1 **Intraspecific variation in flight metabolic rate in the bumblebee *Bombus impatiens*:**

2 **repeatability and functional determinants in workers and drones**

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14 Running title: Flight metabolic rate repeatability

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.

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 impact muscle cell metabolic properties (Crabtree, 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 (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 *Bombus impatiens*. This work follows a series of investigations on intraspecific variation in energetic properties during flight (Skandalis et al., 2011; Skandalis 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.

Material 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). 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

1   numbered tag glued on the thorax. The bees were then placed back in their colony, and a second

2   series of the same measurements was performed 48 hours later.

3   *Flight measurements*

4   Flight metabolic rate measurements were performed using a FoxBox flow-through

5   respirometry system (Sable Systems International, SSI, Las Vegas, NV, USA), where the animal

6   was placed in a 500 millilitre chamber (500 ml glass bottle with side arm) to hover. The system

7   was connected to a laptop computer and data were acquired and analyzed using Expedata (SSI).

8   Only the rate of CO_2 production was considered as all bee species studied so far appear to power

9   flight using carbohydrates as a sole fuel source (Suarez et al., 2005). The CO_2 detector was

10   calibrated daily, using nitrogen and a 402ppm span gas. The baseline CO_2 level was measured

11   before and after each measurement. Dry air was pushed into the 500 ml glass flask using

12   PharmMed® BPT tubing (Fisher Scientific) at a rate of 500 ml per minute. Flight durations of 3

13   to 5 minutes were sufficient to obtain a period of good quality flight, where individuals hovered

14   away from walls (Skandalis and Darveau, 2012). We accepted flight metabolic rate

15   measurements that showed a stable CO_2 production rate for more than 30 seconds of good

16   quality flight during the flight trial.

17   Wingbeat frequency measurements were performed simultaneously using an optical

18   detector under the flight chamber. The signal was acquired and analyzed using the software Trex

19   2.0 Transient Waveform Recorder (Moore Scientific). Measurement period was set to 0.5 s, and

20   individual value is the average of 10 measurements taken during the flight trial.

21   *Morphological measurements*

22   Following the flight measurements, individuals were immobilized by placing them in the

23   refrigerator (4°C) for 10-15 minutes, depending on their size. Wings were removed for

24   morphological measurements and animals were stored at -80°C for enzyme assays. Digital

25   images of the wings were taken using a camera connected to a dissecting microscope (SteREO

26   Discovery V8, Zeiss, Güttingen, Germany). Wing length and surface area were measured using

27   Axio Vision software (Zeiss). Wing loading was calculated and expressed as body mass (mg) per

28   total wing area (mm^2).

1 *Enzyme assays*

2   For each individual, the head, abdomen, and legs were removed with scissors and the

3 remaining thorax was weighed to the nearest 0.1 mg. The thorax was then minced with scissors

4 and homogenized in 19-volumes of ice-cold homogenization buffer. All further manipulations

5 were carried out on ice. The homogenization buffer used consisted of 25 mM Tris-potassium

6 phosphate pH 7.8 at 4°C, 2 mM ethylene diamine tetraacetic acid (EDTA), 5 mM dithiothreitol

7 (DTT) and 0.5% (v/v) Triton-X-100. Minced thoraces were homogenized three times for 10

8 seconds at 10 000 rpm with 30 seconds cooling intervals using an Omni-prep multi-sample

9 homogenizer (Omni International, Kennesaw, GA, USA). Homogenates were then sonicated

10 three times for 10 seconds with 30 seconds cooling intervals using a sonicator (VC750

11 Ultrasonic Processor, Newtown, CT, USA) equipped with a six horns probe and set to low

12 intensity (20%). Finally, homogenates were centrifuged for 5 min at 5000 rpm at 4°C (Sorvall

13 Legend Micro 21R, Thermo Scientific, Osterode, Germany), and supernatants were used for

14 assays.

