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RESEARCH ARTICLE

Gas exchange and dive characteristics of the free-swimming backswimmer *Anisops deanei*

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ABSTRACT

Many aquatic insects utilise air bubbles on the surface of their bodies to supply O_2 while they dive. The bubbles can simply store O_2, as in the case of an 'air store', or they can act as a physical 'gas gill', extracting O_2 from the water. Backswimmers of the genus *Anisops* augment their air store with O_2 from haemoglobin cells located in the abdomen. The O_2 release from the haemoglobin helps stabilise bubble volume, enabling backswimmers to remain near neutrally buoyant for a period of the dive. It is generally assumed that the backswimmer air store does not act as a gas gill and that gas exchange with the water is negligible. This study combines measurements of dive characteristics under different exotic gases (N_2, He, SF_6, CO) with mathematical modelling, to show that the air store of the backswimmer *Anisops deanei* does exchange gases with the water. Our results indicate that approximately 20% of O_2 consumed during a dive is obtained directly from the water. Oxygen from the water complements that released from the haemoglobin, extending the period of near-neutral buoyancy and increasing dive duration.

KEY WORDS: Aquatic insect, Air store, Buoyancy, Exotic gases, Gas exchange

INTRODUCTION

The air-filled tracheal system of insects evolved in the terrestrial environment (Pritchard et al., 1993), yet there are several groups of diving insects that interface their tracheae with bubbles brought from the surface. The bubbles, termed 'air stores', supply O_2 during a dive, but may also function as 'gas gills' by passively extracting dissolved O_2 from the water (Ege, 1915; Rahn and Paganelli, 1968). Compressible gas gills have a limited lifetime because of O_2 consumption by the insect and outward diffusion of N_2 into the water, so the bubble requires periodic renewal at the surface. Backswimmers (Hemiptera: Notonectidae) of the genera *Anisops* and *Buenoa* augment their air store with O_2 released from haemoglobin cells located in the abdomen, which serves to delay the collapse of the bubble during a period of near-neutral buoyancy (Matthews and Seymour, 2006; Miller, 1966a). This mechanism allows these predatory insects to occupy a mid-water niche that is otherwise unavailable to aquatic insects that are either highly positively or negatively buoyant (Miller, 1964). Buoyancy control works best if bubble volume is constant, and so it is assumed that the air store of backswimmers does not act as a gas gill, and that gas exchange with the water is effectively zero (Matthews and Seymour, 2008; Miller, 1966a). This assumption is associated with the observation that the abdominal grooves and hydrophobic hairs that hold the bubble in position during dives reduce the surface area for gas exchange. However, the validity of this assumption has not been tested.

Oxygen release from the haemoglobin during dives depends on binding kinetics. Backswimmer haemoglobin shows extremely high cooperativity (Hill's $n \approx 15$), which permits unloading within a very narrow range of P_{O_2} and produces a phase of relatively stable near-neutral buoyancy (Matthews and Seymour, 2011; Miller, 1966a; Wawrowski et al., 2012; Wells et al., 1981). Because of the relatively high affinity of backswimmer haemoglobin for O_2 (P_{50}=2.4–5.3 kPa), backswimmers must theoretically collect a bubble that is 17% larger than that required for neutral buoyancy to ensure that O_2 release coincides with the phase of neutral buoyancy (Matthews and Seymour, 2008). Data from tethered backswimmers indicate three phases of a dive: a positive buoyancy phase before haemoglobin begins to unload O_2, a near-neutral buoyancy phase during unloading, and a phase of rapid decrease in buoyancy after the haemoglobin is exhausted (Miller, 1964, 1966a; Matthews and Seymour, 2006, 2008). However, changes in buoyancy in free-swimming backswimmers have not been adequately quantified (Miller, 1964), and it is not known how close these insects come to coordinating O_2 release and neutral buoyancy. The convective environment surrounding the bubble is fundamentally different in tethered and free-swimming backswimmers. As free-swimming backswimmers move through the water, the boundary layer becomes thinner and resistance to gas exchange is potentially reduced in comparison to tethered backswimmers. This could cause a more rapid decline in buoyancy through N_2 loss and enhance O_2 uptake, both of which would influence buoyancy and dive duration.

This study combines experimental manipulation of the air store system and mathematical modelling to investigate the gas exchange and dive characteristics of free-swimming backswimmers *Anisops deanei*. Exotic gases were used to test for gas exchange with the water and to investigate patterns of buoyancy change, leg stroke frequency and surfacing duration. Backswimmers were exposed to normoxia (P_{O_2} ~21 kPa) balanced by three different carrier gases: a nitrogen control (N_2-control), a helium (He) treatment to decrease bubble longevity and increase O_2 diffusion rates, and a sulphur hexafluoride (SF_6) treatment to increase bubble longevity and decrease O_2 diffusion rates. A fourth treatment, consisting of air with 1.5% carbon monoxide (CO), was used to prevent O_2–haemoglobin binding. Dive duration and buoyancy results were then compared with a mathematical model based on a compressible gas gill (Rahn and Paganelli, 1968) and an O_2 equilibrium curve for backswimmer haemoglobin (Matthews and Seymour, 2011). The model incorporates O_2 consumption measurements from a closed respirometry system and morphological measurements from live tethered and preserved backswimmers, as well as data from previous studies.

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MATERIALS AND METHODS

Animals

Backswimmers, identified as *Anisops deanei* (Brooks 1951) (Andersen and Weir, 2004), were collected from Balhannah, South Australia, and kept in a 72 l aquarium (20°C) receiving natural light. Backswimmers were fed live mosquito larvae daily. They were dried with a paper towel and weighed to an accuracy of 10 µg on an analytical balance (AE 163, Mettler, Greifensee, Switzerland). After experiments, backswimmers were sexed and found to be 30% female in the N_2-control, He and CO treatments, and 20% female in SF_6.

Experimental setup

Backswimmers were placed into gas-tight, clear acrylic, cylindrical dive chambers (100 mm diameter×150 mm high). Two setups were run simultaneously alongside one another, because backswimmers aggregate naturally in groups and a pilot study found that pairs undertake longer dives than solitary individuals (Miller, 1966b; Bailey, 1987; Gilbert et al., 1999). Each dive chamber contained 850 ml of reverse osmosis water and 75 ml of gas space. The chamber tops were sealed with thick rubber stoppers with gas inlet and outlet tubes. Chambers were submerged in a 32 l water-filled aquarium for temperature control and to eliminate optical distortion. A white aquarium background and a blue chamber background, together with uniform lighting, provided good contrast for video recording. A video camera (GX-PX1, JVC, Yokohama, Japan), set to 59 frames s^{-1}, was placed in front of the aquarium with an angled mirror placed underneath that allowed backswimmers to be viewed from below.

Experimental protocol

Gases supplied to the dive chambers were regulated through mass flow controllers (Model GFC171, 0–100 and 0–1000 ml min^{-1}; and GFC171S, 0–10 l min^{-1}, Aalborg Instruments & Controls, Inc., Orangeburg, NY, USA) operated with a Microsoft Excel macro and analog output board (ProfessorDAQ™ and PowerDAQ™ PD2-AO, United Electronic Industries, Walpole, MA, USA). Four cohorts of backswimmers were exposed to one of four gas treatments (Table 1). Water for He and SF_6 treatments was first de-oxygenated by bubbling N_2 through air stones until O_2 reached 0.2–0.1% saturation (~20 min) as measured with a fibre-optic O_2-sensing optode (Sensor model PSt1, meter model TX-3, PreSens GmbH, Regensburg, Germany), prior to re-oxygenation with the gas treatment mixtures. Treatment mixtures were bubbled through the water until normoxia (~21 kPa) was achieved (~20 min). Correct gas mixtures for He and SF_6 treatments were initially determined using an O_2 analyser (FC-2 Sable Systems, Las Vegas, NV, USA) and the CO treatment involved mixing 1.5% CO with air. After desired gas conditions in the water were attained, backswimmers were placed into the chambers and a 2 min washout of the gas above the water with the treatment mixture occurred. The system was then closed and backswimmers were left to adjust for 15 min. After this period, backswimmers were left undisturbed and video recording commenced for 2 h, followed by chloroform euthanasia and weight measurement.

