

RESEARCH ARTICLE

Intraspecific variation in flight metabolic rate in the bumblebee *Bombus impatiens*: repeatability and functional determinants in workers and drones

Charles-A. Darveau*, Fannie Billardon and Kasandra Bélanger

ABSTRACT

The evolution of flight energetics requires that phenotypes be variable, repeatable and heritable. We studied intraspecific variation in flight energetics in order to assess the repeatability of flight metabolic rate and wingbeat frequency, as well as the functional basis of phenotypic variation in workers and drones of the bumblebee species *Bombus impatiens*. We showed that flight metabolic rate and wingbeat frequency were highly repeatable in workers, even when controlling for body mass variation using residual analysis. We did not detect significant repeatability in drones, but a smaller range of variation might have prevented us from finding significant values in our sample. Based on our results and previous findings, we associated the high repeatability of flight phenotypes in workers to the functional links between body mass, thorax mass, wing size, wingbeat frequency and metabolic rate. Moreover, differences between workers and drones were as predicted from these functional associations, where drones had larger wings for their size, lower wingbeat frequency and lower flight metabolic rate. We also investigated thoracic muscle metabolic phenotypes by measuring the activity of carbohydrate metabolism enzymes, and we found positive correlations between mass-independent metabolic rate and the activity of all enzymes measured, but in workers only. When comparing workers and drones that differ in flight metabolic rate, only the activity of the enzymes hexokinase and trehalase showed the predicted differences. Overall, our study indicates that there should be correlated evolution among physiological phenotypes at multiple levels of organization and morphological traits associated with flight.

KEY WORDS: Repeatability, Metabolic rate, Flight, Wingbeat frequency, Metabolism, Muscle, Enzyme, Hexokinase, Trehalase, Bumblebee, Worker, Drone

INTRODUCTION

Insect species vary widely in flight performances, from long-distance flight in groups such as butterflies to highly manoeuvrable flight achieved by bees. Species diversity in flight energetics has been shown to be associated with wing and body morphology (Casey, 1976; Bartholomew and Casey, 1978; Casey et al., 1985; Byrne et al., 1988; Harrison and Roberts, 2000; Lehmann, 2002; Darveau et al., 2005a), and such variation can have an impact on muscle cell metabolic properties (Crabtree and Newsholme, 1972; Beenakkers et al., 1975; Saktor, 1975; Suarez, 2000; Darveau et al., 2005b). Broad comparisons among insect groups have highlighted

basic principles of energetic and metabolic design, but how it translates to closely related species or within species have yet to be fully resolved.

Studies conducted on a group of orchid bees have shown that flight wingbeat frequency decreased with species body mass, and genera with larger wings have a lower wingbeat frequency and flight metabolic rate (Casey et al., 1985). It was further shown that after controlling for body mass and phylogenetic relatedness, correlations between wing proportion (wing loading), wingbeat frequency and mass-specific metabolic rate were found among species within and across genera (Darveau et al., 2005a). Moreover, species variation in metabolic rate was shown to affect flight muscle metabolic phenotype, mainly the activity of the glycolytic enzyme hexokinase (Darveau et al., 2005b). These studies show strong links between form and function in flight energetics among closely related species, where a macroevolutionary framework suggests correlated evolution among morphological, kinematic, metabolic and cellular traits.

Macroevolutionary studies suggesting correlated evolution among traits are useful to investigate functional association between physiological traits. Such patterns must arise from variation within a species and evolutionary mechanisms acting on such variation (Bennett, 1987; Clark and Wang, 1994). Within a bumblebee species (*Bombus impatiens*), individuals with larger wings had lower wingbeat frequency during flight, metabolic rate was positively correlated with wingbeat frequency, and the activity of some flight muscle enzymes positively correlated with metabolic rate (Skandalis and Darveau, 2012). This shows that the main traits associated with flight energetics in bees vary within species and are functionally linked; thus selection acting on traits affecting body size or wing size can drive the evolution of a suite of physiological traits. Nevertheless, we still do not know the stability of these traits over time (its repeatability), and ultimately the heritability of these functionally associated variables.

Repeatability of metabolic rate has been investigated in many groups of animals under various conditions. Nespolo and Franco (Nespolo and Franco, 2007) conducted a meta-analysis of the repeatability of metabolic rate in a range of animals and in resting and active states. The vast majority of studies reviewed showed that metabolic rate is a repeatable trait, but most investigated metabolic rate in a resting state, few studied insect species, and none investigated flight metabolic rate in insects. Other aspects of insect flight performance have been shown to be repeatable, such as flight time and distance (Tanaka, 2009), suggesting that individual characteristics, in part, dictate flight performance in a predictable way.

The aim of this study was to test for the repeatability of flight energetics traits, namely hovering flight wingbeat frequency and metabolic rate, using the bumblebee species *B. impatiens*. This work follows a series of investigations on intraspecific variation in energetic properties during flight (Skandalis et al., 2011; Skandalis

Department of Biology, University of Ottawa, 30 Marie Curie, Ottawa, ON, K1N 6N5, Canada.

*Author for correspondence (cdarveau@uottawa.ca)

Received 31 May 2013; Accepted 7 October 2013

List of abbreviations

CS	citrate synthase
GP	glycogen phosphorylase
HK	hexokinase
PGI	phosphoglucosomerase
PK	pyruvate kinase
TR	trehalase

and Darveau, 2012). This work also further tests intraspecific variation by investigating sexual dimorphism known to be present between workers and drones in other bee species (Radloff et al., 2003). This approach allows further testing of how wing and body size affect wingbeat frequency, metabolic rate and thoracic metabolic phenotype.

RESULTS**Interindividual variation, sexual dimorphism and repeatability of flight measurements**

Individual *B. impatiens* varied in body mass from 50 to 226 mg. Consecutive measurements of body mass separated by 2 days showed a correlation coefficient of 0.925 ($P < 0.001$). Wing surface area was strongly dependent on body mass (Fig. 1A, Tables 1, 2; mass: $P < 0.001$, sex: $P < 0.001$, mass \times sex: $P = 0.001$; $r^2 = 0.945$), but wing surface of drones increased with body mass with a shallower slope than workers (scaling exponent b values of 0.25 and 0.82, respectively). Similarly, forewing length showed a strong dependence on body mass and difference between sexes (Tables 1, 2; mass: $P < 0.001$, sex: $P < 0.001$, mass \times sex: $P = 0.001$; $r^2 = 0.958$). The calculated wing loading increased with body mass with a steeper slope in drones than workers (Fig. 1B, Table 2; mass: $P < 0.001$, sex: $P < 0.001$, mass \times sex: $P = 0.001$; $r^2 = 0.617$). Thorax mass increased with body mass (Fig. 1C, Table 2; mass: $P < 0.001$, sex: $P < 0.001$, mass \times sex: $P < 0.001$; $r^2 = 0.932$) with a shallower slope in drones ($b = 0.41$) than workers ($b = 0.97$). Wing surface area increased with thorax mass with the same slopes but different intercepts in workers and drones (Fig. 2A; mass: $P < 0.001$, sex: $P < 0.001$; $r^2 = 0.923$). In addition, residual variation accounting for body mass and sex shows a strong positive association between wing surface area and thorax mass in both workers and drones (Fig. 2B; combined: $r = 0.628$, $P < 0.001$; workers: $r = 0.452$, $P = 0.018$; drones: $r = 0.789$, $P < 0.001$).

