

# Size constrains oxygen delivery capacity within but not between bumble bee castes

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## ABSTRACT

Bumble bees are eusocial, with distinct worker and queen castes that vary strikingly in size and life-history. The smaller workers rely on energetically-demanding foraging flights to collect resources for rearing brood. Queens can be 3 to 4 times larger than workers, flying only for short periods in fall and again in spring after overwintering underground. These differences between castes in size and life history may be reflected in hypoxia tolerance. When oxygen demand exceeds supply, oxygen delivery to the tissues can be compromised. Previous work revealed hypermetric scaling of tracheal system volume of worker bumble bees (*Bombus impatiens*); larger workers had much larger tracheal volumes, likely to facilitate oxygen delivery over longer distances. Despite their much larger size, queens had relatively small tracheal volumes, potentially limiting their ability to deliver oxygen and reducing their ability to respond to hypoxia. However, these morphological measurements only indirectly point to differences in respiratory capacity. To directly assess size- and caste-related differences in tolerance to low oxygen, we measured critical PO<sub>2</sub> ( $P_{crit}$ ; the ambient oxygen level below which metabolism cannot be maintained) during both rest and flight of worker and queen bumble bees. Queens and workers had similar  $P_{crit}$  values during both rest and flight. However, during flight in oxygen levels near the  $P_{crit}$ , mass-specific metabolic rates declined precipitously with mass both across and within castes, suggesting strong size limitations on oxygen delivery, but only during extreme conditions, when demand is high and supply is low. Together, these data suggest that the comparatively small tracheal systems of queen bumble bees do not limit their ability to deliver oxygen except in extreme conditions; they pay little cost for filling body space with eggs rather than tracheal structures.

## 1. Introduction

Adult insects are often highly tolerant to hypoxic conditions (Harrison et al., 2006; Hoback and Stanley, 2001; Schmitz and Harrison, 2004). In the lab, insects survive levels of oxygen as low as 0.5 kPa for long time periods (Callier and Nijhout, 2011; Greenlee and Harrison, 2004; Van Voorhies, 2009), and many species can even be reared in hypoxia (Abdelrahman et al., 2014; Klok et al., 2010; Vandenbrooks et al., 2018). In the wild, social (e.g., ants, eusocial bees) and burrowing insects may experience hypoxia due to high colony densities and limited natural air flow (Anderson and Ultsch, 1987; Hoback and Stanley, 2001). Honey bees coordinate wing flapping to ventilate hives both for thermoregulation and removal of CO<sub>2</sub> (O'Donnell and Foster, 2001; Southwick and Moritz, 1987). Some insects even live in underground

burrows surrounded completely by water and must sporadically pump air into their burrows to deal with bouts of hypoxia (Wang et al., 2001). Furthermore, hypoxia is a constant challenge for insects at high altitude (Dillon et al., 2006), potentially even more so for those in colonies and/or underground.

The remarkable tolerance of insects to hypoxia (see e.g., Dillon and Dudley, 2014) is often attributed to the oxygen delivery capacity of the tracheal respiratory system (Greenlee and Harrison, 1998; Hoback and Stanley, 2001; Snyder et al., 1995). The insect tracheal system is comprised of a series of branching tubes that decrease in size from their opening to the atmosphere to their termination in individual cells. Air moves through the spiracles, valved openings on the body wall, into the tracheal tubes, and finally to the tracheoles, blind-ended tubules that deliver oxygen directly to the tissues (Harrison, 2009). Gas exchange

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through the respiratory system is controlled by opening and closing of the spiracles and is driven both by diffusion and, in many insects, by convective airflow generated by coordinated muscular movements (Greenlee et al., 2013; Harrison, 2009; Harrison et al., 2013; Socha et al., 2008).

The capacity for oxygen delivery is in part determined by tracheal volume, which varies strikingly with body size among diverse insects. In general, larger insects invest a greater percentage of body volume to tracheal structures (Greenlee et al., 2009; Kaiser et al., 2007), whereas smaller insects devote a much smaller fraction of body volume to the tracheal system (Schmitz and Perry, 1999). This general pattern, which is particularly pronounced in body compartments distant from the spiracles (Kaiser et al., 2007) may reflect limitations of diffusion over distance—longer distances for oxygen delivery in large insects may require larger diameter tubes to facilitate diffusion and larger air sacs to facilitate convection to more distant tissues. These patterns suggest that larger insects may be more constrained by oxygen delivery, a constraint for which relatively larger tracheal volumes may only partially compensate (Vogt and Dillon, 2013).

The common eastern bumble bee, *Bombus impatiens*, is a primitively eusocial species that nests underground in large social groups composed of a queen and workers. In the fall, colonies produce males (drones) and new queens. After mating, the new queens burrow underground, where they overwinter (Goulson, 2003; Plath, 1934). Thus, both in the colony and while overwintering, bumble bees may have to contend with periodic hypoxia and therefore likely have tracheal systems with safety margins that allow them to do so. Among *B. impatiens* workers (sisters with 75% genetic similarity) that varied over 4-fold in body size, tracheal volume scaled hyperallometrically, such that larger workers had relatively larger tracheal volumes (Vogt and Dillon, 2013). Queens, however, had much smaller tracheal volumes than would have been predicted for their body size (Vogt and Dillon, 2013). Further, larger bumble bees do not invest more in tracheoles (Vogt et al., 2014), which would help facilitate oxygen delivery at the tissues. These findings that queens have relatively smaller tracheal volumes and similar tracheole investment suggest that queens may have reduced oxygen delivery capacity compared to workers. However, queens have larger diameter main tracheal trunks (Vogt and Dillon, 2013), which may enhance diffusion and reduce resistance to convection (Harrison et al., 2006). We hypothesized that, due to their striking deficiency in total tracheal system volume, queen bumble bees have reduced oxygen delivery capacity compared to workers.