15   Enzyme activities were measured in triplicate using a Sinergy 2 Multi-Detection

16 Microplate Reader (Biotek® Instruments Inc., Winooski, VT, USA) adjusted to maintain

17 temperature at 37°C. Glycogen phosphorylase (GP), trehalase (TR), hexokinase (HK) and

18 phosphoglucoisomerase (PGI) reactions were monitored using the rate of appearance of

19 nicotinamide adenine dinucleotide phosphate (NADPH), and pyruvate kinase (PK) using the rate

20 of disappearance of nicotinamide adenine dinucleotide (NADH) monitored at 340 nm using a

21 millimolar extinction coefficient (ε) of 6.22. The citrate synthase (CS) reaction was monitored by

22 following the appearance of 5-thio-2-nitrobenzoic acid from 5,5'-dithiobis-(2-nitrobenzoic acid)

23 (DTNB) monitored at 412 nm using $\varepsilon = 13.6$. Background activity, without the presence of one

24 substrate, was assessed and subtracted when observed. Enzyme activities are expressed in U g^{-1}

25 thorax, where U = μmol min^{-1}.

26   Enzyme assay conditions were as follows: GP: 100 mM potassium phosphate pH 7.1 at

27 37°C, 10 mM MgCl$_2$, 4 mg ml^{-1} glycogen (omitted for control), 0.75 mM NADP$^+$, 4 μM glucose

28 1,6-biphosphate, 2 mM AMP, 5 U ml^{-1} phosphoglucomutase and 5 U ml^{-1} glucose-6-phosphate

29 dehydrogenase. TR: 50 mM potassium phosphate pH 6.6 at 37°C, 1.1 mM MgCl$_2$, 10 mM

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1   trehalose (omitted for control), 0.75 mM $NADP^+$, 1.1 mM ATP, 5 U ml^{-1} hexokinase and 5 U

2   ml^{-1} glucose-6-phosphate dehydrogenase. HK: 100 mM Tris-imidazole pH 8.1 at 37°C, 100 mM

3   KCl, 10 mM $MgCl_2$, 5 mM D-glucose (omitted for control), 1 mM $NADP^+$, 5 mM ATP and 5 U

4   ml^{-1} glucose-6-phosphate dehydrogenase. PGI: 50 mM Tris-imidazole pH 8.1 at 37°C, 5 mM

5   KCl, 10 mM $MgCl_2$, 16 mM fructose-6-phosphate (omitted for control), 0.75 mM NADP and 5

6   U ml^{-1} glucose-6-phosphate dehydrogenase. PK: 100 mM Tris-imidazole pH 8.1 at 37°C, 10 mM

7   KCl, 5 mM $MgCl_2$, 5 mM phosphoenolpyruvate (omitted for control), 0.15 mM NADH, 0.4 mM

8   ADP, 0.75 mM fructose-1,6-bisphosphate and 5 U ml^{-1} lactate dehydrogenase. CS: 50 mM Tris-

9   HCl pH 7.4 at 37°C, 0.5 mM oxaloacetate (omitted for control), 0.3 mM acetyl-CoA, 0.1 mM

10   DTNB. All chemicals were purchased from Sigma-Aldrich (Oakville, ON, Canada), except

11   acetyl-CoA (Bioshop Canada Inc., Burlington, ON, Canada) and ATP (Calbiochem, Darmstadt,

12   Germany).

13   *Data analysis*

14   All statistical analyses were performed using the software SYSTAT 12.0 (Chicago, IL,

15   USA). To determine the effect of body mass on flight metabolic rate, wing beat frequency,

16   thorax mass and wing morphology parameters, ANCOVAs were performed using body mass as

17   covariate and sex (workers versus drones) as a factor. For these analyses, the average of both sets

18   of flight measurements were used and presented, but all analyses were also performed

19   individually for both flight measurements and yielded the same results. Data fulfilled the

20   normality and equal variance assumptions of the linear model, except for thorax mass for which

21   data were log-transformed to fulfil the equal variance assumption and confirm the results of

22   analyses. Following ANCOVA analyses where a sex effect was detected, workers and drones

23   were analyzed separately to identify the nature of the difference. To assess the changes in

24   proportion in body morphology between workers and drones, we performed regressions on log

25   transformed body mass, wing surface area, wing loading and thorax mass in order to obtain the

26   scaling exponents *b* from the equation $Y = aX^b$. Finally, given the unbalanced proportion of

27   workers and drones sampled (n=27 vs 18), we performed all statistical tests using a random sub-

28   sample of 18 workers and found only a few differences that we report in the text.