Determination of dive characteristics

To determine buoyancy change during dives, backswimmers in the video footage were tracked in the vertical axis in each frame using two components of Motion-Based Multiple Object Tracking in Matlab, foreground detection and blob analysis (Matlab R2014a 8.3.0.532, MathWorks, Inc., Natick, MA, USA). Inspection of the backswimmers in the angled mirror confirmed that individuals passively ascended and descended in a straight vertical motion when not actively moving. To accommodate for parallax, the backswimmers were assumed to move randomly in the two horizontal axes, and the known water depth at the centre of the dive chambers was used as a scale. Data were smoothed over the leading 30 frames to reduce noise due to small fluctuations in the position of the centroid (mean centre position of the backswimmer image), then converted from pixels s^{-1} to mm s^{-1}, subdivided into each identified dive, and plotted. Video footage was compared with the plots to identify periods of passive movement to determine rates of ascent and descent. Periods of passive movement ranging from 0.5 to 2 s were identified within 10 s periods throughout the duration of each dive. Five randomly selected dives within ±10% of the mean dive duration for each individual backswimmer were selected. This reduced the influence of varying initial bubble volume on the occurrence and duration of each phase.

Dive duration was calculated as the time between surfacing events, which included time spent at the surface renewing the air store. All complete dives during each 2 h experiment were measured, equating to approximately 58 dives per individual under the N_2-control treatment, 88 under He, 40 under SF_6 and 118 under CO (N=10 backswimmers per treatment). Mean dive duration of individual backswimmers was corrected by removing the mean surfacing duration for that individual. Surfacing duration was calculated by counting the number of frames during which the backswimmers were at the surface and given the known camera frame rate. Leg stroke frequency was calculated by analysing videos at 1/3 normal frame rate and manually time stamping each leg stroke, which produced a record of when each stroke occurred during a dive that could be subsequently divided into 5 s periods. Ten randomly selected dives from each backswimmer were used to determine leg stroke frequency and surfacing duration. Backswimmers often moved too frequently for portions of their dive, which inhibited measurement of passive movement associated with buoyancy for all periods during the dive. However, leg stroke frequency was recorded continuously throughout all dives, resulting in differences in recording length for buoyancy and leg stroke frequency under the different treatments.

Table 1. Gas treatment mixtures above and within the water

	Gas composition	
Treatment	In water	Above water
N_2-control	Air, CO_2-free	Air, CO_2-free
He	79% He, 21% O_2	79% He, 21% O_2
SF_6	79% SF_6, 21% O_2	79% SF_6, 21% O_2
CO	Air, CO_2-free	97.5% Air, CO_2-free, 1.5% CO

Gases used: high purity N_2, industrial O_2 (BOC Ltd, NSW, Australia), high purity He, SF_6 and CO (Coregas Pty Ltd, NSW, Australia).

Air store dimensions

The backswimmer air store approximates a half-ellipsoid in shape, split along the long axis (Fig. 1). Semi-axes a and b were determined in ethanol-preserved backswimmers by photographing (correct to scale) the abdominal grooves that hold the air store in place, using a camera mounted on a dissecting microscope (Fig. 1, top). The height of the air store bubble (semi-axis c) was measured from live backswimmers narcotised with pure CO_2 for ca. 10 s, and attached with cyanomethacrylate adhesive to a paperclip so that a swimming position was adopted with the ventral side facing upwards. Backswimmers were then placed into a shallow clear container with the microscope perpendicular to the sagittal plane of the backswimmer. Water was slowly added until the backswimmer became completely submerged and the air store was no longer in contact with the water's surface (Fig. 1, bottom). At this point, a photograph was taken to scale, and thereafter every 60 s for 3 min. After 3 min, the water level was reduced, exposing the backswimmer to the air again. This was done three times for each backswimmer (N=6). Measurements were taken using image processing software (ImageJ 1.47v, Wayne Rasband, National Institutes of Health, USA). Light reflection and refraction may have influenced the measurement of semi-axis c, with studies indicating a ~10% error in measuring bubbles from images (Leifer et al., 2003; Vazquez et al., 2005). However, this has little overall effect on calculation of air store surface area as a ±10% variation around the mean value for semi-axis c results in only a ±3% change in air store surface area.

Respirometry

Backswimmers were placed in a vertical glass vial (13.5 mm diameter×51 mm high) completely filled with air-equilibrated reverse osmosis water, and sealed with a rubber stopper through which two 0.8 mm outer diameter hypodermic needles were placed. The vial containing the backswimmer was then dried on the outside and weighed. A 1 ml syringe was used to inject a 0.5 ml bubble of air into the chamber,

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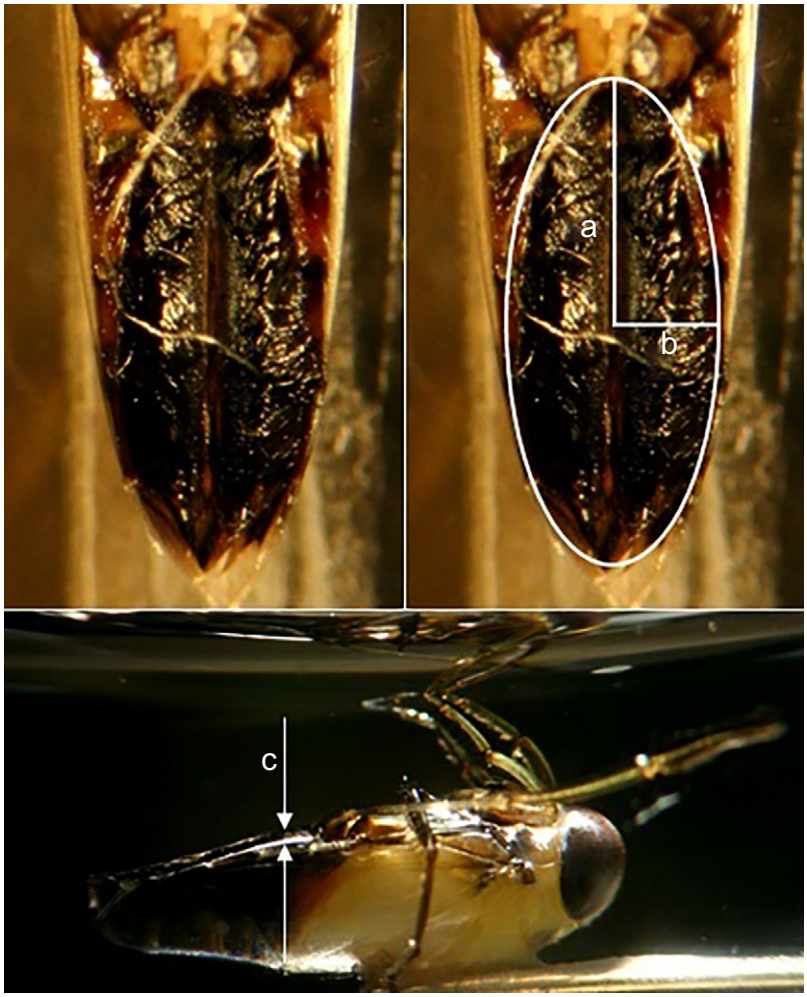