Although measurements of tracheal morphology provide a useful indirect approach to assess oxygen delivery capacity, a complementary and more direct approach is to measure the critical  $PO_2$  ( $P_{crit}$ ), the oxygen level below which metabolism cannot be maintained (Greenlee and Harrison, 2004). Therefore, to test the hypothesis that queens have reduced respiratory system capacity compared to workers, we measured  $P_{crit}$  in both castes by exposing them to progressively reduced ambient oxygen partial pressures ( $PO_2$ ) and measuring metabolic rates. Oxygen demand can vary 100-fold between rest and flight in bumble bees (Darveau et al., 2014; Ellington et al., 1990); such striking differences in oxygen demand may make estimates of  $P_{crit}$  dependent on activity. We therefore estimated  $P_{crit}$  both for resting bees and for bees in flight. Contrary to our hypothesis and despite strong differentiation in tracheal morphology previously described, we found little difference in resting or flight  $P_{crit}$  for bumble bee queens relative to smaller workers.

## 2. Materials and methods

### 2.1. Animals

Eight *Bombus impatiens* colonies, each with one queen and approximately 100 workers, were purchased from a commercial supplier (Koppert Biological Systems) between June and September of 2018. Colonies were placed outside in Fargo, ND, US, from May until August

2018. After August, when conditions were no longer suitable for keeping bees outside, bees were moved to an enclosed metal frame tent in a greenhouse (21.1–26.7 °C). A humidifier in the tent maintained relative humidity around 70%. Each colony was housed in a commercial enclosure provided by the supplier. The containers were affixed with modified lids, which allowed for visual inspection of the colony and easy removal of individual bees, and secondary shields to protect the colonies from sunlight. Bees were provided ad lib access to fresh-frozen honey bee pollen (Koppert Biological Systems, Sacramento, CA) and a sugar-water solution (1:1). The sugar-water and pollen were replaced every other day.

For respirometry, bees were haphazardly collected from colonies. We did not track age of individual bees, but ensured that they were at least 24 h old (i.e., no longer callow; Within 24 h of emergence they are light in color, because the pile has not yet fully melanized.). Workers were collected using nets outside the colonies. Queens used in the trials were all gynes and were collected from enclosures with forceps and placed individually in plastic deli cups (5.5 oz) for no more than 36 h until used in respirometry. Because the storage was for a short time and because we were not measuring respiratory exchange ratios, bees were provided with sponges (approx. 1 in. × 1 in.) soaked in sugar water and stored in an incubator at 27 °C and 70% relative humidity.

### 2.2. Respirometry

#### 2.2.1. Resting metabolic rates

Oxygen delivery capacity was determined by measuring  $P_{crit}$  using flow-through respirometry as previously described (Greenlee and Harrison, 2004; Greenlee and Harrison, 2005; Greenlee et al., 2007; Owings et al., 2014). Briefly, a  $CO_2$  analyzer (Li-Cor 6252, Lincoln, NE) continuously measured  $CO_2$  production while bees were exposed to stepwise decreases in oxygen partial pressure ( $PO_2$ ). The  $CO_2$  analyzer was calibrated daily with a two-point calibration using dry,  $CO_2$ -free air to zero (Balston purge gas generator, Haverhill, MA) and a premixed standard as the span gas (300.9 ppm  $CO_2$ , balance  $N_2$ ). Hypoxic gas mixtures were produced by diluting dry,  $CO_2$ -free air with  $N_2$  using two mass flow meters (Sierra Instruments, Monterrey, CA) controlled by a mass flow controller (MFC-4; Sable Systems, Inc., Las Vegas, NV). To measure resting metabolic rate, individual bees were placed in a 50 mL syringe with the plunger adjusted to 15 mL with a flow rate of 2 l/min.  $CO_2$  emission (ppm) was measured in queens ( $n = 9$ ), and workers ( $n = 9$ ), with each bee exposed for 10 min at each  $PO_2$  (21, 10, 7, 5, 3, 2, 1, and 0 kPa  $PO_2$ ). These levels were selected based on our previous research showing that insects can maintain metabolic rates at higher  $PO_2$  values (Greenlee and Harrison, 2004; Greenlee and Harrison, 2005; Greenlee et al., 2007; Owings et al., 2014).

Excurrent gas was directed through the  $CO_2$  analyzer via Tygon™ tubing to minimize issues of  $CO_2$  and  $H_2O$  absorption (Lighton, 2008). Analog signals generated from the  $CO_2$  analyzer were digitized and recorded using Sable Systems hardware (UI2) and data acquisition software (ExpeData version 1.9.22 Sable Systems, Inc., Las Vegas, NV). Empty chamber baselines were recorded before and after every trial and used for baseline correction.

Insects were left inside the chamber at 21 kPa  $PO_2$  in total darkness until metabolic rate stabilized, around 10 min. Metabolic rate was considered to be stable when we observed discontinuous gas exchange cycles (DGC). Briefly, DGC is a pattern of respiration characterized by periodic release of  $CO_2$  into the excurrent airstream. This state was identified by observing large spikes in  $CO_2$  emission followed by periods of very low  $CO_2$  emission (Quinlan and Gibbs, 2006).