1 Flight metabolic rate and wingbeat frequency repeatability was assessed using Pearson

2 product-moment correlation analysis on residuals obtained from ANCOVA analyses, accounting

3 for body mass and sex. Repeatability estimates were also reported as intraclass correlation

4 coefficients as calculated in Lessells and Boag (1987). Additional correlation analyses on

5 ANCOVA residuals were conducted to evaluate the relationship between wingbeat frequency,

6 metabolic rate, thorax mass and wing surface area. Flight metabolic rate residuals were

7 correlated with GP, PK and CS enzyme activity, and TR, HK and PGI residuals accounting for

8 differences found between sexes. For these correlations probabilities reported account for

9 multiple comparisons using Bonferroni adjustments. All correlation analyses were further

10 performed on workers and drones separately to investigate differences between sexes.

11 **Results**

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

13 Individual *B. impatiens* varied in body mass from 50 to 226 mg. Consecutive

14 measurements of body mass separated by 2 days showed a correlation coefficient of 0.925

15 ($p<0.001$). Wing surface area was strongly dependent on body mass (Fig. 1A, Table 1 and 2;

16 mass: $p<0.001$, sex: $p<0.001$, mass * sex: $p=0.001$; $r^2=0.945$), but wing surface of drones

17 increased with body mass with a shallower slope than workers (scaling exponents *b* of 0.25 and

18 0.82 respectively). Similarly, forewing length showed a strong dependence on body mass and

19 difference between sexes (Table 1 and 2; mass: $p<0.001$, sex: $p<0.001$, mass * sex: $p=0.001$;

20 $r^2=0.958$). The calculated wing loading increased with body mass with a steeper slope in drones

21 than workers (Fig.1B, Table 2; mass: $p<0.001$, sex: $p<0.001$, mass * sex: $p=0.001$; $r^2=0.617$).

22 Thorax mass increased with body mass (Fig. 1C, Table 2; mass: $p<0.001$, sex: $p<0.001$, mass *

23 sex: $p<0.001$; $r^2=0.932$) with a shallower slope in drones (*b*=0.41) than workers (*b*=0.97). Wing

24 surface area increased with thorax mass with the same slopes but different intercepts in workers

25 and drones (Fig. 2A; mass: $p<0.001$, sex: $p<0.001$; $r^2=0.923$). In addition, residual variation

26 accounting for body mass and sex show the strong positive association between wing surface

27 area and thorax mass in both workers and drones (Fig. 2B; combined: *r*=0.628, $p<0.001$;

28 workers: *r*=0.452, p=0.018; drones: *r*=0.789, $p<0.001$).

1   Among all bees, wingbeat frequency varied from 149 to 248 Hz and decreased

2   significantly with body mass. At any given body mass, drones flew with lower wingbeat

3   frequencies than did workers (Fig. 3A; mass: p=0.001, sex: p<0.001, r^2=0.678). When analyzed

4   separately, the relationship with body mass was significant for workers (p=0.006) but not for

5   drones (p=0.125). Both sets of wingbeat frequency measurements were strongly correlated

6   (r=0.911, p<0.001). Using residuals accounting for the effects of mass and sex, wingbeat

7   frequency remained highly repeatable for all bees combined (r=0.743; Fig. 4A, Table 3).

8   Analyses performed on workers and drones separately show that this relationship remained

9   highly significant for workers (r=0.843, p<0.001), but not for drones (r=0.071, p=0.779).

