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RESEARCH ARTICLE

Ventilation and gas exchange before and after voluntary static surface breath-holds in clinically healthy bottlenose dolphins, *Tursiops truncatus*

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ABSTRACT

We measured respiratory flow ($\dot{V}$), breathing frequency (f_R), tidal volume (V_T), breath duration and end-expired O_2 content in bottlenose dolphins (*Tursiops truncatus*) before and after static surface breath-holds ranging from 34 to 292 s. There was considerable variation in the end-expired O_2, V_T and f_R following a breath-hold. The analysis suggests that the dolphins attempt to minimize recovery following a dive by altering V_T and f_R to rapidly replenish the O_2 stores. For the first breath following a surface breath-hold, the end-expired O_2 decreased with dive duration, while V_T and f_R increased. Throughout the recovery period, end-expired O_2 increased while the respiratory effort (V_T, f_R) decreased. We propose that the dolphins alter respiratory effort following a breath-hold according to the reduction in end-expired O_2 levels, allowing almost complete recovery after 1.2 min.

KEY WORDS: Spirometry, End-expired gas, Breathing frequency, Tidal volume, Marine mammal, Diving physiology

INTRODUCTION

Marine mammals forage underwater but need to return to the surface to replenish O_2 stores and to remove the CO_2 produced during dives. While holding their breath underwater, they use the O_2 in their blood and tissues to fuel aerobic metabolism, which produces CO_2. Consequently, the O_2 levels decrease and the CO_2 levels increase during a dive (Scholander, 1940). Marine mammals have developed several strategies to increase time underwater and, therefore, foraging efficiency. For example, the O_2 stores are increased by elevated haematocrit, blood volume, muscle mass and muscle myoglobin levels (Ponganis, 2015). The lungs in most species are not comparatively larger than those in terrestrial species; instead, deep divers seem to have relatively smaller lungs to minimize gas exchange during diving and thereby decompression sickness (Piscitelli et al., 2010; Scholander, 1940).

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Diving mammals also seem to increase foraging efficiency by minimizing time at the surface (Boutilier et al., 2001; Carbone and Houston, 1996; Fahlman et al., 2008; Halsey et al., 2003). The length of time required at the surface is constrained by the efficiency of recovery of blood and tissue gas levels. As the diver returns to the surface, cardiac output, which has been reduced during the dive, begins to increase, and the ascent tachycardia that increases perfusion is thought to help prepare the diver to more efficiently restore O_2 and remove CO_2 (Thompson and Fedak, 1993). It has also been proposed that this pre-surface tachycardia helps remove dissolved N_2, which helps to reduce the risk of gas emboli (Fahlman et al., 2007). Empirical data suggest that the animals incur an O_2 debt during a dive bout that is not repaid until the recovery period at the end of the dive bout. The dive behaviours observed in pinnipeds (Fahlman et al., 2008; Kooyman et al., 1973) are consistent with optimal foraging models, which indicate that the length of the surface interval is determined by the need to restore O_2 stores (Carbone and Houston, 1996; Halsey et al., 2003; Kramer, 1988). While insufficient uptake of O_2 would reduce the aerobic dive duration, insufficient removal of CO_2 could result in acid–base balance disturbances and lactate build-up. Thus, it has been proposed that that removal of CO_2 is the main driving force regulating surface interval duration (Boutilier et al., 2001; Fahlman et al., 2008; Reed et al., 1994, 2000). Recent reports that marine vertebrates may experience gas emboli and suffer decompression sickness (Fernández et al., 2005, 2017; García-Párraga et al., 2014) also suggest that at times accumulation of blood and tissue N_2 levels may limit diving and force animals to extend surface duration of the interval (Fahlman et al., 2014; Hooker et al., 2009). Thus, variation in the surface interval duration may have numerous causes, and changes in dive activity and behaviour may alter the duration at the surface. For example, if a species has to dive deeper to find food, this may result in accumulation of CO_2 and N_2, which may result in longer surface intervals to remove the additional gas taken up or produced. Consequently, understanding the variables that limit the surface interval and how gases are managed and recovered may help provide clues to improve our understanding of cardiorespiratory limitations.

The recovery time at the surface is reduced by increased cardiac output in bottlenose dolphins (*Tursiops truncatus*) (Miedler et al., 2015). Changes in ventilation following diving, in contrast, are not as obvious or not reported (Kooyman et al., 1971; Kooyman et al., 1973; Reed et al., 1994). Thus, the surface interval is a complex dynamic state that requires sufficient gas exchange to enable efficient foraging and the interaction between ventilation and perfusion will significantly alter gas exchange.

In the current study, we investigated the changes in respiratory effort [respiratory flow ($\dot{V}$), breathing frequency (f_R) and tidal

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List of abbreviations

f_R	breathing frequency
$i\dot{V}_{O_2}$	instantaneous rate of oxygen consumption
P_{CO_2}	carbon dioxide partial pressure (gas) or tension (fluid)
P_{O_2}	oxygen partial pressure (gas) or tension (fluid)
$s V_T$	mass-specific tidal volume
TLC_{est}	estimated total lung capacity
$\dot{V}$	respiratory flow
$\dot{V}_E$	expired minute ventilation
$\dot{V}_{exp}$	expiratory flow
$\dot{V}_{insp}$	inspiratory flow
$\dot{V}_{O_2}$	rate of oxygen consumption
V_A	alveolar volume
V_D	dead space volume
V_T	tidal volume
$V_{T,exp}$	expiratory tidal volume
$V_{T,insp}$	inspiratory tidal volume
ΔO_2	difference between inspired and expired O_2

volume (V_T)] and gas exchange (expired O_2) in the bottlenose dolphin before and after periods of surface breath-hold ranging from 34 to 292 s. Our results indicate a complex relationship between respiratory effort and end-expired O_2 content that helps minimize recovery duration following breath-holds. Thus, bottlenose dolphins appear to vary V_T, f_R and breath duration depending on breath-hold duration to enhance gas exchange and minimize recovery duration. From our results, we estimate that a 196 kg bottlenose dolphin can restore its O_2 stores in approximately 1.2 min, following a surface breath-hold of 157 s.