#### 2.2.2. Flight metabolic rates

To measure flight metabolic rates, individual bees were placed in a glass pint jar (~473 mL) plumbed with Tygon™ tubing, perfused at a flow rate of 4 l/min<sup>-1</sup>. Hypoxic gas mixtures were produced as described above.  $CO_2$  emission rates were measured in one of four different  $PO_2$

levels (21, 15, 10, and 7 kPa PO<sub>2</sub>; n = 10 for each PO<sub>2</sub>). Each bee was measured at a single gas concentration to avoid potential effects of prior exposure to hypoxia and of fatigue for individuals flying for long periods. Metabolic rate is highly repeatable within and between individual workers for *Bombus impatiens* (Darveau et al., 2014), so we expected this approach to yield comparable measurements between treatments. For each bee, metabolic rate was monitored during a 6-minute flight bout, with the bee kept in flight by regularly tilting the jar to prevent bees from gripping the sides of the jar, ensuring they were supporting their weight in free flight (Combes et al., 2020; Woods et al., 2005).

Data acquisition and analysis followed the approach described for resting metabolic rates, and empty chamber baselines before and after every six-minute trial were used for baseline correction.

### 2.3. Analysis of metabolic rate and calculation of critical PO<sub>2</sub>

#### 2.3.1. Rest

Metabolic rates and P<sub>crit</sub> were calculated and determined using previously described methods (Bennett et al., 2013; Greenlee and Harrison, 2004; Greenlee and Harrison, 2005; Owings et al., 2014). First, for each individual bee, mean and 95% confidence intervals of CO<sub>2</sub> emission rate were determined for each ambient PO<sub>2</sub>, including only the last eight minutes of each PO<sub>2</sub> exposure, because bees frequently became agitated immediately after stepwise decreases in PO<sub>2</sub> (Fig. 1). We then used these data to identify the P<sub>crit</sub> based on the following statistical comparisons: 1) the 95% confidence interval at the P<sub>crit</sub> did not overlap with previous or subsequent 95% confidence intervals; 2) the 95% confidence interval at the P<sub>crit</sub> was lower than 95% confidence intervals calculated at all higher PO<sub>2</sub> values; 3) the 95% confidence interval at the P<sub>crit</sub> was greater than or equal to the calculated 95% confidence intervals at all lower PO<sub>2</sub> values (Fig. 1). These values of P<sub>crit</sub> for individual bees could then be compared between worker and queen castes.

#### 2.3.2. Flight

To determine the P<sub>crit</sub> for flight, we first identified stable, 30 second intervals of CO<sub>2</sub> emission from the beginning, middle, and end of each six-minute recording of flight metabolic rate as previously done (Bennett

et al., 2015; Bennett et al., 2013; Snelling et al., 2012). From those three measures, we calculated mean CO<sub>2</sub> emission rate during flight for each bee (Fig. 2). Limiting selections to these stable intervals helped avoid spikes or dips in CO<sub>2</sub> emissions associated with brief landings or agitation, both of which were evident from behavioral observations. Because each bee was only measured at one PO<sub>2</sub> level, we could not calculate individual P<sub>crit</sub> values. Instead, we calculated the mean and 95% confidence interval for CO<sub>2</sub> emission rate for bees of each caste at each PO<sub>2</sub>. Within each caste, we compared the confidence intervals and identified the PO<sub>2</sub> at which the confidence interval decreased without overlap. The next higher PO<sub>2</sub> was designated as the P<sub>crit</sub>.

