

RESEARCH ARTICLE

Fluctuations in oxygen influence facultative endothermy in bumblebees

Edward M. Dzialowski^{1,*}, Glenn J. Tattersall², Stewart C. Nicol³ and Peter B. Frappell⁴**ABSTRACT**

Bumblebees are facultative endotherms, having the ability to elevate thorax temperature above ambient temperature by elevating metabolism. Here, we investigated the influence of hypoxia on metabolic demands and thermoregulatory capabilities of the bumblebee *Bombus terrestris*. We measured thorax temperature, rates of oxygen consumption and carbon dioxide production, and abdominal pumping rates of bees randomly exposed to oxygen levels of 20, 15, 10 and 5 kPa at 26°C. Under normoxia, bumblebees maintained an elevated mean thorax temperature of 35.5°C. There was no significant change in thorax temperature at 15 kPa O₂ (33.4°C). Mean thorax temperature decreased significantly at 10 kPa O₂ (31.6°C) and 5 kPa O₂ (27.3°C). Bees were able to maintain an elevated metabolic rate at 15 and 10 kPa O₂. In normoxia, endothermic bees exhibited periods of rapid abdominal pumping (327 min⁻¹) interspaced by periods of no abdominal pumping. At 10 kPa O₂, abdominal pumping rate decreased (255 min⁻¹) but became more continuous. Upon exposure to 5 kPa, metabolic rate and abdominal pumping rate (152 min⁻¹) decreased, although the animals continued abdominal pumping at the reduced rate throughout the exposure period. Bumblebees are able to meet the energetic demands of endothermy at 15 kPa O₂, but become compromised at levels of 10 kPa O₂ and below.

KEY WORDS: Thermoregulation, *Bombus terrestris*, Hypoxia, Ventilation, Metabolism

INTRODUCTION

Low oxygen (hypoxia) is well recognized for its influence on thermoregulation in animals (Bicego et al., 2007). In numerous taxa, hypoxic exposure augments external heat exchange (Tattersall and Milsom, 2003), lowers thermal preference (Dupré and Wood, 1988; Dupré and Owen, 1992; Tattersall and Boutilier, 1997; Wiggins and Frappell, 2002; Cadena and Tattersall, 2009), reduces metabolic rate (Barros et al., 2001), and inhibits shivering (Barros et al., 2001) and non-shivering heat production (Madden and Morrison, 2005). The prevailing hypothesis is that low environmental oxygen reduces thermoregulatory thresholds for the activation of thermoeffector responses (Steiner and Branco, 2002; Bicego et al., 2007; Tattersall and Milsom, 2009). By augmenting heat loss through physiological or behavioral means, and minimizing heat production, animals exposed to low oxygen are capable of rapidly lowering body

temperature. These physiological mechanisms support the notion of a flexible thermoregulatory system, wherein key elements of thermoregulatory control respond to specific stressors to reduce thermosensitivity and thresholds for defense of body temperature (Tattersall and Milsom, 2009). Insects have extraordinary hypoxia tolerance, partly due to the highly efficient mode of gas exchange and partly because respiratory gases are efficiently regulated via spiracular opening and closing (Contreras and Bradley, 2009). Perhaps as a result of their efficient control over spiracular and tracheolar conductance, insects typically exhibit quite low critical P_{O_2} values (P_{crit} , the P_{O_2} below which metabolic rate begins to conform to the inspired level of oxygen); P_{crit} values in many insects are typically between 0.7 and 5 kPa (Harrison and Roberts, 2000; Greenlee and Harrison, 2005; Lease et al., 2012). Exposure to mild hypoxia does not necessarily lead to oxygen limitation at the tissue level, because of tight regulation of tracheolar oxygen through changes in systemic conductance (Hetz and Bradley, 2005). In insects, higher temperatures may diminish the ability of oxygen delivery to keep pace with oxygen demand (Frazier et al., 2001), although recent work suggests that oxygen delivery meets demand even at high temperatures (Mölich et al., 2012). In endothermic insects, where elevated metabolism (i.e. thermogenesis) increases body temperature above ambient temperature, insufficient oxygen delivery relative to demand may occur at higher environmental oxygen levels (a higher P_{crit}) or comparatively lower temperatures, relative to ectothermic insects. Examining thermogenesis in endothermic insects, such as bumblebees, in response to hypoxia allows for a test of whether there is a systemically regulated decline in heat production versus an oxygen limitation at the level of the heat-producing tissues.

As facultative endotherms, bumblebees are highly suited for examination of the thermoregulatory consequences of hypoxia. Provided they have access to adequate energy stores, even in the absence of movement of the wings and thorax, non-flying bees are able to maintain elevated thorax temperatures, especially during brooding and flight warm-up (Heinrich and Kammer, 1973; Heinrich, 1972a; Heinrich, 1972b; Heinrich, 1972c; Heinrich, 1974). Not only do they exhibit spiracular control over oxygen conductance (Contreras and Bradley, 2009) but also they exhibit abdominal pumping, which plays an important role in ventilating tracheolar gases as well as possibly assisting in hemolymph flow (Heinrich, 1971). Abdominal pumping correlates well with levels of carbon dioxide production and has been proposed as an indirect measure of body temperature (T_b) (Heinrich, 1976).

Abdominal pumping has been implicated in altering hemolymph flow (Mänd et al., 2006), as well as in the regulation of thorax and abdominal temperatures in bumblebees. Under conditions of artificial heating (i.e. when bees are warmed above their regulated temperature), abdominal pumping increases with an associated decrease in thorax temperature (Heinrich, 1976). This suggests that under thermal stress, abdominal pumping serves to facilitate heat

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List of symbols and abbreviations

FE_{CO_2}	excurrent CO_2 fraction
FE_{O_2}	excurrent O_2 fraction
FI_{CO_2}	incurrent CO_2 fraction
FI_{O_2}	incurrent O_2 fraction
P_{O_2}	partial pressure of oxygen (kPa)
P_{crit}	P_{O_2} below which metabolic rate begins to decrease (kPa)
$T_{ab,s}$	abdomen surface temperature
T_a	air temperature
T_{th}	thorax temperature
$T_{th,s}$	thorax surface temperature
\dot{V}_{CO_2}	carbon dioxide production rate ($ml\ CO_2\ h^{-1}$)
\dot{V}_I	flow rate ($ml\ h^{-1}\ STP$)
\dot{V}_{O_2}	oxygen consumption rate ($ml\ O_2\ h^{-1}$)

loss by redistributing heat between the thorax and abdomen. Heat exchange between the thorax and abdomen depends on hemolymph flow (Heinrich, 1976): the bumblebee heart and aorta pump hemolymph anteriorly, while returning hemolymph passes posteriorly around the aorta. Heinrich (Heinrich, 1976) proposed that hemolymph flow operates as a counter-current mechanism, conserving heat produced within the thorax. In order to diminish the counter-current mechanism and specifically distribute heat to the abdomen, bumblebees make use of abdominal and diaphragm pumping to lock hemolymph flow between the thorax and abdomen out of phase, so that heat is not retained in the thorax but redirected to the abdomen. Similar differential control over thorax and abdomen temperature through hemolymph distribution has also been observed in the facultatively endothermic sphingid moth *Manduca sexta* (Heinrich, 1971).