MATERIALS AND METHODS
Animals
Eleven adult bottlenose dolphins, *Tursiops truncatus* (Montagu 1821), of varying age and size (Table 1) were used for all experiments. The dolphins were housed in a facility in either Hawaii (Dolphin Quest – Oahu) or Las Vegas (Siegfried and Roy's Secret Garden). The ID, body mass (M_b, measured within 2 weeks of each experiment), straight length (rostrum to midline fluke notch) and age of the animals are summarized in Table 1. Prior to initiating the study, animals were desensitized to the equipment and trained for novel research-associated behaviours. Therefore, all experimental trials were performed with the dolphins using operant conditioning and participation by the dolphins was voluntary (the animals were not

restrained and could refuse to participate or withdraw at any point during the experimental trial). Each experiment (trial) began with one animal remaining stationary in the water, allowing placement of the appropriate equipment (see fig. 1 in Fahlman et al., 2015), breathing spontaneously while continuous measurements were made for between 210 and 400 s (including a 90 s period during which the dolphin was allowed to calm down, 'pre-breath-hold'). This approach allowed collection of experimental data on lung function in dolphins that were in a relaxed, physiologically normal state.

Next, the dolphins were trained to turn upside-down (ventrum towards the water surface) and keep their blowhole submerged while holding their breath without moving ('breath-hold'). Breath-holds of pre-determined duration were terminated by a signal from the trainer. Breath-hold durations were based on the history and comfort level of the individual dolphin. No animal was restrained, so they could terminate the breath-hold and/or withdraw from a trial at any time. At the end of the breath-hold, when the dolphin righted itself, the spirometry equipment was immediately placed over the blowhole, allowing measurement of $\dot{V}$ and expired O_2 content (see below) during recovery ('recovery'). For this study, the recovery period lasted 300 s from the first breath after the apnoeic period (please note that in four trials the recovery terminated early, with a minimum recovery period of 181 s, as the dolphin decided to end the session; see Fig. S1 for the layout of a trial).

Respiratory flow
The procedures and equipment were identical to those used in our previous studies (Fahlman et al., 2015, 2018a,c), and the procedure is briefly summarized here. $\dot{V}$ was measured using a custom-made Fleisch-type pneumotachometer (Micah Brodsky, V.M.D. Consulting, Miami, FL, USA; Mellow Design, Valencia, Spain), which housed a low-resistance laminar flow matrix (item no. Z9A887-2, Merriam Process Technologies, Cleveland, OH, USA). A low-resistance diffuser was added to homogenize the flow (Fahlman et al., 2018c), which helped resolve the difference in calibration factors for inspired ($\dot{V}_{insp}$) and expired ($\dot{V}_{exp}$) flow (Fahlman et al., 2015). A differential pressure transducer (Spirometer Pod, ML 311, ADInstruments, Colorado Springs, CO, USA) was connected to the pneumotachometer with two firm-walled, flexible tubes (310 cm length×2 mm i.d.). The differential pressure transducer was connected to a data acquisition system (Powerlab 8/35, ADInstruments), and the data were captured at 400 Hz and displayed on a laptop computer running LabChart (v.8.1,

Table 1. Morphometric data and resting metabolic rate and lung function parameters from bottlenose dolphins before a surface breath-hold

Animal ID	M_b (kg)	Age (m/y)	N	$\dot{V}_{O_2}$ (l min^{-1})	End-expired O_2 (%)	$\dot{V}$ (l s^{-1}) Expiratory	Inspiratory	f_R (breaths min^{-1})	$V_{T,exp}$ (l)
9FL3	242	18 (10/97)	9	1.070±0.265	11.1±1.7[167]	26.2±12.6	14.6±2.5	5.6±2.4	4.3±1.9
99L7	178	21 (7/94)	7	1.289±0.365	11.8±1.4[143]	20.5±7.3	14.0±1.9	5.7±2.4	4.7±1.5
63H4	184	25 (3/91)	5	0.732±0.248	12.2±1.6[91]	22.8±11.7	14.6±1.9	6.7±2.9	3.9±1.5
9ON6	180	15 (9/00)	8	0.893±0.363	10.0±1.3[156]	21.6±7.5	15.8±2.2	5.6±3.5	3.4±1.1
6JK5	196	21 (1/95)	10	0.932±0.176	12.2±1.9[138]	37.5±25.5	18.5±4.6	4.8±2.4	6.0±3.2
01L5	149	31 (1/85)	2	0.731±0.338	10.1±2.6[26]	21.8±7.9	10.1±2.6	5.0±3.6	4.3±1.7
M0001	226	(8/03)	5	0.619±0.351	13.2±1.8[54]	16.5±7.9	13.1±3.2	6.8±4.1	3.8±1.6
M0002	215	(8/78)	1	0.518	10.6±0.78[10]	17.0±3.4	9.8±1.9	2.7±1.2	3.8±1.4
F0003	192	(10/05)	2	0.952±0.144	10.8±3.5[24]	19.7±10.7	13.5±2.3	6.6±4.4	3.7±2.0
M0004	155	(8/11)	2	1.652±0.380	12.6±1.3[32]	29.8±11.7	18.3±3.2	6.5±4.4	6.9±3.7
M0003	238	(8/05)	2	0.788±0.034	10.6±2.4[38]	33.0±10.8	20.2±3.2	2.3±1.2	8.1±3.2
Grand mean	196±31		5±3	0.925±0.322	11.4±1.1	24.2±6.7	14.8±1.5	5.3±1.5	4.9±1.5

Animal ID, body mass (M_b), approximate age, number of post-prandial metabolic measurements (N), mean (±s.d.) pre-breath-hold rate of oxygen consumption ($\dot{V}_{O_2}$), end-expired O_2, respiratory flow ($\dot{V}$), breathing frequency (f_R) and expiratory tidal volume ($V_{T,exp}$) are shown. Superscript numbers in the end-expired O_2 column are the number of breaths analysed for end-expired O_2, $\dot{V}$, f_R and V_T.

ADInstruments). The differential pressure was used to determine $\dot{V}$ and was calibrated using a 7.0 l calibration syringe (Series 4900, Hans-Rudolph Inc., Shawnee, KS, USA). The signal was integrated and the $\dot{V}$ determined as detailed previously (Fahlman et al., 2015).

A normal breath was considered a respiration that began with an exhalation followed by an immediate inspiration. Some breaths were partial, i.e. only exhalation or inhalation, and these were excluded in the analysis specifically looking at the first breath following the breath-hold.

