieving full biochemical or mechanical competence. Honeybee workers (*Apis mellifera*) begin their first orientation flights well before they begin foraging (Herold and Borei, 1963), and prior to attaining maximum tissue metabolic capacity (enzyme activities and cytochrome content: Harrison, 1986; Harrison and Fewell, 2002; Herold and Borei, 1963). Biochemically immature blowfly (*Protophormia terraenovae*) males start flying even though some enzymes only have 40% capacity (Collatz et al., 1981). After emergence, enzyme trajectories frequently mirror life-history transitions. The loss of glycolytic enzyme capacity corresponds to the loss of flight in *P. terraenovae* males (Collatz et al., 1981). The conversion from nursing to foraging behaviour in honeybee workers coincides with upregulation of enzymes involved in glycolytic and mitochondrial metabolism (Schippers et al., 2006; Wolschin and Amdam, 2007). The regulation of metabolic enzymes is therefore a dynamic activity in the lifetime of the adult insect, and not necessarily permanently established at emergence.

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We examined how both morphological and biochemical maturation profiles compare to the initiation of flight in bumblebee workers (*Bombus impatiens*). *B. impatiens* is endemic to North America and raised as a pollinator for greenhouses. New workers are distinguished by their high degree of wing curvature and their silvery grey pile which yellows in about 24 h. Heinrich (1979) estimated that new workers begin flying around 48 h after emergence, and we therefore focused on characterising the maturation of glycolytic enzymes from 0 h to 48 h intensively, and daily thereafter. We examined the complete suite of glycolytic enzyme activities to examine their coordinated changes during maturation. We also examined changes in activities of enzymes involved in substrate recruitment (glycogen phosphorylase and trehalase), and those involved in biochemical pathways linked to glycolysis (glucose-6-phosphate dehydrogenase and αGPDH). Finally, citrate synthase and isolated muscle fibre respiration rates were studied as representative of the tricarboxylic acid cycle and mitochondrial maturation.

2. Materials and methods

2.1. Housing colonies

Colonies of *B. impatiens* were obtained from a local greenhouse supplier (BioBest Canada, Ltd.) and maintained in their housing box. Ambient temperature was 25–26 °C, with a 16:8 light–dark cycle. Bees were fed *ad libitum* on sucrose solution and pollen balls (unlyophilised), presented directly to the colony.

New workers were collected daily while their silver pelosity distinguished them from mature adults. Each individual was marked with a coloured and numbered card disc, glued between the tegulae with resin glue, and then replaced in the colony. Individuals' ages were recorded as the time in hours post-emergence from their cocoons. Data for the age at first flight was obtained from three colonies held in separate free-flight enclosures. Colonies were stimulated daily by shaking, which resulted in workers performing guarding sorties. Flying guards were captured, and their identities recorded. The age at first flight was the first day an individual was observed; the ages were known only to within 24 h. The age at which flight could be sustained was also tested directly by dropping individuals from a height of ~1 m, which was successful when bees could sustain hovering.

2.2. Collection of individuals

The physiological maturation of workers was studied in two phases. A fine time scale of measurements was made with individuals from a single colony aged 0, 3, 6, 12, 18, 24, 48 and >180 h post-emergence. The ages of bees collected for time intervals between 0 and 6 h old were known exactly: they were taken while emerging from their cocoon. Otherwise, ages of individuals collected between 12 and 24 h old were known to within 1 h; ages of individuals collected at 48 h were known to within 3 h; ages of individuals collected after 180 h or older were known to within 24 h. A second set of measurements was made on a coarser time scale, using individuals from a different colony, collected at ages from 0 to 6 days old. 0 day old individuals were collected as they emerged from their cocoons, and otherwise ages were known to within 12 h.

Workers' wings were photographed in profile, and analysed in ImageJ software (Rasband, 1997–2009) to find the time course of wing unfurling. A dimensionless wing curvature coefficient was found by dividing the area under the wing, from tegula to tip, by the square of the wing length. This value was log-transformed for statistical analyses. Maturation of wing beat frequency was tested in a group of males tested repeatedly over the first five days after emergence. Adults immediately emerged from the cocoon cannot fly, so wing beat frequency was acquired by grasping a hind limb with a pair of forceps, inducing a struggling wing beat response ($f_{struggle}$). $f_{struggle}$ was measured with an optical tachometer (Moore Scientific) and Trex 2.0 Transient Waveform Recorder. In mature adults, inter-individual variation in wing beat frequency during flight is correlated with $f_{struggle}$ (linear correlation coefficient, $r = 0.73$, $p < 0.001$, data not shown). Therefore, although $f_{struggle}$ is an indirect measure of flight wing beat frequency, it is a reasonable approximation of changes in thoracic properties. A limitation is that males might differ in the time course of maturation. However, the initiation of flight occurs roughly at the same time between the two sexes (certainly within the resolution of the observations) and so should also be representative of females.