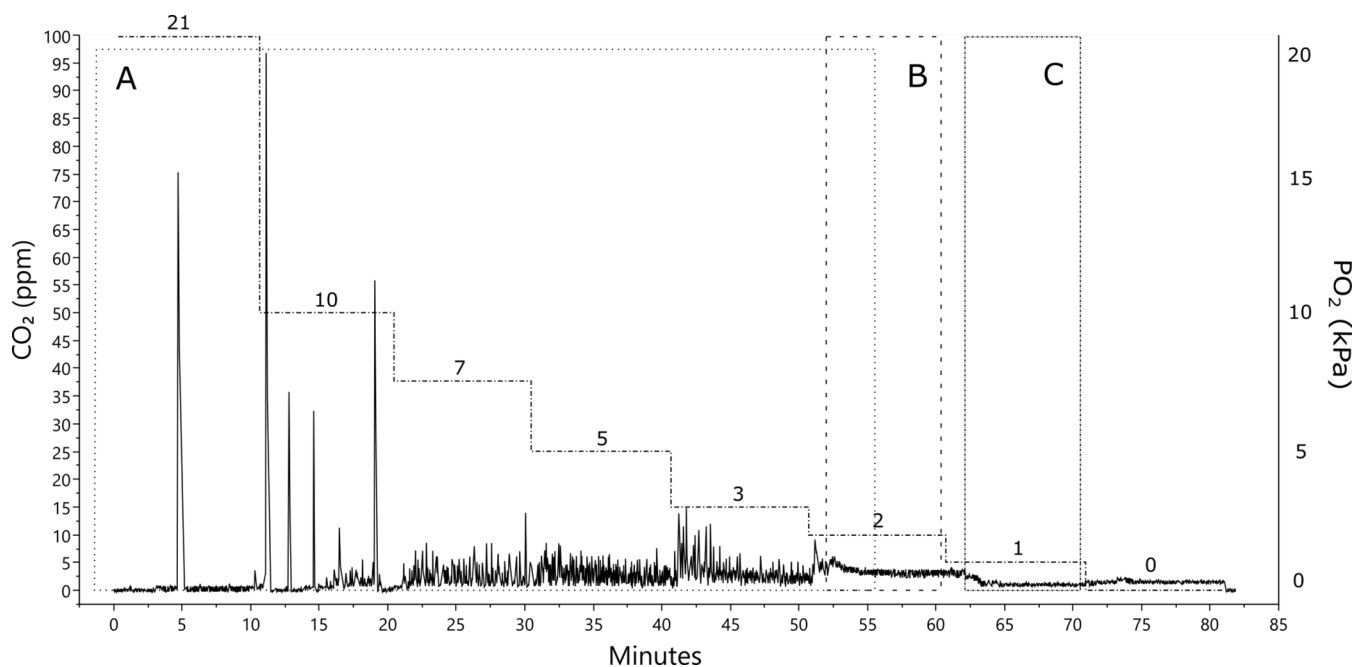
### 2.4. Statistical analysis

IBM SPSS (Version 19) and JMP Pro (version 14.0.0) were used for statistical analysis. Figures were created using JMP Pro (14.0.0) and Sigmaplot (version 11). Data are presented as means ± S.E.M. throughout. The metabolic rate data were log-transformed to meet the assumption of equal variance for ANOVA. To determine if resting metabolic rate varied among caste and PO<sub>2</sub>, repeated measures ANOVA was conducted with PO<sub>2</sub> as the within-subjects factor and caste as the between-subjects factor. Flight metabolic rates were compared using ANOVA, and P<sub>crit</sub> and body mass were analyzed using t-tests. To determine scaling relationships, CO<sub>2</sub> emission rates and body mass were log-transformed and subjected to linear regression. Differences were considered significant if p < 0.05.

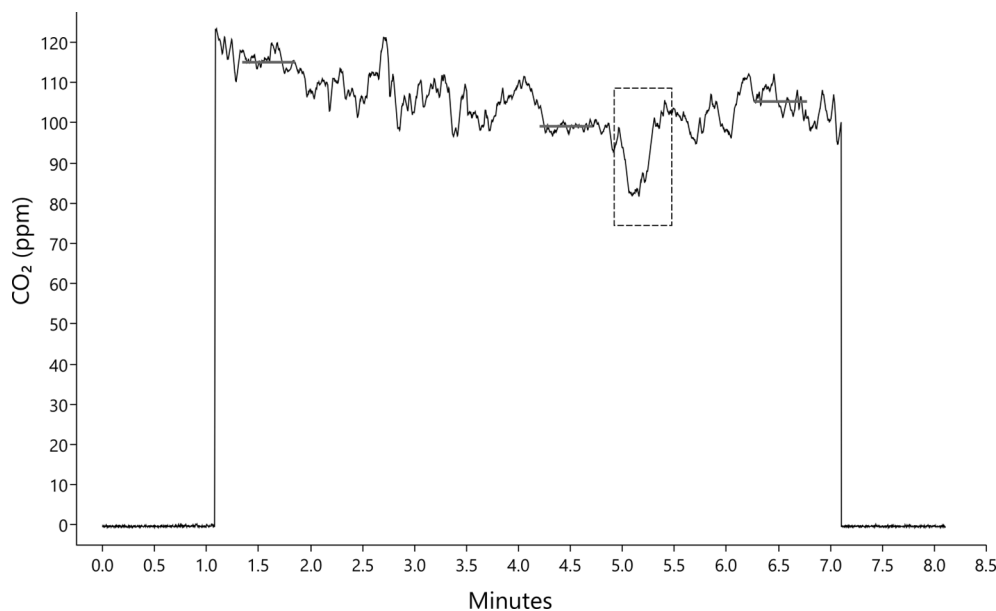
## 3. Results

### 3.1. Resting metabolic rate

For bees at rest, both absolute (F<sub>7,10</sub> = 9.17, p < 0.002) and mass-specific metabolic rates (F<sub>7,10</sub> = 9.8, p < 0.001) decreased as atmospheric oxygen decreased (Fig. 3). Overall, queens had higher absolute metabolic rates than workers (Fig. 3; F<sub>1,16</sub> = 28.93, p < 0.0001), but mass-specific metabolic rate did not differ between castes (Fig. 3; F<sub>1,16</sub> = 0.23, p = 0.6383). Workers (2.78 ± 0.32 kPa) and queens (2.44 ± 0.58



**Fig. 1.** Raw CO<sub>2</sub> emission data (solid, black line) from queen 3. CO<sub>2</sub> emission in Box C (dark gray, dotted line) is significantly lower than that in Box B (black, dashed line), and lower than CO<sub>2</sub> emission combined across all higher PO<sub>2</sub>s (Box A, light gray, dotted line). PO<sub>2</sub> values (right axis; dashed and dotted line) are indicated above the line. The first two minutes at each PO<sub>2</sub> were excluded from the analysis.



**Fig. 2.** Raw CO<sub>2</sub> emission from a worker bumble bee flying in air with a PO<sub>2</sub> of 15 kPa (solid black line). Horizontal gray lines indicate sampling locations for beginning, middle, and end time points used to calculate mean flight metabolic rate at this PO<sub>2</sub>. Dashed box denotes a dip in CO<sub>2</sub> emission that corresponds to bee landing and was excluded from the analysis.

kPa) had similar  $P_{crit}$  values (Fig. 4;  $t = 0.50$ ,  $p = 0.623$ ).

Both queens and workers showed large variation in CO<sub>2</sub> emissions when exposed to 10 and 21 kPa PO<sub>2</sub> (Fig. 3) that could have affected estimates of  $P_{crit}$ . This large variation was likely due to the amplitude and frequency of CO<sub>2</sub> emission peaks during discontinuous gas exchange for some individuals (see DGC peaks in Fig. 1) or to individuals that resumed activity during resting trials. To ensure that this variation was not causing us to overestimate  $P_{crit}$ , we reanalyzed data in two ways: 1) after excluding CO<sub>2</sub> emission estimates for 10 and 21 kPa PO<sub>2</sub> treatments and 2) after excluding two individuals showing high amounts of variation. As in the full analysis, absolute metabolic rate differed between castes when data from 10 and 21 kPa PO<sub>2</sub> were excluded ( $F_{1,16} = 28.9$ ,  $p < 0.0001$ ) and when individuals with highly variable metabolic rates at those PO<sub>2</sub> values were excluded ( $F_{1,14} = 22.7$ ,  $p < 0.001$ ). Mass-specific metabolic rate did not vary between castes when data from 10 and 21 kPa PO<sub>2</sub> treatments were excluded ( $F_{1,16} = 0.23$ ,  $p = 0.6383$ ) or when highly variable individuals were excluded ( $F_{1,14} = 0.02$ ,  $p = 0.8811$ ). No differences between castes were observed when reanalyzing  $P_{crit}$  without those highly variable individuals ( $t = 0.58$ ,  $p < 0.7155$ ).