Respiratory gas composition
The concentration of expired O_2 was subsampled via a port in the pneumotachometer and passed through a firm-walled, flexible tube (310 cm length×2 mm i.d.) connected to 30 cm length of 1.5 mm i.d. Nafion tubing, fed into a fast-response gas analyser (ML206, Harvard Apparatus, Holliston, MA, USA) at a flow rate of 200 ml min^{-1}. The gas analyser was connected to the data acquisition system and sampled at 400 Hz. The gas analyser was calibrated before and after the experiment using a commercial mixture of 5% O_2, 5% CO_2 and 90% N_2 (product no. 17L-340, GASCO, Oldsmar, FL, USA). Ambient air was used to check the calibration before and after each experimental trial.

We used $\dot{V}$ and expired O_2 to estimate the rate of O_2 consumption ($\dot{V}_{O_2}$) as previously detailed (Fahlman et al., 2015). To investigate the dynamics of gas exchange, we calculated the instantaneous $\dot{V}_{O_2}$ (i$\dot{V}_{O_2}$) from the volume of O_2 taken up since the last breath.

Mean (±s.d.) air temperature and humidity during trials were 28.2±1.9°C (n=80, range: 22.6–33.0°C) and 62.0±7.0% (44–76%) in Hawaii, and 35.6±5.9°C (n=33, range: 21.3–39.0°C) and 17.1±9.5% (10–45%) in Las Vegas. The average water temperature in the lagoon in Hawaii was 26.0±0.6°C (range: 25.6–26.7°C) and in Las Vegas it was 25.0±0.9°C (range: 22.7–26.0°C).

Data and statistical analysis
We removed the first 90 s of data at the beginning of the trial before the breath-hold from the time the pneumotachometer was placed over the blowhole to allow the animal to settle down. Metabolic data are reported as the mean $\dot{V}_{O_2}$ before a breath-hold. The relationship between a dependent variable and experimental covariates was analysed using linear-mixed effects models (lme, R v.3.1.0, 2014, http://www.R-project.org/). The individual animal was treated as a random effect, which accounted for the correlation between repeated measurements on the same individual (Littell et al., 1998). Best models were chosen by the log-likelihood ratio test against nested models. In this study, $P \leq 0.05$ was considered as significant and $P \leq 0.1$ was considered a trend. Data are presented as the mean±s.d., unless stated otherwise.

As V_T, f_R, $\dot{V}$, end-expired O_2 and i$\dot{V}_{O_2}$ decayed exponentially throughout the recovery period, we log$_{10}$-transformed these variables to linearize the relationship with the number of breaths following the breath-hold. This allowed us to determine how M_b and dive duration affected the recovery following a breath-hold.

Lung health assessment
The six dolphins at the Dolphin Quest site underwent thorough clinical and diagnostic assessment to establish general and pulmonary health within 2 weeks of the experiments. This included radiographic (x-ray) and/or ultrasonographic imaging of the thorax with evaluation by one of two board-certified veterinary radiologists with experience in marine mammal diagnostic imaging. Additional testing included complete blood count, serum chemistry, sedimentation rate and sputum cytology. The health of the five dolphins at Siegfried and

Roy's Secret Garden – Las Vegas was evaluated from their medical records, which included routine radiographs every 6 months (except for animal M00001). These routine radiographs were evaluated by one of the veterinary radiologists.

RESULTS
Data from a total of 879 spontaneous breaths before and 1017 breaths following voluntary surface breath-holds were collected from 11 adult male bottlenose dolphins (Tables 1 and 2) housed at Dolphin Quest – Oahu in May 2016 or at Siegfried and Roy's Secret Garden – Las Vegas in May 2015. A total of 42 breath-holds were performed with a mean±s.d. breath-hold duration of 157±60 s (Table 2).

$\dot{V}$ and expired gas concentration before breath-hold
End-expired O_2 concentration (Table 1), expiratory and inspiratory duration (expiratory: 370±49 ms; inspiratory: 421±58 ms), $\dot{V}$ ($\dot{V}_{exp}$: 24.9±6.6 l s^{-1}; $\dot{V}_{insp}$: 15.5±2.6 l s^{-1}) and V_T (expiratory: 4.9±1.5 l; inspiratory: 5.0±1.5 l) did not change with breath number (χ^2<2.5, d.f.=1, P>0.1 for all). $\dot{V}_{exp}$ was significantly higher than $\dot{V}_{insp}$ (paired t-test, t-value=7.11, d.f.=10, P<0.01), but there was no difference in inspiratory ($V_{T,insp}$) or expiratory ($V_{T,exp}$) V_T (paired t-test, d.f.=10, t-value=1.20, P>0.1). The duration of the expiratory phase was significantly shorter than that of the inspiratory phase (paired t-test, d.f.=10, t-value=3.4, P<0.01). The average f_R and expired minute ventilation ($\dot{V}_E$) before breath-hold were 5.3±1.5 breaths min^{-1} (range: 2.3–6.8 breaths min^{-1}) and 23.0±8.8 l min^{-1} (range: 4.1–38.7 l min^{-1}), respectively. All but two short breath-holds (<60 s) began after a normal respiratory cycle of exhalation followed by inspiration.

The mean $\dot{V}_{O_2}$ before breath-hold was 0.925±0.322 l O_2 min^{-1}, and the average mass-specific $\dot{V}_{O_2}$ was 4.9±2.3 ml O_2 min^{-1} kg^{-1}.

$\dot{V}$ and expired gas concentration following breath-hold
Following a breath-hold, neither the inspiratory breath duration (χ^2=1.9, d.f.=1, P>0.1, 524±400 ms, n=12) nor total breath duration (χ^2=2.1, d.f.=1, P>0.1, 866±447 ms) changed with breath number ($N_{breaths}$) or time to recovery (P>0.1), but there was a slight decrease in expiratory duration with each breath (χ^2=8.1, d.f.=1, P<0.01, expiratory duration=348−0.6×$N_{breaths}$).

