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‘Futile cycle’ enzymes in the flight muscles of North American bumblebees

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Summary

In the flight muscles of European bumblebees, high activities of fructose-1,6-bisphosphatase (FbPase) relative to phosphofructokinase (PFK) have suggested a thermogenic ‘futile cycle’ important for regional endothermy. We find generally low activities of FbPase (0.7–19.7 units g^{-1} thorax) in North American *Bombus* species, with the exception of *Bombus rufocinctus*, where activity (43.1 units g^{-1} thorax) is comparable with that of European congeners. These data, taken with estimates of maximal rates of heat production by cycling, do not support a significant thermogenic role for the PFK/FbPase cycle. In agreement with earlier studies, both PFK and FbPase activities were found to scale allometrically with body size (allometric exponents –0.18 and –1.33, respectively). The cycle may serve to supplement thermogenesis or amplify glycolytic flux in rest-to-flight transitions, especially in smaller bees.

Key words: thermogenesis, flight muscle, fructose-1,6-bisphosphatase, phosphofructokinase, futile cycle, enzyme, bumblebee, *Bombus*.

Introduction

It has long been recognized that many insects can regulate the temperature of body regions in the face of changes in ambient temperature (T_a). This pattern of thermoregulation requires mechanisms of heat production (thermogenesis), retention, dissipation and internal distribution. Bumblebees (Hymenoptera; *Bombus* and closely related genera) are generally good thermoregulators. Given their wide geographical distribution it is not surprising, however, to note varying thermoregulatory abilities in this group (Bishop and Armbruster, 1999; Heinrich and Vogt, 1993). During bumblebee flight, most heat is generated as a by-product of flight muscle metabolism, specifically actinomyosin ATPase and oxidative phosphorylation. Considerable thermogenesis is also seen at rest, and this heat may be used to incubate broods (Heinrich and Vogt, 1993), to raise thoracic temperature (T_{th}), allowing for flight at T_as as low as 2.5°C (Heinrich, 1975; Stone and Willmer, 1989), or to maintain elevated T_{th} in non-flying bees, for example when foraging by walking on mass flowers (Prys-Jones, 1985). Within a matter of minutes, resting bumblebees can raise their T_{th} from ~7°C to the 35–40°C necessary for flight muscles to produce sufficient power for flight (Goller and Esch, 1990; Stone and Willmer, 1989).

The mechanisms of heat production in non-flying bees have been debated since the 1970s. One theory holds that heat is generated by an enzymatic ‘futile cycle’ within the flight muscles. Newsholme et al. (1972) found high activities of both phosphofructokinase (PFK) and fructose-1,6-bisphosphatase (FbPase) in the flight muscles of several European *Bombus* species. It was hypothesized that the unusually high (for muscle) activities of FbPase would rapidly hydrolyse fructose-1,6-bisphosphate produced by PFK, providing more substrate for PFK and resulting in a cycle between the two enzymes (Fig. 1). Each turn of this cycle would result in the net hydrolysis of 1 ATP. If it were to function at high rates, this cycle would produce significant amounts of heat that could warm the thorax in the absence of muscular contraction. This mechanism is thought to produce heat in malignant hyperthermia in pigs (Clark et al., 1973b). In *Bombus affinis*, the rate of cycling in resting bees increases as T_a decreases and completely stops when flight is initiated (Clark et al., 1973a). The cycle can be deactivated during flight, as bumblebee FbPase is inhibited by Ca^{2+} (Grieve and Surholt, 1990; Storey, 1978) that is released into the flight muscle cytoplasm when activated by stretching or motor nerve action potentials. Further support for this theory comes from observations that the activities of FbPase among European *Bombus* species correlate negatively with body mass (Newsholme et al., 1972) and positively with foraging activity on massed flowers (Prys-Jones, 1985). From this, it was concluded that bees that foraged primarily by walking on massed flowers could not rely on heat generated by flight muscle contractions and thus there would be selective pressures favouring alternative mechanisms of thermogenesis (Prys-Jones, 1985).