Fig. 1. Dimensions of the backswimmer air store. Top left, unobstructed view of the air store on the ventral side of an ethanol-preserved backswimmer with the hydrophobic hair layer covering the air store. Top right, semi-axes a and b of the ellipsoid shown with an overlain oval representing the approximate shape of the air store. Bottom, a live tethered backswimmer submerged below the water's surface showing the air store bubble height between the arrows, representing semi-axis c of the ellipsoid.

displacing an equal volume of water. The chamber was dried and reweighed to verify air volume. The chamber was placed in a brace and lowered into a 6 l aquarium with the top of the chamber 10–20 mm underwater. The hypodermic needles were used as guides for insertion and positioning of O_2-sensing optodes housed within syringes with 0.25 mm outer diameter hypodermic needles. One optode was placed into the air bubble while the other was placed into the water. Each optode entered the chamber through two concentric needles filled with water to prevent O_2 contamination from the atmosphere while allowing for pressure change.

The decline in P_{O_2} of both the air and water was measured over a 3.5 h period and recorded using computer software (OxyView TX3, V5.31, PreSens GmbH). The backswimmers were then removed and the P_{O_2} measured in the vacant chamber for a further 3.5 h to determine sensor drift and background respiration. This contributed 3.5±3.9% of the decline in P_{O_2} and was used to correct O_2 consumption slopes. P_{O_2} decline was unstable for the first hour, so data were taken between 1 and 3.5 h of each run. Oxygen consumed was calculated from the O_2 capacitance of air (451 µmol l^{-1} kPa^{-1}) and water (13.7 µmol l^{-1} kPa^{-1}) at 20°C (Dejours, 1981), the volumes of the media, and the rate of P_{O_2} change. Chamber and water barrier integrity were checked by filling the chamber with pure gaseous N_2 and measuring the increase in P_{O_2} over a 24 h period. Oxygen increased by 0.07 kPa h^{-1} with a gradient of 16–20 kPa. In comparison, backswimmers in the chamber produced mean values of 1.02 kPa h^{-1} in water and 1.13 kPa h^{-1} in air.

Statistics

Means are reported with 95% confidence intervals (CI). ANOVA, Tukey's *post hoc*, and polynomial, segmented linear and linear regressions were conducted using GraphPad statistical software (GraphPad Prism 6, La Jolla, CA, USA). Breakpoint analysis and ANCOVA were performed according to Yeager and Ultsch (1989) and Zar (1998), respectively.

RESULTS

Morphology

The overall mean mass of backswimmers used in this study was 10.18±0.36 mg (N=40). Measurements of the abdominal grooves of backswimmers preserved in ethanol show that the air store's long axis (semi-axis a, Fig. 1) was 1.13±0.05 mm, and the short axis (semi-axis b) was 0.42±0.02 mm (N=10, body mass=9.81±0.55 mg). The height of the air store (semi-axis c), in live tethered backswimmers over a 180 s period of submergence, did not change with time: 0.22±0.03 mm at 0 s, 0.21±0.04 mm at 60 s, 0.22±0.03 mm at 120 s and 0.22±0.04 mm at 180 s (ANOVA, P=0.16). The pooled value for semi-axis c was 0.22±0.03 mm (N=6, body mass=9.89±0.32 mg).

Dive and surfacing duration

Dive and surfacing durations varied depending on the exotic gas treatment. Mean dive duration was 124±11 s for the N_2-control, 85±14 s for the He treatment, 183±28 s for the SF_6 treatment and 60±10 s for the CO treatment (Fig. 2A). All four treatments were significantly different from one another (ANOVA, P<0.0001; Tukey's *post hoc*, P<0.05), except for the He and CO treatments (P>0.05). Surfacing durations were 0.46±0.11 s for the N_2-control, 0.18±0.02 s for the He treatment, 1.84±0.48 s for the SF_6 treatment and 5.05±1.60 s for the CO treatment (Fig. 2B). All treatments

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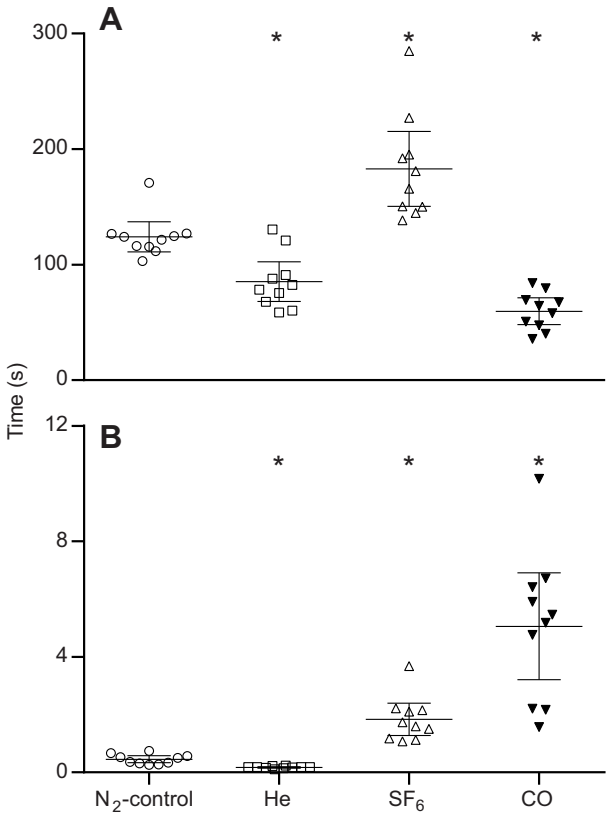

Fig. 2. Backswimmer dive and surfacing durations. (A) Mean±95% confidence interval (CI) dive duration under the four gas treatments (*N*=10 individuals for each group). Each dive duration point is the mean value of all complete dives over the 2 h experimental period for each individual backswimmer. (B) Mean surfacing duration under the four gas treatments (*N*=10). Each surfacing duration point is the mean value of 10 randomly selected dives of an individual backswimmer. *Significantly different from the N_2-control treatment.

