© 2017. Published by The Company of Biologists Ltd | Journal of Experimental Biology (2017) 220, 4432-4439 doi:10.1242/jeb.168187

RESEARCH ARTICLE

Flight metabolic rate of *Locusta migratoria* in relation to oxygen partial pressure in atmospheres of varying diffusivity and density

Edward P. Snelling[1,2,*], Rebecca Duncker[2], Karl K. Jones[2], Erinn P. Fagan-Jeffries[2] and Roger S. Seymour[2]

ABSTRACT

Flying insects have the highest mass-specific metabolic rate of all animals. Oxygen is supplied to the flight muscles by a combination of diffusion and convection along the internal air-filled tubes of the tracheal system. This study measured maximum flight metabolic rate (FMR) during tethered flight in the migratory locust *Locusta migratoria* under varying oxygen partial pressure (P_{O_2}) in background gas mixtures of nitrogen (N_2), sulfur hexafluoride (SF_6) and helium (He), to vary O_2 diffusivity and gas mixture density independently. With N_2 as the sole background gas (normodiffusive–normodense), mass-independent FMR averaged 132±19 mW $g^{-0.75}$ at normoxia (P_{O_2}=21 kPa), and was not limited by tracheal system conductance, because FMR did not increase in hyperoxia. However, FMR declined immediately with hypoxia, oxy-conforming nearly completely. Thus, the locust respiratory system is matched to maximum functional requirements, with little reserve capacity. With SF_6 as the sole background gas (hypodiffusive–hyperdense), the shape of the relationship between FMR and P_{O_2} was similar to that in N_2, except that FMR was generally lower (e.g. 24% lower at normoxia). This appeared to be due to increased density of the gas mixture rather than decreased O_2 diffusivity, because hyperoxia did not reverse it. Normoxic FMR was not significantly different in He-SF_6 (hyperdiffusive–normodense) compared with the N_2 background gas, and likewise there was no significant difference between FMR in SF_6-He (normodiffusive–hyperdense) compared with the SF_6 background gas. The results indicate that convection, not diffusion, is the main mechanism of O_2 delivery to the flight muscle of the locust when demand is high.

KEY WORDS: Convection, Critical P_{O_2}, Diffusion, Tracheal system, Locust, Regulation index

INTRODUCTION

The high metabolic demands of insect flight are met by the tracheal system, which consists of air-filled, semi-rigid tubes, where respiratory gases (i.e. O_2 and CO_2) move rapidly in the gas phase between the atmosphere and the cells. The gas transport occurs by two mechanisms, diffusion (the passive net movement of a gas from a high concentration to a lower concentration as a result of molecular collisions) and convection (the usually active movement of gases by bulk flow in a medium from a high total pressure to a lower pressure, also called advection). Together, these mechanisms determine the conductance of the tracheal system, which is the ease of respiratory gas exchange in the system. Diffusion alone is thought to be sufficient to meet the flight metabolic rate (FMR) in very small insects (e.g. mosquitos); however, in larger insects (bees, dragonflies, locusts), ventilation creates convective flow in the lateral regions of the tracheal system, thereby reducing the diffusion distance and maintaining a pressure gradient for diffusion along the terminal parts of the tracheal system. Ventilation of the tracheal system occurs by abdominal pumping and thoracic autoventilation (Weis-Fogh, 1964a,b). Abdominal pumping affects haemolymph pressure, resulting in the compression and expansion of large tracheae and air sacs (large, modified tracheae), and the bulk movement of luminal air. Thoracic autoventilation occurs with deformation of the thorax by the wing muscles during flight, resulting in the pumping of air through the tracheal system at wingbeat frequency (Komai, 1998; Wasserthal, 2001, 2015; Weis-Fogh, 1964b, 1967). The degree to which these ventilation mechanisms are employed varies between species. For instance, ventilation in dragonflies, which have little capacity for abdominal movement, occurs almost exclusively by thoracic autoventilation (Weis-Fogh, 1967), while bees favour abdominal pumping (Komai, 2001). Locusts, in contrast, use both abdominal pumping and thoracic autoventilation to varying degrees (Harrison et al., 2013). Thoracic autoventilation services the flight muscles, pumping air at approximately 250 ml g^{-1} h^{-1}, and it is supplemented by abdominal pumping, which delivers air at approximately 70 ml g^{-1} h^{-1} (Miller, 1960; Weis-Fogh, 1967).

Diffusion occurs throughout the tracheal system, but it is most important in the terminal regions, particularly the blind-ended tracheoles (Harrison et al., 2013). Oxygen diffuses down its concentration gradient, theoretically according to Fick's first law of diffusion. The rate of flux depends on the anatomy of the system (cross-sectional area and distance), the difference in O_2 partial pressure (P_{O_2}) along a given distance, and Krogh's coefficient for O_2 diffusion in the gaseous medium (normally air). Krogh's coefficient for O_2 depends on temperature, barometric pressure and the nature of the background gases. For a given P_{O_2} differential, the O_2 diffusion rate should increase at low barometric pressure or in an artificial gas mixture where nitrogen (N_2), the background gas of normal air, is replaced with a gas of low molecular weight, such as helium (He). Conversely, the rate would decrease at high barometric pressure or in a gas mixture where N_2 is replaced with a gas of high molecular weight, such as sulfur hexafluoride (SF_6). These effects are in contrast to the convective mechanism of ventilation in which O_2 flux is largely independent of background gases, except for the added effort to move high-density gases compared with the reduced effort to move low-density gases.

The low metabolic rate of resting insects is satisfied easily by a tracheal system capable of achieving metabolic rates during flight that are 20–100 times higher than at rest (Ellington, 1985). The conductance of the O_2 supply pathway is often revealed by

[1]Brain Function Research Group, School of Physiology, University of the Witwatersrand, Johannesburg, Gauteng 2193, South Africa. [2]Department of Ecology and Environmental Science, School of Biological Sciences, University of Adelaide, Adelaide, SA 5005, Australia.

*Author for correspondence (edward.snelling@wits.ac.za)

E.P.S., 0000-0002-8985-8737; K.K.J., 0000-0002-0478-8021; R.S.S., 0000-0002-3395-0059

Received 15 August 2017; Accepted 28 September 2017

Journal of Experimental Biology

Journal of Experimental Biology (2017) 220, 4432-4439 doi:10.1242/jeb.168187

measuring metabolic rate over a range of ambient P_{O_2} and defining a point, called the critical P_{O_2} (P_C), below which metabolic rate declines. Resting adult insects tolerate severe hypoxia as low as 1–5 kPa without exhibiting a decline in metabolic rate (Greenlee and Harrison, 2004a,b; Greenlee et al., 2007; Harrison et al., 2006; Joos et al., 1997; Klok et al., 2010; Rascón and Harrison, 2005; Van Voorhies, 2009). During flight, this apparently large reserve capacity or 'safety margin' for O_2 delivery decreases significantly (Harrison et al., 2006). The P_C is approximately 10 kPa in hovering honeybees, *Apis mellifera* (Joos et al., 1997), and between 10 and 21 kPa in tethered flying locusts *Schistocerca americana* (Rascón and Harrison, 2005). The P_C values for 11 species of flying dragonfly appear to be about 10–15 kPa (Henry and Harrison, 2014), but P_C is difficult to define in one dragonfly species, *Erythemis simplicicollis*, because only a slight change in metabolic rate occurs between 5 and 50 kPa, and the rate at 5 kPa is only 18% below that at 21 kPa (Harrison and Lighton, 1998). In most studies, the relationship between metabolic rate and P_{O_2} is curved, rather than an ideal independence above the P_C and proportional dependence below it. This curved relationship introduces uncertainties in determining P_C, and calls into question whether a single definitive P_C even exists. Instead of calculating P_C from broken stick regression models, the dependence of metabolic rate on P_{O_2} can be evaluated with a new metric, the Regulation Index, which is a value that has clearly defined limits, from perfect oxy-conformity (0) to perfect oxy-independence (1), but does not force the data to follow any particular pattern (Mueller and Seymour, 2011).