### 3.2. Flight metabolic rate

During flight, absolute metabolic rates of queens and workers responded differently to decreases in oxygen, with queens having overall higher metabolic rates (Fig. 5; caste  $\times$  oxygen interaction:  $F_{3,72} = 7.39$ ,  $p < 0.0001$ ). However, mass-specific flight metabolic rate did not vary significantly between castes (Fig. 5;  $F_{3,72} = 2.19$ ,  $p = 0.0965$ ). Mass-specific metabolic rate decreased as PO<sub>2</sub> decreased (Fig. 5;  $F_{3,72} = 23.53$ ,  $p < 0.0001$ ). Flight  $P_{crit}$  was estimated to be 10 kPa for both castes.

Queens were significantly larger than workers across all ambient PO<sub>2</sub> treatments (Fig. 6;  $F_{1,72} = 774$ ,  $p < 0.001$ ). For workers, we detected significant variation in mass among PO<sub>2</sub> treatments (Fig. 6; effect of PO<sub>2</sub>:  $F_{3,36} = 3.6$ ,  $p < 0.03$ ). Due to the haphazard selection of bees, workers in the 15 kPa PO<sub>2</sub> treatment were significantly smaller than workers in the 7 kPa PO<sub>2</sub> treatment (Tukey HSD,  $p < 0.02$ ).

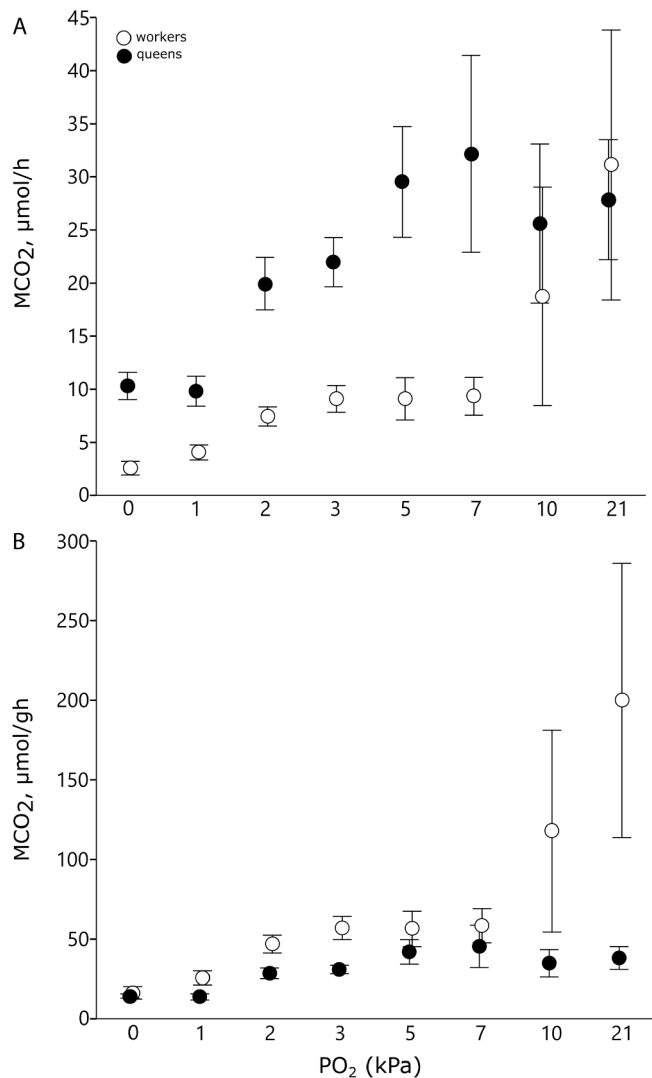
### 3.3. Mass-scaling relationships

At rest, in normoxia, absolute metabolic rate was not correlated with body mass within or across castes (Fig. 7A). Mass-specific metabolic rates did not scale with body mass within castes, but scaled with mass to the  $-0.69$  power when all bees were grouped together (Fig. 7C,  $r^2 = 0.23$ ,  $p < 0.05$ ). Near the  $P_{crit}$  at 5 kPa, absolute resting metabolic rate scaled with mass to the  $0.83$  power when all bees were analyzed together (Fig. 7B), but mass-specific metabolic rate did not vary with mass (Fig. 7D).

During flight in normoxia, absolute metabolic rate scaled to the  $0.9$  power with mass across castes (Fig. 8A). Within queens, absolute metabolic rate trended toward a correlation with mass, but that of workers did not (Fig. 8A). Mass-specific metabolic rate did not scale with mass across all bees or within either of the castes (Fig. 8C). Bees flying in 10 kPa PO<sub>2</sub>, showed stronger scaling relationships, with absolute metabolic rate of all bees scaling with mass to the  $0.83$  power ( $r^2 = 0.91$ ,  $p < 0.0001$ ; Fig. 8B). Within queens, absolute metabolic rate scaled positively with mass but with a much shallower and marginally significant slope (Fig. 8B). Mass-specific flight metabolic rates near the  $P_{crit}$  scaled with body mass in all cases (Fig. 8D). Mass-specific metabolic rate clearly decreased with body mass across castes, and the pattern was even stronger within castes, with mass-specific metabolic rate scaling with mass to approximately  $0.68$  for both workers and queens (Fig. 8D).