Although elegant, the futile cycle theory has been challenged. Some investigators estimate that the rates of heat production, either at observed *in vivo* cycling rates (Clark et

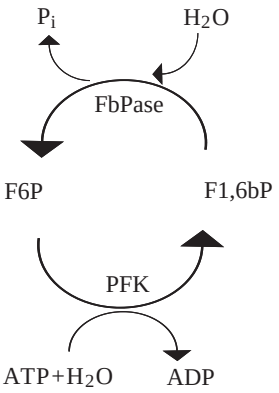

Fig. 1. The reactions catalyzed by phosphofructokinase (PFK) and fructose-1,6-bisphosphatase (FbPase). PFK phosphorylates fructose-6-phosphate (F6P), hydrolyzing ATP in the process. FbPase catalyses the hydrolysis of fructose-1,6-bisphosphate (F1,6bP) to F6P, releasing inorganic phosphate (P_i).

al., 1973a) or at cycling rates defined by maximal PFK and FbPase activities, are insufficient to account for the observed rate of thoracic warming (Kammer and Heinrich, 1978; Newsholme and Crabtree, 1976). Beyond this, it is clear that, during pre-flight warm-up, action potentials are delivered to the flight muscles of *Bombus terrestris*, although there is no apparent movement of the wings (Surholt et al., 1990) and only minute deformations of the thorax (Esch et al., 1991). This has been interpreted as the simultaneous tetanic contraction of the dorsoventral and dorsal longitudinal flight muscles (Esch and Goller, 1991). In this way, metabolic rate and heat production are stimulated through actinomyosin hydrolysis of ATP, but no 'shivering' *per se* is obvious.

If the PFK/FbPase futile cycle is a common thermogenic mechanism, one would predict that activities of both enzymes, especially FbPase, would be high among bumblebees. Although PFK and FbPase activities have been determined in seven *Bombus* species found in western Europe (Newsholme et al., 1972), only one North American species has been examined to our knowledge (Clark et al., 1973a; Newsholme et al., 1972). The purpose of the present study is to measure these enzymes in several bumblebee species from central North America and to relate them to animal mass.

Materials and methods

Animals

North American bees were caught between May and September using butterfly nets and plastic vials with air-holes. Only actively foraging workers were used in this study. They were identified using a dichotomous key (Laverty and Harder, 1988). *Bombus impatiens* (Cresson), *Bombus bimaculatus* (Cresson), *Bombus vagans* (Smith), *Bombus rufocinctus* (Cresson) and *Bombus affinis* (Cresson) were caught in London, Ontario, Canada (42°59′ N, 81°14′ W). *Bombus perplexus* (Cresson), *Bombus griseocollis* (DeGeer) and *Psithyrus citrinus* (Smith) were caught on Amherst Island, Ontario (44°10′ N, 76°44′ W). The European *Bombus terrestris* L.? were obtained from a commercial supplier (Koppert Biological Systems, Haverhill, Suffolk, UK). Bees were fasted overnight in a refrigerator (4°C) and were then placed in a freezer (–20°C). After chill coma was induced (approximately 2 min in freezer), all visible pollen was removed and whole-animal mass was recorded. The thoraxes were dissected and the wings and legs were removed. The thoraxes were then weighed and frozen in liquid nitrogen. Frozen thoraxes were placed in pre-chilled screw-top centrifuge tubes ('cryovials') at –70°C for no more than two months before assay.

Enzyme assays

All assays were performed at 37°C using a Varian DMS80 dual-beam spectrophotometer. The PFK assay was based on the methods of Suarez et al. (1996). Thoraxes were homogenized in nine volumes of buffer containing 25 mmol l^{-1} Tris-KH_2PO_4 (pH 7.8 at 4°C), 2 mmol l^{-1} EDTA, 0.5% (v/v) Triton X-100, 1 mmol l^{-1} fructose-6-phosphate and 0.1% (v/v) β-mercaptoethanol. Glucose-6-phosphate (G6P) was omitted from the homogenization medium to allow the assay of both PFK and FbPase in the same homogenate. Preliminary experiments showed that the omission of G6P did not affect the activity of PFK. Thoraxes were minced with scissors and homogenized on ice with three 15 s passes (30 s between passes) of a small homogenizer (Tissue Tearor). The homogenate was then sonicated on ice using three 15 s pulses (30 s between pulses). The homogenate was then centrifuged at 10 000 ***g*** for 5 min at 4°C and the supernatant was used for enzyme assays.