Among all bees, wingbeat frequency varied from 149 to 248 Hz and decreased significantly with body mass. At any given body mass, drones flew with lower wingbeat frequencies than did workers (Fig. 3A; mass: $P = 0.001$, sex: $P < 0.001$, $r^2 = 0.678$). When analyzed separately, the relationship with body mass was significant for workers ($P = 0.006$) but not for drones ($P = 0.125$). Both sets of wingbeat frequency measurements were strongly correlated ($r = 0.911$, $P < 0.001$). Using residuals accounting for the effects of mass and sex, wingbeat frequency remained highly repeatable for all bees combined ($r = 0.743$; Fig. 4A, Table 3). Analyses performed on workers and drones separately show that this relationship remained highly significant for workers ($r = 0.843$, $P < 0.001$), but not for drones ($r = 0.071$, $P = 0.779$).

Metabolic rate measurements ranged from 2.0 to 24.5 ml CO₂ h⁻¹ per animal, or 41.7 to 163.3 ml CO₂ h⁻¹ g⁻¹ when expressed on a mass-specific basis. Metabolic rate increased significantly with body mass, and drones had lower metabolic rate than workers (Fig. 3B; mass: $P < 0.001$, sex: $P < 0.001$, $r^2 = 0.617$). The relationship with body mass was significant for both sexes when analyzed separately. Mass-specific metabolic rate was size invariant, but differed between sexes (mass: $P = 0.349$, sex: $P < 0.001$, $r^2 = 0.364$). Analysis of

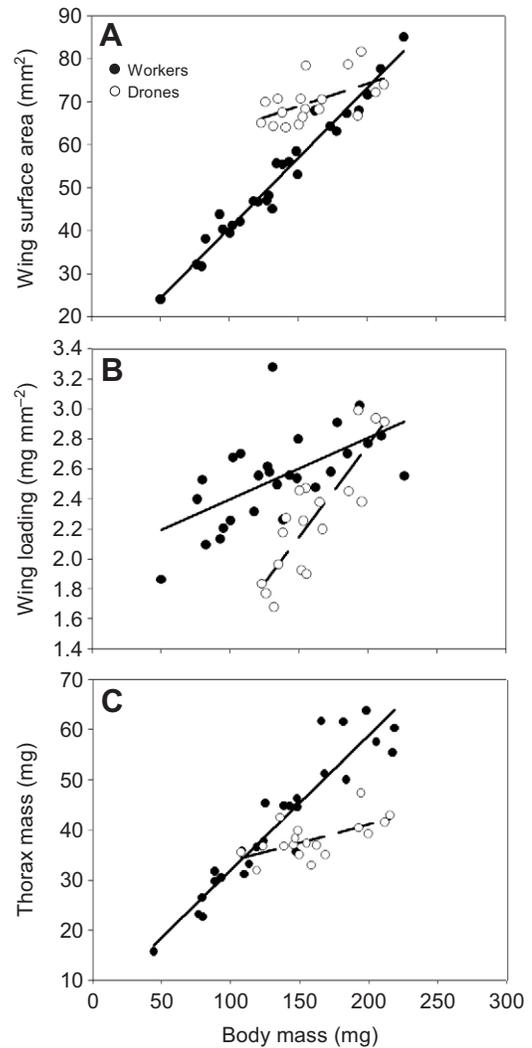


Fig. 1. Allometry of morphological variation in worker and drone bumblebees. Relationship between body mass and (A) wing surface area, (B) wing loading and (C) thorax mass in worker and drone bumblebees (*Bombus impatiens*). For all variables, the slope of the relationship differed between workers and drones (see Results and Table 1 for statistics).

metabolic rate performed using balanced samples showed a significant interaction term where drones follow a shallower slope than workers (not shown; mass: $P < 0.001$, sex: $P = 0.292$, mass \times sex: $P = 0.009$; $r^2 = 0.770$). Consecutive measurements of metabolic rate were correlated with a coefficient of 0.855 ($P < 0.001$), and remained correlated when expressed on a mass-specific basis ($r = 0.772$, $P < 0.001$). Residuals of whole-animal metabolic rate accounting for both body mass and sex were positively correlated with a coefficient of 0.624 (Fig. 4B, Table 3). Repeatability analyses performed on workers and drones separately show that the correlation remained significant for workers ($r = 0.726$, $P < 0.001$), but not for drones ($r = 0.232$, $P = 0.355$).

Wingbeat frequency residuals accounting for body mass and sex effects were not correlated with wing surface area residuals or other wing size measures. Metabolic rate residuals were positively correlated with thorax mass residuals (Fig. 5A; $r = 0.451$, $P = 0.002$) and with wingbeat frequency residuals (Fig. 5B; $r = 0.617$, $P < 0.001$). Analysis performed with both variables combined confirmed that individuals with both relatively larger thorax and higher wingbeat

Table 1. Mean (\pm s.e.m.) values of morphological, flight energetics and thorax metabolic properties measured in worker and drone bumblebees (*Bombus impatiens*)

Variable	Workers (N=27)	Drones (N=18)	P
Body mass (mg)	135.6 \pm 8.6	160.5.5 \pm 6.5	0.041 ^a
Wing surface area (mm ²)	52.14 \pm 2.9	70.0 \pm 1.2	<0.001 ^b
Forewing length (mm)	9.18 \pm 0.26	10.68 \pm 0.11	<0.001 ^b
Wing loading (mg mm ⁻²)	2.54 \pm 0.06	2.27 \pm 0.09	<0.001 ^b
Thorax mass (mg)	41.2 \pm 2.6	38.2 \pm 0.9	<0.001 ^b
Wingbeat frequency (Hz)	200.8 \pm 4.1	160.9 \pm 1.5	<0.001 ^c
Metabolic rate (ml CO ₂ h ⁻¹)	15.5 \pm 1.3	11.6 \pm 0.6	<0.001 ^c
Glycogen phosphorylase (U g ⁻¹ thorax)	8.0 \pm 0.5	7.8 \pm 0.4	0.818 ^a
Trehalase (U g ⁻¹ thorax)	31.9 \pm 1.2	28.0 \pm 1.2	0.032 ^a
Hexokinase (U g ⁻¹ thorax)	78.9 \pm 2.5	70.2 \pm 1.5	0.012 ^a
Phosphoglucosomerase (U g ⁻¹ thorax)	335.1 \pm 19.9	423.0 \pm 24.0	0.007 ^a
Pyruvate kinase (U g ⁻¹ thorax)	95.6 \pm 5.0	90.3 \pm 5.1	0.478 ^a
Citrate synthase (U g ⁻¹ thorax)	359.0 \pm 18.9	321.7 \pm 10.8	0.140 ^a

^aP-values represent the effect of sex.

^bP-values indicate a significant interaction term between body mass and sex, or difference in slope.

^cP-values indicate the effect of sex with body mass as a significant covariate, or difference in intercept.

frequency had relatively higher flight metabolic rates (thorax mass residuals: $P < 0.001$; wingbeat frequency residuals: $P < 0.001$; $r^2 = 0.556$). Analyses performed on workers and drones separately show that the relationship between metabolic rate and thorax mass remain for both sexes (workers: $r = 0.455$, $P = 0.017$; drones: $r = 0.574$, $P = 0.013$), but with wingbeat frequency it was significant only for workers ($r = 0.729$, $P < 0.001$) and not drones ($r = -0.367$, $P = 0.134$).