An important question in insect respiration concerns how the structure and function of the respiratory system are matched to the gas exchange requirements of the animal. One working hypothesis, called symmorphosis, predicts that the respiratory system should be constructed according to economic design principles (Weibel et al., 1998). Symmorphosis posits that every step of the O_2 cascade should be equal in its capacity to transport O_2 from the atmosphere towards the mitochondria that use it (Weibel et al., 1991). Flying insects are arguably the most appropriate group to study, because their mass-specific metabolic rates are the highest of all animals (Schippers et al., 2010; Suarez, 2000). Symmorphosis is evident in the hopping muscle of *Locusta migratoria* by the parallel scaling of maximum muscle metabolic rate, tracheolar radial diffusing capacity, mitochondrial volume and inner mitochondrial membrane surface area (Snelling et al., 2011b). Symmorphosis also holds for the locust flight muscle, except for an apparent mitochondrial oversupply (Snelling et al., 2012b). Other studies indirectly show parallel development of tracheal capacity and metabolic rate (Greenlee and Harrison, 2004a; Harrison et al., 2005; Hartung et al., 2004; Kaiser et al., 2007). One test for symmorphosis is to assess the matching of tracheal O_2 transport capacity with maximum O_2 consumption rate by the mitochondria. This can be achieved by analysing the response of maximum metabolic rate to varying ambient P_{O_2} levels. An increase in maximum O_2 consumption rate under hyperoxia would indicate that the maximum rate is normally limited by the conductance of the tracheal system. Conversely, the maintenance of maximum O_2 consumption rate under hypoxia would indicate a conductance oversupply of the tracheal system, possibly to maintain a reserve capacity for carrying added loads or for bouts of energy-intensive acrobatic flight not normally performed.

Another experimental approach to test for matching between maximum O_2 transport and maximum O_2 consumption is to vary the O_2 diffusivity of the gaseous medium. The diffusion rate of O_2 in the tracheal system is dependent, in part, upon the molecular weights of all gas species present. The diffusivity of a gas increases with decreasing molecular weight, according to Graham's law; however, it is also affected by a range of other factors, including temperature, pressure and the viscous properties of the other gas molecules through which the gas diffuses. If two gases are involved, then binary diffusion coefficients can be used to estimate the rate, but if three gases are involved, then ternary coefficients must be used (Cussler, 2009; Poling et al., 2001). Manipulating the molecular weights of gases also affects the density of the gas mixture, with density being directly proportional to molecular weight. This relationship is the opposite of the relationship between molecular weight and diffusivity and, as such, density is also inversely (though not linearly) proportional to diffusivity.

Given the inverse relationship between diffusivity and density parameters, it is difficult to determine conclusively which factor, or whether a combination of both factors, affects the metabolic rate of insects flying in artificial gas mixtures. It has been assumed that increased O_2 diffusivity does not have a significant effect on O_2 consumption rate during flight (Roberts et al., 2004); however, there are no data to support this idea. Previous studies measuring FMR of insects under variable barometric pressure (e.g. Withers, 1981) are inconclusive, because altering barometric pressure has three opposing influences on flight metabolism. For example, low barometric pressure reduces atmospheric density, which should increase the power required for flight and therefore increase FMR. Low barometric pressure decreases atmospheric P_{O_2}, which should decrease the driving force for diffusion and therefore decrease FMR. Low total pressure increases O_2 diffusivity, which should increase FMR. No previous study has examined the effect of decreased molecular diffusivity in isolation. By selecting specific gas combinations, it is possible to separate the confounding effects of diffusivity and density through the creation of mixtures with normal atmospheric diffusivity and varying density, and vice versa, while holding the P_{O_2} at normal levels (normoxia).

The main objective of this study was to determine the metabolic limitations of tethered flight in the migratory locust *Locusta migratoria*. Conductance limitations of the tracheal system during flight were tested four ways. Firstly, maximum FMR was measured across a range of ambient P_{O_2} to evaluate the influence of O_2-supply limitation with N_2 as the sole background gas. Secondly, FMR was measured across a range of ambient P_{O_2} with SF_6 as the sole background gas to decrease O_2 diffusivity in the tracheal system. In theory, the effects of low diffusivity due to the presence of SF_6 can be offset by an adequate increase in ambient P_{O_2}. Thirdly, FMR was measured across ambient P_{O_2} with He as the sole background gas to increase O_2 diffusivity in the tracheal system. Fourthly, to distinguish between the O_2 diffusivity and density effects of the background gas, ternary gas mixtures of O_2, He and SF_6 were used to adjust these properties independently while holding P_{O_2} constant at normoxia.

MATERIALS AND METHODS

Insect rearing

Gregarious-phase migratory locusts *Locusta migratoria* (Linnaeus 1758) were reared from egg to adulthood at the University of Adelaide, under a regulated temperature (33±2°C) and photoperiod regime (12 h:12 h light–dark cycle), and with *ad libitum* access to wheatgrass and wheat germ, as previously described (Snelling et al., 2011a). Only adult males 2–5 weeks post-final moult were used, as by this age the flight muscles are fully developed (Mizisin and Ready, 1986).

Journal of Experimental Biology (2017) 220, 4432-4439 doi:10.1242/jeb.168187

Flight respirometry

Flow-through respirometry was used to measure metabolic rate during tethered flight exercise in a constant-temperature cabinet set to 35±2°C, the upper-preferred temperature of locust flight (Weis-Fogh, 1967). The respirometer consisted of a clear cylindrical acrylic flight chamber (93×100 mm, inner diameter×length) through which artificial gas mixtures were pushed at a rate of 1000 ml min^{-1}. Gas exiting the chamber passed through a small column of desiccant (Drierite; W. A. Hammond Drierite Co. Ltd, Xenia, OH, USA) prior to entering a CO_2 gas analyser (LI-820; Li-COR Biosciences, Lincoln, NE, USA) and then an O_2 gas analyser (FC-2 Oxzilla; Sable Systems, Las Vegas, NV, USA). The analog outputs of both gas analysers were recorded with a PowerLab data acquisition system running LabChart software (ADInstruments, Bella Vista, NSW, Australia). Analyser baselines were set using a bypass around the chamber before and after flight measurements.