The PFK assay medium contained 50 mmol l^{-1} Tris (pH 8.0 at 37°C), 10 mmol l^{-1} $MgCl_2$, 100 mmol l^{-1} KCl, 0.1% (v/v) β-mercaptoethanol, 20 mmol l^{-1} fructose-6-phosphate (omitted for determination of control rates), 2 mmol l^{-1} ATP, 0.01 mmol l^{-1} fructose-2,6-bisphosphate, 0.15 mmol l^{-1} NADH, 0.3 units ml^{-1} aldolase (Sigma, St Louis, MO, USA), 3.6 units ml^{-1} triosephosphate isomerase (Sigma) and 0.5 units ml^{-1} glycerol-3-phosphate dehydrogenase (Sigma).

The FbPase assay was based on the method of Storey (1978). FbPase was assayed in the same homogenate used for PFK assays. Preliminary experiments showed no significant difference in activity when compared with homogenates prepared according to Storey (1978). Measuring both PFK and FbPase activities in the same homogenate allowed us to examine the ratio of the activities of the two enzymes among individual animals. The FbPase assay medium contained 50 mmol l^{-1} Tris (pH 7.4 at 37°C), 6 mmol l^{-1} $MgCl_2$, 0.2 mmol l^{-1} fructose-1,6-bisphosphate (omitted for determination of control rates), 0.2 mmol l^{-1} NADP, 10 units ml^{-1} phosphoglucose isomerase (Roche, Laval, QC, Canada) and 2 units ml^{-1} glucose-6-phosphate dehydrogenase (Roche). Coupling enzymes from another supplier (Sigma) appeared to be contaminated with FbPase, giving significant

changes in optical density in the absence of muscle homogenate. In preliminary experiments, supernatants from the crude homogenates were centrifuged through a desalting column to remove small-molecular-mass metabolites (Helmerhorst and Stokes, 1980), but this had no significant effect on FbPase activity.

Data analysis

Enzyme activities were calculated relative to thorax mass. Activities of PFK and FbPase and ratios of FbPase/PFK were compared among species by one-way analysis of variance (ANOVA; α=0.05). Thorax mass-specific enzyme activities and whole-animal masses were $\log_{10}$ transformed, and the relationships analysed by least-squares regression. All statistical calculations and transformations were performed by SigmaStat (version 2.03, SPSS Inc.).

Results

Activities of PFK are significantly higher in *B. vagans* than in *B. terrestris*, *B. perplexus*, *B. griseocollis*, *B. bimaculatus* and *P. citrinus* (P<0.05; Fig. 2; for *B. affinis* N=2, precluding statistical analysis). There are no other significant differences in PFK activity among the species examined. We report activities relative to total thorax mass, and approximately 75% of bumblebee thorax mass is comprised of flight muscle (Nachtigall et al., 1995). From our data, this corresponds to PFK activities of 99.0±9.8 units g^{-1} muscle (N=10) in *B. terrestris*. This value is somewhat higher than those previously reported (62 units g^{-1} muscle) by Newsholme et al. (1972). This is probably due to better preservation of PFK activity through inclusion of substrates and anti-oxidants during homogenization in this study. The values reported here are comparable with those measured in honeybees (*Apis mellifera* L.) using a similar method (Suarez et al., 1996).

FbPase activities in North American bees are generally low, only 0.02–0.3-fold those of *B. terrestris* (Fig. 2). The one exception is *B. rufocinctus*, where FbPase activities are not significantly different from those of *B. terrestris*. FbPase activity could not be detected in *P. citrinus* thoraxes. In *B. terrestris*, our FbPase data correspond to 54.9±4.2 units g^{-1} muscle (N=10), close to the 65 units g^{-1} muscle reported by Newsholme et al. (1972). As reported by other investigators (Newsholme et al., 1972; Storey, 1978), we found *B. terrestris* FbPase to be sensitive to Ca^{2+} (33% inhibition at 0.125 mmol l^{-1}) and fructose-2,6-bisphosphate (41% inhibition at 0.05 mmol l^{-1}; data not shown). No detailed enzyme kinetic studies were performed in this experiment.

The ratio of FbPase to PFK calculated for individual animals is depicted in Fig. 3. Relative to PFK, there is significantly more FbPase in *B. terrestris* than in any of the North American species analysed. The ratio in *B. rufocinctus* is significantly higher than in the other North American bees.