Metabolic rate and metabolic enzyme activity

The activity of all enzymes measured was correlated with flight metabolic rate (Fig. 6); analyses were performed using residuals accounting for variation associated with body mass and sex for metabolic rate, and sex for enzymes that differed between workers and drones, namely trehalase (TR), hexokinase (HK) and phosphoglucosomerase (PGI) activity (Table 1). Using balanced samples yielded similar results, except that we lost significance for citrate synthase (CS) and TR, which also did not differ between sexes. Finally, when sexes were analyzed separately, all relationships remained significant for workers, but no relationships were significant for drones.

DISCUSSION

Repeatability of flight metabolic rate and wingbeat frequency

Short-term repeatability of metabolic rate and wingbeat frequency was high in flying bumblebees but appeared significant only for

workers and not for drones. This work shows that, at least for workers, consistent individual variation in flight energetics is observed and probably the outcome of morphological and physiological traits. The high repeatability estimates found here provide an upper limit to heritability estimates of these plastic physiological traits (Falconer and Mackay, 1996) (but see Dohm, 2002; Naya, 2010), and the potential response to selection of these flight phenotypes ultimately gives rise to species physiological traits (Casey, 1976; Casey et al., 1985; Darveau et al., 2005a).

Repeatability of metabolic rate during locomotion in insects has been documented during running in beetles (Rogowitz and Chappell, 2000), and a recent study reported significant repeatability for peak flight metabolic rate in a butterfly species (Niitepöld and Hanski, 2013). These studies showed repeatability estimates for mass-independent metabolic rate that are generally high, and our findings support high repeatability of metabolic rate during locomotion in another insect species. In addition, wingbeat frequency has been reported to be repeatable in free-flying mosquitoes, suggesting it is heritable (Robertson et al., 2002). Other flight properties have been investigated, such as the study of Tanaka (Tanaka, 2009) who showed that beetles measured on a flight mill have significant repeatability for traits such as total flight time, longest and mean single flight, presence or absence of flights and number of flights. Such flight properties have also been shown to be heritable and to respond to selection (Tanaka, 2009; Han et al., 2009), but the extent to which this applies to traits such as metabolic rate remains unknown.

Table 2. Relationship between body mass, morphological parameters and flight energetics measurements for worker and drone bumblebees (*Bombus impatiens*)

Variable	Gender	a	b	r ²	P
Wing surface area (mm ²)	Workers	-0.04 (0.07)	0.82 (0.03)	0.96	<0.001
	Drones	1.31 (0.195)	0.25 (0.09)	0.32	0.014
Forewing length (mm)	Workers	0.09 (0.05)	0.41 (0.02)	0.92	<0.001
	Drones	0.73 (0.12)	0.13 (0.06)	0.27	0.026
Wing loading (mg mm ⁻²)	Workers	-0.09 (0.11)	0.23 (0.05)	0.47	<0.001
	Drones	-1.57 (0.31)	0.87 (0.14)	0.71	<0.001
Thorax mass (mg)	Workers	-0.45 (0.89)	0.97 (0.04)	0.96	<0.001
	Drones	0.68 (0.23)	0.41 (0.10)	0.49	0.001
Wingbeat frequency (Hz)	Workers	2.62 (0.11)	-0.15 (0.05)	0.24	0.009
	Drones	2.40 (0.12)	-0.09 (0.05)	0.15	0.116
Flight metabolic rate (ml CO ₂ h ⁻¹)	Workers	-1.71 (0.40)	1.35 (0.19)	0.67	<0.001
	Drones	-0.89 (0.47)	0.89 (0.21)	0.52	0.001

Least-squares regression analyses were performed using log-transformed data such that $\log Y = \log a + b \log X$, where X is body mass in mg. Standard errors are presented in parentheses.

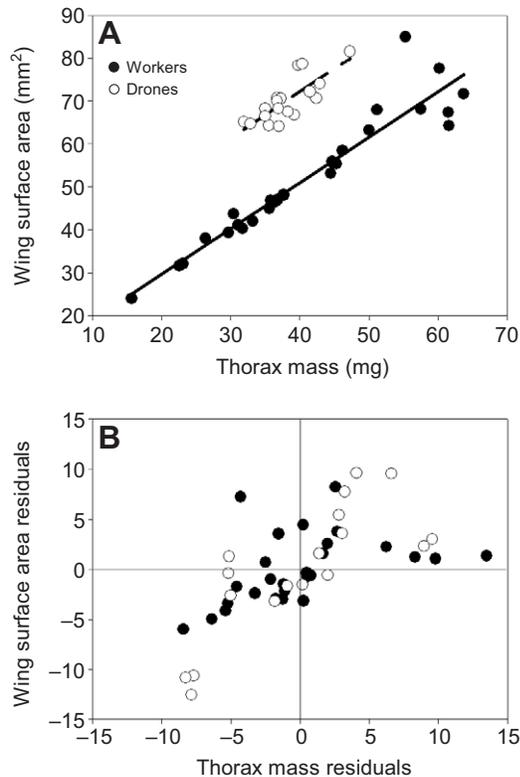


Fig. 2. Wing surface area variation is associated with thorax mass.

(A) Relationship between thorax mass and wing surface area in worker and drone *Bombus impatiens*. (B) Correlation between residuals obtained from the regression with body mass for thorax mass and wing surface area. Correlations were significant when workers and drones were analyzed separately or combined ($P < 0.05$).

The 2-day period separating our measurements potentially influenced the repeatability estimates we obtained. Other studies reporting repeatability of locomotion in insects also used a period of 2–3 days between measurements (Rogowitz and Chappell, 2000; Tanaka, 2009; Niitepõld and Hanski, 2013), allowing comparison with our results. The study of Rogowitz and Chappell (Rogowitz and Chappell, 2000) reported repeatability estimates for running metabolic rate, and for one beetle species measurements separated by 2 or 4 days yielded similar repeatability values of 0.64–0.69. Comparison of studies performed by Nespolo and Franco (Nespolo and Franco, 2007) also suggest that the time between measurements did not have an impact on repeatability estimates, which appears supported by other studies in mammals (Konarzewski et al., 2005). Repeatability of metabolic rate (resting and recovery from exercise in fish) has also been shown to gradually decline over time (Norin and Malte, 2011), and a recent study suggested that this may be a general tendency (White et al., 2013). Metabolic phenotype during locomotion in bumblebees is probably relatively stable in adult life stage, averaging 60 days in workers of this species (Gradish et al., 2010), similar to recent observations on another flying insect (Niitepõld and Hanski, 2013). Nevertheless, transitions during maturation (Skandalis et al., 2011) and possibly senescence can affect repeatability estimates.