A tether descending from the roof of the flight chamber was used to position the locust centrally and toward the incoming stream. A small magnetic disk (3 mm diameter) was affixed to the pronotum of each locust to allow temporary, but firm, attachment to the tether head. The disk was affixed using melted depilatory wax (Klorane, Boulogne, France) and care was taken to ensure the wax was not excessively hot upon application (locusts seemed unperturbed). Once tethered within the flight chamber, individual locusts were placed in the constant-temperature cabinet for at least 10 min before each experiment to acclimate. Then, flight was initiated through rapid removal of a flexible perch beneath the locust, which elicited a tarsal flight response (Weis-Fogh, 1956). Flight bouts typically lasted 5–10 min, including an initial washout period before the perch was removed. No more than four flights per locust per day were conducted as there was an apparent decrease in flight effort thereafter. At least 1 h recovery was given to each locust between flight bouts. After each flight, locusts were weighed to the nearest 0.01 mg on a digital balance (AE163; Mettler, Greifensee, Switzerland) and adjusted later to account for the weight of the magnetic disk and wax.

The raw recordings of CO_2 production were instantaneously corrected by applying an empirically derived washout constant for the chamber using previously described principles (Seymour et al., 1998). Instantaneous CO_2 production rates (μmol s^{-1}) were calculated as the product of incurrent flow rate and the fraction of CO_2 added to the excurrent air (Lighton, 2008), and then converted to instantaneous metabolic rate (mW) assuming a respiratory exchange ratio of 0.86, as determined in pilot experiments, and a conversion factor of 0.54 J μmol^{-1} (Withers, 1992). Finally, the mean maximum FMR for each locust was calculated by averaging the highest instantaneous metabolic rate over 30 s of flight exercise, consistent with earlier studies (Kirkton et al., 2005; Snelling et al., 2012a). Because mass-specific data do not eliminate the effect of body mass, the data are presented in mass-independent units (mW g$^{-0.75}$) to standardise the values. The exponent of 0.75 comes from a previous study of locusts in which the maximum metabolic rate during tethered flight (FMR, μmol^{-1} O_2 h^{-1}) scales with body mass (M_b, g) according to the equation FMR=994 $M_b^{0.75}$ (Snelling et al., 2012a). As the mean body mass of locusts used in this study was 1.01 g, the mass-specific, mass-independent and absolute metabolic rates are all approximately equal.

Mixing of oxygen with exotic background gases

The artificial gas mixtures that were pushed through the flight chamber were generated using a combination of mass flow controllers (10, 100, 500, 1000 and 10,000 ml min^{-1}; Aalborg Instruments and Controls, Orangeburg, NY, USA), calibrated for each gas with a bubble flow meter (Gilibrator; Sensidyne, Clearwater, FL, USA), and regulated by a spreadsheet-controlled 16-bit analog output board (PowerDAQ PD2-AO and Professor DAQ software; United Electronic Industries, Watertown, MA, USA). The gases were mixed at an elevation of ∼50 m and a barometric pressure of ∼101 kPa (Adelaide, Australia) and so the oxygen percentage (% O_2) is approximately numerically equivalent to the P_{O_2} expressed in kPa.

Binary gas mixtures consisting of selected levels of O_2 balanced with N_2, He or SF_6 were used to test the dependency of locust FMR on P_{O_2} in the various background gases. The chosen nominal compositions (±1%) were as follows: 5, 8, 10, 12, 15, 17, 19, 21, 32, 43% O_2 balanced in N_2; 5, 10, 15, 21, 44% O_2 balanced in He; and 5, 10, 15, 21, 26, 30, 37, 44% O_2 balanced in SF_6. Ternary gas mixtures consisting of 21% O_2 in varying levels of both He and SF_6 allowed independent manipulation of the O_2 diffusivity and density parameters of the gas mix while holding P_{O_2} constant. Determination of the desired gas compositions required the calculation of both binary ($D_{A,B}$; Eqn 1) and ternary ($D_{A,mix}$ Eqn 2) diffusion coefficients.

The diffusivity of a binary gas mixture (i.e. gas_A in gas_B) can be calculated, with relative accuracy, by use of a simplification of the Chapman–Enskog equation, which gives the diffusivity of the target gas (i.e. O_2) in a two-component gas mixture (Poling et al., 2001):

$$D_{A,B} = \frac{0.00266\ T^{3/2}}{P\ M_{A,B}^{1/2}\ \sigma_{A,B}^{2}\ \Omega_D}. \quad (1)$$

Here, $D_{A,B}$ is the diffusivity of gas_A in gas_B (cm^2 s^{-1}), T is temperature (K), P is atmospheric pressure (bar=100 kPa≈1 atm), $M_{A,B}=2[(1/M_A)+(1/M_B)]^{-1}$, where M_A and M_B are the respective molar masses (g mol^{-1}), $\sigma_{A,B}$ is the characteristic length (Å=0.1 nm) and Ω_D is the diffusion collision integral (dimensionless). The characteristic length (Eqn 1.1) and diffusion collision integral (Eqn 1.2) were calculated from experimental data (Poling et al., 2001):

$$\sigma_{A,B} = \frac{\sigma_A + \sigma_B}{2}, \quad (1.1)$$

$$\Omega_D = \frac{1.06036}{(T^*)^{0.15610}} + \frac{0.19300}{\exp(0.47635\ T^*)} + \frac{1.03587}{\exp(1.52996\ T^*)} + \frac{1.76474}{\exp(3.89411\ T^*)}. \quad (1.2)$$

Here, $T^*=kT/\varepsilon_{A,B}$, where k is Boltzmann's constant (1.3805×10^{-23} J K^{-1}) and $\varepsilon_{A,B}=(\varepsilon_A\varepsilon_B)^{1/2}$, where ε values were obtained from experimental data (Poling et al., 2001).

The diffusivity of a ternary gas mixture (i.e. gas_A in gas_{mix}) can be approximated according to the multi-component diffusivity equation, which incorporates the binary diffusion coefficients of each individual gas component (Eqn 1) and gives the diffusivity of the target gas (i.e. O_2) in the multi-component gas mixture (Worth and Piiper, 1978):

$$D_{A,mix} = \frac{1}{1 - F_A} \cdot \sum_{y=1}^{n} F_N \cdot D_{A,N}. \quad (2)$$

Here, $D_{A,mix}$ is the diffusivity of gas_A in the gas mixture comprising n gas species (cm^2 s^{-1}), F_A is the mole fraction of gas_A (the target gas), F_N is the mole fraction of gas_N (one of the background gases), and $D_{A,N}$ is the binary diffusion coefficient of gas_A in gas_N (cm^2 s^{-1}).