## 4. Discussion

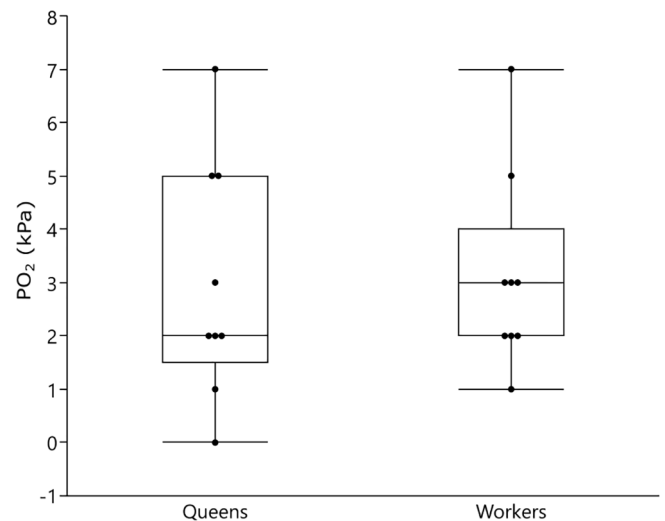
In this study, we measured critical PO<sub>2</sub> ( $P_{crit}$ ) of worker and queen bumble bees to test the hypothesis that castes differ in oxygen delivery capacity. Previous work found pronounced hyperallometric scaling of tracheal volume among workers; larger bees devoted progressively more of their body volume to tracheal structures (tracheal volume scaled with mass<sup>2.6</sup>; Vogt and Dillon, 2013). Queens, however, were not simply large workers, because they had surprisingly small tracheal volumes for their body size (Vogt and Dillon, 2013). Based on this and the isometry of tracheole dimensions across body size and caste (Vogt et al., 2014), we hypothesized that oxygen delivery in queens is likely compromised, making them less tolerant of low oxygen conditions. Surprisingly, we found no difference in  $P_{crit}$  between castes either when at rest or during



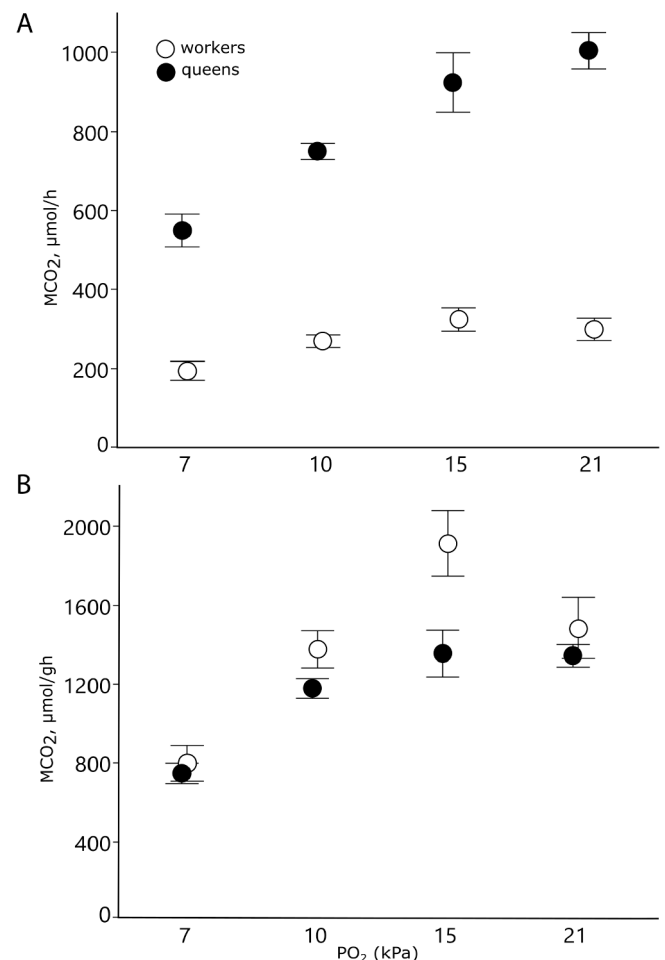
**Fig. 3.** Absolute (A) and mass-specific (B) CO<sub>2</sub> emission rates of queens (filled) and workers (open) at rest as a function of ambient PO<sub>2</sub>. Absolute metabolic rates differed significantly between queens and workers, but mass-specific metabolic rate did not (see text for statistical details).

more energetically demanding flight (Figs. 3–5), suggesting that despite reduced tracheal system volumes, queens are tolerant of hypoxia. Possibly including more PO<sub>2</sub> treatments, particularly in flight experiments, and larger sample sizes would reveal  $P_{crit}$  differences between castes, particularly given the striking differences in tracheal volume previously documented (Vogt and Dillon, 2013). Regardless, both worker and queen bumble bees appear to have a remarkable capacity for oxygen delivery, given that they can sustain metabolism down to ambient PO<sub>2</sub> of 2–3 kPa at rest.