Determinants of individual flight energetics

In previous work on the same species we showed an association between body mass, wing morphology and individual variation in

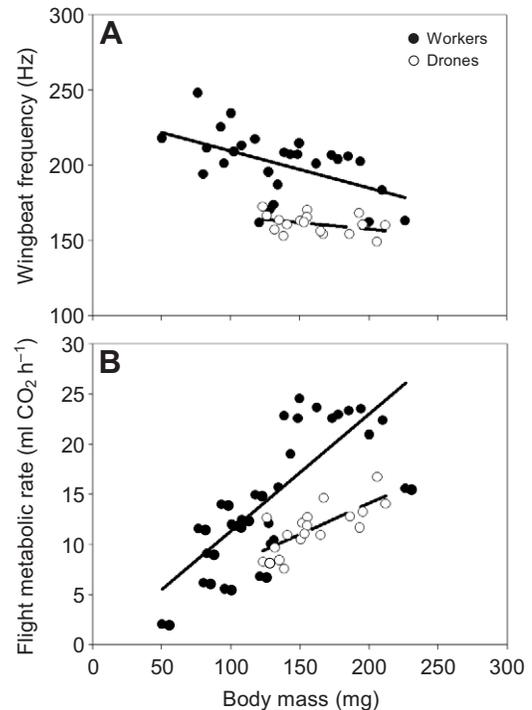


Fig. 3. Allometry of flight energetics variation in worker and drone bumblebees. Relationship between body mass and (A) wingbeat frequency and (B) metabolic rate during hovering flight for worker and drone *Bombus impatiens*. Significant effects of body mass and sex were found for both flight variables (see Results and Table 1 for details).

wingbeat frequency (Skandalis and Darveau, 2012), which was similar to patterns observed among species of bees (Darveau et al., 2005a). This association could not be detected in the current study, probably due to the relatively small sample size ($N=27$ for workers) as a correlation between wing surface area and wingbeat frequency residuals was found by Skandalis and Darveau (Skandalis and Darveau, 2012) and was reproduced using an independent data set including 352 workers (F.B. and C.-A.D., unpublished observations). Therefore, we suggest that the repeatability in wingbeat frequency is due to individual differences in wing morphology, a fixed trait for the majority of the life of these animals. The substantial wing wear occurring over the lifetime of a bumblebee (Foster and Cartar, 2011) should have an impact on the extent of repeatability of that trait.

The significant repeatability in flight metabolic rate for workers can also be explained by the functional association linking flight form and function in insects (Casey, 1976; Casey et al., 1985; Darveau et al., 2005a; Skandalis and Darveau, 2012), and as presented in Fig. 5. The relationship observed between wingbeat frequency and metabolic rate, independent of body size, illustrates the effect of individual variation in muscle contraction frequency on metabolic energy expenditure. It is noteworthy that variation in residual flight metabolic rate is also associated with residual variation in thorax mass, which is another trait probably fixed over the majority of the adult life stage.

Muscle metabolic phenotype was characterized using the activity of a suite of metabolic enzymes based on previous work (Suarez, 2000; Darveau et al., 2005b; Suarez et al., 2005a; Suarez et al., 2005b; Skandalis and Darveau, 2012). We found a high number of positive correlations, including the enzyme glycogen phosphorylase (GP), TR and HK as predicted from interspecific

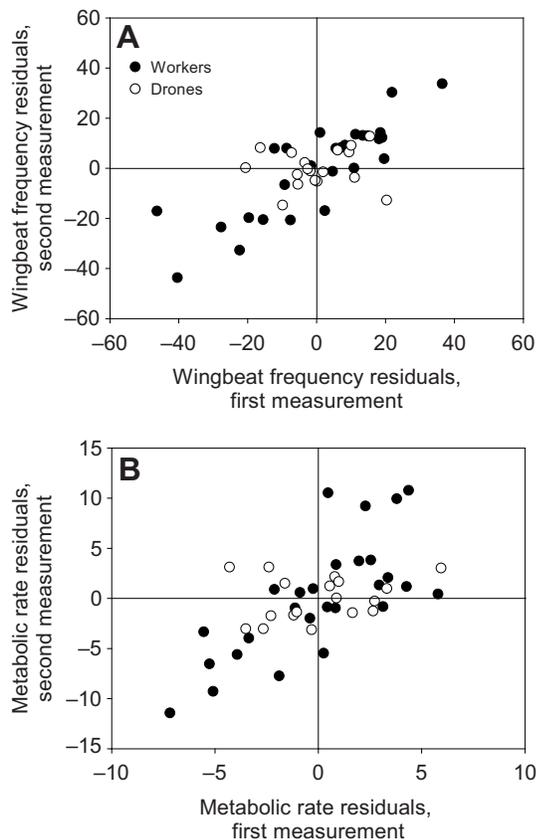


Fig. 4. Repeatability of flight energetics in a bumblebee. Repeatability of (A) wingbeat frequency and (B) metabolic rate measurements during hovering flight in worker and drone *Bombus impatiens* (see Table 3 for details of the repeatability estimates and statistics).

and intraspecific studies (Darveau et al., 2005b; Skandalis and Darveau, 2012), but also the activity of other glycolytic enzymes (PGI and pyruvate kinase) and the mitochondrial enzyme CS. These results suggest that individuals with high metabolic rate show an overall increase in enzyme content, at least in workers, supporting the hypothesis that metabolic enzymes may form a correlated suite (Clark and Wang, 1994). In previous work on the same species we found that metabolic rate correlated with the activity of TR and HK but not PGI (Skandalis and Darveau, 2012), but variation among multiple colonies or even experimental variation in activity estimates could explain these differences. These findings warrant further work on the coordinated changes among functionally linked enzymes.

Differences between workers and drones

Drones are distinct from workers in many respects. Morphologically, the wing surface area of drones increases with body mass, but with a shallower slope than in workers. Wing surface area of workers departs from isometry and shows negative allometry, but this estimate is probably influenced by the modest sample size, as previous studies involving a larger number of individuals showed isometric exponent values (Buchwald and Dudley, 2010; Skandalis and Darveau, 2012). Nevertheless, it is clear that drones have a shallower slope with negative allometry, which might impose constraints on larger individuals and advantages to smaller ones as wing loading of drones follows steeper changes with body mass than workers. Our data show a point of convergence between workers and drones at around 200 mg, which coincides with the largest drones collected in our sample. Body mass of worker and drone could differ due to different proportions of honey stomach content, as in the species *Bombus terricola* nectar load corresponds to 10 and 30% of body mass in workers and drones, respectively (Heinrich and Heinrich, 1983). A similar difference in our species would magnify the proportion of wing surface area to body mass, but would not be likely to explain the difference in slope between sexes. In addition, it appears that the differences between sexes in the relationship of wing size to body mass is in part driven by thorax size as the difference in slope disappears when wing surface area is related to thorax mass. Nevertheless, workers and drones remain clearly distinct, with drones having larger wings for any given thorax mass. Developmental allometry and common mechanisms guiding thorax and wing growth are probably at play to explain these sex differences (Hartfelder and Engels, 1992; Nijhout and Grunert, 2010; Perrard et al., 2012).

The largest drones have similar size wings to workers, but have smaller thoraxes. A 100 mg drone has a thorax to body mass proportion of 31% and a 200 mg individual only 20%, while for workers this ratio varies only from 32 to 29% over the same body mass range. Comparisons of drone and worker mean values for several species of Asian honeybees (Radloff et al., 2003) and the European honeybee (Coelho, 1991) show that drones are larger, they have larger wings as they show lower wing loading, and the thorax mass to body mass ratio is slightly higher. In a bumblebee, we also find lower wing loading in drones but they do not have larger thorax mass. Our study further shows that interpretations of mean values alone should be taken with caution, given that workers and drones can follow different scaling trajectories.