Journal of Experimental Biology (2017) 220, 4432-4439 doi:10.1242/jeb.168187

Table 1. Composition, O_2 diffusivity and density of the binary and ternary gas mixtures at 35°C

Background gas	O_2 (%)	N_2 (%)	He (%)	SF_6 (%)	O_2 diffusivity ($cm^2\ s^{-1}$)	Density ($kg\ m^{-3}$)
N_2 (normodiffusive–normodense)	21.0	79.0	0	0	0.22	1.14
He (hyperdiffusive–hypodense)	21.0	0	79.0	0	0.80	0.39
SF_6 (hypodiffusive–hyperdense)	21.0	0	0	79.0	0.10	4.83
He-SF_6 mix (hyperdiffusive–normodense)	21.0	0	65.6	13.4	0.68	1.14
SF_6-He mix (normodiffusive–hyperdense)	21.0	0	13.0	66.0	0.22	4.10

Diffusivity was calculated according Eqns 1 and 2, and density was calculated according to Eqn 3.

The density of a gas mixture can be calculated by applying a modification of the Ideal gas law:

$$\rho_{mix} = (P\,\bar{M}_{mix})\ /\ (R\,T). \quad (3)$$

Here, ρ_{mix} is the gas mixture density ($kg\ m^{-3}$), $\bar{M}_{mix}$ is the weighted average molar mass of the gas mixture ($g\ mol^{-1}$) and R is the universal gas constant ($0.08314\ l\ bar\ K^{-1}\ mol^{-1}$). Mixtures were produced, correct to 35°C, with normal atmospheric O_2 diffusivity (~$0.22\ cm^2\ s^{-1}$) and normal atmospheric air density (~$1.14\ kg\ m^{-3}$), as well as mixtures of variable diffusivity and density (Table 1). The O_2 component of all artificial gas mixtures was verified with the FC-2 Oxzilla O_2 gas analyser.

Flight kinematics

Wingbeat frequency and wing stroke amplitude were measured in a second cohort of similarly aged adult male locusts, also at 35±2°C. These individuals were flown in binary gas mixtures of 21% O_2 balanced with either N_2 or SF_6 background gases, using the same respirometry apparatus, and under the same conditions, as described previously. A high-speed digital video camera (Xacti VPC-FH1; Sanyo Electric Co., Osaka, Japan) was positioned lateral to the tethered locust in the chamber, and flight bouts were recorded at 600 frames s^{-1}. Tracking the tips of the forewing and hindwing during slow-motion playback was facilitated by bright lighting, the application of small black dots on the wing tips (marked with felt pen), and a white background behind the flight chamber.

Videos were analysed early in the flight bouts corresponding to periods of FMR measured at the same time. Mean wingbeat frequency (f, Hz) was calculated over five complete wing cycles as f=(600×5)/N, where 600 is the frame rate (Hz), 5 is the number of complete wing cycles analysed, and N is the number of frames taken over the five complete wing cycles. Mean wing stroke amplitude of the forewing and hindwing was determined as the angle (degrees) between the upper and lower reversal points of the wings. This angle was not measured directly, but calculated from the distance between the upper and lower reversal points of the wing tips as a cosine function of wing length (Fischer et al., 2002; Snelling et al., 2012a). The distance between the reversal points was measured from superimposed images of the wings in these extreme positions captured from the high-speed video footage, and corrected to scale using a high-definition photograph of a 1 mm incremented ruler positioned in the same plane. Because mean wing stroke amplitude did not differ significantly between the forewings and hindwings measured in air, the angles from five dual measurements were averaged for each locust.

Statistics

Significance between means, tests for normality and equal variance, and curve fitting of the data were undertaken using graphing and statistical software (Prism 5; GraphPad Software, La Jolla, CA, USA). The Regulation Index (RI) was calculated as the ratio A/B, where A is the area bounded by the regression curve of FMR below P_{O_2} of 21 kPa and a diagonal line between FMR at 21 kPa and the origin, and B is the triangular area above the diagonal line and below a horizontal line intersecting FMR at 21 kPa (Mueller and Seymour, 2011). Thus, RI varies between 0 representing no regulation (i.e. linear dependence of FMR on P_{O_2} below 21 kPa), and 1 representing perfect regulation (i.e. ideal independence of FMR down to P_{O_2} of 0 kPa).

RESULTS

The mean body mass (M_b) of the 42 locusts used for flights in the binary gas mixtures of variable P_{O_2} balanced with either N_2 or SF_6 was 1.01±0.08 g (mean±95% confidence interval, CI). For comparison with other studies, the mean mass-specific FMR measured in normal air at normoxia (P_{O_2}=21 kPa) from 12 locusts (M_b=1.08 g) was 1009±138 µmol $O_2\ g^{-1}\ h^{-1}$ and 847±131 µmol $CO_2\ g^{-1}\ h^{-1}$, giving a respiratory exchange ratio of 0.84±0.03.

The mean mass-independent FMR measured over a range of P_{O_2} revealed curves that plateaued above normoxia in both N_2 and SF_6 as the sole background gas (Fig. 1). With N_2 as the sole background gas, FMR did not increase significantly above 21 kPa, but dropped precipitously below it, decreasing 25% from 132 to 99 mW $g^{-0.75}$ between P_{O_2} of 21 and 15 kPa. Regressions involving sigmoid curves fitted the data better than one-phase associations and allowed the dependence of FMR on P_{O_2} to be evaluated with the RI. The RI value was 0.19 with N_2 as the sole background gas and 0.18 with SF_6 as the sole background gas, both of which indicate very low regulatory ability.

The general trend showed that FMR tended to be lower in SF_6 than in N_2 background gases at P_{O_2} levels above approximately 10 kPa (Fig. 1). At 21 kPa, FMR was 24% lower in SF_6

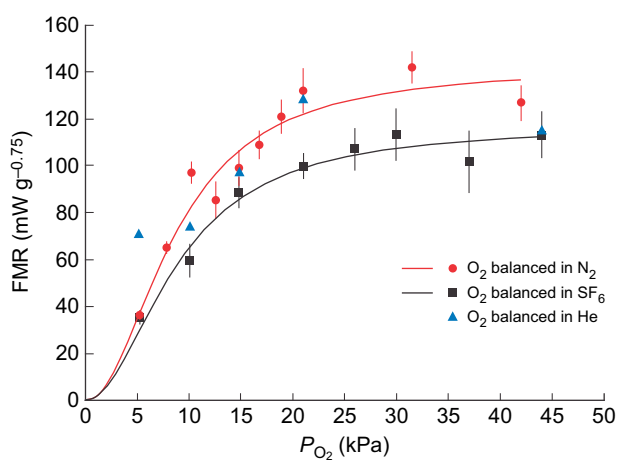

Fig. 1. Mean±s.e.m. mass-independent flight metabolic rate (FMR) of tethered *Locusta migratoria* in relation to ambient P_{O_2} in background gases of N_2, SF_6 and He. Superimposed on the data are allosteric sigmoid curves of the form FMR=(a×$P_{O_2}{}^b$)/(c+$P_{O_2}{}^b$), where a=142, b=2.04 and c=81.8 for N_2 background gas (R^2=0.93) and a=117, b=2.00 and c=79.3 for SF_6 background gas (R^2=0.97).