The end-expired O_2 concentration (Fig. 1A), instantaneous $\dot{V}_{O_2}$ (Fig. 1B), V_T (Fig. 2A), f_R (Fig. 2B), $\dot{V}_E$ (Fig. 2C) and $\dot{V}$ (Fig. 3) changed during the recovery period. Following a breath-hold, there was considerable variation in the end-expired O_2 concentration (Fig. 1A). The first post-breath-hold breath was

Table 2. Trial details for each dolphin

Animal ID	n	BHD (s)
9FL3	5	154±66
99L7	4	132±50
63H4	5	177±32
9ON6	6	230±39
6JK5	11	211±65
01L5	2	191±96
M0001	3	208±88
M0002	1	191
F0003	1	34
M0004	1	111
M0003	2	87±48
Mean	4±3	157±60

Number of breath-hold trials for each dolphin. Animal ID, number of breath-holds (n) and mean (±s.d.) breath-hold duration (BHD) are shown.
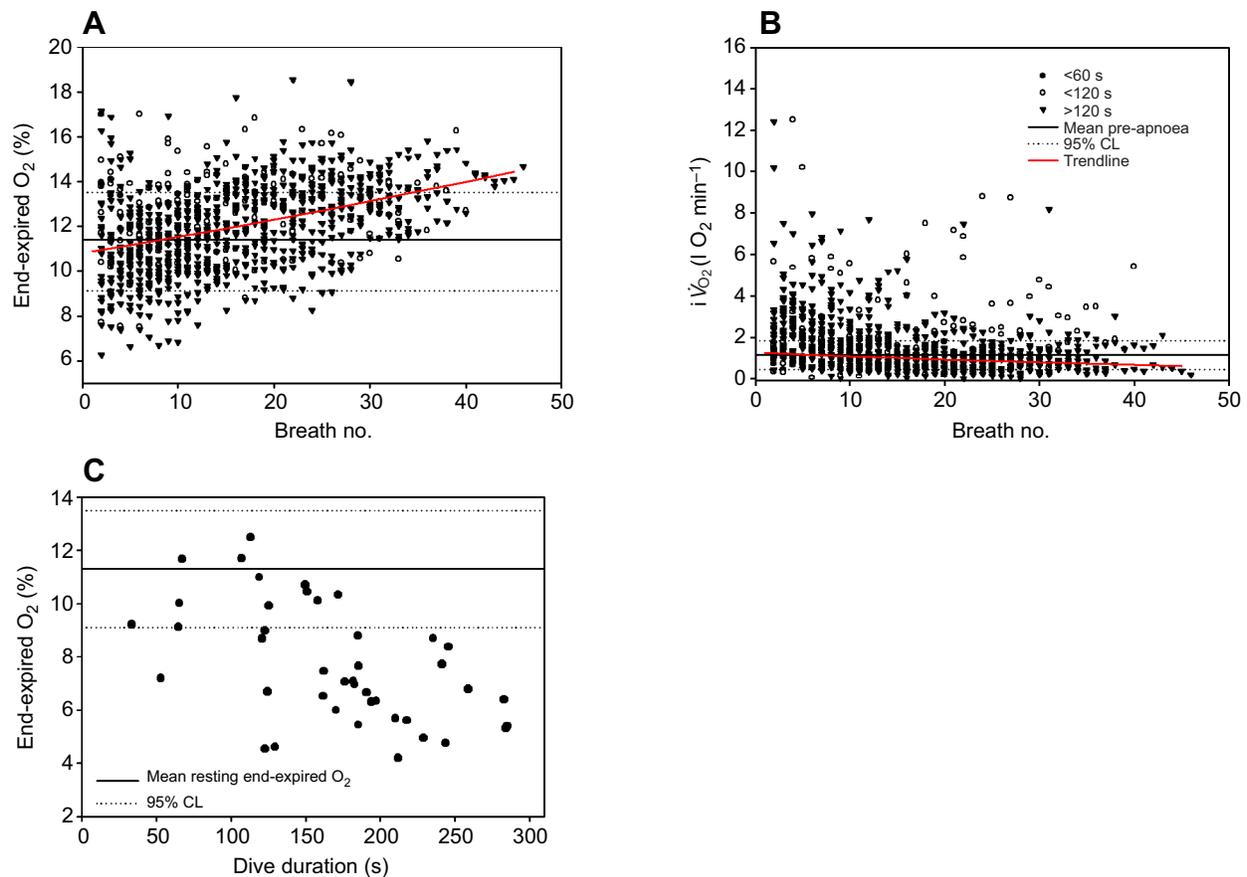
Fig. 1. Expired O$_2$ concentration and rate of O$_2$ consumption following a static surface breath-hold. (A) End-expired O$_2$ and (B) instantaneous rate of oxygen consumption (i$\dot{V}_{O_2}$) versus breath number following a surface breath-hold for all dolphins and breaths used in the study. Data are separated into three different breath-hold durations: less than 60 s (<60 s), more than 60 s but less than 120 s (<120 s), or more than 120 s (>120 s). Solid and dotted black lines are mean and 95% confidence limits (CL), respectively, before the breath-hold (apnoea). Solid red line is a trend line from the model in Table 3. The trend line is for a 196 kg dolphin, following a 157 s breath-hold, and with a mean expired minute ventilation ($\dot{V}_E$) of 23 l min^{-1}. (C) End-expired O$_2$ for the first breath following a breath-hold against dive duration.

successfully sampled from a total of 31 breath-holds, and the end-expired O$_2$ decreased significantly with the duration of the breath-hold (one-way ANOVA, F=4.8, P<0.05) from an average of 15.5± 4.0% for a breath-hold less than 1 min to 10.4±3.1% for a breath-hold over 3 min (Fig. 1C). The best equation that explained the variation in the end-expired O$_2$ data included duration of the breath-hold, breath number following the breath-hold and increasing $\dot{V}_E$ (Table 3). i$\dot{V}_{O_2}$ decreased with breath number and the duration of the breath-hold, and increased with increasing $\dot{V}_E$ (Table 3).