The objectives of this study were to understand the thermoregulatory response to changing oxygen availability in endothermic European bumblebees, *Bombus terrestris* (Linnaeus 1758). We tested three hypotheses: (1) hypoxia reduces thermogenesis, and thereby eliminates endothermy in the bumblebee, the effect being reversible when hypoxia is removed; (2) reduction in thermogenesis is related to O_2 limitation, and thus thermogenesis will exhibit a linear relationship with oxygen; (3) physiological responses geared toward augmenting oxygen delivery (e.g. abdominal pumping) will accompany the metabolic and thermoregulatory responses to hypoxia.

RESULTS

Body mass

The mean mass of bumblebees used for thorax temperature and respiration measurements in November 2010 was 789 ± 148 mg ($N=7$). The mean mass of bumblebees used for thermal imaging measurements in February 2011 was 348 ± 141 mg ($N=7$).

Effects of oxygen level on thorax and abdomen temperatures

Oxygen availability significantly influenced bumblebee thermoregulation ($F_{3,16}=28.1$; $P<0.001$). Bumblebees maintained an elevated thorax temperature (T_{th}) under normoxic conditions (Fig. 1). Thorax temperature was unchanged at 15 kPa oxygen exposure. At 10 kPa oxygen, T_{th} decreased significantly when compared with 20 kPa. At 5 kPa oxygen, the animal no longer elevated T_{th} above ambient temperature (T_a).

Variability of T_{th} decreased with decreasing oxygen exposure (Fig. 2). At 20 and 15 kPa, the variability in T_{th} was relatively large as the animals tended to regulate their T_{th} between a relatively high maximum and minimum. During exposure to 10 kPa oxygen, the

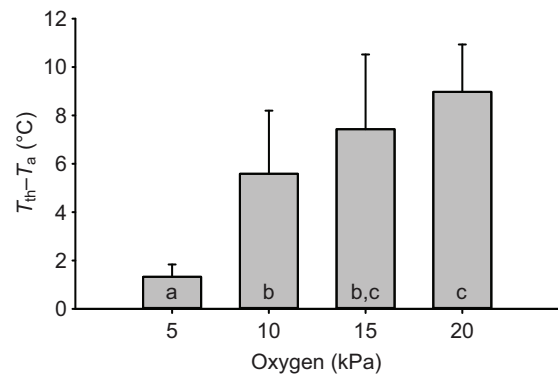


Fig. 1. Thorax temperature of endothermic bumblebees in normoxia and hypoxia. Difference between thorax temperature (T_{th}) and ambient temperature (T_a , 26°C) in endothermic bumblebees. Groups that are not significantly different share a letter.

variability decreased significantly, and at 5 kPa oxygen, T_{th} decreased to 27°C and showed almost no variability.

The response of surface abdominal and thoracic temperature ($T_{ab,s}$ and $T_{th,s}$) to hypoxia was dependent upon the level of oxygen

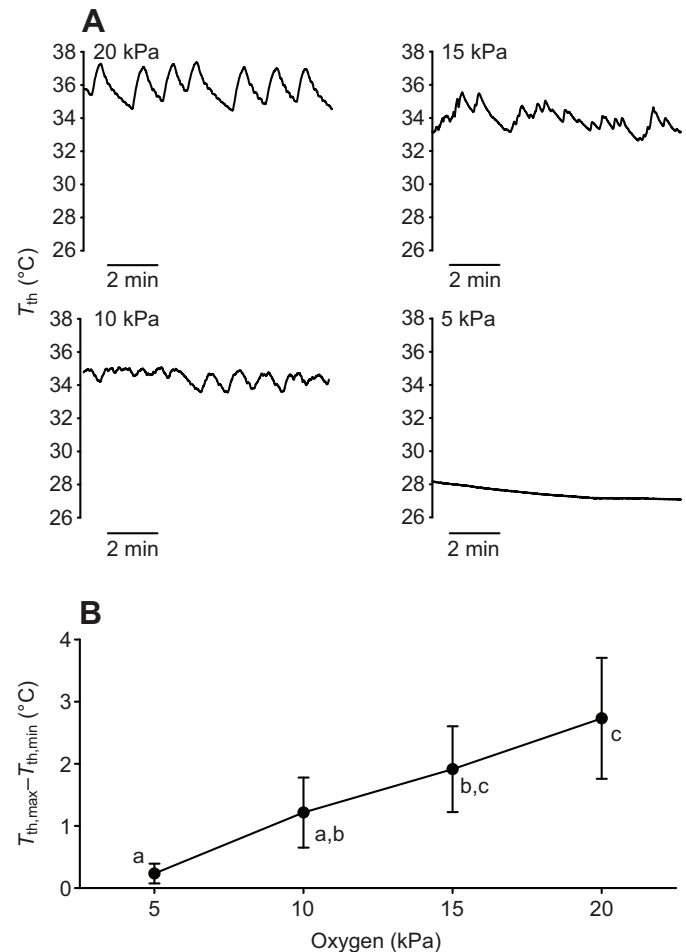


Fig. 2. Variability in T_{th} decreases with decreasing levels of oxygen. (A) Example T_{th} from a bumblebee exposed to 20, 15, 10 and 5 kPa O_2 . Temperature traces are taken from the same individual during the last portion of each oxygen exposure. (B) Mean difference between the maximum T_{th} and minimum T_{th} as a measure of the variability of T_{th} in response to 20, 15, 10 and 5 kPa O_2 . Groups that are not significantly different share a letter.