10   Metabolic rate measurements ranged from 2.0 to 24.5 ml CO_2 h^{-1} per animal, or 41.7 to

11   163.3 ml CO_2 h^{-1} g^{-1} when expressed on a mass-specific basis. Metabolic rate increased

12   significantly with body mass, and drones had lower metabolic rate than workers (Fig.3B; mass:

13   p<0.001, sex: p<0.001, r^2=0.617). The relationship with body mass was significant for both sexes

14   when analyzed separately. Mass-specific metabolic rate was size-invariant but differed between

15   sexes (mass: p=0.349, sex: p<0.001, r^2=0.364). Analysis of metabolic rate performed using

16   balanced samples showed a significant interaction term where drones follow a shallower slope

17   than workers (not shown; mass: p<0.001, sex: p=0.292, mass*sex: p=0.009; r^2=0.770).

18   Consecutive measurements of metabolic rate were correlated with a coefficient of 0.855

19   (p<0.001), and remained correlated when expressed on a mass-specific basis (r=0.772, p<0.001).

20   Residuals of whole-animal metabolic rate accounting for both body mass and sex were positively

21   correlated with a coefficient of 0.624 (Fig. 4B; Table 3). Repeatability analyses performed on

22   workers and drones separately show that the correlation remained significant for workers

23   (r=0.726, p<0.001), but not for drones (r=0.232, p=0.355).

24   Wingbeat frequency residuals accounting for body mass and sex effects were not

25   correlated with wing surface area residuals or other wing size measures. Metabolic rate residuals

26   were positively correlated with thorax mass residuals (Fig. 5A; r=0.451, p=0.002) and with

27   wingbeat frequency residuals (Fig. 5B; r=0.617, p<0.001). Analysis performed with both

28   variables combined confirmed that individuals with both relatively larger thorax and higher

29   wingbeat frequency had relatively higher flight metabolic rates (thorax mass residuals: p<0.001;

30   wingbeat frequency residuals: p<0.001; r^2=0.556). Analyses performed on workers and drones

1   separately show that the relationship between metabolic rate and thorax mass remain for both

2   sexes (workers: $r=0.455$, p=0.017; drones: $r=0.574$, p=0.013), but with wingbeat frequency it

3   was significant only for workers ($r=0.729$, p<0.001) and not drones ($r=-0.367$, p=0.134).

4   *Metabolic rate and metabolic enzyme activity*

5   The activity of all enzymes measured was correlated with flight metabolic rate (Fig. 6A-

6   F); analyses were performed using residuals accounting for variation associated with body mass

7   and sex for metabolic rate, and sex for enzymes that differed between workers and drones,

8   namely TR, HK and PGI activity (Table 1). Using balanced samples yielded similar results,

9   except that we lost significance for CS and TR, which also did not differ between sexes. Finally,

10 when sexes were analyzed separately, all relationships remained significant for workers, but no

11 relationships were significant for drones.

12 **Discussion**

13 *Repeatability of flight metabolic rate and wingbeat frequency*

14 Short-term repeatability of metabolic rate and wingbeat frequency was high in flying

15 bumblebees but appeared significant only for workers and not for drones. This work shows that,

16 at least for workers, consistent individual variation in flight energetics is observed and likely the

17 outcome of morphological and physiological traits. The high repeatability estimates found here

18 provide an upper limit to heritability estimates of these plastic physiological traits (Falconer and

19 Mackay, 1996; but see Dohm, 2002; Naya, 2010), and the potential response to selection of these

20 flight phenotypes ultimately gives rise to species physiological traits (Casey, 1976; Casey et al.,

21 1985; Darveau et al., 2005a).

22 Repeatability of metabolic rate during locomotion in insects has been documented during

23 running in beetles (Rogowitz and Chappell, 2000), and a recent study reported significant

24 repeatability for peak flight metabolic rate in a butterfly species (Niitepõld and Hanski, 2013).

25 These studies showed repeatability estimates for mass-independent metabolic rate that are

26 generally high, and our findings support high repeatability of metabolic rate during locomotion

27 in another insect species. In addition, wingbeat frequency has been reported to be repeatable in

28 free-flying mosquitoes, suggesting it is heritable (Robertson et al., 2002). Other flight properties

have been investigated, such as the study of 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.