There was large variation in both the expired and inspired V_T following breath-hold, but there were no differences in the mean± s.d. $V_{T,insp}$ (5.0±1.5 l) or $V_{T,exp}$ (4.9±1.5 l) (paired t-test, t-value: 1.3, d.f.=10, P>0.1). We therefore only analysed and report the $V_{T,exp}$. $V_{T,exp}$ increased with the duration of the breath-hold and with M_b, but decreased with breath number following the breath-hold (Table 3). The $V_{T,exp}$ following the breath-hold, expressed as a percentage of the estimated total lung capacity (TLC$_{est}$) from an equation based on excised lungs from a range of species (Fahlman et al., 2011; Kooyman, 1973) or from an estimate from a diving dolphin (Ridgway et al., 1969), was 30% (range: 4–106%) and 54% (range: 7–184%), respectively. f_R increased with the duration of the breath-hold and decreased with breath number (Table 3). The average inspiratory flow ($\dot{V}_{insp}$: 15.1±2.5 l s^{-1}) was significantly lower than the average expiratory flow ($\dot{V}_{exp}$: 27.7±4.8 l s^{-1}, paired t-test, t-value: 7.1, d.f.=10, P<0.01). The average $\dot{V}_{exp}$

(31.7±6.8 l s^{-1}, range 3.3–64.5 l s^{-1}, n=11, t-value: 2.61, P<0.05) and $\dot{V}_{insp}$ (20.2±2.8 l s^{-1}, range 0.8–46.6 l s^{-1}, t-value: 4.14, P<0.01) for the first breath following the breath-hold was significantly greater than the average $\dot{V}$ before the breath-hold (Table 1). Both $\dot{V}_{insp}$ and $\dot{V}_{exp}$ decreased with breath number and scaled allometrically with M_b (Table 3). The inspiratory duration did not change with breath number following a breath-hold (χ^2=2.02, P>0.1), but both expiratory and total breath duration increased with the duration of the breath-hold and decreased with breath number (Table 3).

Lung health assessment

Thoracic radiographs showed no evidence of clinically active pulmonary or other intrathoracic disease in any dolphin. In two cases (9FL3 and 99L7), a very mild interstitial pattern was identified on radiographs. Another animal (63H4) had two small pulmonary nodules, suggestive of incidental granulomas in this species. The findings were not considered to be clinically relevant and may indicate incidental age-related changes or very mild fibrosis from a previous pulmonary insult.

Three animals had foci of surface lung pathology characterized ultrasonographically as alveolar-interstitial syndrome (63H4, 99L7 and 9FL3). This finding is abnormal but non-specific, and can be seen with lung fibrosis (i.e. from previous scarring), early pneumonia, atelectasis or pulmonary oedema (Lichtenstein et al.,
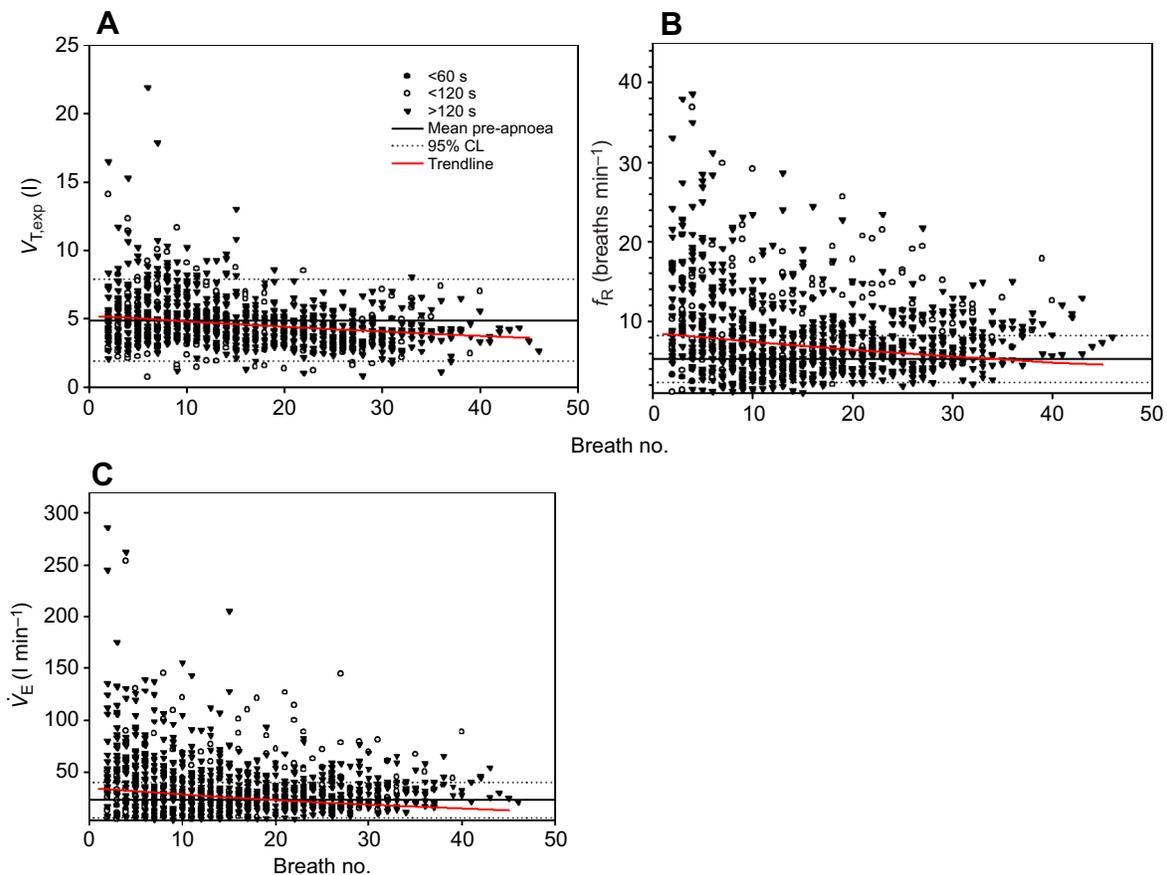
Fig. 2. Tidal volume, breathing frequency and minute ventilation following a static surface breath-hold. (A) Expired tidal volume ($V_{T,exp}$), (B) breathing frequency (f_R) and (C) $\dot{V}_E$ versus breath number following a surface breath-hold for all dolphins and breaths used in the study. Recovery data are separated into breath-hold duration: less than 60 s (<60 s), more than 60 s but less than 120 s (<120 s), or more than 120 s (>120 s). Solid and dotted black lines are mean and 95% CL, respectively, before breath-hold. Solid red line is a trend line from the model in Table 3. The trend line is for a 196 kg dolphin, following a 157 s breath-hold.