There is an approximately 10-fold range in body mass of bees used in this study, allowing for allometric analysis of enzyme activities among individuals. From Figs 4, 5, it is evident that there is a significant negative allometric relationship between body mass and thorax-mass-specific activity of PFK (P=0.011) and FbPase (P=0.002). For PFK, the allometric exponent is –0.18 (r^2=0.13), while for FbPase it is –1.33 (r^2=0.21).

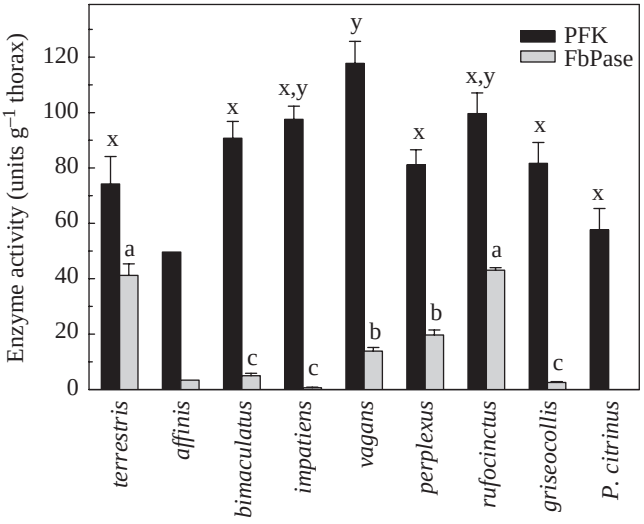

Fig. 2. Activities of phosphofructokinase (PFK) and fructose-1,6-bisphosphatase (FbPase) in bumblebee thoracic flight muscle. Values are means ± S.E.M. Species with the same letters are not significantly different from each other (ANOVA). Sample sizes are as follows: *B. terrestris*, *B. bimaculatus*, *B. impatiens*, *B. vagans*, 10; *B. perplexus*, 5; *B. rufocinctus*, 6; *B. griseocollis*, 5; *B. affinis*, 2; *Psithyrus citrinus*, 3.

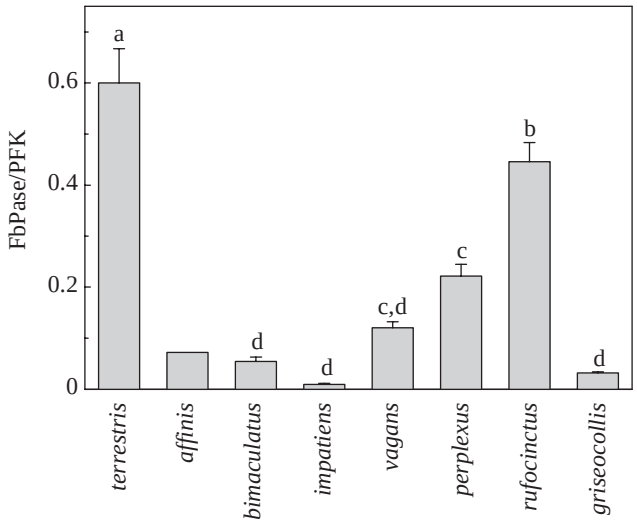

Fig. 3. Ratio of phosphofructokinase (PFK) and fructose-1,6-bisphosphatase (FbPase) activities in flight muscle of individual bumblebees. Values are means ± S.E.M. Species with the same letters are not significantly different from each other (ANOVA). For sample sizes, see Fig. 2.

Discussion

Flight in *B. terrestris* (Bertsch, 1984) and other bees (Rothe and Nachtigall, 1989) is expensive and is powered exclusively by carbohydrate oxidation. Therefore, the primary function of the flight muscle glycolytic pathway is probably to provide pyruvate for mitochondrial oxidative phosphorylation. Indeed, in flying honeybees, flux through PFK is 57% of maximal capacity (Suarez et al., 1996). During tethered flight, *B. terrestris* workers consume oxygen at a rate of 27.5 ml g^{-1} body mass h^{-1} (Wolf et al., 1996). Assuming that virtually all of this oxygen is consumed by flight muscles and that flight muscle comprises 32.4% of body mass (Surholt et al., 1991), this corresponds to a glycolytic flux of 10.5 μmol hexose g^{-1} muscle min^{-1}. This value is within the range of 7.6–19.1 μmol hexose g^{-1} muscle min^{-1} estimated for free-flying male (Surholt et al., 1991) and queen (Silvola, 1984) *B. terrestris*, respectively. This glycolytic flux represents only 10.6% of the maximal PFK catalytic capacity measured in the present study, a low fractional velocity (pathway flux/maximal enzymatic capacity) compared with honeybees (Suarez et al., 1997) and the 26.1% observed in *B. impatiens* workers (J. F. Staples and R. P. Maloney, unpublished data). Due to differences in allometry between PFK activity and whole-animal oxygen consumption (see below), we predict that PFK fractional velocities are higher in smaller bumblebees.