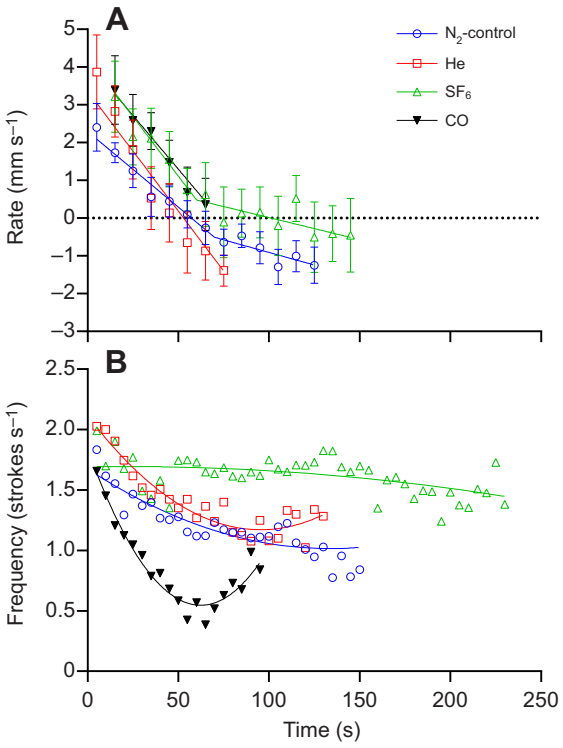

Fig. 3. Backswimmer ascent and descent rates, and leg stroke frequencies during dives. (A) Mean±95% CI ascent and descent rate throughout the duration of a dive under N_2-control (*N*=7 individuals), He (*N*=9), SF_6 (*N*=7) and CO (*N*=9) treatments. Five dives were performed per individual. Segmented linear regression was applied to the N_2-control and SF_6 treatment, and linear regression to the He and CO treatments. Breaks in regressions were determined with breakpoint analysis (Yeager and Ultsch, 1989). Only points for which there were >6 input values are plotted. (B) Mean leg stroke frequency (*f*; strokes s^{-1}) in relation to time (*t*; s) across dives under the N_2-control and three gas treatments. Second-order polynomial regressions are: N_2-control, $f=1.675-9.871\times10^{-3}t+3.694\times10^{-5}t^2$; He, $f=2.116-0.0198t+1.038\times10^{-4}t^2$; SF_6, $f=1.692+2.472\times10^{-4}t-5.687\times10^{-6}t^2$; CO, $f=1.839-0.04147t+3.326\times10^{-4}t^2$. Mean values were derived from the leg stroke frequency of 10 randomly selected dives of six backswimmers. Each point represents an average derived from 10–60 input values. Points with <10 input values were excluded from the analysis.

were significantly different from one another once data were $\log_{10}$-transformed and normally distributed (ANOVA, $P<0.0001$; Tukey's *post hoc*, $P<0.05$). Mean temperature during swimming experiments was 20.5±0.3°C with no significant difference between treatments (ANOVA, $P=0.43$).

Buoyancy

The rate of buoyancy decline during dives occurred in two phases in both the N_2-control and SF_6 treatment, but only one phase was detected in the He and CO treatments (Fig. 3A). Buoyancy declined significantly more rapidly in the He treatment (-0.064 mm s^{-2}) and in the first phase of the SF_6 treatment (-0.063 mm s^{-2}) compared with the first phase of the N_2-control (-0.040 mm s^{-2}) (Table 2). Buoyancy decline in the CO treatment (-0.057 mm s^{-2}) was not significantly different from that in the first phase of the N_2-control, although the elevation of the regression was significantly higher. The elevation of the SF_6 first phase regression was significantly higher than that of the He treatment, and no significant difference in the rate of decline existed between the second phase of the N_2-control (-0.013 mm s^{-2}) and the SF_6 (-0.012 mm s^{-2}) treatment, although the SF_6 treatment had a higher regression elevation. Breakpoints between the two phases occurred at 70 s with a descent rate of -0.55 mm s^{-1} in the N_2-control, and at 60 s with an ascent rate of $+0.48$ mm s^{-1} in the SF_6 treatment (Fig. 3A).

Leg stroke frequency

Leg stroke frequency in all treatments tended to be high at the beginning of dives (1.6–2.0 strokes s^{-1}), but then followed different patterns over the course of the dive (Fig. 3B). In the N_2-control treatment, leg stroke frequency decreased initially but eventually stabilised. The He treatment showed a similar pattern, but with an increasing trend toward the end of the dive. Leg stroke frequency during the SF_6 treatment was fairly constant for the first 150 s of the dive, decreasing slightly thereafter. CO-treated backswimmers showed lower leg stroke frequencies than the other three treatments, and exhibit a pronounced U-shaped curve over time.

Respirometry

Mean mass of the backswimmers used for respirometry was 9.64±0.51 mg (*N*=7). At a temperature of 19.9±0.1°C, the O_2 consumption rate of the backswimmers was $5.81\times10^{-3}\pm5.66\times10^{-4}$ µmol min^{-1}, and the mass-specific value was 0.604±0.058 µmol min^{-1} g^{-1}.

DISCUSSION

Free-swimming backswimmers

The N_2-control experiments in this study revealed a range of dive characteristics of freely swimming backswimmers. Under these

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Table 2. ANCOVA comparisons between regressions of buoyancy phases identified between gas treatments

Regression comparison	Slope	Elevation	*F*-value
N_2-control 1st phase vs SF_6 1st phase	P=0.0155*	n/a	$F_{1,185}$=5.969
N_2-control 1st phase vs He	P=0.0106*	n/a	$F_{1,300}$=6.608
SF_6 1st phase vs He	P=0.9201	P<0.001*	$F_{1,271}$=15.54
N_2-control 2nd phase vs SF_6 2nd phase	P=0.6072	P<0.001*	$F_{1,221}$=33.99
N_2-control 1st phase vs CO	P=0.066	P<0.001*	$F_{1,243}$=3.411

*Statistically significant.

conditions, backswimmers surfaced for approximately 0.46 s to replenish the air store, before diving for an average duration of 124 s (Fig. 2). Once underwater, the backswimmers' buoyancy declined in two phases, with the first phase associated with a rapid decline from positively buoyant to negatively buoyant, and the second phase characterised by a less rapid decline as backswimmers became progressively more negatively buoyant. The transition between the two buoyancy phases occurred at approximately 70 s into the dive (Fig. 3A). Leg stroke frequency was typically high at the commencement of dives, before declining and stabilising prior to dive termination (Fig. 3B).

The backswimmers' dive characteristics are intimately linked to the flux of gases to and from the air store. The surfacing duration reflects the time required for the air store to be replenished. The first phase of rapidly declining buoyancy is associated with O_2 consumption from the bubble (Matthews and Seymour, 2006) and a small amount of N_2 loss to the water. The second phase of slow buoyancy change arises because O_2 released from the haemoglobin tends to stabilise bubble volume. The transition between the two phases represents the point at which O_2 starts to unload from the haemoglobin.

The occurrence of the transition between the two buoyancy phases while negatively buoyant indicates that backswimmers are collecting bubbles smaller than that required for the O_2 release to correspond with attaining neutral buoyancy (Fig. 3A). Previously, the predicted initial volume of the backswimmer bubble was 17% above that required for neutral buoyancy, because this corresponds to the approximate volume of O_2 required to be consumed before haemoglobin begins to release its O_2 (Matthews and Seymour, 2008). However, achieving as close to neutral buoyancy as possible may not be as important for backswimmers as previously thought. Perfect neutral buoyancy can only be achieved momentarily because of the binding characteristics of the haemoglobin. The haemoglobin saturation curve, although steep, is not vertical and is therefore unable to maintain a constant bubble volume through O_2 release. Additional N_2 loss to the water further reduces the ability to maintain neutral buoyancy, producing an appreciable decline in buoyancy during the second phase.

Previous studies have identified a third phase of buoyancy in backswimmer dives during which buoyancy decline becomes more rapid before termination of the dive (Miller, 1964; Matthews and Seymour, 2006). Miller (1964) provided ascent and descent rates of free-swimming *Anisops pellucens* that indicate the third phase, but sample size was restricted to only two full dives. Interspecific variation may be responsible for the difference between *A. pellucens* and *A. deanei*, although a more comprehensive study of free-swimming *A. pellucens* would be required to confirm these buoyancy relationships. Matthews and Seymour (2006, 2008) also showed the third phase in tethered *A. deanei*. Tethered *A. deanei* are probably forced into the third phase, but under natural conditions they may not enter this phase as they become too negatively buoyant prior to this point, resulting in termination of the dive.