Journal of Experimental Biology

Table 2. Mean±95% confidence interval (CI) mass-independent flight metabolic rate (FMR) of tethered *Locusta migratoria* in normoxia (P_{O_2}=21 kPa) and selected background gases and gas mixtures

			P		
Background gas	FMR (mW $g^{-0.75}$)	*N*	SF_6	He-SF_6 mix	SF_6-He mix
N_2 (normodiffusive–normodense)	132±19	12	**0.023**	0.093	**0.034**
SF_6 (hypodiffusive–hyperdense)	100±11	18	–	0.999	0.974
He-SF_6 mix (hyperdiffusive–normodense)	100±23	8	–	–	0.979
SF_6-He mix (normodiffusive–hyperdense)	95±17	8	–	–	–

Ordinary one-way ANOVA was performed ($F_{3,42}$=3.99, *P*=0.014) and the results of the Tukey's *post hoc* tests for comparison of FMR between treatments are given as adjusted *P*-values. Locusts did not fly consistently in normoxia with He as the sole background gas and so we did not perform ANOVA on this limited dataset (FMR=129±14 mW $g^{-0.75}$, *N*=2).

(100 mW $g^{-0.75}$, *N*=18) than in N_2 (132 mW $g^{-0.75}$, *N*=12) (*t*-test, two-tailed, unpaired: $t_{1,28}$=3.03, *P*=0.0052). Only eight successful flight bouts were obtained with He as the sole background gas because, for some reason, the locusts were reluctant to fly. Mean FMR data from three insects flown in five treatments of varying P_{O_2} with He as the background gas are superimposed in Fig. 1. Although not statistically adequate, these points suggest that the He background did not enhance FMR, except possibly at 5 kPa.

Comparisons of FMR measured in normoxia (P_{O_2}=21 kPa) and balanced in four different background gases, or gas mixtures, are shown in Table 2. FMR with N_2 as the sole background gas was significantly higher than that with SF_6 as the sole background gas (ANOVA: $F_{3,42}$=3.99, *P*=0.014; Tukey's *post hoc* test: *P*<0.05). FMR with N_2 was also significantly higher than that with the SF_6-He background mixture (*P*<0.05), but it was not significantly different from that of flights in the He-SF_6 background mixture (*P*>0.05; Table 2).

There were marked differences in the measured kinematics of locusts flying in normoxia with N_2 as the sole background gas compared with SF_6 as the sole background gas (Table 3). In SF_6, wingbeat frequency decreased by 15% and wing stroke amplitude by 12% (*t*-tests, two-tailed, unpaired: *P*<0.01).

DISCUSSION

Maximum metabolic rate during tethered flight

The aim of this study was to determine how the gaseous environment affects O_2 delivery to the flight muscles of locusts under conditions of maximum O_2 demand. To achieve this aim, it was first necessary to establish that flight efforts were maximal. Tethering is thought to underestimate the FMR of free flight, because it removes the requirement of insects to support their own body weight, alters flight kinematics and interrupts sensory feedback loops (Dudley and Ellington, 1990). Past comparisons of the flight mechanics of locusts undertaking tethered flight versus free flight suggested significant tethering effects, including lower wingbeat frequencies (Baker et al., 1981; Gewecke, 1975; Gewecke and Kutsch, 1979) and measured lift that was less than body mass (Gee and Robertson, 1998; Kutsch and Gewecke, 1979; Rascón and Harrison, 2005; Robertson and Johnson, 1993; Wolf, 1993). Furthermore, the FMR of hovering species of insect (bees and moths) is approximately 3-fold higher than that of tethered locusts (Snelling et al., 2012a).

Despite the possibility of significant tethering effects, the results of our study indicate that FMR during tethered flight of locusts was indeed maximal, at least during the initial period of intense exercise. If the locusts had the potential to increase FMR further for free flight, then we would expect that the tethered individuals would maintain the same FMR in mild hypoxia as in normoxia. However, FMR began to decrease immediately as P_{O_2} was reduced (Fig. 1). Several lines of recent evidence support this conclusion for locusts (Snelling et al., 2012a). Firstly, locusts tethered to a vertical force transducer with air flowing over them at velocities comparable to those of forward flight (ca. 4 to 5 m s^{-1}) can create average lift that is equivalent to body mass (previous work on locusts in stagnant air may have prevented the lift generated by aerofoils in forward flight). Secondly, during these periods of peak lift, wingbeat frequencies and wing stroke amplitudes are similar to those in free flight. Thirdly, locust FMR does not increase with the addition of weights to the wings. And lastly, the approximately 3-fold higher FMR of hovering bees and moths compared with tethered locusts can be largely accounted for by the ∼4-fold greater mitochondrial investment in the flight muscles of the hovering insects compared with those of the locusts (Snelling et al., 2012a).

Symmorphosis: matching tracheal system conductance to peak O_2 demand

FMR during tethered flight in the locusts measured under a range of P_{O_2} with N_2 as the sole background gas shows a pattern of independence above 21 kPa and steeply declining rates below it (Fig. 1). The steepness of the decline suggests a P_C near normal atmospheric levels, and the low RI value of 0.19 reveals high dependence of FMR under hypoxia. These results indicate that the conductance of the tracheal system is matched to peak O_2 demand under normal atmospheric conditions, neither being limited by convection or diffusion nor having excess supply. Such results are expected according to the economical design hypothesis of symmorphosis.

The pattern of O_2 dependence of FMR in our locusts is similar to that in the American locust *S. americana*, but quite different from that of hovering bees and dragonflies (Fig. 2). The locusts have very low RI values and show immediate oxy-conforming metabolism, while the bees and dragonflies have moderate to high RI values that indicate a considerable reserve capacity or 'safety margin'. The

Table 3. Mean±95% CI wingbeat frequency and wing stroke amplitude of tethered *L. migratoria* in normoxia (P_{O_2}=21 kPa) and selected background gases

Background gas	Frequency (Hz)	*N*	*P*	Amplitude (deg)	*N*	*P*
N_2 (normodiffusive–normodense)	24.6±1.5	10	**0.0030**	100±5	8	**0.0035**
SF_6 (hypodiffusive–hyperdense)	21.0±1.3	9		88±4	8	

The results of unpaired two-tailed *t*-tests for both frequency ($t_{1,17}$=3.45) and amplitude ($t_{1,14}$=3.50) are given as *P*-values.