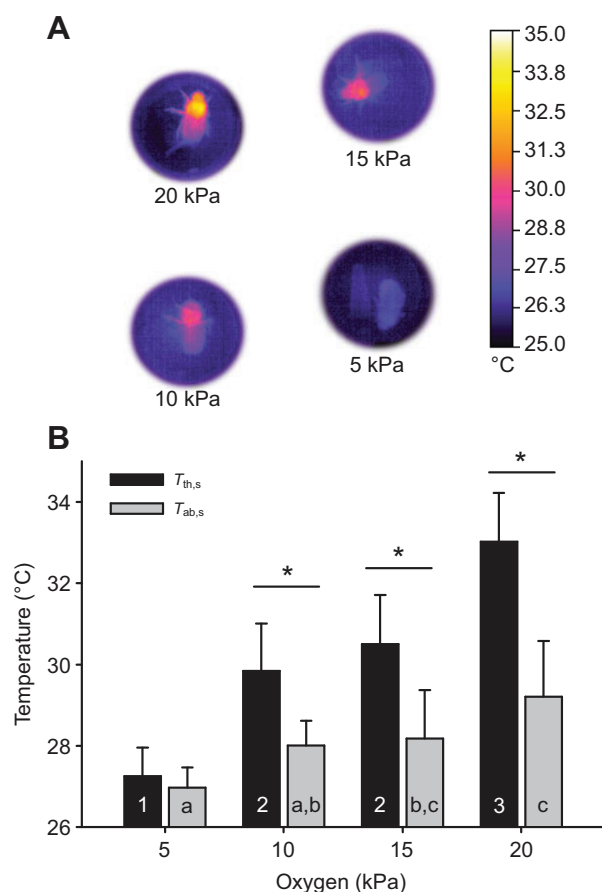


Fig. 3. Thorax and abdomen surface temperatures of thermoregulating bumblebees in normoxia and hypoxia. (A) Example of recorded thermographic images of a thermoregulating bumblebee at 20, 15, 10 and 5 kPa oxygen. (B) Mean thorax and abdomen surface temperatures ($T_{th,s}$ and $T_{ab,s}$, respectively) of bumblebees. Within a body location, groups with different letters (abdomen) or numbers (thorax) are significantly different between treatments. Within an oxygen level, asterisks represent significant differences between the two locations.

(Fig. 3A,B; $F_{3,18}=11.6$, $P<0.001$). Within an oxygen level, $T_{th,s}$ was significantly higher than $T_{ab,s}$ except at 5 kPa, where they did not differ. $T_{th,s}$ was highest at 20 kPa oxygen; it remained elevated at 15 and 10 kPa, but was significantly lower than at 20 kPa. $T_{th,s}$ was significantly lower at 5 kPa oxygen when compared with the higher levels of oxygen. $T_{ab,s}$ exhibited similar changes in response to decreasing oxygen.

The pattern of warming and cooling of $T_{th,s}$ and $T_{ab,s}$ differed upon exposure to 5 kPa oxygen and subsequent return to normoxia (Fig. 4). Upon exposure to 5 kPa oxygen, $T_{th,s}$ cooled gradually and on return to normoxia it increased rapidly (Fig. 4A). From the cooling and warming curves in Fig. 4A, we estimated thermal time constants as the time it took the $T_{th,s}$ excursion to reach 63.2% of the asymptotic value. The thermal time constant for cooling in response to a change from 20 kPa O_2 to 5 kPa was 153 s. In comparison, bumblebees actively warmed more quickly when returned to normoxia, with a thermal time constant of 77 s. Changes in $T_{th,s}$ and $T_{ab,s}$ did not appear to be coupled during 5 kPa oxygen cooling or reoxygenation warming (Fig. 4B). During cooling, $T_{th,s}$ decreased upon exposure to 5 kPa O_2 while $T_{ab,s}$ remained constant. On return to normoxia, $T_{th,s}$ began to increase without a change in $T_{ab,s}$. The abdominal temperature excess was

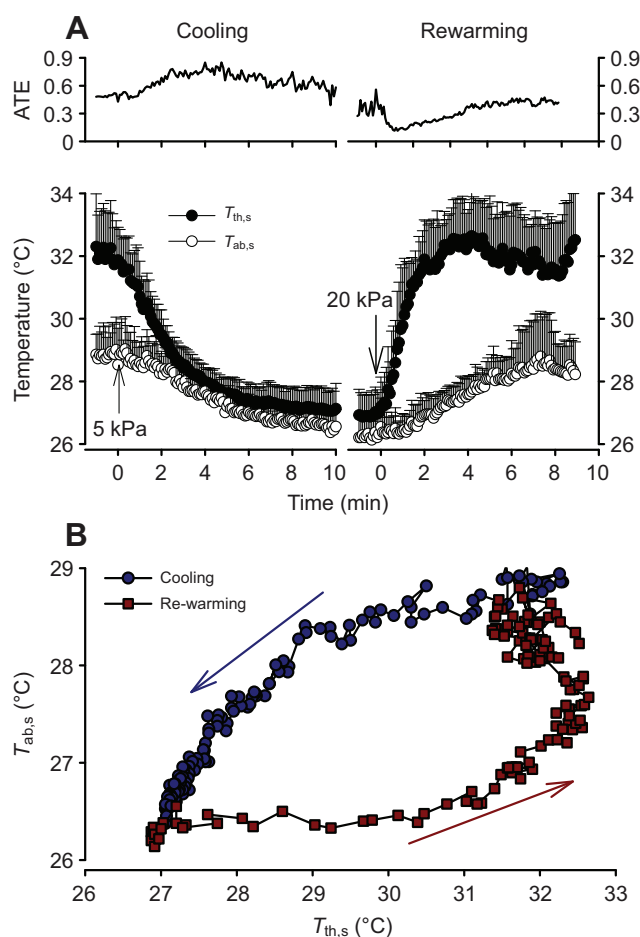


Fig. 4. Initial bumblebee surface temperature responses upon exposure to 5 kPa oxygen and subsequent reoxygenation (20 kPa). (A) Mean $T_{th,s}$ and $T_{ab,s}$ upon exposure to 5 kPa O_2 followed by 20 kPa O_2 . The corresponding abdominal temperature excess (ATE) ratio for each time point during the hypoxic and normoxic exposures is shown at the top. (B) Relationship between $T_{ab,s}$ and $T_{th,s}$ during 5 kPa O_2 exposure (cooling) and subsequent 20 kPa O_2 exposure (warming).

low (<0.2) on initial return to normoxia, indicating that changes in $T_{th,s}$ and $T_{ab,s}$ were uncoupled.

Effects of oxygen level on \dot{V}_{O_2} and \dot{V}_{CO_2}

There was a significant effect of oxygen level on the metabolism of endothermic bumblebees (Fig. 5; O_2 consumption rate, \dot{V}_{O_2} : $F_{3,15}=9.668$, $P<0.001$; CO_2 production rate, \dot{V}_{CO_2} : $F_{3,15}=14.3$, $P<0.001$). Bumblebees were able to maintain elevated \dot{V}_{O_2} and \dot{V}_{CO_2} at 20 and 15 kPa O_2 . There was an insignificant drop in \dot{V}_{O_2} and \dot{V}_{CO_2} at 10 kPa O_2 . At 5 kPa O_2 , \dot{V}_{O_2} and \dot{V}_{CO_2} were significantly lower than at higher O_2 levels. The respiratory exchange ratio was close to unity and was not significantly different between O_2 levels ($P=0.443$).