Gas exchange between the air store and the water

Previous work on notonectids has assumed that, after the air store is renewed at the surface, there is negligible exchange with the water (Matthews and Seymour, 2008, 2011; Miller, 1966a). This idea is supported by the observation that *Anisops* has a reduced bubble surface in comparison to some other diving insects that rely heavily on O_2 uptake through the physical gas gill. In contrast to the large, naked bubbles of corixids, the air store of backswimmers is held within narrow grooves on the abdomen and covered with a film of hairs (Fig. 1). Furthermore, it would be disadvantageous to have free exchange with the water, because N_2 would be lost at a high rate, greatly decreasing the ability to remain at near-neutral buoyancy while haemoglobin unloads its O_2. Nevertheless, the findings of the present study indicate that there is appreciable gas exchange between the air store and the water. This is evident in the significant differences between dive durations under the N_2-control, He and SF_6 treatments (Fig. 2A). Compared with the N_2-control runs, rapidly diffusing He produces shorter dives, and slowly diffusing SF_6 results in longer dives, which would not occur if the air store were completely sealed.

Dive duration

Rahn and Paganelli (1968) predicted with their model of a naked compressible gas gill that the replacement of N_2 with He would cause a 33% decrease in dive duration, and replacement of N_2 with SF_6 would cause a 400% increase. The present study shows that He caused a 30% decrease and SF_6 a 150% increase in dive duration. However, these results are not directly comparable to Rahn and Paganelli's predictions, because their model assumes that insects terminate their dives when the bubble is completely exhausted. Free-swimming insects terminate their dives earlier, when the P_{O_2} within the bubble becomes too low to support metabolism. The stimulus for dive termination may also vary between species and environmental conditions.

The rate of buoyancy decline would be expected to be fastest in He, slower in the N_2-control and slowest in SF_6 because of the rates at which these gases diffuse into the water (Rahn and Paganelli, 1968). The buoyancy of backswimmers in the He treatment did decline more rapidly than in the N_2-control (Fig. 3A, Table 2). However, the decline in the first phase of the SF_6 treatment was more rapid than in the N_2-control, and the rate in the second phase was similar to that in the N_2-control, indicating that other factors may influence buoyancy. Both He- and SF_6-treated backswimmers began their dives more buoyant than N_2-controls (Fig. 3A) and may compensate with a higher leg stroke frequency (Fig. 3B). This would presumably increase O_2 consumption and boundary layer ventilation, facilitating a more rapid decline in buoyancy. SF_6-treated backswimmers remained positively buoyant for a moderate part of the dive and may compensate with more frequent leg strokes. Conversely, He-treated backswimmers may maintain a high stroke frequency, because buoyancy declined more rapidly. The 60 s breakpoint in the SF_6 treatment provides further evidence that O_2 begins to unload from the haemoglobin around this time (Fig. 3A). This also means that backswimmers under the He treatment terminate their dives (ca. 85 s) just as O_2 begins to release from the haemoglobin, which suggests that severe negative buoyancy, rather than low O_2, is the primary trigger for dive termination.

It is not clear why backswimmers were more positively buoyant in the He, SF_6 and CO treatments than in the N_2-controls. One

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expects that the volume of O_2 in the air store would be similar in all treatments. Differences in gas density cannot explain this, because He is less dense, and SF_6 more dense, than N_2, but both gases produce greater buoyancy. Further work is necessary to determine the factors that influence the volume of gas renewed at the surface.

The dive duration observed in this study is half that recorded for free-swimming *A. deanei* previously (Matthews and Seymour, 2008). These differences may result from variation among different source populations, time of year or methodology. Dive chambers in the present study were more confined and well lit than the aquarium used by Matthews and Seymour (2008), and collisions with the sides of the chambers did occur at a higher rate than in the larger holding aquarium. Within the dive chambers, the two backswimmers were able see each other, which could have increased dive duration by reducing activity associated with searching for conspecifics (Matthews and Seymour, 2008). However, confinement and high light levels may also contribute to agitation and a higher metabolic rate, thus resulting in shorter dive duration. Confinement within the respirometry chambers is likely to have also contributed to an elevated metabolic rate.

Leg stroke frequency

Although the initial high leg stroke frequency in all treatments is almost certainly associated with positive buoyancy and the desire to reach depth after surfacing, the leg stroke frequency remained relatively high for the rest of the dive, except for the CO treatment (Fig. 3B). Miller (1964) presented leg stroke frequency data for an exceptionally long dive of *A. pellucens*, where initial frequency was 0.77 strokes s^{-1}, declining to 0.10 strokes s^{-1} at 4.5 min, and then increasing again to 0.63 strokes s^{-1} at 8 min. This U-shaped relationship was attributed to the three phases of buoyancy, with high stroke frequency occurring at both extreme positive and negative buoyancies, and lower frequency in between. Other behaviours may also contribute to a high stroke frequency throughout dives of *A. deanei*, such as ventilation of the boundary layer or searching for prey or other backswimmers for social interactions.

Surfacing duration

Minimising surfacing duration appears important to backswimmers because of the danger of predation (Miller, 1964); however, sufficient time is necessary to renew the air store volume and saturate the haemoglobin. Surfacing durations under the different treatments provide insight into the factors that affect this time. Surfacing involves the placement of the abdominal tip to the water's surface and the opening of the narrow connection between the air and air store. On occasions, backswimmers allowed the hydrophobic hairs along the abdomen to open, exposing the whole air store, which appeared more frequent in CO-treated backswimmers. Surfacing duration was shortest in He, longer in N_2 and longest in SF_6, and was thus negatively correlated with O_2 diffusivity (cm^2 s^{-1}) in each carrier gas (Fig. 4; Poling et al., 2001). These results suggest that backswimmers have some ability to sense O_2, and wait at the surface long enough for the O_2 to be replenished before beginning their next dive. The surfacing duration may reflect the time taken for O_2 to diffuse from the air into the abdominal grooves and through the tracheal system to the body and haemoglobin cells. Oxygen may reach a critical value somewhere in the body that stimulates commencement of the next dive. However, despite surfacing duration being inversely related to the diffusion coefficient, the relationship is not linear, indicating other factors are involved in determining surfacing duration.

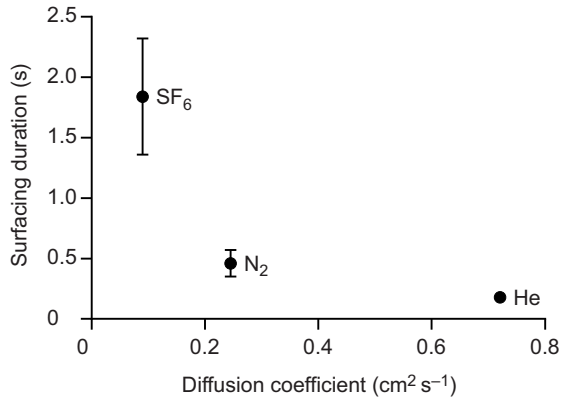

Fig. 4. Correlation between diffusion coefficient and surfacing duration. Mean±95% CI surfacing duration under N_2-control, He and SF_6 treatments plotted against the diffusion coefficient for O_2 within each carrier gas, calculated with the Chapman–Enskog equation for the diffusivity of binary gases, assuming 20°C and 101.3 kPa (Poling et al., 2001). Helium treatment error bars are not visible.