Journal of Experimental Biology

Journal of Experimental Biology (2017) 220, 4432-4439 doi:10.1242/jeb.168187

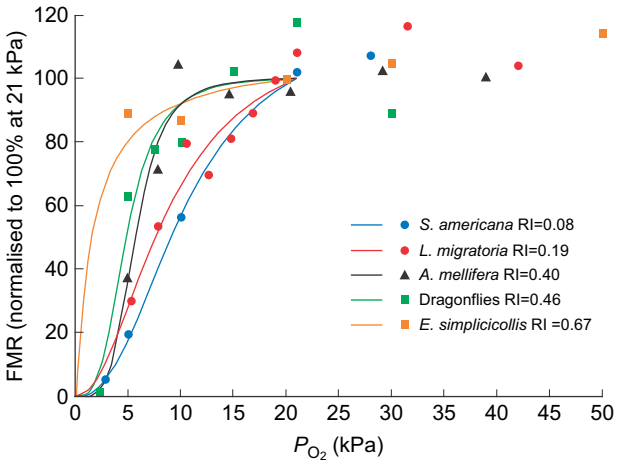

Fig. 2. Flight metabolic rate in ambient P_{O_2} during tethered flight in locusts (*Schistocerca americana* and *Locusta migratoria*), hovering in honeybees (*Apis mellifera*) and hovering in dragonflies (species including *Erythemis simplicicollis*). Data were sourced from this study and previous work (Harrison and Lighton, 1998; Henry and Harrison, 2014; Joos et al., 1997; Rascón and Harrison, 2005). Points were digitised from figures or tables in the literature and normalised as a percentage of the value calculated by regression at P_{O_2}=21 kPa. Sigmoid curves were fitted to all data except for *E. simplicicollis* (Michaelis–Menten), resulting in the best fits (R^2=0.93–0.99). The Regulation Index (RI) was calculated for each group (see Materials and methods) and the value is indicated in the figure. The RI is on a scale from 0 to 1 (i.e. no regulation to perfect regulation).

results may be related to the fact that locusts do not carry significant extra weight during flight, while the predatory dragonflies and foraging bees commonly do (Marden, 2000).

Effects of exotic background gases

FMR was 24% lower during normoxic flights with O_2-SF_6 compared with O_2-N_2 (Fig. 1). The reduction does not appear to be related strictly to the lower O_2 diffusivity in the dense SF_6, because hyperoxia in SF_6 did not result in higher FMR. Hyperoxia not only increases the P_{O_2} differential but also decreases the SF_6 concentration, decreasing the density of the mixture and increasing O_2 diffusivity, all of which would augment the rate of O_2 diffusion. Instead of increasing, however, FMR in all O_2-SF_6 treatments appeared lower than in O_2-N_2 treatments. The curves fitted to the data are similar in shape, with virtually identical RI values of 0.18 and 0.19, respectively. The few successful runs with the hyperdiffusive–hypodense O_2-He mixtures were largely coincident with those for O_2-N_2 (Fig. 1), tentatively suggesting that increased O_2 diffusivity does not augment FMR above the maximum measured in air.

In an attempt to separate the effects of O_2 diffusivity and density on FMR, and to determine why there is a significant difference between normoxic flights with SF_6 as the sole background gas compared with N_2 as the sole background gas, we created normoxic hyperdiffusive–normodense and normoxic normodiffusive–hyperdense ternary gas mixtures with O_2, He and SF_6. Our results indicate that gas mixture density has a greater impact on FMR than O_2 diffusivity (Table 2). FMR in SF_6 (hypodiffusive-hyperdense) and in SF_6-He (normodiffusive–hyperdense) was significantly lower than that in N_2 (normodiffusive–normodense). There was no significant difference between FMR in He-SF_6 (hyperdiffusive–normodense) background gases compared with that in N_2, and, rather than augmenting FMR, the He-SF_6 data were nearly significantly lower (P=0.093). We conclude that O_2 diffusivity has very little effect on tracheal conductance in flying locust. These experiments may be improved upon, because the calculation of the diffusivity of ternary gas mixtures (Eqn 2) is not always confirmed by direct measurement, and may be better calculated as the summation of concentration-weighted reciprocal binary diffusion coefficients (Fairbanks and Wilke, 1950; Piiper and Worth, 1980; Worth and Piiper, 1978).

Our conclusion that diffusivity is having a negligible effect on the locusts during normoxic flight is consistent with gas mixing experiments on dogs which show that molecular diffusivity plays only a minor role under conditions of high ventilation (Klocke et al., 1990). Another factor to consider is the effect of high-frequency vibrations during flight on the overall effective diffusivity of the system. Vibrations occurring in the tracheal system during locust flight could produce ‘facilitated diffusion’ whereby high-frequency, small-volume, non-directional oscillations enhance gas diffusivity, as has been shown in the lungs of dogs (Bohn et al., 1980). The high ventilation rates and thoracic vibrations of locusts during flight apparently overwhelm any limitations in molecular diffusivity of O_2.

Flight kinematics

The effect of dense background gases on FMR is probably related to changes in flight kinematics affecting the work rate of the flight muscles. Locust flight in normoxia revealed significantly decreased wingbeat frequency and wing stroke amplitude in SF_6 compared with N_2 background gases (Table 3). The mean wingbeat frequency of locusts flown in normal air is 24.6±1.5 Hz. This is comparable to 23.7 Hz recorded in a similar study using tethered locusts (Snelling et al., 2012a) and 22.9 Hz recorded from locusts in free flight (Baker et al., 1981). By comparison, the wingbeat frequency in SF_6 is 21.0±1.3 Hz. The mean wing stroke amplitude of the forewings and hindwings in normal air is 100±5 deg. Once again, this compares well with previous estimates of 105 deg for forewings and 96 deg for hindwings in the same species during tethered flight in normal air (Snelling et al., 2012a). In contrast, mean wing stroke amplitude is 88±4 deg in SF_6.

Changing the density of the flight medium consequently alters flight kinematics. Decreasing density appears to increase the level of mechanical work required of the wings to produce enough lift to offset body mass and maintain flight, while increasing density appears to have the opposite effect (Chai and Dudley, 1999; Dudley, 1998). These changes in mechanical work often exhibit as respective changes in wingbeat frequency and/or wing stroke amplitude. For instance, low density flight experiments (e.g. heliox, 21% O_2 and 79% He) show increases in metabolic rate and wing stroke amplitude in both carpenter bees (Roberts et al., 2004) and hummingbirds (Chai and Dudley, 1996, 1999). In contrast, insects flown in high density atmospheres (e.g. high barometric pressures) can produce the same amount of lift as they would in normodense air by changing a range of wing mechanics, including angle of attack, wingbeat frequency and wing stroke amplitude (Withers, 1981). For example, wingbeat frequency of *Drosophila* decreases with experimental increases in barometric pressure (Chadwick and Williams, 1949), and the O_2 consumption rate of honeybees decreases linearly with increasing barometric pressure (Withers, 1981). Certainly, it would seem that the 24% decrease in locust FMR measured here in SF_6 relative to that in normal air must be related to the 15% decrease in wingbeat frequency (Ellington, 1985; Pennycuick and Rezende, 1984) and the 12% decrease in wing stroke amplitude.

Changing the density of the flight medium also affects the mechanical work of ventilation, with ventilation becoming easier at

lower densities, and more difficult at higher densities (Dudley, 2000). The decrease in flight performance in SF_6 could be related to a decrease in active ventilation, autoventilation and facilitated diffusion, consequently impairing O_2 transport through the tracheal system. Thus, the effects of density can be multiple and complex. The effect on ventilation could be explored further by creating variable P_{O_2} in ternary gas mixtures of different densities.