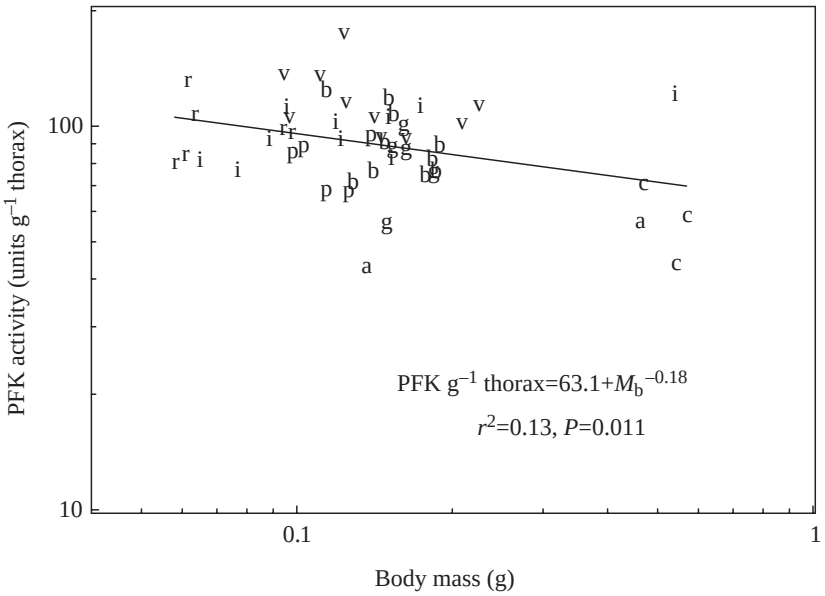

Fig. 4. Allometric scaling of phosphofructokinase (PFK) activity in North American bumblebees. Abbreviations: b, *B. bimaculatus*; i, *B. impatiens*; v, *B. vagans*; r, *B. rufocinctus*; p, *B. perplexus*; g, *B. griseocollis*; c, *Psithyrus citrinus*; a, *B. affinis*; M_b, body mass.

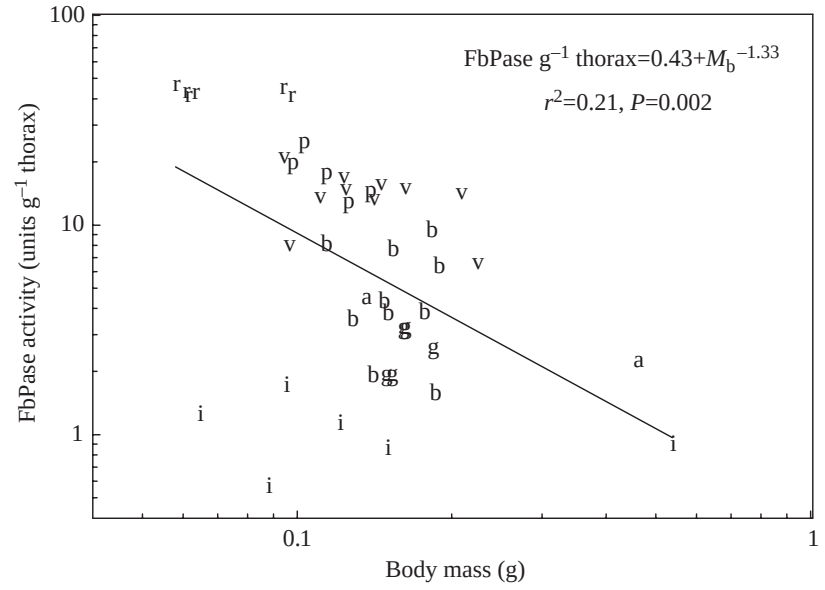

Fig. 5. Allometric scaling of fructose-1,6-bisphosphatase (FbPase) activity in North American bumblebees. For abbreviations, see Fig. 4.