Effect of CO

Despite CO inhibiting the O_2 storage ability of haemoglobin and influencing the passage of electrons through the electron transport chain, backswimmers, like other insects, appear to tolerate high levels of CO (Miller, 1964, 1966a; Matthews and Seymour, 2006; Harvey and Williams, 1958; Baker and Wright, 1977). The decline in air store P_{O_2} in the first phase of control and CO-treated backswimmers is similar (Matthews and Seymour, 2006, 2008), and dive duration, although shortened, is independent of CO concentration between 6% and 20% (Miller, 1966a). Both of these results are consistent with CO not influencing metabolism through the electron transport chain. In the present study, dive duration declined by 50% when backswimmers were treated with CO, suggesting a 50% contribution of O_2 from the haemoglobin (Fig. 2A). This is in agreement with the calculated contribution in *A. deanei* of 0.26 µl of O_2 from the air store and 0.25 µl of O_2 from the haemoglobin (Matthews and Seymour, 2008). However, CO-treated backswimmers had a reduced leg stroke frequency with a prominent U-shaped pattern (Fig. 3B). Given that control and CO-treated backswimmers showed a similar decline in buoyancy in the first phase of the dive, although beginning the dive more buoyant (Fig. 3A, Table 2; Matthews and Seymour, 2006, 2008), it would be expected that the initial leg stroke frequencies would also be similar or higher. The other obvious effect of CO on swimming behaviour is that the surfacing duration was 10 times longer than in the N_2-control (Fig. 2B). Long surfacing durations in CO-treated backswimmers have previously been observed (Miller, 1964). The difference may relate to problems with O_2 sensing. CO is known to regulate large-conductance Ca^{2+}-sensitive potassium channels, which are implicated in O_2 sensing, and are found in most cells (Williams et al., 2004; Wicher et al., 2001). Treatment with CO may interfere with the function of these channels. Additionally, blockage of the haemoglobin binding sites with CO may disrupt oxygenation, leading to longer surfacing durations.

Mathematical models

To test our experimental results, we developed a mathematical model to replicate gas fluxes to and from the backswimmer air store over the duration of a dive. This model incorporates a compressible gas gill model (Rahn and Paganelli, 1968) with a model developed to estimate the O_2–haemoglobin saturation curves of *A. deanei* from

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in vivo measurements of P_{O_2} in the air store (Matthews and Seymour, 2011). In Rahn and Paganelli's model, the insect begins its dive with a bubble of a given volume. Oxygen is consumed from the bubble, leading to a decline in bubble P_{O_2}. P_{N_2} then increases according to Dalton's law of partial pressures that states that the total pressure within a given volume is the sum of the partial pressures of the component gases. As CO_2 is readily lost to the water, it contributes little to total bubble pressure, which is assumed to be constant at a given depth (Ege, 1915; Rahn and Paganelli, 1968). The P_{O_2} within the gas gill then declines below that of the surrounding water, while P_{N_2} in the gas gill rises above that of the water. O_2 and N_2 then diffuse down their respective partial pressure gradients according to Fick's law of diffusion, where the rate of diffusion is related to the Krogh's coefficient for the respective gas (capacitance×diffusivity), the surface area for gas exchange, the boundary layer thickness, and the partial pressure difference. The effective boundary layer thickness is a measure of the fluid layer thickness next to a surface, like a bubble, where the dissolved gas content is less than the surrounding bulk fluid, providing resistance to gas diffusion across the bubble–water interface (Seymour and Matthews, 2013).

Our model was produced using modelling software (Stella, Version 6.0.1, High Performance Systems, Hanover, NH, USA) with the simulation duration set to 200 s at 1 s increments. This encompasses the mean experimentally determined dive duration +10% under all gas treatments. The results of the simulations include the decline in air store P_{O_2} across dive time, and the change in mean density of the backswimmer (due to the change in bubble volume), which can be used as an indication of buoyancy. Comparisons were made for the different gas treatments (N_2-control, He, SF_6 and CO), and changes in surface area and boundary layer thickness. Manipulations of the model involved changing the Krogh's coefficient of diffusion for N_2 with that of He and SF_6 to make comparisons between gas treatments. The CO treatment in the model involved setting the initial O_2 volume in the haemoglobin to zero. The base model from which manipulations were made had a boundary layer thickness of 0.04 mm and a surface area of half an ellipsoid, calculated using the values in Table S1. For the reduced conductance comparisons, boundary layer thickness was altered from 0.04 to 0.10 mm, then 0.60 mm, and surface area was reduced to 90%, 60% and 10% of that used in the control simulation. See Table S2 for full details of assumptions and equations used in the model.

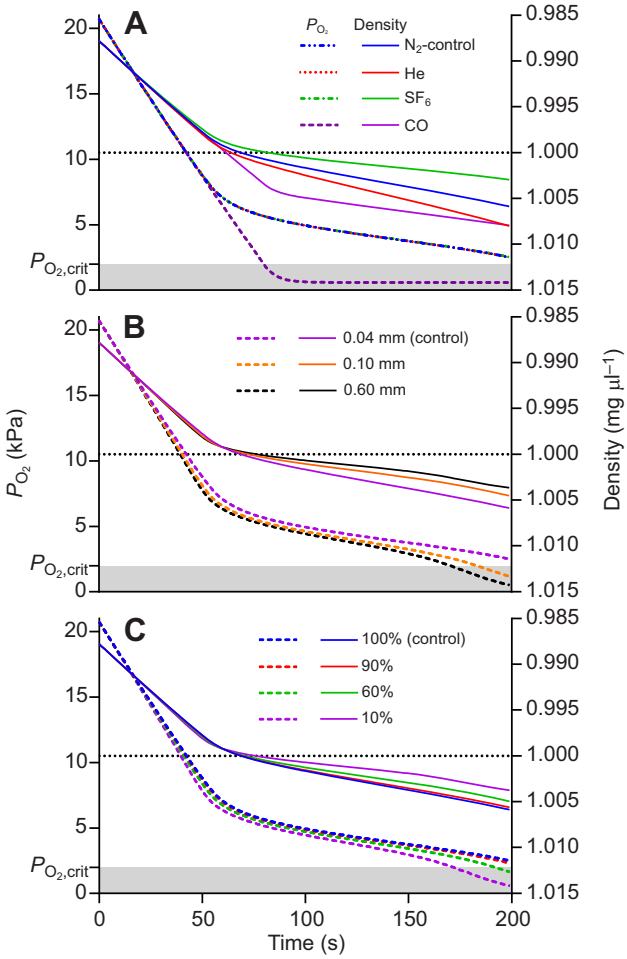

Fig. 5. Mathematical model of the decline in buoyancy and air store P_{O_2}. (A) Decline in air store P_{O_2} and density (buoyancy) of the N_2-control, He, SF_6 and CO treatments, as predicted by the model (see Discussion; Tables S1, S2). Solid lines represent density, dashed/dotted lines represent P_{O_2}, and the dotted horizontal line represents neutral buoyancy, where density equals 1 mg µl^{-1}. $P_{O_2,crit}$ is the estimated critical P_{O_2} (2 kPa) at which metabolic rate becomes limited by O_2 delivery. Note that the P_{O_2} lines for the N_2-control, He and SF_6 treatments lie on top of one another. (B) Decline in air store P_{O_2} and density of the base model when boundary layer thickness is increased from 0.04 mm, to 0.10 mm, then 0.60 mm. (C) Decline in air store P_{O_2} and density of the base model when the surface area for gas exchange is reduced from 100% to 90%, 60% and 10%.