The changes observed in metabolism mirrored those observed in T_{th} . There was a significant positive relationship between mean T_{th} and \dot{V}_{CO_2} across the different levels of oxygen exposure (Fig. 6; $F_{1,21}=156.3$, $P<0.001$, $r^2=0.88$).

Effects of oxygen level on ventilation rates

Abdominal pumping frequency varied significantly with the level of oxygen exposure (Fig. 7A; $F_{3,12}=33.56$, $P<0.001$). During normoxic exposure, endothermic bees maintained a high mean abdominal

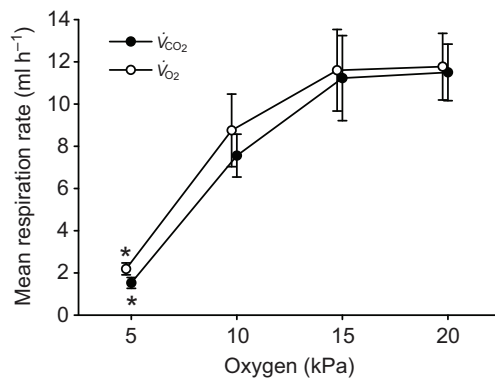


Fig. 5. Metabolism and system conductance of thermoregulating bumblebees in normoxia and hypoxia. Mean rate of oxygen consumption (\dot{V}_{O_2}) and carbon dioxide production (\dot{V}_{CO_2}) measured in thermoregulating bumblebees at 20, 15, 10 and 5 kPa O_2 . Asterisks indicate a significant difference from the 20 kPa value.

pumping rate of 313 min^{-1} (5.2 Hz; Fig. 7). As oxygen levels decreased, abdominal pumping rate decreased significantly at 10 and 5 kPa when compared with that at 20 kPa. At 5 kPa oxygen, bees maintained abdominal pumping, albeit at a much reduced frequency, even though they were unable to maintain an elevated T_{th} . Across all oxygen levels and experiments, there was a significant correlation between abdominal pumping rate and T_{th} (Fig. 7B; $F_{1,17}=195.6$, $P<0.001$, $r^2=0.86$).

The pattern of abdominal pumping changed as animals were exposed to different oxygen levels. Fig. 8 shows a representative response of T_{th} , abdominal pumping and \dot{V}_{CO_2} during 20 and 10 kPa oxygen exposures in an individual endothermic bumblebee. The ventilation pattern at 20 kPa oxygen showed periods of intense abdominal pumping during which T_{th} and \dot{V}_{CO_2} increased. These were followed by a period of repeating short bursts of abdominal pumping during which T_{th} decreased and \dot{V}_{CO_2} appeared to be discontinuous. During the extended periods of active ventilation lasting $34.3 \pm 3.9 \text{ s}$ (mean \pm s.d. of six bouts) as the animal was warming, abdominal pumping frequency increased from 5.25 to 6 Hz. During the shorter periods of abdominal pumping, lasting an average of $4.7 \pm 0.8 \text{ s}$ ($N=11$ bouts) during the cooling phase, the frequency began at 5.5 Hz and decreased with each successive burst to 5 Hz. Each of the brief bouts of abdominal pumping during the cooling phase was followed by a period without pumping lasting $4.9 \pm 0.8 \text{ s}$ ($N=11$ bouts). The relative ventilation volume was lower during short bursts of abdominal pumping when compared with longer periods of pumping. In this same animal,

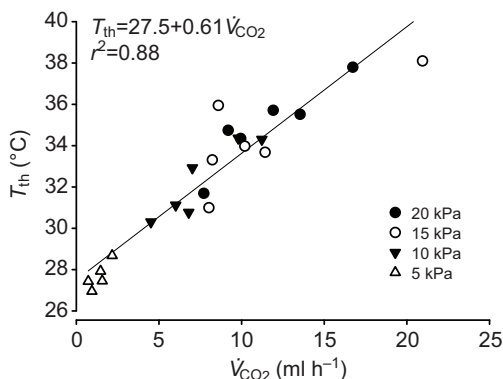


Fig. 6. Relationship between mean T_{th} and \dot{V}_{CO_2} in endothermic bumblebees exposed to 20, 15, 10 and 5 kPa O_2 .

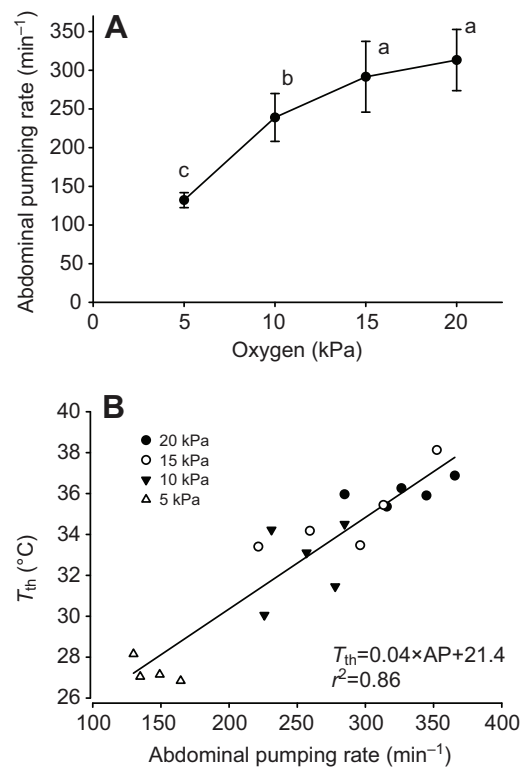


Fig. 7. Abdominal pumping rates of thermoregulating bumblebees at 20, 15, 10 and 5 kPa oxygen. (A) Abdominal pumping rate plotted against P_{O_2} . Oxygen levels with different letters are significantly different from each other. (B) T_{th} plotted against abdominal pumping rate (AP).

exposure to 10 kPa oxygen abolished the variability in abdominal pumping frequency and the repeating short bursts observed at 20 kPa oxygen. Abdominal pumping frequency became invariant around 4.25 Hz during exposure to 10 kPa oxygen. At 10 kPa, there were periods of continuous abdominal pumping associated with warming. This was followed by cooling period of $\sim 30 \text{ s}$ during which the animal had only one short burst of abdominal pumping.

Pumping frequency decreased as oxygen levels declined from 20 to 5 kPa (Fig. 9). The spectrogram revealed during the first 10 min of exposure that the animal continuously pumped its abdomen. This was followed by intermittent bursts of abdominal pumping. During the periods of 5 kPa oxygen exposure, the bumblebees continued to pump their abdomen, maintaining \dot{V}_{CO_2} above 0 (Fig. 10). Normoxic non-endothermic resting bees exhibited discontinuous gas exchange with periods of abdominal pumping and respiration followed by longer periods during which CO_2 release approached 0 (Fig. 10A). In contrast, endothermic bumblebees with an elevated T_{th} prior to exposure to 5 kPa O_2 maintained abdominal pumping and a continuous \dot{V}_{CO_2} when exposed to 5 kPa (Fig. 10B).