In assessing sexual dimorphism of the flight apparatus, Radloff et al. (Radloff et al., 2003) used the excess power index, which is calculated as a ratio of thorax mass to body mass divided by wing loading (Hepburn et al., 1998; Hepburn et al., 1999), and emphasized that differences in morphology between workers and

Table 3. Repeatability of metabolic rate and wingbeat frequency measurements separated by 48 h in hovering bumblebees (*Bombus impatiens*)

Variable	Gender	Repeatability estimate	
		Pearson correlation coefficient (<i>r</i>)	Intraclass correlation coefficient (τ)
Wingbeat frequency	Workers (<i>N</i> =27)	0.843*	0.846*
	Drones (<i>N</i> =18)	0.071	0.096
	Combined	0.743*	0.745*
Metabolic rate	Workers (<i>N</i> =27)	0.726*	0.647*
	Drones (<i>N</i> =18)	0.232	0.254
	Combined	0.624*	0.706

Note: repeatability estimates were assessed on residual values taken from ANCOVAs accounting for body mass and gender.

**P*<0.001.

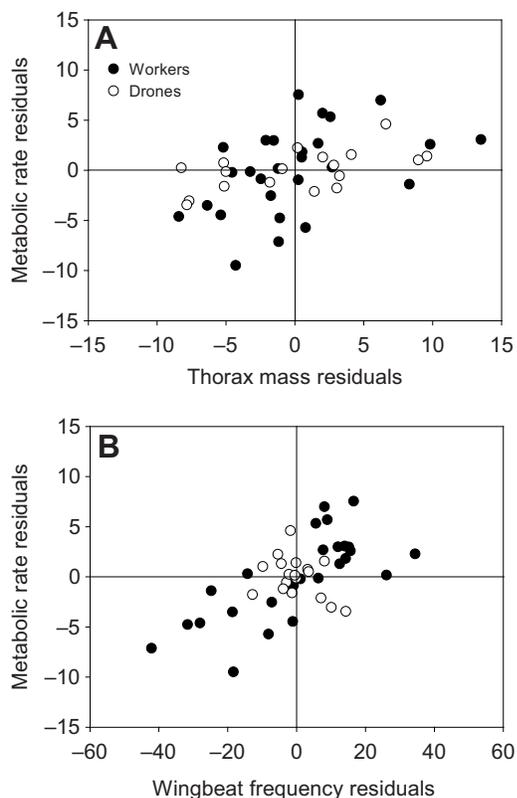


Fig. 5. Flight metabolic rate variation is associated with thorax mass and wingbeat frequency. Correlation between residuals obtained from the body mass relationship for flight metabolic rate and (A) residual thorax mass and (B) residual wingbeat frequency. Correlations were significant for workers, but for drones only the correlation with thorax mass was significant (see Results for details).

drones should have some functional impact such as reduced flight cost that may enhance flight duration. The most common mate-location mechanism in bumblebees consists of males patrolling regular circuits (Goulson, 2010). In this case, drones would benefit from increased forward flight duration and distance, which might in part explain differences of flight morphology and energetics. Our previous work based on interspecific and intraspecific studies showed reduced flight metabolic rate associated with lower wing loading (Darveau et al., 2005a; Skandalis and Darveau, 2012), which could ultimately increase flight duration, assuming constant nectar loads. Additional measures that capture the breadth of flight performance such as speed, duration and distance, would help identify if there are indeed advantages and constraints associated with worker and drone morphology.

Sexual dimorphism offers further insights into flight energetics. Drones have larger wings, lower flight wingbeat frequency and lower metabolic rate than workers. Moreover, larger drones converge with workers for wingbeat frequency and wing surface area (Fig. 1A, Fig. 3A), supporting the functional association between wing morphology and flight kinematics previously documented in this species (Skandalis and Darveau, 2012) and among species of bees (Darveau et al., 2005a). However, larger drones appear to diverge from workers for flight metabolic rate (Fig. 3B), as well as thorax mass (Fig. 1C), in line with the association found between those variables (Fig. 5A). The lower flight metabolic rate of drones is also associated with lower activity of the metabolic enzymes TR and HK expressed per unit thorax mass. This suggests that flight muscle

metabolic phenotype differs between sexes assuming that the proportion of thoracic muscle mass is maintained. These differences support the association between metabolic rate and flight muscle phenotype previously documented in this species (Skandalis and Darveau, 2012) and among bee species (Darveau et al., 2005b). Nevertheless, PGI was higher in drones, which is opposite to the predicted difference. What imposes sex-dependent differences in this muscle glycolytic enzyme and how they are associated with metabolism is not clear. The mechanisms driving these apparent differences in flight muscle metabolic phenotypes remain unresolved, but plasticity following development of individuals or sexes with different wing size remains the first candidate.

Conclusion

Flight metabolic rate and wingbeat frequency are highly repeatable in bumblebee workers, fulfilling basic conditions for these flight energetics traits to respond to selection. The lack of repeatable estimates in drones should be interpreted with caution, as the narrower range of variation alone, rather than a lack of common mechanisms observed in workers, could explain these differences. Repeatability estimates using intraclass correlation analyses over multiple repeated measures would help clarify this point. The repeatability of these traits is probably associated with their functional associations with morphological traits, namely body mass, thorax mass and wing size. The functional links among those traits are further supported by differences observed between workers and drones. Sexual dimorphism and its impact on flight metabolic rate also had the expected impact on the thorax metabolic phenotype, showing lower activity of the enzymes HK and TR. Our study further supports the correlated evolution of physiological phenotypes at multiple levels of organization and morphological phenotypes associated with flight.

MATERIALS AND METHODS

Animals and repeatability measurements

Bumblebees (*Bombus impatiens* Cresson 1863) used in this study were purchased from a commercial supplier (Biobest Canada Ltd, Leamington, ON, Canada). A total of 45 individuals were used for measurements, where 27 workers and 18 drones were considered. The colony was kept in its housing box in a room maintained at 25°C, with access to sucrose solution and pollen.

Individuals were captured, and flight measurements were performed within minutes. The bees were then immobilized by placing them in the refrigerator at 4°C, weighed to the nearest 0.1 mg using an analytical balance (Mettler Toledo, Greifensee, Switzerland), and identified using a numbered tag glued on the thorax. The bees were then placed back in their colony, and a second series of the same measurements was performed 48 h later.

Flight measurements

Flight metabolic rate measurements were performed using a FoxBox flow-through respirometry system (Sable Systems International, Las Vegas, NV, USA), where the animal was placed in a 500 ml chamber (500 ml glass bottle with side arm) to hover. The system was connected to a laptop computer and data were acquired and analyzed using Expedata (Sable Systems International). Only the rate of CO₂ production was considered as all bee species studied so far appear to power flight using carbohydrates as a sole fuel source (Suarez et al., 2005a). The CO₂ detector was calibrated daily, using nitrogen and a 402 ppm span gas. The baseline CO₂ level was measured before and after each measurement. Dry air was pushed into the 500 ml glass flask using PharmMed BPT tubing (Fisher Scientific, Pittsburgh, PA, USA) at a rate of 500 ml min⁻¹. Flight durations of 3–5 min were sufficient to obtain a period of good quality flight, where individuals hovered away from walls (Skandalis and Darveau, 2012). We accepted flight metabolic rate measurements that showed a stable CO₂ production rate for more than 30 s of good quality flight during the flight trial.