Model outcomes

Both the experimental and modelling results show two phases of buoyancy, with the transition between phases occurring at approximately the same time; 60–70 s for experimental dives (Fig. 3A) and approximately 60 s in modelling simulations (Fig. 5A). Both the experimental and modelling results also agree that the rate of decline in the second phase is reduced compared with that in the first phase. These phases occur in the model simulations for the same reasons as that outlined for the experiments. However, the second phase observed in the CO simulation (Fig. 5A) does not occur experimentally, because the backswimmers surface before this takes place (Fig. 3A; Matthews and Seymour, 2006; Miller, 1964). In the CO simulations, the second buoyancy phase occurs because the air store P_{O_2} declines below the critical P_{O_2} ($P_{O_2,crit}$), which is the O_2 partial pressure at which metabolic rate becomes limited. This results in a balance between the rate of O_2 consumption by the insect and O_2 diffusion from the water, and so only N_2 loss to the water contributes to buoyancy decline. The occurrence of the transition between phases in the inert gas simulations, when the model backswimmers are slightly positively buoyant, results from the initial bubble volume being 17% above that required for neutral buoyancy. A reduced initial volume would cause the transition to occur while less buoyant, further indicating that experimental backswimmers collect initial bubble volumes that are slightly less than that required for the second phase of buoyancy to coincide with neutral buoyancy.

Two phases are also evident in the P_{O_2} decline of the inert gas simulations, although the rate of decline is identical between treatments (Fig. 5A). P_{O_2} decline is the same irrespective of carrier gas because O_2 conductance does not vary among the simulations. This provides further evidence that the He-treated backswimmers terminate their dives due to negative buoyancy rather than O_2 limitation. He-treated backswimmers terminate their dives before or during the beginning of the second phase (Fig. 3A), when, according to the simulations, there remains sufficient O_2 that could be used for the dive.

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The identical P_{O_2} declines in the inert gas simulations also provides an explanation for the non-linear relationship between the O_2 diffusion coefficient of the carrier gas and surfacing duration recorded in the experiments (Fig. 4). This result is surprising as one would expect surfacing duration to be directly and inversely proportional to the O_2 diffusion coefficient, if all dives end with similar P_{O_2}. However, He-treated backswimmers appear to terminate their dives at a higher air store P_{O_2} than N_2-control backswimmers, and higher again than SF_6-treated backswimmers. The difference in air store P_{O_2} at dive termination might then influence the time taken to replenish the air store with O_2 during surfacing, whereby a high air store P_{O_2} just prior to surfacing requires a short replenishment period (i.e. He), but a low air store P_{O_2} requires a long replenishment period (i.e. SF_6).

Limitation of air store conductance either by increasing boundary layer thickness or decreasing surface area for gas exchange reduces the difference in the rate of buoyancy decline between the N_2-control, He and SF_6 treatments (Fig. 6). Fig. 6 simulates the effect of an increase in boundary layer thickness to 0.60 mm or a reduction in surface area by 95% from the base model. Under these conditions, both the buoyancy and P_{O_2} declines are essentially the same between treatments. If exchange with the water was restricted to this extent, which is effectively negligible gas exchange with the water, no differences between the experimentally determined dive duration, buoyancy decline and leg stroke frequencies would be expected; however, this is not the case.

When the N_2-control simulation is terminated at the $P_{O_2,crit}$ (2 kPa; Table S2) or at the experimentally determined N_2-control dive duration (124 s), the O_2 contribution from the water is approximately 20% of that consumed during the dive. This, with a boundary layer of 0.04 mm, assumes high levels of convection associated with swimming activity. If the backswimmers were inactive in stagnant water, this level of O_2 uptake would not occur. Experimentally measured boundary layers are thicker than this, ranging from 0.1 to 0.8 mm; however, boundary layer thickness varies depending on the species and convective conditions (Seymour et al., 2015; Seymour and Matthews, 2013).

If conductance is reduced in the simulations, either through reduced surface area or increased boundary layer thickness, with N_2 as the carrier gas, three changes occur (Fig. 5B,C): (1) the rate of buoyancy decline in the second phase is reduced and it becomes more stable, (2) the duration of the second phase is shortened and (3) a third phase appears, where buoyancy decline becomes more rapid. These changes are most evident when surface area is reduced to ≤60% of the control simulation or boundary layer is increased to ≥0.1 mm. The changes occur because loss of the carrier gas is reduced, O_2 gain from the water is reduced, and the haemoglobin is exhausted more rapidly. The simulations give a good understanding of the benefits and detriments of gas exchange with the water for the backswimmers during their dives. By having some gas exchange with the water, the dive duration and the second phase of buoyancy can be extended as O_2 diffusion from the water subsidises the O_2 release from the haemoglobin. However, with some N_2 loss to the water, the rate of buoyancy decline is increased in comparison to a sealed bubble.

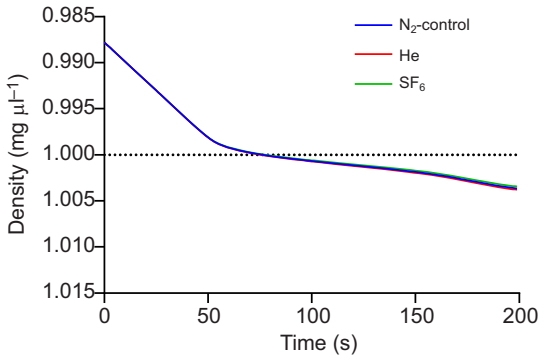

Fig. 6. Mathematical model of the decline in buoyancy with reduced air store conductance. Decline in backswimmer density (buoyancy) of the N_2-control, He and SF_6 treatments, as predicted by the model with air store conductance reduced equivalent to a boundary layer thickness of 0.60 mm, or a 95% reduction in surface area for gas exchange compared with the control simulation.

Conclusions

This study shows for the first time that the backswimmer air store has some capacity to behave as a compressible gas gill, although its effectiveness is much less than in many other aquatic insects. As indicated by the model simulations, O_2 uptake from the water is predicted to contribute approximately 20% of that consumed during a dive. By comparison, other insects with compressible gas gills can gain up to 88% of their O_2 from the water (Matthews and Seymour, 2010; Rahn and Paganelli, 1968). There may be an evolutionary trade-off between the ability to maintain near-neutral buoyancy, with some N_2 loss, and extending dive duration through O_2 uptake from the water. Initially, backswimmers utilising compressible gas gills may have acquired haemoglobin that extended dive duration by storing and releasing O_2. Selection would favour haemoglobin that has a high cooperativity, releasing most of the O_2 over a narrow P_{O_2} range, where the lower end of that range remains above the $P_{O_2,crit}$. This characteristic maintains the largest P_{O_2} gradient possible between the water and the bubble, increasing the rate of O_2 diffusion and facilitating more O_2 uptake from the water. These characteristics are pre-adaptations for maintaining a stable bubble volume, enabling backswimmers to maintain more consistent buoyancy over a longer period.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

K.K.J. is largely responsible for the whole project, working from a concept from R.S.S. E.P.S. assisted with experimental design and data analysis. A.P.W. developed the Matlab tracking script. All authors drafted and revised the manuscript.

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Supplementary information

Supplementary information available online at
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Table S1. Variables and constants used in the mathematical model.