The two 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 two to three days between measurements (Rogowitz and Chappell, 2000; Tanaka, 2009; Niitepõld and Hanski, 2013), allowing comparison with our results. The study of 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 to 0.69. Comparison of studies performed by Nespolo and Franco (2007), also suggest that the time between measurements did not impact 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 likely relatively stable in adult life stage, averaging 60 days in worker of this species (Gradish et al., 2010), similar to recent observation 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 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 likely due to the relatively small sample size (n=27 for workers) as a correlation between wing surface area and wingbeat frequency residuals was found in Skandalis and Darveau (2012) and was reproduced using an independent data set including 352 workers (Billardon and Darveau, unpublished). Therefore, we

11

1   suggest that the repeatability in wingbeat frequency is due to individual differences in wing

2   morphology, a fixed trait for the majority of the life of these animals. The substantial wing wear

3   occurring over the lifetime of a bumblebee (Foster and Carter, 2011) should impact the extent of

4   repeatability of that trait.

5   The significant repeatability in flight metabolic rate for workers can also be explained by

6   the functional association linking flight form and function in insects (Casey, 1976; Casey et al.,

7   1985; Darveau et al., 2005a; Skandalis and Darveau, 2012), and as presented in Figure 5. The

8   relationship observed between wingbeat frequency and metabolic rate, independent of body size,

9   illustrates the effect of individual variation in muscle contraction frequency on metabolic energy

10 expenditure. It is noteworthy that variation in residual flight metabolic rate is also associated

11 with residual variation in thorax mass, which is another trait likely fixed over the majority of the

12 adult life-stage.

13   Muscle metabolic phenotype was characterized using the activity of a suite of metabolic

14 enzymes based on previous work (Suarez, 2000, Suarez et al., 2005a,b; Darveau et al, 2005b,

15 Skandalis and Darveau, 2012). We found a high number of positive correlations, including the

16 enzyme glycogen phosphorylase, trehalase, and hexokinase as predicted from interspecific and

17 intraspecific studies (Darveau et al, 2005b; Skandalis and Darveau, 2012), but also the activity of

18 other glycolytic enzymes (phosphoglucoisomerase and pyruvate kinase) and the mitochondrial

19 enzyme citrate synthase. These results suggest that individuals with high metabolic rate show an

20 overall increase in enzyme content, at least in workers, supporting the hypothesis that metabolic

21 enzymes may form a correlated suite (Clark and Wang, 1994). In previous work on the same

22 species we found that metabolic rate correlated with the activity of trehalase and hexokinase but

23 not phosphoglucoisomerase (Skandalis and Darveau, 2012), but variation among multiple

24 colonies or even experimental variation in activity estimates could explain these differences.

25 These findings warrant further work on the coordinated changes among functionally linked

26 enzymes.

27 *Differences between workers and drones*

28   Drones are distinct from workers in many respects. Morphologically, drones wing surface

29 area increases with body mass, but with a shallower slope than workers. Wing surface area of

workers depart from isometry and show negative allometry, but this estimate is likely influenced by the modest sample size as previous studies involving a larger number of individuals showed isometric exponent values (Skandalis and Darveau, 2012; Buchwald and Dudley, 2010). 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 200mg, 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 likely 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 likely at play to explain these sex differences (Nijhout and Grunert, 2010, Hartfelder and Engels, 1992, Perrard et al., 2012).

The largest drones have similar size wings as workers but have smaller thoraces. 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. (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, 1999), and emphasized that differences in morphology between

1   workers and drones should have some functional impact such as reduced flight cost that may

2   enhance flight duration. The most common mate-location mechanism in bumblebees consists of

3   males patrolling regular circuits (Goulson, 2010). In this case, drones would benefit from

4   increased forward flight duration and distance, which might in part explain differences of flight

5   morphology and energetics. Our previous work based on interspecific and intraspecific studies

6   showed reduced flight metabolic rate associated with lower wing loading (Darveau et al., 2005a;

7   Skandalis and Darveau, 2012), which could ultimately increase flight duration, assuming

8   constant nectar loads. Additional measures that capture the breadth of flight performance such as

9   speed, duration and distance, would help identify if there are indeed advantages and constraints

10 associated with worker and drone morphology.