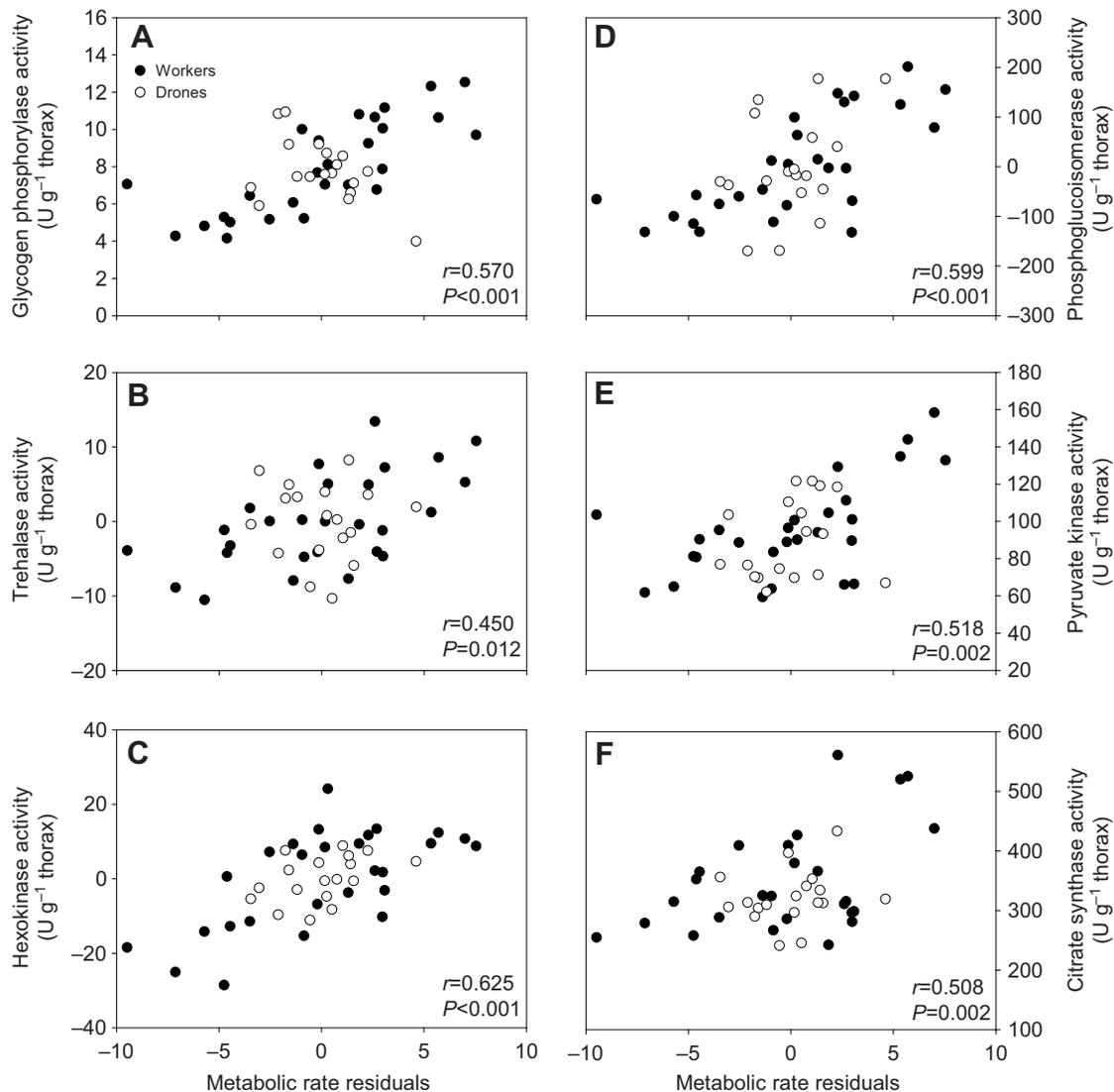


Fig. 6. Flight metabolic rate variation is associated with muscle metabolic enzyme activities. Correlation between flight metabolic rate residuals and the activity of the flight muscle enzymes (A) glycogen phosphorylase, (B) trehalase, (C) hexokinase, (D) phosphoglucosomerase, (E) pyruvate kinase and (F) citrate synthase. For enzymes that differed between sexes (trehalase, hexokinase and phosphoglucosomerase) residuals accounting for sex were analyzed. Significant correlations were detected for all enzymes in workers, but none for drones (see Results for details).

Wingbeat frequency measurements were performed simultaneously using an optical detector under the flight chamber. The signal was acquired and analyzed using the software Trex 2.0 Transient Waveform Recorder (Moore Scientific). Measurement period was set to 0.5 s, and the individual value is the average of 10 measurements taken during the flight trial.

Morphological measurements

Following the flight measurements, individuals were immobilized by placing them in the refrigerator (4°C) for 10–15 min, depending on their size. Wings were removed for morphological measurements and animals were stored at –80°C for enzyme assays. Digital images of the wings were taken using a camera connected to a dissecting microscope (SteREO Discovery V8, Zeiss, Göttingen, Germany). Wing length and surface area were measured using Axio Vision software (Zeiss). Wing loading was calculated and expressed as body mass (mg) per total wing area (mm²).

Enzyme assays

For each individual, the head, abdomen and legs were removed with scissors and the remaining thorax was weighed to the nearest 0.1 mg. The thorax was then minced with scissors and homogenized in 19 volumes of

ice-cold homogenization buffer. All further manipulations were carried out on ice. The homogenization buffer used consisted of 25 mmol l⁻¹ Tris-potassium phosphate, pH 7.8 at 4°C, 2 mmol l⁻¹ ethylene diamine tetraacetic acid (EDTA), 5 mmol l⁻¹ dithiothreitol (DTT) and 0.5% (v/v) Triton X-100. Minced thoraces were homogenized three times for 10 s at 10,000 r.p.m. with 30 s cooling intervals using an Omni-prep multi-sample homogenizer (Omni International, Kennesaw, GA, USA). Homogenates were then sonicated three times for 10 s with 30 s cooling intervals using a sonicator (VC750 Ultrasonic Processor, Newtown, CT, USA) equipped with a six horns probe and set to low intensity (20%). Finally, homogenates were centrifuged for 5 min at 2400 g at 4°C (Sorvall Legend Micro 21R, Thermo Scientific, Osterode, Germany), and supernatants were used for assays.

Enzyme activities were measured in triplicate using a Sinergy 2 Multi-Detection Microplate Reader (Biotek Instruments, Winooski, VT, USA) adjusted to maintain temperature at 37°C. GP, TR, HK and PGI reactions were monitored using the rate of appearance of nicotinamide adenine dinucleotide phosphate (NADPH) and pyruvate kinase (PK) using the rate of disappearance of nicotinamide adenine dinucleotide (NADH) monitored at 340 nm using a millimolar extinction coefficient (ϵ) of 6.22. The CS

reaction was monitored by following the appearance of 5-thio-2-nitrobenzoic acid from 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) monitored at 412 nm using $\epsilon=13.6$. Background activity, without the presence of one substrate, was assessed and subtracted when observed. Enzyme activities are expressed in $U\ g^{-1}\ thorax$, where $U=\mu mol\ min^{-1}$.