1997). One other dolphin (01L5) had evidence of marginal lymph node enlargement, suspected to be due to a reactive process. The marginal lymph nodes are the primary lymphatic drainage site for the lung parenchyma in dolphins, and can readily be assessed with ultrasound (Cowan and Smith, 1999; Martony et al., 2017).

At the time of this study, there was no significant respiratory disease present that would require medical therapy for any of the dolphins in this study. This assessment statement was based on the judgement of the attending veterinarians at each facility, physical examination, cytological evaluation and thorough assessment of standard diagnostic laboratory parameters, and radiographic and ultrasonographic evaluation.

DISCUSSION

In the current study, we showed that bottlenose dolphins increase $\dot{V}$, f_R and V_T following a static surface breath-hold. The $\dot{V}$ during recovery increased more with increasing breath-hold duration, which helped to reduce the number of breaths before the end-expired O_2 had returned to levels equivalent to those before the breath-hold. Our results show that a 196 kg dolphin requires approximately 10 breaths to recover from a 159 s breath-hold, or approximately 1.2 min of recovery. Our results provide information about pulmonary function and gas exchange before and after a breath-hold in clinically healthy dolphins, which can be used as a comparison for wild populations.

One advantage of studying physiological function in animals under managed care is that the health of the study participants can be

evaluated and monitored on an individual animal basis. In the current study, we confirmed that all animals were considered clinically healthy based on physical examination by experienced marine mammal veterinarians at each facility, and diagnostic imaging interpreted by one of the veterinary radiologists. In addition, studying trained animals under stimulus control that are participating voluntarily allows researchers to obtain baseline values with minimal stress. While some researchers rightly consider results obtained from animals under human care not to be perfectly representative of their free-ranging counterparts, certain information is virtually impossible to obtain from wild populations where controlled studies are extremely challenging to carry out. For that reason, we previously compared lung function values and metabolic rates in bottlenose dolphins under managed care, with both near-shore and pelagic populations, and have shown that results obtained with trained cetaceans do provide useful physiological information (Fahlman et al., 2015, 2018a,c). Thus, the data from the current study provide baseline lung function estimates from healthy dolphins under a situation with minimal stress.

One disadvantage of studying trained animals is that the training itself has the potential to cause a bias, which can be difficult to assess. For example, the large variation in V_T following a breath-hold may be the result of bias stemming from a dolphin anticipating additional requests for specific behaviours from the trainer following the breath-hold. At the beginning of the trial, the initial 90 s of the pre-breath-hold period were removed. Following this, there were no systematic
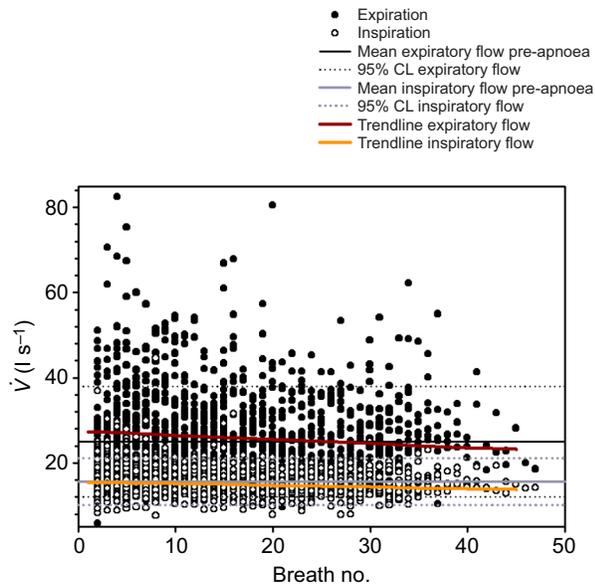
Fig. 3. Expired or inspired respiratory flow ($\dot{V}$) versus breath number following a breath-hold for all dolphins and breaths used in the study. Solid and dotted black (expiratory) and grey (inspiratory) lines are mean and 95% CL, respectively, before breath-hold. Solid red and orange lines are trend lines for expiratory and inspiratory flow from the model in Table 3. The trend line is for a 196 kg dolphin, following a 157 s breath-hold.

changes in end-expired gas concentration or respiratory variables. If the dolphins had been stressed or uncomfortable with the procedure, we would have expected to see systematic changes with time. In addition, the f_R was within the range previously reported for bottlenose dolphins (Fahlman et al., 2015, 2018a,c), suggesting that the data reported prior to a breath-hold (Table 1) were normal.

During a surface breath-hold, gas is exchanged between the lungs and the blood; O_2 is taken up and CO_2 diffuses from the blood into the lungs. Thus, the pulmonary O_2 should be lower following the surface breath-hold and then increase as the blood and tissue O_2 levels are restored. Having the dolphins perform the breath-hold at the surface allowed us to determine how the pulmonary O_2 changes with minimal impact on ambient pressure and the shunt that develops during diving to depth (Kooyman and Sinnett, 1982; Ridgway et al., 1969). Thus, our experimental design allowed us to specifically look at changes in pulmonary gas content due to the breath-hold, and how the dolphins manage respiration during recovery.

The end-expired O_2 of the first breath following a breath-hold decreased with dive duration from an average of 9.4% for a

breath-hold of up to 70 s to 6.4% when the breath-hold exceeded 180 s (Fig. 1C). The lowest end-expired O_2 was 4.2% following a 212 s breath-hold. Following the first breath, the end-expired O_2 increased throughout the recovery period, and increased with increasing $\dot{V}_E$ (Fig. 1A, Table 3). There was a significant increase in end-expired O_2 and a decrease in $i\dot{V}_{O_2}$, V_T, f_R and $\dot{V}_E$ with increasing recovery time after the breath-hold (Figs 1–3). The end-expired O_2 increased with the number of breaths following the breath-hold and increased with increasing $\dot{V}_E$ (Table 3, Figs 1 and 3). Thus, the recovery from a breath-hold was enhanced by increased respiratory effort. The V_T and end-expired O_2 following a dive were similar to those reported in the bottlenose dolphin [mass-specific V_T (sV_T)=36–43 ml kg^{-1}, end-expired O_2=5.4–13.2%; Ridgway et al., 1969], the harbour porpoise (*Phocoena phocoena*, sV_T=35–41 ml kg^{-1}, end-expired O_2≈9.5–13.5%; Reed et al., 2000) and the grey seal (*Halichoerus grypus*, sV_T=30–39 ml kg^{-1}, end-expired O_2≈8.6–10.1%; Reed et al., 1994). As dive duration increased, more O_2 was consumed, which depleted the O_2 stores. Following the breath-hold, the dolphins increased f_R and V_T to help replenish the O_2 stores.