DISCUSSION

Bumblebees are well known for their ability to maintain a high T_{th} across a wide range of ambient temperatures. Maintenance of T_{th} by endothermic heat generation requires a high level of energy expenditure and sufficient oxygen supply to sustain high levels of aerobic metabolism. Few studies have examined constraints on energetics and thermal responses of endothermic insects during hypoxia. We found that aerobic metabolism and endothermic

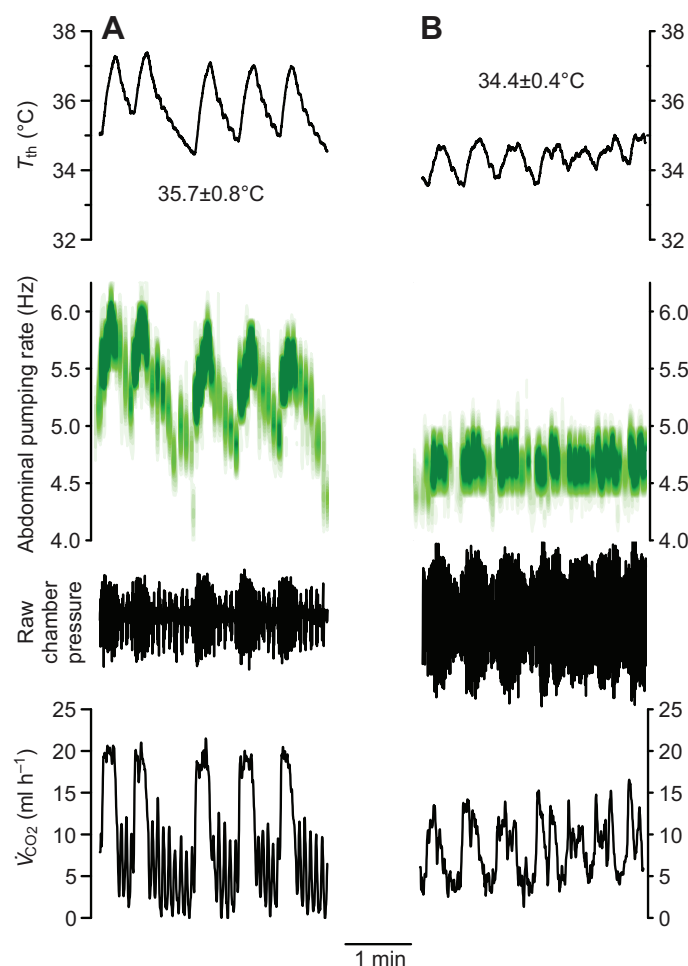


Fig. 8. Sample traces of T_{th} , abdominal pumping rate, raw chamber pressure and \dot{V}_{CO_2} at different P_{O_2} . Traces are from the same bumblebee at (A) 20 kPa and (B) 10 kPa. T_{th} is given as mean \pm s.d. Abdominal pumping frequency was calculated using fast Fourier transform (FFT) from the raw chamber pressure and is presented as a spectrogram showing the power (light green, low; dark green, high) for abdominal pumping frequencies between 4 Hz (240 min⁻¹) and 6.25 Hz (375 min⁻¹).

capacity in bumblebees are limited as oxygen availability decreases. In response to increasing hypoxia, both T_{th} and \dot{V}_{CO_2} decreased significantly; however, the critical oxygen tensions resulting in these changes were not identical, as metabolic rate was maintained down to lower oxygen levels. Ventilation rate, in contrast, decreased in the highest hypoxic exposure, with intermediate levels of oxygen driving slower but more continuous abdominal pumping.

Effects of oxygen on body temperature

Hypoxic exposure constrains the thermoregulatory capacity of endothermic bumblebees. These results are in accord with previous work that has shown that honeybees were able to maintain T_{th} above ambient temperature at P_{O_2} levels as low as 4.9 kPa (Joos et al., 1997). In the case of both large and small bumblebees, this manifested as a slight reduction in T_{th} and $T_{th,s}$ in mild hypoxia, followed by a profound reduction at lower P_{O_2} levels coincident with the depression of metabolic heat production. In short, this response is similar to what has been observed in numerous other ectothermic and endothermic animals, wherein the apparent body temperature set-point is reduced in severe hypoxia (Steiner and Branco, 2002; Bicego et al., 2007; Tattersall and Milsom, 2009).

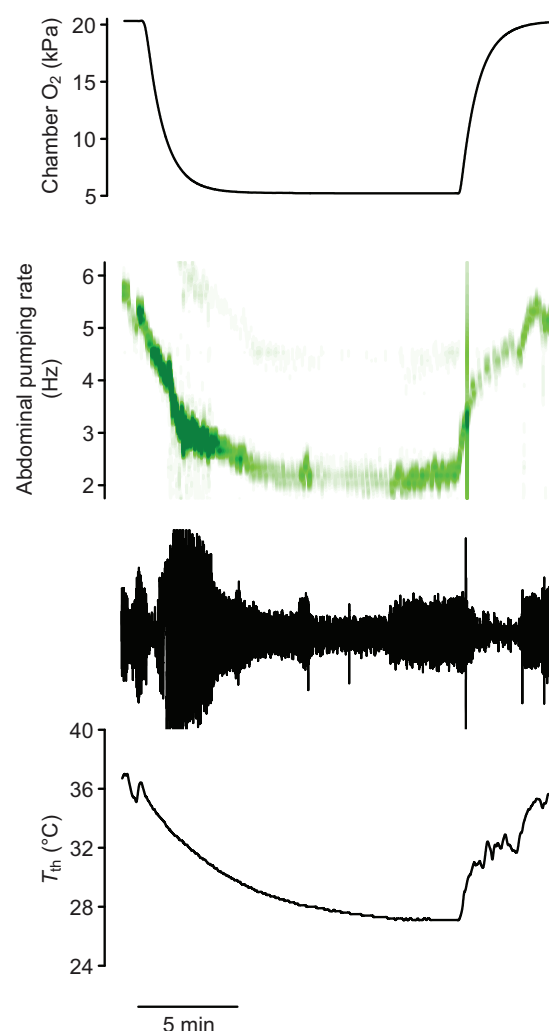


Fig. 9. Sample traces of chamber oxygen, abdominal pumping rate and T_{th} of a bumblebee switching from 20 to 5 kPa O_2 . Abdominal pumping frequency is presented as in Fig. 8.