Enzyme assay conditions were as follows: GP: 100 $mmol\ l^{-1}$ potassium phosphate, pH 7.1 at 37°C, 10 $mmol\ l^{-1}$ $MgCl_2$, 4 $mg\ ml^{-1}$ glycogen (omitted for control), 0.75 $mmol\ l^{-1}$ $NADP^+$, 4 $\mu mol\ l^{-1}$ glucose 1,6-bisphosphate, 2 $mmol\ l^{-1}$ AMP, 5 $U\ ml^{-1}$ phosphoglucosmutase and 5 $U\ ml^{-1}$ glucose-6-phosphate dehydrogenase; TR: 50 $mmol\ l^{-1}$ potassium phosphate, pH 6.6 at 37°C, 1.1 $mmol\ l^{-1}$ $MgCl_2$, 10 $mmol\ l^{-1}$ trehalose (omitted for control), 0.75 $mmol\ l^{-1}$ $NADP^+$, 1.1 $mmol\ l^{-1}$ ATP, 5 $U\ ml^{-1}$ HK and 5 $U\ ml^{-1}$ glucose-6-phosphate dehydrogenase; HK: 100 $mmol\ l^{-1}$ Tris-imidazole, pH 8.1 at 37°C, 100 $mmol\ l^{-1}$ KCl, 10 $mmol\ l^{-1}$ $MgCl_2$, 5 $mmol\ l^{-1}$ D-glucose (omitted for control), 1 $mmol\ l^{-1}$ $NADP^+$, 5 $mmol\ l^{-1}$ ATP and 5 $U\ ml^{-1}$ glucose-6-phosphate dehydrogenase; PGI: 50 $mmol\ l^{-1}$ Tris-imidazole, pH 8.1 at 37°C, 5 $mmol\ l^{-1}$ KCl, 10 $mmol\ l^{-1}$ $MgCl_2$, 16 $mmol\ l^{-1}$ fructose-6-phosphate (omitted for control), 0.75 $mmol\ l^{-1}$ $NADP$ and 5 $U\ ml^{-1}$ glucose-6-phosphate dehydrogenase; PK: 100 $mmol\ l^{-1}$ Tris-imidazole, pH 8.1 at 37°C, 10 $mmol\ l^{-1}$ KCl, 5 $mmol\ l^{-1}$ $MgCl_2$, 5 $mmol\ l^{-1}$ phosphoenolpyruvate (omitted for control), 0.15 $mmol\ l^{-1}$ NADH, 0.4 $mmol\ l^{-1}$ ADP, 0.75 $mmol\ l^{-1}$ fructose-1,6-bisphosphate and 5 $U\ ml^{-1}$ lactate dehydrogenase; CS: 50 $mmol\ l^{-1}$ Tris-HCl, pH 7.4 at 37°C, 0.5 $mmol\ l^{-1}$ oxaloacetate (omitted for control), 0.3 $mmol\ l^{-1}$ acetyl-CoA, 0.1 $mmol\ l^{-1}$ DTNB. All chemicals were purchased from Sigma-Aldrich (Oakville, ON, Canada), except for acetyl-CoA (Bioshop Canada Inc., Burlington, ON, Canada) and ATP (Calbiochem, Darmstadt, Germany).

Data analysis

All statistical analyses were performed using the software SYSTAT 12.0 (Systat Software, Chicago, IL, USA). To determine the effect of body mass on flight metabolic rate, wing beat frequency, thorax mass and wing morphology parameters, analyses of covariance (ANCOVAs) were performed using body mass as covariate and sex (workers versus drones) as a factor. For these analyses, the average of both sets of flight measurements were used and presented, but all analyses were also performed individually for both flight measurements and yielded the same results. Data fulfilled the normality and equal variance assumptions of the linear model, except for thorax mass for which data were log-transformed to fulfil the equal variance assumption and confirm the results of analyses. Following ANCOVA analyses where a sex effect was detected, workers and drones were analyzed separately to identify the nature of the difference. To assess the changes in proportion in body morphology between workers and drones, we performed regressions on log transformed body mass, wing surface area, wing loading and thorax mass in order to obtain the scaling exponent b from the equation $Y=aX^b$. Finally, given the unbalanced proportion of workers and drones sampled ($N=27$ versus 18), we performed all statistical tests using a random sub-sample of 18 workers and found only a few differences that we report in the text.

Flight metabolic rate and wingbeat frequency repeatability was assessed using Pearson product moment correlation analysis on residuals obtained from ANCOVA analyses, accounting for body mass and sex. Repeatability estimates were also reported as intraclass correlation coefficients as calculated in Lessells and Boag (Lessells and Boag, 1987). Additional correlation analyses on ANCOVA residuals were conducted to evaluate the relationship between wingbeat frequency, metabolic rate, thorax mass and wing surface area. Flight metabolic rate residuals were correlated with GP, PK and CS enzyme activity, and TR, HK and PGI residuals accounting for differences found between sexes. For these correlations probabilities reported account for multiple comparisons using Bonferroni adjustments. All correlation analyses were further performed on workers and drones separately to investigate differences between sexes.

Acknowledgements

The authors would like to acknowledge Dimitri Skandalis and Bénédicte Rivière for their help in the laboratory. We thank Enrique Rodríguez, Kim Borg and John Lewis for their helpful comments, as well as the thorough review of anonymous referees that greatly improved the manuscript.

Competing interests

The authors declare no competing financial interests.

Author contributions

C.-A.D. designed the research; C.-A.D., F.B. and K.B. performed the experiments; C.-A.D., F.B. and K.B. analysed the data; C.-A.D. and F.B. wrote the manuscript.

Funding

This research was supported by a Natural Sciences and Engineering Research Council of Canada Discovery grant, Canadian Foundation for Innovation and Ontario Research Fund grants to C.-A.D.