11   Sexual dimorphism offers further insights into flight energetics. Drones have larger

12 wings, lower flight wingbeat frequency and lower metabolic rate than workers. Moreover, larger

13 drones converge with workers for wingbeat frequency and wing surface area (Figs. 1A and 3A),

14 supporting the functional association between wing morphology and flight kinematics previously

15 documented in this species (Skandalis and Darveau, 2012) and among species of bees (Darveau

16 et al., 2005a). On the other hand, larger drones appear to diverge from workers for flight

17 metabolic rate (Fig. 3B), as well as thorax mass (Fig. 1C), in line with the association found

18 between those variables (Fig. 5A). The lower flight metabolic rate of drones is also associated

19 with lower activity of the metabolic enzymes trehalase and hexokinase expressed per unit thorax

20 mass. This suggests that flight muscle metabolic phenotype differs between sexes assuming that

21 the proportion of thoracic muscle mass is maintained. These differences support the association

22 between metabolic rate and flight muscle phenotype previously documented in this species

23 (Skandalis and Darveau, 2012) and among bee species (Darveau et al., 2005b). Nevertheless,

24 phosphoglucoisomerase was higher in drones which is opposite to the predicted difference. What

25 imposes sex-dependent differences in this muscle glycolytic enzyme and how they are associated

26 with metabolism is not clear. The mechanisms driving these apparent differences in flight muscle

27 metabolic phenotypes remain unresolved, but plasticity following development of individuals or

28 sexes with different wing size remains the first candidate.

29 *Conclusion*

14

1   Flight metabolic rate and wingbeat frequency are highly repeatable in bumblebee

2 workers, fulfilling basic conditions for these flight energetics traits to respond to selection. The

3 lack of repeatable estimates in drones should be interpreted with caution, as the narrower range

4 of variation alone, rather than a lack of common mechanisms observed in workers, could explain

5 these differences. Repeatability estimates using intraclass correlation analyses over multiple

6 repeated measures would help clarify this point. The repeatability of these traits is likely

7 associated with their functional associations with morphological traits, namely body mass, thorax

8 mass and wing size. The functional links among those traits are further supported by differences

9 observed between workers and drones. Sexual dimorphism and its impact on flight metabolic

10 rate also had the expected impact on the thorax metabolic phenotype, showing lower activity of

11 the enzymes hexokinase and trehalase. Our study further supports the correlated evolution of

12 physiological phenotypes at multiple levels of organization and morphological phenotypes

13 associated with flight.

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Author contributions

20 C.-A.D. designed the research; C.-A.D., F.B. and K.B. performed the experiments; C.-A.D., F.B.

21 and K.B. analysed the data; C.-A.D. and F.B. wrote the manuscript.

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27

1   **List of abbreviations**

2   CS: citrate synthase

3   GP: glycogen phosphorylase

4   HK: hexokinase

5   PGI: phosphoglucoisomerase

6   PK: pyruvate kinase

7   TR: trehalase

8

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

Variable	Mean±SE		*P*
	Workers (*N*=27)	Drones (*N*=18)	
Body mass (mg)	135.6±8.6	160.5.5±6.5	0.041[a]
Wing surface area (mm^2)	52.14±2.9	70.0±1.2[1]	<0.001[b]
Forewing length (mm)	9.18±0.26	10.68±0.11[1]	<0.001[b]
Wing loading ($mg\ mm^{-2}$)	2.54±0.06	2.27±0.09[1]	<0.001[b]
Thorax mass (mg)	41.2±2.6	38.2±0.9[1]	<0.001[b]
Wingbeat frequency (Hz)	200.8±4.1	160.9±1.5	<0.001[c]
Metabolic rate (ml $CO_2\ hr^{-1}$)	15.5±1.3	11.6±0.6	<0.001[c]
GP (U g^{-1} thorax)	8.0±0.5	7.8±0.4	0.818[a]
TR (U g^{-1} thorax)	31.9±1.2	28.0±1.2	0.032[a]
HK (U g^{-1} thorax)	78.9±2.5	70.2±1.5	0.012[a]
PGI (U g^{-1} thorax)	335.1±19.9	423.0±24.0	0.007[a]
PK (U g^{-1} thorax)	95.6±5.0	90.3±5.1	0.478[a]
CS (U g^{-1} thorax)	359.0±18.9	321.7±10.8	0.140[a]

4 [a] P-values represent the effect of sex.