There was considerable variation in the end-expired O_2 and $\dot{V}_E$ following a breath-hold (Figs 1 and 2), and when all post-breath-hold data were analysed, the end-expired O_2 increased with dive duration. While this may seem counterintuitive, the longer breath-holds also caused greater V_T, f_R and $\dot{V}_E$, which may shorten the recovery and counteract the greater decrease in pulmonary O_2 during longer dives (Fig. 1C, Table 3). Thus, the animals compensated for the reduced O_2 levels by increasing respiratory effort to reduce recovery time. The end-expired O_2 returned to normal values within approximately 10 breaths (Fig. 1A), which is similar to the data reported by Ridgway et al. (1969). Similar responses were observed in the freely diving Weddell seal (*Leptonychotes weddellii*) where both $i\dot{V}_{O_2}$ (Kooyman et al., 1973) and $\dot{V}_E$ (Kooyman et al., 1971) changed throughout the recovery period. In the grey seal, in contrast, changes in V_T were not observed following breath-holds (Reed et al., 1994). It is possible that the changes in end-expired O_2 with breath number are affected by the changes in V_T.

During a breath with a V_T that is greater than the dead space volume (V_D), the measured O_2 content initially decreases to a plateau. This plateau reflects alveolar gas content, which, in a healthy lung, indicates arterial gas tension (West, 2012). If $V_T<V_D$, the measured O_2 content reflects a mixture of gas in the dead space. Consequently, as V_T changed with the number of breaths during recovery, it is possible that the temporal changes seen in end-expired O_2 are an artefact of these changes. To evaluate this, we estimated the V_D for each dolphin by assuming it was similar to the average minimum air volume (MAV=7% of TLC$_{est}$) (Fahlman et al., 2011;

Table 3. Results from generalized least square analysis of the data from bottlenose dolphins

Dependent variable	Intercept	Duration of surface breath-hold	Breath number	log(M_b)	log($\dot{V}_E$)	χ^2
log(O_2)	0.959±0.019	0.000225±0.000038	0.00280±0.00021	–	0.0292±0.0066	52.1
log($i\dot{V}_{O_2}$)	−0.903±0.057	−0.000496±0.000153	−0.00693±0.00085	–	0.798±0.027	10.4
log($V_{T,exp}$)	−0.955±0.563	0.000191±0.000098	−0.00364±0.00052	0.711±0.245	–	3.9
log(f_R)	0.878±0.074	0.000337±0.00016	−0.00618±0.00083	–	–	16.8
log($\dot{V}_E$)	54.2±5.0	0.00037±0.000185	−0.00936±0.00090	−23.0±2.2	–	4.1
log($\dot{V}_{insp}$)	−0.040±0.458	–	−0.0012±0.00025	0.537±0.200	–	4.8
log($\dot{V}_{exp}$)	0.0268±0.253	–	−0.00167±0.00045	0.616±0.110	–	13.6
Inspiratory duration	0.416±0.024	–	–	–	–	–
Expiratory duration	0.334±0.017	0.0000946±0.0000482	−0.000696±0.000237	–	–	3.9
Total breath duration	0.735±0.040	0.000273±0.000136	−0.00160±0.000674			4.0

Generalized least square regression results for log$_{10}$-transformed end-expired O_2 [log(O_2)], instantaneous $\dot{V}_{O_2}$ [log($i\dot{V}_{O_2}$)], expired tidal volume [log($V_{T,exp}$)], expired minute volume [log($\dot{V}_E$)], and inspired ($\dot{V}_{insp}$) and expired ($\dot{V}_{exp}$) respiratory flow. Shown are the parameters (±s.e.) for the best model and the χ^2 value against nested models.

Kooyman and Sinnett, 1979) measured from water displacement of excised lungs from a range of marine mammals, including dolphins. Using these results, we calculated $V_A=V_T-V_D$, where V_A is the volume of V_T from the alveolar space. Between 0.5% and 2% (depending on which method was used to obtain TLC_{est}) of all breaths during recovery from a breath-hold were negative, representing breaths of lower volume than the V_D. However, while V_T changed throughout the recovery period, the proportion of breaths below the V_D remained constant with either method. Consequently, the changes in end-expired O_2 were not dependent on an increasing proportion of breaths not representing alveolar gas content, and we suggest that the end-expired O_2 can be used as a reflection of changes in arterial O_2 content. Looking at Fig. 1A, we can estimate that a 196 kg dolphin has recovered its end-expired O_2 content after 10 breaths following a 157 s breath-hold, i.e. when the red trend line crosses the average end-expired O_2 content before the breath-hold. The duration of this recovery will depend on the f_R. From Fig. 2B, we can see that f_R is about 8 breaths min^{-1} during the first 10 breaths, so this dolphin would have recovered its arterial O_2 content in about 1.2 min.

Reed et al. (1994) showed end-expired O_2 and CO_2 recovery curves that changed with time in an opposite pattern in seals. For O_2, the levels were initially high, then decreased and finally increased again at the end. It was suggested that this could indicate reduced gas exchange at the lung level and/or reduced tissue perfusion (Reed et al., 1994). Either situation would reduce pulmonary gas uptake, and the partial pressures in the lung would remain high for O_2 and low for CO_2. As blood flow and gas exchange resume during the post-breath-hold surface interval, the venous oxygen (P_{O_2}) and CO_2 tension (P_{CO_2}) would be lower and higher, respectively, causing a drop in pulmonary O_2 and an increase in CO_2. As O_2 is restored and CO_2 is removed, the partial pressures again approach normal values. Interestingly, this U-shaped pattern was not reported in the harbour porpoise (Reed et al., 2000), in the bottlenose dolphins in the current study or in the work by Ridgway et al. (1969). A new hypothesis suggesting how cetaceans control pulmonary gas exchange through management of ventilation–perfusion matching was recently published (García-Párraga et al., 2018). Ventilation–perfusion matching would enable active pulmonary shunting, which would allow cetaceans to selectively access pulmonary gases, facilitating exchange of O_2 and CO_2 with minimal exchange of N_2. Thus, the variation in results may reflect differences in the mechanisms by which marine mammals manage gases during diving.