References

- Bartholomew, G. A. and Casey, T. M. (1978). Oxygen consumption of moths during rest, pre-flight warm-up, and flight in relation to body size and wing morphology. *J. Exp. Biol.* **76**, 11–25.
- Beenackers, A. M. T., van den Broek, A. T. M. and de Ronde, T. J. A. (1975). Development of catabolic pathways in insect flight muscles. A comparative study. *J. Insect Physiol.* **21**, 849–859.
- Bennett, A. F. (1987). Interindividual variability: an underutilized resource. In *New Directions in Ecological Physiology* (ed. M. E. Feder, A. F. Bennett, W. W. Burggren and R. B. Huey), pp. 147–169. New York, NY: Cambridge University Press.
- Buchwald, R. and Dudley, R. (2010). Limits to vertical force and power production in bumblebees (Hymenoptera: *Bombus impatiens*). *J. Exp. Biol.* **213**, 426–432.
- Byrne, D. N., Buchmann, S. L. and Spangler, H. G. (1988). Relationship between wing loading, wing stroke frequency and body mass in homopterous insects. *J. Exp. Biol.* **135**, 9–23.
- Casey, T. M. (1976). Flight energetics of sphinx moths: power input during hovering flight. *J. Exp. Biol.* **64**, 529–543.
- Casey, T. M., May, M. L. and Morgan, K. R. (1985). Flight energetics of euglossine bees in relation to morphology and wing stroke frequency. *J. Exp. Biol.* **116**, 271–289.
- Clark, A. G. and Wang, L. (1994). Comparative evolutionary analysis of metabolism in 9 *Drosophila* species. *Evolution* **48**, 1230–1243.
- Coelho, J. R. (1991). Heat transfer and body temperature in honey bee (Hymenoptera: Apidae) drones and workers. *Environ. Entomol.* **20**, 1627–1635.
- Crabtree, B. and Newsholme, E. A. (1972). The activities of phosphorylase, hexokinase, phosphofructokinase, lactate dehydrogenase and the glycerol 3-phosphate dehydrogenases in muscles from vertebrates and invertebrates. *Biochem. J.* **126**, 49–58.
- Darveau, C.-A., Hochachka, P. W., Welch, K. C., Jr, Roubik, D. W. and Suarez, R. K. (2005a). Allometric scaling of flight energetics in Panamanian orchid bees: a comparative phylogenetic approach. *J. Exp. Biol.* **208**, 3581–3591.
- Darveau, C.-A., Hochachka, P. W., Roubik, D. W. and Suarez, R. K. (2005b). Allometric scaling of flight energetics in orchid bees: evolution of flux capacities and flux rates. *J. Exp. Biol.* **208**, 3593–3602.
- Dohm, M. R. (2002). Repatability estimates do not always set an upper limit to heritability. *Funct. Ecol.* **16**, 273–280.
- Falconer, D. S. and Mackay, T. F. (1996). *Introduction to Quantitative Genetics*, 4th edn. Harlow: Longmans.
- Foster, D. J. and Cartar, R. V. (2011). What causes wing wear in foraging bumble bees? *J. Exp. Biol.* **214**, 1896–1901.
- Goulson, D. (2010). *Bumblebees Behaviour, Ecology and Conservation*. New York, NY: Oxford University Press.
- Gradish, A. E., Scott-Dupree, C. D., Shipp, L., Harris, C. R. and Ferguson, G. (2010). Effect of reduced risk pesticides for use in greenhouse vegetable production on *Bombus impatiens* (Hymenoptera: Apidae). *Pest Manag. Sci.* **66**, 142–146.
- Han, L. Z., Gu, H. N., Zhai, B. P. and Zhang, X. X. (2009). Genetic effects on flight capacity in the beet armyworm, *Spodoptera exigua* (Lep., Noctuidae). *J. Appl. Entomol.* **133**, 262–271.
- Harrison, J. F. and Roberts, S. P. (2000). Flight respiration and energetics. *Annu. Rev. Physiol.* **62**, 179–205.
- Hartfelder, K. and Engels, W. (1992). Allometric and multivariate analysis of sex and caste polymorphism in the neotropical stingless bee, *Scaptotrigona postica*. *Insectes Sociaux* **39**, 251–266.
- Heinrich, B. and Heinrich, M. J. E. (1983). Heterothermia in foraging workers and drones of the bumblebee *Bombus terricola*. *Physiol. Zool.* **56**, 563–567.
- Hepburn, H. R., Radloff, S. E., Steele, G. R. and Brown, R. E. (1998). Dimensional aspects of flight in the honeybees of Africa. *J. Apic. Res.* **37**, 147–154.
- Hepburn, H. R., Radloff, S. E. and Fuchs, S. (1999). Flight machinery dimensions of honeybees, *Apis mellifera*. *J. Comp. Physiol. B* **169**, 107–112.
- Konarzewski, M., Książek, A. and Lapo, I. B. (2005). Artificial selection on metabolic rates and related traits in rodents. *Integr. Comp. Biol.* **45**, 416–425.
- Lehmann, F. O. (2002). The constraints of body size on aerodynamics and energetics in flying fruit flies: an integrative view. *Zoology* **105**, 287–295.
- Lessells, C. M. and Boag, P. T. (1987). Unrepeatable repeatabilities: a common mistake. *Auk* **104**, 116–121.
- Naya, D. E. (2010). Why may repeatability of highly flexible traits say little about their evolutionary potential? *Open Ecology J.* **3**, 26–28.
- Nespolo, R. F. and Franco, M. (2007). Whole-animal metabolic rate is a repeatable trait: a meta-analysis. *J. Exp. Biol.* **210**, 2000–2005.
- Nitépöld, K. and Hanski, I. (2013). A long life in the fast lane: positive association between peak metabolic rate and lifespan in a butterfly. *J. Exp. Biol.* **216**, 1388–1397.

- Nijhout, H. F. and Grunert, L. W.** (2010). The cellular and physiological mechanism of wing-body scaling in *Manduca sexta*. *Science* **330**, 1693-1695.
- Norin, T. and Malte, H.** (2011). Repeatability of standard metabolic rate, active metabolic rate and aerobic scope in young brown trout during a period of moderate food availability. *J. Exp. Biol.* **214**, 1668-1675.
- Perrard, A., Villemant, C., Carpenter, J. M. and Baylac, M.** (2012). Differences in caste dimorphism among three hornet species (Hymenoptera: Vespidae): forewing size, shape and allometry. *J. Evol. Biol.* **25**, 1389-1398.
- Radloff, S. E., Hepburn, H. R. and Koeniger, G.** (2003). Comparison of flight design of Asian honeybee drones. *Apidologie* **34**, 353-358.
- Robertson, S. P., Caprio, M. A. and Faver, M. K.** (2002). Heritability of wing-beat frequency in *Anopheles quadrimaculatus*. *J. Am. Mosq. Control Assoc.* **18**, 316-320.
- Rogowitz, G. L. and Chappell, M. A.** (2000). Energy metabolism of eucalyptus-boring beetles at rest and during locomotion: gender makes a difference. *J. Exp. Biol.* **203**, 1131-1139.
- Saktor, B.** (1975) Biochemistry of insect flight – utilization of fuels by muscle. In *Insect Biochemistry and Function* (ed. D. J. Candy and B. A. Kilby), pp. 1-81. London: Chapman and Hall Ltd.
- Skandalis, D. A. and Darveau, C.-A.** (2012). Morphological and physiological idiosyncrasies lead to interindividual variation in flight metabolic rate in worker bumblebees (*Bombus impatiens*). *Physiol. Biochem. Zool.* **85**, 657-670.
- Skandalis, D. A., Roy, C. and Darveau, C.-A.** (2011). Behavioural, morphological, and metabolic maturation of newly emerged adult workers of the bumblebee, *Bombus impatiens*. *J. Insect Physiol.* **57**, 704-711.
- Suarez, R. K.** (2000). Energy metabolism during insect flight: biochemical design and physiological performance. *Physiol. Biochem. Zool.* **73**, 765-771.
- Suarez, R. K., Darveau, C.-A., Welch, K. C., Jr, O'Brien, D. M., Roubik, D. W. and Hochachka, P. W.** (2005a). Energy metabolism in orchid bee flight muscles: carbohydrate fuels all. *J. Exp. Biol.* **208**, 3573-3579.
- Suarez, R. K., Darveau, C.-A. and Hochachka, P. W.** (2005b). Roles of hierarchical and metabolic regulation in the allometric scaling of metabolism in Panamanian orchid bees. *J. Exp. Biol.* **208**, 3603-3607.
- Tanaka, K.** (2009). Genetic variation in flight activity of *Ophraella communa* (Coleoptera: Chrysomelidae): heritability estimated by artificial selection. *Environ. Entomol.* **38**, 266-273.
- White, C. R., Schimpf, N. G. and Cassey, P.** (2013). The repeatability of metabolic rate declines with time. *J. Exp. Biol.* **216**, 1763-1765.