Bumblebees demonstrate almost complete metabolic regulation, as well as constancy in heat production, between 10 and 20 kPa O_2 , despite observing surface temperature differences between 20 and 10 kPa O_2 . In other words, oxygen impacts differently on the various thermoregulatory pathways in the bumblebee and thermolysis occurring prior to metabolic downregulation. This situation is similar to what has been observed in small mammals (Tattersall et al., 2002; Tattersall and Milsom, 2009). Exposure to low O_2 levels, however, does not appear to have a long-term consequence in terms of thermoregulatory ability. Bumblebees that were exposed to the lowest levels of O_2 for 20 min rapidly rewarmed upon exposure to 20 kPa O_2 (Figs 4 and 9). Similar rapid physiological responses to changing P_{O_2} from 5 to 20 kPa have been observed in honeybees. Upon return to normoxia, the honeybees that were incapable of flying at 5 kPa O_2 were able to fly immediately upon return to 20 kPa (Joos, et al., 1997).

The hysteresis that exists in the timing of cooling in response to 5 kPa O_2 and rewarming when returned to 20 kPa suggests bumblebees are cooling passively in response to 5 kPa whereas as soon as they are re-exposed to 20 kPa, they begin to warm their thorax actively while the abdomen temperature lags behind (Fig. 4).

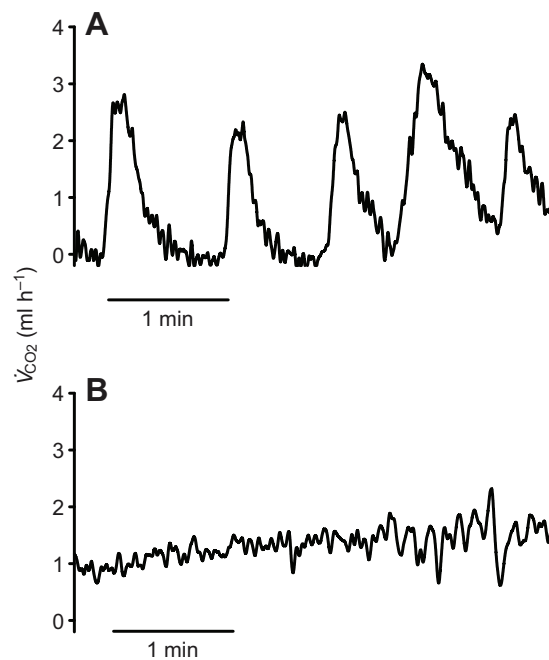


Fig. 10. Sample traces of \dot{V}_{CO_2} in bumblebees at 20 and 5 kPa O_2 . (A) A resting, normoxic bumblebee that was exhibiting little thermogenesis. (B) Another bumblebee that had been endothermic prior to exposure to 5 kPa O_2 .

Bumblebees have a countercurrent exchange mechanism that allows them to retain heat preferentially in the thorax when warming (Heinrich, 1976). This countercurrent exchanger can be short circuited to allow for the transfer of heat from the thorax into the abdomen. It appears from the independent changes in surface temperatures at the abdomen and thorax that thermal responses to changes in O_2 are uncoupled in the thorax and abdomen when animals are cooling or rewarming. During rewarming, it was not until $T_{\text{th},s}$ reached its plateau that $T_{\text{ab},s}$ began to rise, suggesting that the animals may be using their countercurrent exchanger to conserve heat when they are relatively cool but activating thoracic thermogenesis.

Effects of oxygen on metabolic rates

In bumblebees, during endothermic bouts, \dot{V}_{CO_2} can rise to between 42 and 50 times higher than observed in quiescent, endothermic individuals. Our findings show that between 15 and 10 kPa O_2 both \dot{V}_{CO_2} and \dot{V}_{O_2} begin to decline. As P_{O_2} decreases further from 10 to 5 kPa, the endothermic bumblebees significantly decrease \dot{V}_{CO_2} and \dot{V}_{O_2} (Fig. 5A). The critical P_{O_2} for endothermic levels of metabolism lies somewhere between 10 and 5 kPa. Bumblebee \dot{V}_{CO_2} levels rise to an even greater level upon flight (Kammer and Heinrich, 1974). It has been shown that flight and \dot{V}_{CO_2} are influenced by oxygen levels in the dragonfly *Erythemis simplicicollis* (Harrison and Lighton, 1998). The ability to fly decreased significantly in these dragonflies at P_{O_2} values somewhere between 10 and 5 kPa. Similar decreases in flight \dot{V}_{CO_2} at P_{O_2} values of 10 kPa O_2 and lower were observed in the locust (Rascón and Harrison, 2005). These animals lose the ability for sustained flight somewhere between 21 and 10 kPa O_2 ; carbon dioxide production during flight or attempted flight decreased between 20 and 10 kPa O_2 . In the American locust, first minute jumping rate and endurance time were significantly reduced at 5 kPa O_2 across the 2nd, 4th and 6th instar and adults (Kirkton, et al., 2005). Honeybees exposed to 4.9 kPa O_2 were unable to maintain flight (Joos et al., 1997) and their \dot{V}_{CO_2} levels

decreased to those observed in our 5 kPa O_2 bumblebees. Given the higher levels \dot{V}_{CO_2} needed for flight in the bumblebee compared with maintaining endothermy alone, flight should be constrained to a greater extent by low oxygen levels than only maintaining endothermy. In summary, during energetically expensive activities, \dot{V}_{CO_2} and \dot{V}_{O_2} of insects become limited by oxygen availability.

In contrast, resting metabolic rates in insects are not strongly influenced by oxygen levels. In the American locust, *Schistocerca americana*, there was virtually no effect of atmospheric oxygen, at least down to below 2 kPa (Greenlee and Harrison, 1998). The P_{crit} of American locust adults at rest was later determined to be between 2.5 and 1.3 kPa (Greenlee and Harrison, 2004a; Greenlee and Harrison, 2004b). In a study of seven Tenebrionidae and seven Scarabidae beetles, the P_{crit} was found to be lower than 3 kPa for all species (Lease et al., 2012). It appears that, at rest, there is sufficient oxygen exchange to support metabolic rate at very low P_{O_2} . However, under hypoxia, as energy demands increase (e.g. increased activity or thermogenesis), the increased requirement for O_2 cannot be met by the oxygen delivery capacity of the insects.

Insight into the importance of intracellular P_{O_2} regulation and flight metabolism comes from research performed on bumblebee muscle. During flight, muscle P_{O_2} levels are tightly regulated at ~6 kPa, through increases in abdominal pumping (pre-flight and during flight) and changes in the duration of spiracle opening (Komai, 2001). It would appear that bumblebees regulate flight muscle oxygen levels primarily during periods of elevated metabolism, as during rest, muscle P_{O_2} values can vary from almost zero to 7 kPa (Komai, 2001). If similar levels are maintained during endothermic bouts like those measured in the current study, then as environmental P_{O_2} approaches 6 kPa, the animals should begin to become oxygen limited at the tissue level. This would facilitate a supply-driven decrease in the metabolic rate of hypoxic animals.