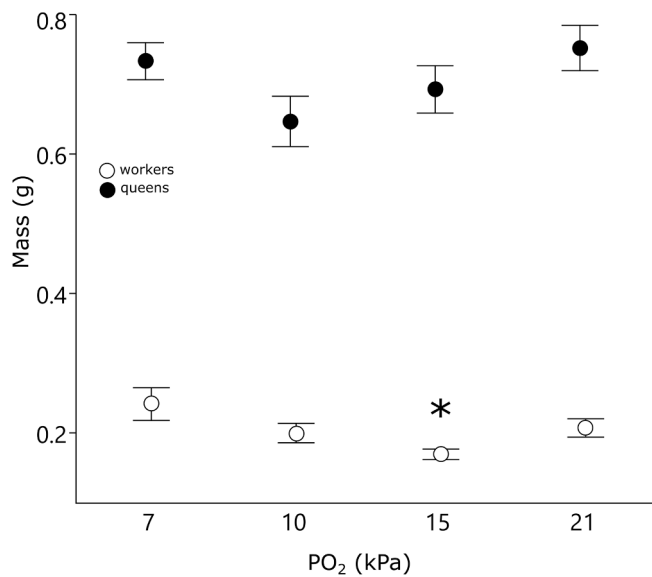
Although no significant differences in  $P_{crit}$  were observed, *B. impatiens* was highly tolerant to hypoxia, with similar  $P_{crit}$  values as other terrestrial insect species (Harrison et al., 2006; Hoback and Stanley, 2001; Lease et al., 2012; Owings et al., 2014). The rationale for our original hypothesis was based on the tracheal physiology of the queens, with their tracheal system volume being smaller than expected for their large body size (Vogt and Dillon, 2013), potentially indicating a lower oxygen delivery capacity. Despite the tracheal system proportion differences, we saw no difference in  $P_{crit}$  between workers and queens, which begs the question: do workers have a larger than required tracheal system? Workers and queens may have similar tracheal volumes because the queens require more body space to accommodate for eggs, as predicted in previous work (Greenlee et al., 2009; Kaiser et al., 2007). Other



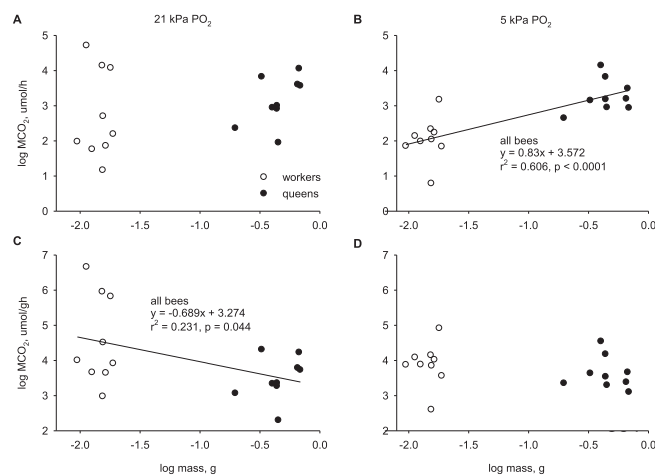
**Fig. 4.** Resting critical PO<sub>2</sub> did not differ significantly between queens and workers. Bars indicate the max and min, box borders indicate the first and third quartiles, and the line indicates the median.



**Fig. 5.** Absolute (A) and mass-specific (B) CO<sub>2</sub> emission rates of queens (filled) and workers (open) during flight as a function of ambient PO<sub>2</sub>. Absolute metabolic rates differed significantly between queens and workers, but mass-specific metabolic rate did not (see text for statistical details).

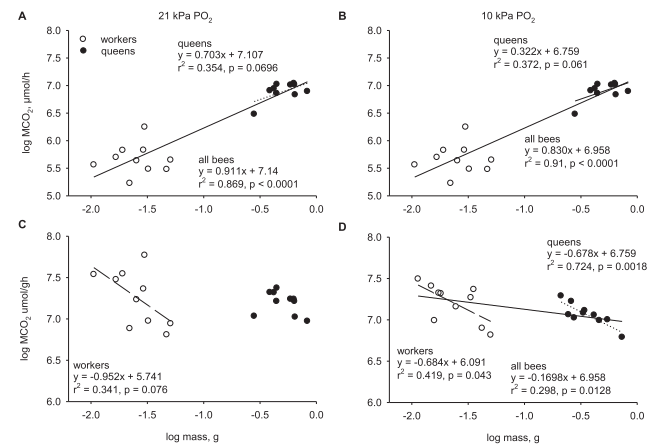


**Fig. 6.** Body mass of queens and workers used in flight critical  $\text{PO}_2$  experiments. Workers at 15 kPa  $\text{PO}_2$  were significantly smaller than workers at 7 kPa  $\text{PO}_2$  denoted by asterisk ( $p < 0.02$ ).



**Fig. 7.** Log-log plots of absolute (A, B) and mass-specific (C, D)  $\text{CO}_2$  emission rate versus mass for each caste in 21 kPa  $\text{PO}_2$  (left column) and 5 kPa  $\text{PO}_2$  (right column) at rest. Regression equations (solid lines) are shown for the significant relationships among all bees in B and C.

studies evaluating  $P_{\text{crit}}$  in insects found no differences in oxygen delivery capacity relating to body size (Greenlee and Harrison, 2004; Greenlee et al., 2007; Lease et al., 2012), but these were not eusocial species. This may suggest that *B. impatiens* queens have a small safety margin for gas exchange while workers have a much larger safety margin for oxygen delivery. The data on mass-scaling further support this idea. The high variability observed in resting metabolic rates at ambient  $\text{PO}_2$  was reduced at 7 kPa  $\text{PO}_2$ , suggesting slight constraints of reduced  $\text{O}_2$ . This constraining effect of  $\text{PO}_2$  was most evident when demand for oxygen was high, i.e., during flight. We saw less variability in metabolic rate for flying bumble bees at ambient  $\text{PO}_2$ , and at 10 kPa  $\text{PO}_2$ , flight metabolic rate became even more constrained and mass-dependent. The low  $P_{\text{crit}}$  values for bumble bees here suggest that queens and workers alike are overbuilt – workers perhaps need to have a large respiratory system capacity to power flight for foraging and queens possibly because they have to store eggs in lieu of taking up body volume with tracheae. Workers are likely “smaller queens” that devote more body volume to tracheae and air sacs, because that volume is not as necessary for egg