5 [b] P-values indicate a significant interaction term between body mass and sex, or difference in
6 slope.

7 [c] P-values indicate the effect of sex with body mass as a significant covariate, or difference in
8 intercept.

9

1 Table 2. Relationship between body mass, morphological parameters and flight energetics
2 measurements for worker and drone bumblebees, *Bombus impatiens*. Least-squares
3 regression analyses were performed using log-transformed data such that logY = log$a$+$b$logX,
4 where X is body mass in mg. Standard errors are presented in parenthesis.

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

5

6

1 Table 3. Repeatability of metabolic rate and wingbeat frequency measurements separated by 48 hours

2 in hovering bumblebees (*Bombus impatiens*).

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

3 Note: Repeatability estimates were assessed on residual values taken from ANCOVAs accounting for

4 body mass and gender. * *P*<0.001

5

1 Figure 1. Relationship between body mass and A) wing surface area, B) wing loading and C)

2 thorax mass in worker and drone bumblebees (*Bombus impatiens*). For all variables, the slope of

3 the relationship differed between workers and drones (see Results and Table 1 for statistics).

4 Figure 2. A) Relationship between thorax mass and wing surface area in worker and drone

5 *Bombus impatiens*. B) Correlation between residuals obtained from the regression with body

6 mass for thorax mass and wing surface area. Correlations were significant when workers and

7 drones were analyzed separately or combined (p<0.05).

8 Figure 3. Relationship between body mass and A) wingbeat frequency and B) metabolic rate

9 during hovering flight for worker and drone *Bombus impatiens*. Significant effects of body mass

10 and sex were found for both flight variables (see Results and Table 1 for details).

11 Figure 4. Repeatability of A) wingbeat frequency and B) metabolic rate measurements during

12 hovering flight in worker and drone *Bombus impatiens* (see Table 3 for details of the

13 repeatability estimates and statistics).

14 Figure 5. Correlation between residuals obtained from the body mass relationship for flight

15 metabolic rate and A) residual thorax mass, and B) residual wingbeat frequency. Correlations

16 were significant for workers, but for drones only the correlation with thorax mass was significant
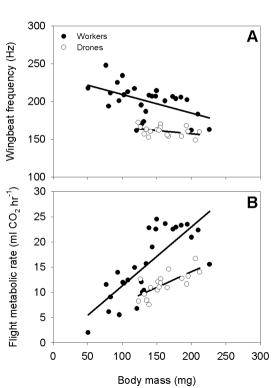
17 (see Results for details).

18 Figure 6. Correlation between flight metabolic rate residuals and the activity of the flight muscle

19 enzymes A) glycogen phosphorylase, B) trehalase, C) hexokinase, D) phosphoglucoisomerase,

20 E) pyruvate kinase, and F) citrate synthase. For enzymes that differed between sexes (trehalase,

21 hexokinase and phosphoglucoisomerase) residuals accounting for sex were analyzed. Significant

22 correlations were detected for all enzymes in workers, but none for drones (see Results for
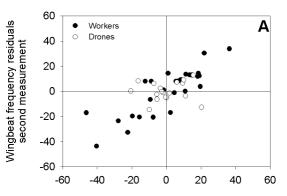
23 details).

24

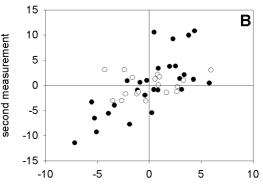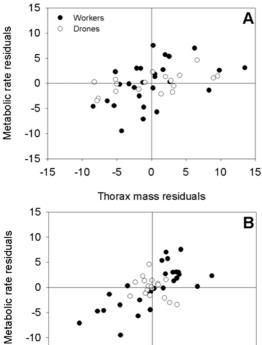Wing surface area (mm²) vs Thorax mass (mg) for Workers (●) and Drones (○).