Bumblebee flight muscle contains very low activities of enzymes from gluconeogenic and pentose phosphate pathways (Clark et al., 1973a; Newsholme et al., 1972), limiting the possible roles of FbPase in this tissue. Given the role of PFK in powering flight metabolism, any capacity for thermogenesis by the PFK/FbPase cycle is probably best reflected by FbPase activity, especially when expressed relative to PFK activity. In a broad sampling of European bumblebee species, the average ratio of FbPase/PFK activities was reported to be 1.10, with a range of FbPase activity of 14–114 units g^{-1} muscle (Newsholme et al., 1972). Because of the higher PFK activities in the present study (probably due to an improved homogenization technique; see Results), the FbPase/PFK for *B. terrestris* (0.6) is lower than the 1.05 reported by Newsholme et al. (1972). Despite this, the ratio is significantly higher in *B. terrestris* than in any North American species measured here (Fig. 3). In the North American *B. rufocinctus*, the FbPase/PFK ratio is significantly lower than *B. terrestris* but is still higher than the other North American species sampled here, and the FbPase activity (43.1 units g^{-1} thorax) is not significantly different from *B. terrestris*. In the present study, we found very low FbPase activity in *B. affinis* (3.4 units g^{-1} thorax; N=2; range 2.2–4.5 units g^{-1} thorax). This contrasts with earlier studies that show high (45.3 units g^{-1} muscle) FbPase activities (Clark et al., 1973a). The reasons for this discrepancy are not immediately obvious but may relate to geographic intraspecific differences (Wisconsin *vs* Ontario), the small sample size available for

this study, and potential contamination of the coupling enzymes used by Clark et al. (1973a) in the FbPase assay (see Materials and methods).

To ascertain whether this substrate cycle could functionally contribute to thermogenesis in a whole bumblebee requires, in part, a calculation of how much heat could be produced by the cycle. It is estimated that heat production at a rate of 92.1 J g^{-1} muscle min^{-1} (22 cal g^{-1} muscle min^{-1}) is required to maintain a thoracic temperature 27°C higher than T_a (Heinrich, 1972; Newsholme and Crabtree, 1976). The PFK/FbPase cycle can produce some heat directly from the hydrolysis of ATP, yielding 30.6 kJ mol^{-1} (7.3 kcal mol^{-1}) ATP. The maximal possible cycling rate is determined by the maximal catalytic capacities of the two enzymes. In the present study, the species with the highest FbPase activity (*B. rufocinctus*) could theoretically support a cycling rate of 57.5 μmol g^{-1} muscle min^{-1}. Heat production from ATP hydrolysis due to cycling (7.3 kcal mol^{-1}) would produce only 1.7 J g^{-1} muscle min^{-1} (0.4 cal g^{-1} muscle min^{-1}). Even during flight, the tissue content of ATP in bumblebee flight muscle decreases only slightly (Newsholme et al., 1972). It is, therefore, reasonable to assume that most of the ADP produced in the cycle would be rephosphorylated through the complete oxidation of glucose. This would produce a further 4.6 J g^{-1} muscle min^{-1} (1.1 cal g^{-1} muscle min^{-1}; assuming 686 kcal and 36 mol ATP mol^{-1} glucose oxidized). In total, we estimate that, at maximal cycling rates, the PFK/FbPase cycle could produce less than 7% of the heat required to maintain thoracic temperature on a cold day. Estimates using ^{14}C- and ^{3}H-labelled glucose suggest that maximum *in vivo* cycling rates are only 10.4 μmol g^{-1} muscle min^{-1} (Clark et al., 1973a). This rate of cycling would produce less than 2% of the heat required to keep a bumblebee thorax warm on a cold day. It is worth noting that another study on *B. terrestris* males has estimated that during the pre-flight 'warm-up' phase cycling occurred at a rate of 249 μmol g^{-1} muscle min^{-1}, and this could contribute significantly to thermogenesis (Surholt et al., 1991). We suspect, however, that this result is an artefact of the isotopic method employed, as the reported cycling rate is ~4-fold greater than PFK and FbPase activities found in *B. terrestris* workers (Newsholme et al., 1972). This is unlikely because in other *Bombus* species FbPase activities in males and workers are comparable (Newsholme et al., 1972).