Effects of oxygen on ventilation rate

Although oxygen moves from the tracheole system to the muscle primarily via diffusion, convection within the tracheoles is effected through the bellows-type movement of the abdomen. Synchronicity in abdominal pumping and spiracle opening has been observed in locusts, dragonflies and beetles (Miller, 1960; Miller, 1962; Miller, 1966), supporting a role for convective air exchange via abdominal pumping in the ventilatory control of tracheolar oxygen levels. Bumblebees are known to employ ventilatory abdominal pumping in response to increased metabolic demand during endothermic bouts. During abdominal pumping, air flows into the abdominal air sac from the abdominal spiracles during inspiration (Heinrich, 1976). During expiration, air flows through the tracheal system from the abdominal air sac and into the thorax (Heinrich, 1976). In normoxic conditions, bumblebees exhibit abdominal pumping rates above 300 min^{-1} that are dependent upon T_{th} – in this study, endothermic bumblebees maintained an abdominal pumping rate of 313 min^{-1} during bouts of continuous pumping and similar high rates have been observed in *Bombus edwardsii* (Heinrich, 2004) and *Anthophora plumipes* (Stone, 1993).

Tracheal system conductance depends on tracheal system morphology and convective movement of gas through the system. In this study, abdominal pumping rates decreased significantly with exposure to decreasing oxygen levels. In contrast, Greenlee and Harrison (Greenlee and Harrison, 2004b) demonstrated that abdominal pumping rate rises with exposure to hypoxia in adult locusts, thereby providing them with greater hypoxia tolerance than earlier instars. However, the oxygen demand of the locust was much lower than the demand of an endothermic bumblebee.

The mean rate of abdominal pumping in bumblebees correlates strongly with the mean T_{th} across all levels of O_2 exposure (Fig. 7B). The increase in abdominal pumping rate correlated with an increase in T_{th} probably occurred via an increase in O_2 delivery to the flight muscles, thereby contributing to heat production in the thorax. Similar correlations between abdominal pumping rate and T_{th} have been observed in other bumblebee species during endothermic bouts. This occurs even though T_{ab} is much lower than T_{th} . There also appears to be a good correlation between instantaneous changes in pumping rate and T_{th} (Fig. 8), with the rise in abdominal pumping preceding the rise in T_{th} . During the cyclic changes in T_{th} in the animal shown in Fig. 8, the abdominal pumping rate follows the same trend. As the thorax is warmed by an increase in \dot{V}_{CO_2} and heat production, the rate of abdominal pumping mirrors this increase. Under hypoxic conditions of 10 kPa O_2 , the abdominal pumping rate appeared to be more consistent (Fig. 8B). At 5 kPa, ventilation rate continued to decline in the bumblebees, reaching levels that were approximately one-third of those during normoxia. In most insect species, decreases in atmospheric oxygen levels prompt an increase in abdominal pumping. In the American locust, for example, ventilation frequency increases from around 50 to 90 breaths min^{-1} as oxygen is decreased from 21 to 5 kPa (Greenlee and Harrison, 2004b). Although the rates of abdominal pumping are higher in the bumblebee compared with the locust, clearly the abdominal pumping responses in these two insects follow opposite patterns, suggesting that the abdominal pumping observed in bumblebees is primarily serving thermoregulatory requirements for oxygen delivery.

Another possible mechanism available to maintain \dot{V}_{CO_2} and \dot{V}_{O_2} in endothermic bees at lowered P_{O_2} may be an increase in ventilation volume associated with the decrease in ventilation rate. Unfortunately, we were unable to measure changes in abdominal volume in sufficient animals to draw any conclusions. However, the animal in Fig. 8 demonstrates a proportional increase in ventilation volume in response to 10 kPa O_2 when compared with the same animal at 20 kPa. While ventilation rate decreased at 10 kPa, the apparent increase in ventilation volume would appear to be an attempt to maintain adequate delivery of oxygen.

Cause and effect

There are two potential responses to a decrease in oxygen. The animal could lower the temperature set-point at lower levels of oxygen, which would result in a decrease in heat production. This decrease in heat production would be seen as a lowering of metabolic rate and associated abdominal pumping. Alternatively, decreases in oxygen availability could cause the oxygen supply to outweigh the oxygen demand of ATP production to fund the heat production, thus leading to a decrease in metabolic rate and associated abdominal pumping. In turn, this would cause T_{th} of the bumblebee to decline. Decreasing levels of oxygen would produce great limits on the supply of oxygen relative to demand, resulting in further decreases in metabolic rate and T_{th} . Given the strong correlations between abdominal pumping, thorax temperature and metabolic rate with respect to oxygen, we posit that common neural centers controlling both heat production and abdominal pumping are similarly affected by hypoxia, leading to a coordinated reduction in energy expenditure.

Perspectives and conclusions

The European bumblebee, *B. terrestris*, was introduced to Tasmania in 1992 and has since spread rapidly throughout the island (Schmid-Hempel et al., 2007), showing clear signs of being a successful

invader. As altitude-related changes in oxygen and temperature are known to limit insect ecogeographic distribution (see Dillon et al., 2006), it is prudent to consider the physiological constraints that may influence the capacity for this species to continue to spread. In this study, we examined the relationships among ventilatory, thermoregulatory and metabolic responses in endothermic bumblebees during exposure to oxygen levels spanning and exceeding the levels associated with normal altitudinal changes within the current range of the European bumblebee, but discovered that thermoregulatory and metabolic changes to hypoxia show different sensitivities. Body temperatures, assessed as thoracic and abdominal temperatures, were significantly lowered by exposure to mild hypoxia (15 kPa), even though metabolic rates remained constant down to between 10 and 5 kPa O_2 . The increased sensitivity of the thermoregulatory system in the endothermic bumblebees does suggest that atmospheric oxygen levels may affect muscle temperature, and therefore flight efficiency, before any effects on power output, and warrants further investigation.

MATERIALS AND METHODS

Animals

European bumblebees (*B. terrestris*) were caught between 08:00 h and 10:00 h while feeding on flowers and immediately transported to the laboratory at the University of Tasmania. Measurements were conducted over the summer: respiratory in November 2010 and thermal imaging in February 2011. Upon arrival at the lab, bumblebees were supplied with local flowers and a honey and water mixture (~50%:50%). Bumblebees were observed feeding on both the flowers and the honey mixture prior to use in an experiment. Experiments began between 1 and 3 h after capture.