2.3. In vitro enzyme kinetics

Enzymes are expressed as their maximum activity *in vitro* (V_{max}), in U g thorax^{-1}, where 1 U = 1 μmol min^{-1}. Assays were performed over multiple days and age groups were distributed among assay days. Frozen thoraces were weighed and then homogenised in 19 volumes of ice-cold homogenisation buffer (25 mM Tris–potassium phosphate (KPO_4), pH 7.8 at 4 °C, 2 mM ethylenediaminetetraacetic acid (EDTA), 5 mM dithiothreitol (DTT) and 0.5% Triton X-100), or 1000 μL, whichever was less (to prevent overflow during homogenisation). Homogenisation was performed in three 10 s bursts with 30 s intervals (six 7 mm heads, 10,000 RPM, OMNI-Prep Homogenizer), and the homogenate was then sonicated in two 5 s bursts with 20 s intervals, at 20% amplitude (Sonics Vibra-cell, six heads). Homogenates of 1000 μL were then brought to 19 volumes total dilution and vortexed, and all samples were spun down at 500 × *g* for 10 min at 4 °C. The supernatant was tested for activities.

Enzyme assays were optimised with respect to reagent concentration and performed at the *in vitro* pH optimum (determined in preliminary experiments), and at 37 °C. Each homogenate was assayed in triplicates of 10 μL diluted homogenate in 240 μL of reaction buffer, on a BioTek Synergy2 plate spectrophotometer (Winooski, VT). The best duplicate measurements were used to calculate enzyme activities. All reagents were obtained from Sigma–Aldrich except acetyl-CoA (BioShop Canada Inc.) and adenosine triphosphate (ATP) (Calbiochem). Protocols for glycogen phosphorylase (GP), trehalase (TRE), hexokinase (HK), phosphoglucose isomerase (PGI), phosphofructokinase (PFK), α-glycerol-3-phosphate dehydrogenase (αGPDH), and citrate synthase (CS) were modified from Darveau et al. (2005); aldolase (ALD), triose phosphate isomerase (TPI), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), phosphoglycerate mutase (PGM), phosphoglycerate kinase (PGK), enolase (ENOL), and pyruvate kinase (PK) from Pierce and Crawford (1994); and glucose-6-phosphate dehydrogenase (G6PDH) from Joanisse and Storey (1994). Coupling enzyme was added at 1.25 U/250 μL, except where noted. Assay conditions, with pH given at 37 °C, are as follows: GP: 100 mM KPO_4 (pH 7.1), 10 mM magnesium chloride ($MgCl_2$), 4 μM glucose 1,6-bisphosphate, 0.75 mM nicotinamide adenine diphosphate (NADP), 2 mM adenosine monophosphate, 1 mg/250 μL glycogen, phosphoglucose mutase and G6PDH. TRE: 100 mM KPO_4 (pH 6.6), 1.1 mM $MgCl_2$, 0.75 mM NADP, 1.1 mM ATP, 10 mM trehalose, HK and G6PDH. G6PDH: 50 mM Tris–Imidazole (pH 7.5), 10 mM $MgCl_2$, 0.1 mM NADP, 1 mM glucose 6-phosphate. HK: 100 mM Tris–Imidazole (pH 8.1), 100 mM potassium chloride (KCl), 10 mM $MgCl_2$, 1 mM NADP, 5 mM ATP, 5 mM D-glucose, G6PDH. PGI: 50 mM Tris–Imidazole (pH 7.8), 5 mM KCl, 10 mM $MgCl_2$, 0.75 mM NADP, 16 mM fructose 6-phosphate (F6P), G6PDH. PFK: 50 mM Tris–Imidazole (pH 8.0), 100 mM KCl, 10 mM $MgCl_2$, 0.2 mM reduced nicotinamide adenine