Variable	Value	Units	Reference
P_b	101.3	kPa	1
ρ_{air}	1.20×10^{-3}	mg µl^{-1}	
P_{H_2O}	2.34	kPa	2
$P_{N_{2,aq}}$	78.12	kPa	2
$P_{O_{2,aq}}$	20.73	kPa	2
K_{O_2}	6.96×10^{-7}	mm^2 s^{-1} kPa^{-1}	3
K_{N_2}	3.18×10^{-7}	mm^2 s^{-1} kPa^{-1}	3
K_{He}	4.91×10^{-7}	mm^2 s^{-1} kPa^{-1}	3
K_{SF_6}	8.18×10^{-8}	mm^2 s^{-1} kPa^{-1}	3
A	1.87	mm^2	4
a	1.13	mm	4
b	0.42	mm	4
c	0.22	mm	4
M_b	10.18	mg	4
$V_{B,\ init}$	0.867	µl	5
V_{bs}	9.44	µl	5
$V_{O_{2,hb}}$	0.25	µl	5
P_{50}	3.90	kPa	6
$P_{O_{2,crit}}$	2.0	kPa	7
$\dot{V}_{O_{2,MR}}$	2.17×10^{-3}	µl s^{-1}	4
X	0.04, 0.10, 0.60	mm	3, 1

References: 1, Seymour and Matthews, 2013; 2, Dejours 1981; 3, Rahn and Paganelli, 1968; 4, present study; 5 Matthews and Seymour, 2008; 6, Matthews and Seymour, 2011; 7, Matthews and Seymour 2010. Symbols: P_b = barometric pressure, ρ_{air} = air density, P_g = pressure of relevant gas, K_g = Krogh's coefficient of diffusion of relevant gas, *A* = surface area for gas exchange, a,b,c = ellipsoid semi-axis values as described in results, M_b = backswimmer body mass, $V_{B,\ init}$ = initial bubble volume, V_{bs} = backswimmer volume, $V_{O_{2,hb}}$ = initial O_2 volume bound to haemoglobin, P_{50} = P_{O_2} at which haemoglobin is 50% saturated, $P_{O_{2,crit}}$ = critical P_{O_2}, $\dot{V}_{O_{2,MR}}$ = O_2 consumption rate, *X* = boundary layer thickness.

Table S2. Mathematical model used to simulate the decline in backswimmer buoyancy and air store P_{O_2} during dives. The model has the following assumptions: (1) surface area for gas exchange (*A*) is defined as the surface area of a half-ellipsoid with semi-axes a, b and c, defined in the results and supplementary Table S1, (2) conductance of each gas remains constant throughout each simulation at a level defined by Fick's law as the Krogh's coefficient (K_g) multiplied by the quotient of *A* and the boundary layer thickness (*X*) (Rahn and Paganelli, 1968; Matthews and Seymour, 2010; supplementary material Table S1), (3) the insect is considered to be just below the water's surface where hydrostatic pressure is negligible and is thus not included (Seymour and Matthews, 2013), (4) bubble surface tension is not included due to its minimal impact on total bubble pressure (Seymour and Matthews, 2013), (5) CO_2 production by the insect is excluded as CO_2 diffuses readily into the water surrounding the air store (Ege, 1915; Rahn and Paganelli, 1968), (6) barometric pressure and temperature are constant at 101.3 kPa and 20°C, respectively, (7) water is well-mixed and in equilibrium with atmospheric gas compositions: P_{O_2} of 20.73 kPa and P_{N_2} of 78.12 kPa (Dejours, 1981; Rahn and Paganelli, 1968), and (8) initial air store composition considers trace gases as part of the N_2 fraction (Dejours, 1981; Rahn and Paganelli, 1968). The equations relating to the O_2 release from the haemoglobin ($\dot{V}_{O_{2,hb}}$), and haemoglobin saturation curves, are outlined in Matthews and Seymour (2011), with values for the P_{50} and O_2 volume stored in haemoglobin ($V_{O_{2,hb}}$) shown in Table S1 of the supplementary material. Density of the insect with the bubble was calculated as the sum of bubble mass (= air density × bubble volume, $\rho_{air} \times V_b$) and body mass (M_b), divided by the sum of bubble volume and body volume (V_{bs}) (supplementary material Table S1).

	Equation	*Description*
(1)	$\dot{V}_g = K_g \frac{A}{X} (\Delta P_g)$	Fick's law describes diffusion of N_2 out of the air store and O_2 in, where $\dot{V}_g$ is the rate of change in volume of a particular gas species (µl s^{-1}), K_g is the Krogh's coefficient (mm^2 s^{-1} kPa^{-1}), which is the product of capacitance (β; mm^3 mm^{-3} kPa^{-1}) and diffusivity (D; mm^2 s^{-1}), *A* is surface area available for gas exchange (mm^2), *X* is thickness of the effective boundary layer (mm), and ΔP_g is the difference between the pressure of the relevant gas species inside and outside the bubble (kPa).
(2)	$V_b(t) = V_b(t - dt) + \left(\dot{V}_{O_{2,hb}} + \dot{V}_{O_{2,H_2O}} - \dot{V}_{O_2} - \dot{V}_{N_{2,H_2O}}\right) \times dt$	Differential Eq. 2 describes the change in bubble volume (V_b; µl) over time (*t*; s), where $V_b(t)$ is bubble volume at time t, $\dot{V}_{O_{2,hb}}$ (µl s^{-1}) is the rate of O_2 release from the haemoglobin (hb), $\dot{V}_{O_{2,H_2O}}$(µl s^{-1}) is the

		rate of O_2 diffusion from the surrounding water, $\dot{V}_{O_2}$ (µl s^{-1}) is the O_2 consumption rate by the backswimmer, and $\dot{V}_{N_{2,H_2O}}$ (µl s^{-1}) is the rate of N_2 loss to the surrounding water.
(3.1) (3.2)	$\dot{V}_{O_2} = P_{O_{2,in}} \times 0.0011$ $\dot{V}_{O_2} = \dot{V}_{O_{2,MR}}$	When air store PO_2 ($P_{O_{2,in}}$; kPa) drops below the critical PO_2 ($PO_{2,crit}$) of 2 kPa, based on measurements from water boatmen (Matthews and Seymour, 2010), the backswimmer's O_2 consumption rate ($\dot{V}_{O_2}$) is dependent on $P_{O_{2,in}}$ (Eq. 5), and is described by Eq. 3.1. The slope of decline in $\dot{V}_{O_{2,MR}}$ (= 0.0011) is the experimentally measured O_2 consumption rate (µl s^{-1}; supplementary material Table S1) from 0 to 2 kPa. However, when $P_{O_{2,in}}$ is greater than the assumed $PO_{2,crit}$, backswimmer $\dot{V}_{O_2}$ is independent of $P_{O_{2,in}}$, and Eq. 3.2 applies.
(4)	$P_{O_2} = \left(\frac{\dot{V}_{O_{2,flux}}}{V_b(t)}\right) \times (P_b - P_{H_2O})$	The incremental change in bubble PO_2 (kPa s^{-1}) is described by Eq. 4, where $\dot{V}_{O_{2,flux}}$ (µl s^{-1}) incorporates the various O_2 fluxes (i.e. $\dot{V}_{O_{2,H_2O}} + \dot{V}_{O_{2,hb}} - \dot{V}_{O_2}$), $V_b(t)$ is bubble volume (µl) at time *t*, P_b is barometric pressure (kPa), and P_{H_2O} is water vapour pressure (kPa).
(5)	$P_{O_{2,in}}(t) = P_{O_{2,in}}(t - dt) + (P_{O_2}) \times dt$	The PO_2 within the air store ($P_{O_{2,in}}$; kPa) is described by a second differential equation.
(6)	$P_{N_{2\,in}} = P_b - P_{O_{2\,in}} - P_{H_2O}$	PN_2 within the air store ($P_{N_{2,in}}$; kPa) relates to Dalton's law.
(7)	$A = \left(4\pi\left(\frac{(ab)^{1.6} + (ac)^{1.6} + (bc)^{1.6}}{3}\right)^{\frac{1}{1.6}}\right)/2$	Surface area for gas exchange (*A*, mm^2) is defined as the approximate equation for the surface area of half an ellipsoid with semi-axes a, b and c (mm; defined in supplementary material Table S1).

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