T_{th} , abdominal pumping and metabolic rate

Bumblebees were sedated with a small drop of isoflurane in an air-tight chamber. Once sedated, bees were weighed. A small hole in the thorax was made with a 23 gauge needle and the tip of a 46 gauge copper constantan thermocouple was inserted to measure T_{th} . The thermocouple was held in place with a small drop of wax. Bees recovered within 5–8 min. Respiration was measured in a flow-through system. The animal was placed in a temperature-controlled glass metabolic chamber (approximate volume, 30 ml) maintained at 26°C. While in the metabolic chamber, the bees would typically walk around and sometimes chew on the chamber fittings. The chamber was too small to allow the bees to fly. The gas stream was pulled through the metabolic chamber, then the first channel of an Applied Electrochemistry oxygen analyzer (S-3A/II, Pittsburgh, PA, USA), followed by a Nafion desiccant tube surrounded by Drierite, the second channel of the O_2 analyzer, and finally an Applied Electrochemistry CO_2 analyzer (CD-3A). Inflow oxygen levels were controlled with a Wösthoff gas mixer (Bochum, Germany) mixing bottled oxygen and nitrogen. A Validyne pressure transducer (MP45-1, Northridge, CA, USA) was attached to the inflow of the respiratory chamber to record pressure changes associated with abdominal pumping to provide a measure of ventilation rate (Mortola and Frappell, 1998). Air temperature (T_a) was recorded with a 46 gauge thermocouple placed within the metabolic chamber. All data were recorded by an 8SP Powerlab and Chart 6.2 (ADInstruments, Bella Vista, NSW, Australia) at 40 Hz. Flow rate was measured using a custom-made, burette-calibrated air float system and corrected to standard temperature and pressure.

Experimental design

The experiment was designed to examine metabolic, thermoregulatory and ventilatory responses of thermoregulating bumblebees to decreasing levels of oxygen. Metabolic rate (rate of oxygen consumption) was measured in bumblebees exposed to 20, 15, 10 and 5 kPa O_2 . Animals were initially exposed to 20 kPa O_2 in the chamber for ~30 min. Upon obtaining baseline metabolic data, the inflow oxygen was changed to one of the experimental oxygen levels and measured for 20–30 min. Animals were exposed to

20 kPa O₂ between each treatment exposure for 10–15 min before being exposed to the next level. The order of oxygen exposure was varied for each animal.

Data analysis

Rates of oxygen consumption (\dot{V}_{O_2} ; ml O₂ h⁻¹) and carbon dioxide production (\dot{V}_{CO_2} ; ml CO₂ h⁻¹) were calculated using the following equations (Withers, 2001):

$$\dot{V}_{O_2} = \dot{V}_I \times \frac{F_{IO_2} - [F_{EO_2} \times (1 - F_{IO_2} - F_{ICO_2})]}{1 - F_{EO_2} - F_{ECO_2}}, \quad (1)$$

$$\dot{V}_{CO_2} = \dot{V}_I \times \left\{ \frac{F_{ECO_2} \times [1 - F_{IO_2} - F_{ICO_2}]}{1 - F_{EO_2} - F_{ECO_2}} - F_{ICO_2} \right\}, \quad (2)$$

where \dot{V}_I is incurrent flow rate (ml h⁻¹ STP), F_{IO_2} and F_{ICO_2} are incurrent O₂ and CO₂ fractions, and F_{EO_2} and F_{ECO_2} are excurrent O₂ and CO₂ fractions in dry gas. To obtain instantaneous respiration traces, z-transformation was conducted on the \dot{V}_{CO_2} data (Lighton and Halsey, 2011).

Ventilation data were analyzed for abdominal pumping frequency and duration. Abdominal pumping frequency was calculated from the pressure transducer traces of five animals by fast Fourier transform on 256 data points using a Hann windowing function with 50% overlap using the spectrum function of LabChart Pro 7.2.1.

Thermal imaging

A separate group ($N=7$) of bumblebees was used for non-invasively monitoring thorax and abdomen temperatures during exposure to hypoxia (20, 15, 10 and 5 kPa O₂). Bees were placed inside a plastic respirometer over which a thin sheet of polyethylene film (Glad Wrap®) was placed, the polyethylene film providing essentially a gas-tight seal and a window that permitted thermal infrared imaging. Infrared images, used to estimate surface temperatures, were collected using a thermal imaging camera (Mikron Model 7515, Oakland, NJ, USA; 320×240 pixels) connected to a computer software package (MikrospecRT), which allowed samples to be taken every 5 s for the duration of the experiments. Bees were initially exposed to 20 kPa O₂ for 30 min, followed by 15–20 min at each of the levels of hypoxia (randomly presented) at a constant temperature of 26°C. As above, in between hypoxic exposures, bees were exposed to 20 kPa O₂ for 10–15 min. These experiments allowed for the steady-state estimates of $T_{th,s}$ and $T_{ab,s}$. Offline software (MikrospecRT) analysis was conducted using graphical tools that enabled the average temperature to be obtained within specific, manually drawn regions of interest. This enabled us to avoid any edge effects in our measurements. The thorax took up roughly 256 pixels, allowing for a sample area 10 times larger than the minimum detectable size for the camera. For steady-state measurements, a minimum of five frames was analyzed at each oxygen level and averaged for each individual. The $T_{th,s}$ and $T_{ab,s}$ changes associated with the transition to the lowest level of oxygen (5 kPa) were followed frame by frame during the first 10 min of 5 kPa O₂ exposure in each animal. These calculations were also repeated following reoxygenation (20 kPa O₂). As an indicator of heat transfer, the abdominal temperature excess ratio was calculated as $(T_{ab,s} - T_a)/(T_{th,s} - T_a)$ during these transitions (Stavenga et al., 1993; May, 1995; Roberts and Harrison, 1999). Values close to 1 indicate that $T_{th,s}$ and $T_{ab,s}$ are similar and values close to 0 indicate an uncoupling of $T_{ab,s}$ from $T_{th,s}$.

Statistical analysis

We used one-way repeated measures ANOVA to compare the responses of \dot{V}_{CO_2} , \dot{V}_{O_2} , abdominal pumping, conductance and T_{th} with changes in oxygen availability. This was followed by Holm–Sidak *post hoc* test for pair-wise comparisons. A two-way repeated measures ANOVA was used when analyzing $T_{th,s}$ and $T_{ab,s}$. Significance was accepted at $P<0.05$. Statistical analyses were performed using SigmaPlot 11.0. All data are presented as means ± s.d.

Acknowledgements

The authors would like to thank Elias Polymeropoulos for technical assistance. Additionally, two reviewers provided useful suggestions.

Competing interests

The authors declare no competing financial interests.

Author contributions

Experiments were designed by all the authors. E.M.D. and G.J.T. carried out the experiments. Statistical analysis was done by E.M.D. and G.J.T. All authors were involved in interpretation and manuscript preparation.

Funding

P.B.F. provided funds to support the experiments and travel.

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