Conclusions

The flight muscles of the locust are supplied by the diffusion and convection of O_2 along the tracheal system. At maximum FMR, O_2 delivery does not appear to be limited at normal atmospheric P_{O_2}, but it does become limited at slightly hypoxic P_{O_2}, consonant with the data from the American locust *S. americana*. This suggests that the conductance of the tracheal system is matched to maximum functional requirements, in line with the hypothesis of symmorphosis. Locusts therefore have little reserve capacity to deliver O_2, compared with bees and dragonflies, which is possibly related to their modest ability to carry additional loads. Exposure to normoxia in background gas mixtures involving different O_2 diffusivities and densities reveals that O_2 supply to the flight muscles is primarily by convection rather than diffusion.

Acknowledgements

The authors thank Jon Harrison of Arizona State University who provided valuable suggestions on an earlier draft of this manuscript.

Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: E.P.S., R.D., R.S.S.; Methodology: E.P.S., R.D., R.S.S.; Validation: R.D.; Formal analysis: R.D., K.K.J., R.S.S.; Investigation: R.D., K.K.J., E.P.F.; Resources: R.S.S.; Data curation: R.D., K.K.J., R.S.S.; Writing - original draft: R.D., R.S.S.; Writing - review & editing: E.P.S., K.K.J., E.P.F., R.S.S.; Supervision: E.P.S., R.S.S.; Project administration: E.P.S., R.S.S.

Funding

E.P.S. holds a South African Claude Leon Foundation Postdoctoral Fellowship. Summer research scholarships were provided generously by the University of Adelaide.

Data availability

Data are available at ResearchGate (www.researchgate.net/profile/Edward_Snelling).

References

Baker, P. S., Gewecke, M. and Cooter, R. J. (1981). The natural flight of the migratory locust, *Locusta migratoria* L. III: wing-beat frequency, flight speed and attitude. *J. Comp. Physiol.* **141**, 233-237.

Bohn, D. J., Miyasaka, K., Marchak, B. E., Thompson, W. K., Froese, A. B. and Bryan, A. C. (1980). Ventilation by high-frequency oscillation. *J. Appl. Physiol.* **48**, 710-716.

Chadwick, L. E. and Williams, C. M. (1949). The effects of atmospheric pressure and composition on the flight of *Drosophila*. *Biol. Bull.* **97**, 115-137.

Chai, P. and Dudley, R. (1996). Limits to flight energetics of hummingbirds hovering in hypodense and hypoxic gas mixtures. *J. Exp. Biol.* **199**, 2285-2295.

Chai, P. and Dudley, R. (1999). Maximum flight performance of hummingbirds: capacities, constraints, and trade-offs. *Am. Nat.* **153**, 398-411.

Cussler, E. L. (2009). *Diffusion: Mass Transfer in Fluid Systems*, 3rd edn, Part 2, pp. 115-234. Cambridge, UK: Cambridge University Press.

Dudley, R. (1998). Atmospheric oxygen, giant Paleozoic insects and the evolution of aerial locomotor performance. *J. Exp. Biol.* **201**, 1043-1050.

Dudley, R. (2000). *The Biomechanics of Insect Flight: Form, Function, Evolution.* Princeton, NJ, USA: Princeton University Press.

Dudley, R. and Ellington, C. P. (1990). Mechanics of forward flight in bumblebees I: kinematics and morphology. *J. Exp. Biol.* **148**, 19-52.

Ellington, C. P. (1985). Power and efficiency of insect flight muscle. *J. Exp. Biol.* **115**, 293-304.

Fairbanks, D. F. and Wilke, C. R. (1950). Diffusion coefficients in multicomponent gas mixtures. *Ind. Eng. Chem.* **42**, 471-475.

Fischer, H., Wolf, H. and Büschges, A. (2002). The locust tegula: kinematic parameters and activity pattern during the wing stroke. *J. Exp. Biol.* **205**, 1531-1545.

Gee, C. E. and Meldrum Robertson, R. (1998). Free-flight ability in locusts recovering from partial deafferentation. *Naturwissenschaften* **85**, 167-170.

Gewecke, M. (1975). The influence of the air-current sense organs on the flight behaviour of *Locusta migratoria*. *J. Comp. Physiol.* **103**, 79-95.

Gewecke, M. and Kutsch, W. (1979). Development of flight behaviour in maturing adults of *Locusta migratoria* I: flight performance and wing-stroke parameters. *J. Insect Physiol.* **25**, 249-253.

Greenlee, K. J. and Harrison, J. F. (2004a). Development of respiratory function in the American locust *Schistocerca americana* I: across-instar effects. *J. Exp. Biol.* **207**, 497-508.

Greenlee, K. J. and Harrison, J. F. (2004b). Development of respiratory function in the American locust *Schistocerca americana* II: within-instar effects. *J. Exp. Biol.* **207**, 509-517.

Greenlee, K. J., Nebeker, C. and Harrison, J. F. (2007). Body size-independent safety margins for gas exchange across grasshopper species. *J. Exp. Biol.* **210**, 1288-1296.

Harrison, J. F. and Lighton, J. R. B. (1998). Oxygen-sensitive flight metabolism in the dragonfly *Erythemis simplicicollis*. *J. Exp. Biol.* **201**, 1739-1744.

Harrison, J. F., Lafreniere, J. J. and Greenlee, K. J. (2005). Ontogeny of tracheal dimensions and gas exchange capacities in the grasshopper, *Schistocerca americana*. *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* **141**, 372-380.

Harrison, J. F., Frazier, M. R., Henry, J. R., Kaiser, A., Klok, C. J. and Rascón, B. (2006). Responses of terrestrial insects to hypoxia or hyperoxia. *Respir. Physiol. Neurobiol.* **154**, 4-17.

Harrison, J. F., Waters, J. S., Cease, A. J., VandenBrooks, J. M., Callier, V., Klok, C. J., Shaffer, K. and Socha, J. J. (2013). How locusts breathe. *Physiology* **28**, 18-27.

Hartung, D. K., Kirkton, S. D. and Harrison, J. F. (2004). Ontogeny of tracheal system structure: a light and electron-microscopy study of the metathoracic femur of the American locust, *Schistocerca americana*. *J. Morphol.* **262**, 800-812.

Henry, J. R. and Harrison, J. F. (2014). Effects of body size on the oxygen sensitivity of dragonfly flight. *J. Exp. Biol.* **217**, 3447-3456.

Joos, B., Lighton, J. R. B., Harrison, J. F., Suarez, R. K. and Roberts, S. P. (1997). Effects of ambient oxygen tension on flight performance, metabolism, and water loss of the honeybee. *Physiol. Zool.* **70**, 167-174.

Kaiser, A., Klok, C. J., Socha, J. J., Lee, W.-K., Quinlan, M. C. and Harrison, J. F. (2007). Increase in tracheal investment with beetle size supports hypothesis of oxygen limitation on insect gigantism. *Proc. Natl. Acad. Sci. USA* **104**, 13198-13203.

Kirkton, S. D., Niska, J. A. and Harrison, J. F. (2005). Ontogenetic effects on aerobic and anaerobic metabolism during jumping in the American locust, *Schistocerca americana*. *J. Exp. Biol.* **208**, 3003-3012.

Klocke, R. A., Saltzman, A. R., Grant, B. J. B., Aquilina, A. T. and Zhang, S. (1990). Role of molecular diffusion in conventional and high frequency ventilation. *Am. Rev. Respir. Dis.* **142**, 802-806.