**Fig. 8.** Log-log plots of absolute (A, B) and mass-specific (C, D)  $\text{CO}_2$  emission rate versus body mass for each caste in 21 kPa  $\text{PO}_2$  during flight. Regression equations shown for the significant relationship among all bees (solid lines in panels A, B, and D), among queens (dotted line in D) and among workers (dashed line in D). Non-significant trends are also shown for queens in 21 kPa (A; dotted line) and in 10 kPa (B; short solid line) and workers in 21 kPa (B; dashed line).

storage. However, workers can develop and lay eggs depending on social and nutritional conditions (Cnaani et al., 2002), so it would be interesting to compare tracheal volumes and  $P_{\text{crit}}$  values of workers and queens with different levels of egg development.

Despite having similar  $P_{\text{crit}}$  values, there was a significant effect of caste on absolute  $\text{CO}_2$  emission rate. Furthermore, queens and workers seemed to respond differently to decreasing levels of oxygen, with queens appearing to decrease metabolic rate more steeply as  $\text{PO}_2$  dropped from 7 to 2 kPa (Fig. 3A). This pattern disappeared when correcting for mass (Fig. 3B), suggesting that the queen’s larger body mass, rather than caste per se, was responsible. To tease apart possible effects of body mass, we calculated scaling coefficients for queens, workers, and all bees together at rest and in flight in normoxia and near the  $P_{\text{crit}}$ , 5 kPa for rest and 10 kPa for flight (Figs. 7–10). We expected to see different scaling relationships with mass for queens when compared to that of workers, but in most cases the relationship between  $\text{CO}_2$  emission and body mass were not significant. Another study measuring metabolic scaling in *B. impatiens* at rest and during flight in normoxia found no difference in mass-scaling between queens and workers, although there was a significant scaling relationship between metabolic rate and body mass (Billardon and Darveau, 2019). Along with the high variability in normoxia, our sample size was much smaller, possibly contributing to the lack of a detectable allometric relationship. The one exception we identified was during flight at 10 kPa, in which metabolic rates of queens and workers both scaled with  $\text{mass}^{-0.68}$ .  $\text{CO}_2$  emission of all bees showed a different relationship with body mass, scaling with  $\text{mass}^{-0.17}$ . Thus, from this study, no clear pattern emerged with respect to body mass effects on metabolic rate.

Bumble bee queens are thought to overwinter underground at depths of 2–15 cm, depending in part on the density of the soil in which the nest is located (Alford, 1969). These overwintering sites may become hypoxic over time, depending on the depth and soil type (Kung et al., 1990; Silver et al., 1999). In dry conditions, soil oxygen concentrations do not generally decrease below what we determined to be the  $P_{\text{crit}}$  for queens (Kung et al., 1990). A study in the tsetse fly, *Glossina pallidipes*, suggested that hypoxia tolerance was likely not related to underground hypoxia exposure (Basson and Terblanche, 2010; Kung et al., 1990). However, queens, especially in northern latitudes, may overwinter in wet and potentially frozen conditions. These conditions could create hypoxic environments, given enough time (Silver et al., 1999). However,

additional research on overwintering sites of queens is needed to draw further conclusions. The queens in this study were from late summer and would be expected to be preparing to overwinter, and as such, likely had no ovary development (Röseler, 1985). Early spring queens emerging from overwintering hibernacula and developing ovaries might differ in hypoxia tolerance, as might queens that have established colonies, ceasing to leave the nest to forage and with fully developed ovaries.

Workers are the primary foragers of the colonies, so frequent or extended periods of flight with frequent stops may be required (Wolf and Moritz, 2008), which could require larger safety margins for gas exchange. Closely related *Bombus* (Pyrobombus) species have estimated foraging ranges of 25–100 m away from their original nest (Geib et al., 2015), which suggests these bees may be making short and frequent trips rather than for extended lengths of time. Flight energetics studies in bees provide mixed results for whether different sized reward loads carry an increase in metabolic rate related to the load size itself (Feuerbacher, 2003; Moffatt, 2000), but a study analyzing pollen versus nectar loads found a difference in flight performance from the two different reward types (Mountcastle et al., 2015). Further, pronounced increases in mechanical power requirements during loading likely necessitate increased energetic demands (Buchwald and Dudley, 2010). Remarkably, recent work suggests that bumble bees can modulate energetic demands of flight with loads dependent on wing beat kinematics (Combes et al., 2020); this capacity might help ameliorate metabolic demand while carrying loads or during maneuvering flights in turbulent environments, which can be costly (Crall et al., 2015). Although these results are intriguing, much remains to be elucidated, including whether the low  $P_{crit}$  values reported here overestimate respiratory capacity, since they involved individuals not carrying loads, and how insect age and division of labor might affect respiration. Hypoxia tolerance may facilitate bumble bee success at high elevations where reduced air density challenges force production and therefore oxygen demand while reduced ambient  $PO_2$  challenges oxygen delivery (Dillon and Dudley, 2014; Dillon et al., 2006).

#### CRedit authorship contribution statement

**Rikki M. Walter:** Methodology, Formal analysis, Investigation, Visualization, Writing - original draft. **Joseph P. Rinehart:** Conceptualization, Methodology, Writing - review & editing, Supervision. **Michael E. Dillon:** Validation, Writing - review & editing. **Kendra J. Greenlee:** Conceptualization, Methodology, Formal analysis, Visualization, Funding acquisition, Supervision, Writing - review & editing.

#### Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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#### Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jinsphys.2021.104297>.

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