Our data do not support a significant thermogenic role for substrate cycling between PFK and FbPase in bumblebees. This leaves open the question of why *B. rufocinctus* and several European *Bombus* species have relatively high levels of FbPase activity in a tissue with no significant capacities for gluconeogenesis or pentose phosphate metabolism. Operation of this cycle at low levels probably allows for greater sensitivity of PFK and/or FbPase to allosteric regulators and therefore greater amplification of net glycolytic flux in the transition from rest to flight (Newsholme and Crabtree, 1973). Such amplification may be possible with the relatively low FbPase activities found in most North American bees in this study (Newsholme and Crabtree, 1973), although the conditions necessary for significant amplification are limited (Fell, 1997). An amplification role for this cycle does not explain, however, why other regionally endothermic flying hymenopterans, such as *Psithyrus* spp. and *Apis mellifera*, have virtually no FbPase (Newsholme et al., 1972) or why European bumblebees generally have higher FbPase activities than most North American congeners.

Our data do not show any apparent pattern of cycling capacity among *Bombus* subgenera. High FbPase activities were found in members of *Cullumanobombus* (*B. rufocinctus*) and *Bombus (sensu strictu)* (*B. terrestris*), while another member of the latter subgenus has low FbPase activities (*B. affinis*; present study). None of the members of the subgenera *Pyrobombus* (*B. bimaculatus*, *B. perplexus*, *B. impatiens*, *B. vagans*) or *Separatobombus* (*B. griseocollis*) have FbPase activities comparable with those of *B. terrestris* or *B. rufocinctus*. Future studies evaluating the capacity for PFK/FbPase cycling in relation to phylogeny would be informative.

The negative allometric relationship (exponent –0.18) between body mass and mass-specific PFK activity (Fig. 4) reflects the primary role of PFK in powering flight. Whole-animal oxygen consumption in euglossine bees scales with a greater allometric exponent of –0.42 (Casey and Ellington, 1990). If the scaling of bumblebee oxygen consumption follows a similar pattern, it would suggest that glycolytic flux in smaller bees is closer to maximal PFK capacity and would result in higher enzyme fractional velocities. Support for this hypothesis will require a rigorous examination of glycolytic flux and enzyme capacities within individuals of *Bombus* species spanning a large mass range. We are currently performing such experiments. PFK activity in European *Bombus* species was found to scale with an allometric exponent of –0.6 (Newsholme et al., 1972), much higher than the exponent reported in the present study. This difference probably reflects the improved homogenization technique used in the present study, which better preserved PFK activity. It is also worth noting that our analysis was confined to workers, while Newsholme et al. (1972) considered all castes.

FbPase activity scales with an allometric exponent of –1.33 (Fig. 5), comparing favourably with the exponent of –1.4 found in European bumblebees (Newsholme et al., 1972). This more intense scaling relative to PFK is consistent with a thermogenic role of the PFK/FbPase cycle – smaller bees cool more quickly, and one may predict that the capacity for thermogenesis by cycling would increase more quickly than the maximal capacity for glycolytic flux (reflected by mass-specific PFK activity). Our data suggest, however, that in *B. rufocinctus* this cycle could contribute, at most, 7% of the required heat to maintain T_{th} on a cold day and that other mechanisms of thermogenesis would be more important. Cycling may contribute more significantly when differences between T_a and T_{th} are less than 27°C. The differential scaling of PFK and FbPase may also indicate that smaller bees have a greater need for amplification of glycolytic flux when moving from rest to flight.

In summary, we found generally low levels of FbPase activity in the flight muscles of North American bumblebees, with the exception of *B. rufocinctus*. This results in low capacities for substrate cycling compared with European *Bombus* species. Combined with our calculation of maximal rates of heat production, these data do not support a significant thermogenic role of a PFK/FbPase cycle. This cycle may serve to amplify glycolytic flux in rest-to-flight transitions. Both PFK and FbPase activities show negative allometric scaling with body mass, but the scaling of FbPase activity is much more intense. These results are consistent with the negative allometric scaling of whole-animal oxygen consumption and a greater role for glycolytic amplification by PFK/FbPase cycling in smaller bees.

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