Klok, C. J., Kaiser, A., Lighton, J. R. B. and Harrison, J. F. (2010). Critical oxygen partial pressures and maximal tracheal conductances for *Drosophila melanogaster* reared for multiple generations in hypoxia or hyperoxia. *J. Insect Physiol.* **56**, 461-469.

Komai, Y. (1998). Augmented respiration in a flying insect. *J. Exp. Biol.* **201**, 2359-2366.

Komai, Y. (2001). Direct measurement of oxygen partial pressure in a flying bumblebee. *J. Exp. Biol.* **204**, 2999-3007.

Kutsch, W. and Gewecke, M. (1979). Development of flight behaviour in maturing adults of *Locusta migratoria* II: aerodynamic parameters. *J. Insect Physiol.* **25**, 299-304.

Lighton, J. R. B. (2008). *Measuring Metabolic Rates: A Manual for Scientists*. Oxford, UK: Oxford University Press.

Marden, J. H. (2000). Variability in the size, composition, and function of insect flight muscles. *Annu. Rev. Physiol.* **62**, 157-178.

Miller, P. L. (1960). Respiration in the desert locust III: ventilation and the spiracles during flight. *J. Exp. Biol.* **37**, 264-278.

Mizisin, A. P. and Ready, N. E. (1986). Growth and development of flight muscle in the locust (*Schistocerca nitens*, Thünberg). *J. Exp. Zool.* **237**, 45-55.

Mueller, C. A. and Seymour, R. S. (2011). The Regulation Index: a new method for assessing the relationship between oxygen consumption and environmental oxygen. *Physiol. Biochem. Zool.* **84**, 522-532.

Pennycuick, C. J. and Rezende, M. A. (1984). The specific power output of aerobic muscle, related to the power density of mitochondria. *J. Exp. Biol.* **108**, 377-392.

Piiper, J. and Worth, H. (1980). Value and limits of Graham's law for prediction of diffusivities of gases in gas mixtures. *Respir.Physiol.* **41**, 233-240.

Poling, B. E., Prausnitz, J. M. and O'Connell, J. P. (2001). *The Properties of Gases and Liquids*, 5th edn, Ch 11, pp. 11.1-11.55. New York City, NY, USA: McGraw-Hill.

Rascón, B. and Harrison, J. F. (2005). Oxygen partial pressure effects on metabolic rate and behavior of tethered flying locusts. *J. Insect Physiol.* **51**, 1193-1199.

Roberts, S. P., Harrison, J. F. and Dudley, R. (2004). Allometry of kinematics and energetics in carpenter bees (*Xylocopa varipuncta*) hovering in variable-density gases. *J. Exp. Biol.* **207**, 993-1004.

Robertson, R. M. and Johnson, A. G. (1993). Collision avoidance of flying locusts: steering torques and behaviour. *J. Exp. Biol.* **183**, 35-60.

Schippers, M.-P., Dukas, R. and McClelland, G. B. (2010). Lifetime- and caste-specific changes in flight metabolic rate and muscle biochemistry of honeybees, *Apis mellifera*. *J. Comp. Physiol. B Biochem. Syst. Environ. Physiol.* **180**, 45-55.

Seymour, R. S., Withers, P. C. and Weathers, W. W. (1998). Energetics of burrowing, running, and free-living in the Namib Desert golden mole (*Eremitalpa namibensis*). *J. Zool. (Lond.)* **244**, 107-117.

Snelling, E. P., Seymour, R. S., Matthews, P. G. D., Runciman, S. and White, C. R. (2011a). Scaling of resting and maximum hopping metabolic rate throughout the life cycle of the locust *Locusta migratoria*. *J. Exp. Biol.* **214**, 3218-3224.

Snelling, E. P., Seymour, R. S., Runciman, S., Matthews, P. G. D. and White, C. R. (2011b). Symmorphosis and the insect respiratory system: allometric variation. *J. Exp. Biol.* **214**, 3225-3237.

Snelling, E. P., Seymour, R. S., Matthews, P. G. D. and White, C. R. (2012a). Maximum metabolic rate, relative lift, wingbeat frequency and stroke amplitude during tethered flight in the adult locust *Locusta migratoria*. *J. Exp. Biol.* **215**, 3317-3323.

Snelling, E. P., Seymour, R. S., Runciman, S., Matthews, P. G. D. and White, C. R. (2012b). Symmorphosis and the insect respiratory system: a comparison between flight and hopping muscle. *J. Exp. Biol.* **215**, 3324-3333.

Suarez, R. K. (2000). Energy metabolism during insect flight: biochemical design and physiological performance. *Physiol. Biochem. Zool.* **73**, 765-771.

Van Voorhies, W. A. (2009). Metabolic function in *Drosophila melanogaster* in response to hypoxia and pure oxygen. *J. Exp. Biol.* **212**, 3132-3141.

Wasserthal, L. T. (2001). Flight-motor-driven respiratory air flow in the hawkmoth *Manduca sexta*. *J. Exp. Biol.* **204**, 2209-2220.

Wasserthal, L. T. (2015). Flight-motor-driven respiratory airflow increases tracheal oxygen to nearly atmospheric level in blowflies (*Calliphora vicina*). *J. Exp. Biol.* **218**, 2201-2210.

Weibel, E. R., Taylor, C. R. and Hoppeler, H. (1991). The concept of symmorphosis: a testable hypothesis of structure-function relationship. *Proc. Natl. Acad. Sci. USA* **88**, 10357-10361.

Weibel, E. R., Taylor, C. R. and Bolis, L. (1998). *Principles of Animal Design: The Optimization and Symmorphosis Debate*. Cambridge, UK: Cambridge University Press.

Weis-Fogh, T. (1956). Biology and physics of locust flight IV: notes on sensory mechanisms in locust flight. *Philos. Trans. R. Soc. Lond. B Biolog. Sci.* **239**, 553-584.

Weis-Fogh, T. (1964a). Diffusion in insect wing muscle, the most active tissue known. *J. Exp. Biol.* **41**, 229-256.

Weis-Fogh, T. (1964b). Functional design of the tracheal system of flying insects as compared with the avian lung. *J. Exp. Biol.* **41**, 207-227.

Weis-Fogh, T. (1967). Respiration and tracheal ventilation in locusts and other flying insects. *J. Exp. Biol.* **47**, 561-587.

Withers, P. C. (1981). The effects of ambient air pressure on oxygen consumption of resting and hovering honeybees. *J. Comp. Physiol.* **141**, 433-437.

Withers, P. C. (1992). *Comparative Animal Physiology*. Philadelphia, PA, USA: Saunders College Publishing.

Wolf, H. (1993). The locust tegula: significance for flight rhythm generation, wing movement control and aerodynamic force production. *J. Exp. Biol.* **182**, 229-253.

Worth, H. and Piiper, J. (1978). Diffusion of helium, carbon monoxide and sulfur hexafluoride in gas mixtures similar to alveolar gas. *Respir. Physiol.* **32**, 155-166.