Past measurements have shown that cetaceans are capable of exchanging as much as 90% of their TLC_{est} (Fahlman et al., 2015, 2017a, 2019; Kooyman and Cornell, 1981; Olsen et al., 1969; Ridgway et al., 1969). From these estimates, numerous studies have assumed that the V_T is close to TLC_{est}, and during exercise or during recovery from a dive it may be logical to assume that V_T is close to TLC_{est} (Dolphin, 1987a; Fahlman et al., 2016; Folkow and Blix, 1992). However, our results suggest that the average V_T during recovery from a breath-hold was only between 30% and 54% of TLC_{est}, depending on which value is used to estimate TLC_{est}. The latter value was based on measured V_T (5–6 l) following diving in a 138 kg bottlenose dolphin. By assuming that the measured V_T represented a diving lung volume of 80% of total lung capacity, TLC_{est} was 6.9 l (Ridgway et al., 1969). Using this TLC_{est}, the V_T during recovery ranged from 7% to 184%. The other equation for TLC_{est} was based on work on excised lungs from a range of marine mammal species ($TLC_{est}=0.135M_b^{0.92}$) (Fahlman et al., 2011; Kooyman, 1973), resulting in a range of V_T from 4% to 106% of TLC_{est}. With either method, it appears that during recovery from a

surface breath-hold, V_T is mostly considerably lower than TLC_{est}. These results are similar to those of our previous study measuring V_T during recovery from a bout of exercise in bottlenose dolphins (Fahlman et al., 2016). Our results indicate that the $V_{T,exp}$ during recovery increased with the length of the surface breath-hold (Table 3, Fig. 2A), but most breaths were still considerably lower than TLC_{est}. Based on these results, it appears that bottlenose dolphins have considerable scope to further increase respiratory effort following a breath-hold. While it is difficult to extrapolate this to freely swimming and diving animals, bottlenose dolphins may be capable of much longer (Fahlman et al., 2018b) or more active breath-holds with similar recovery rates. However, this warrants further study if respiratory variables are to be used to assess field metabolic rate.

Past studies have attempted to estimate field metabolic rate from f_R in a number of species by assuming static respiratory values (Armstrong and Siegfried, 1991; Blix and Folkow, 1995; Christiansen et al., 2014; Dolphin, 1987a; Dolphin, 1987b; Folkow and Blix, 1992; Williams and Noren, 2009), or by measuring the variation in V_T and O_2 exchange fraction (ΔO_2=inspired O_2−expired O_2) following exercise (Fahlman et al., 2016). While this method is attractive in its simplicity, considerable variation in V_T and ΔO_2 yields extensive error (Fahlman et al., 2016, 2017b; Folkow and Blix, 2017). The results from the current study show a large variation in V_T, f_R and end-expired gas content following breath-holds ranging from 34 to 292 s. Maximal and average dive durations in shallow-diving near-shore dolphins are within this range (Fahlman et al., 2018b; Wells et al., 2013), and this ecotype may therefore show similar variation following diving. In addition, the change in respiratory effort following longer breath-holds to reduce the duration of recovery resulted in a complicated relationship between V_T, end-expired gas content and recovery duration. Our results are from static breath-holds and it is possible that the variability in f_R and V_T in free-ranging dolphins diving to depth and breathing while swimming is reduced. New methods are being developed that may help estimation of f_R and V_T in free-ranging marine mammals (Cauture et al., 2019; Sumich, 2001; van der Hoop et al., 2014). These may contribute to our understanding of respiratory effort and expired gas content, allowing us to continue to develop more accurate and useful estimates of field metabolic rate from methods based solely on f_R.

Both $\dot{V}_{insp}$ and $\dot{V}_{exp}$ increased immediately following a breath-hold, and decreased throughout the recovery period, but were not affected by the duration of the breath-hold (Fig. 3, Table 3). $\dot{V}$ of the first breath following the breath-hold was significantly higher than the average $\dot{V}$ before the breath-hold (Fig. 3). The expiratory breath duration increased positively with breath-hold duration and decreased during recovery. Thus, the increased V_T following a surface breath-hold was achieved by increased breath duration and $\dot{V}_{exp}$, but only by increasing $\dot{V}_{insp}$. During voluntary breaths, exhalation is passive and mainly driven by the elastic recoil of the chest, whereas the inspiratory flow is active for both voluntary and forced breaths (see fig. 5A in Fahlman et al., 2017a). As the respiratory effort increases, a greater proportion of the expiratory phase becomes active where the respiratory muscles assist in generating high $\dot{V}$ (see fig. 5B,C in Fahlman et al., 2017a). The relative importance of the diaphragm, cranio-cervical, thoracic and lumbo-pelvic muscles to drive inspiration during rest in dolphins is not known (Cotten et al., 2008; Dearolf, 2003). Understanding the mechanism of respiration both at rest and while actively swimming could provide important information for conservation efforts, and this could be an interesting topic for future studies.

There are conflicting reports in the literature about whether marine mammals dive on inhalation or exhalation, but some studies

clearly show that cetaceans dive on full or partial inhalation (Ridgway, 1986; Ridgway and Howard, 1979; Scholander, 1940). Our results concur with this, and most breath-holds began following a normal breath, which in many cases had a higher than normal $V_{T,insp}$. Following a breath-hold, the initial V_T was highly variable and occasionally the dolphins began with a small exhalation and inhalation. In these cases, a second breath of larger volume followed shortly after, but these were exceptions to the pattern. During the study trials, when a trainer signalled the beginning of a breath-hold, most dolphins took a deep breath before initiating the breath-hold. While the deep breath before the breath-hold may be a learned behaviour to extend the breath-hold, this implies a certain level of behavioural control and it is likely that free-ranging animals perform a similar inspiration before the breath-hold. Assessment of respiratory effort in free-ranging animals could be made possible by phonospirometry (Sumich, 2001), or by assessing respiratory sinus arrhythmia and relating these changes to $V_{T,insp}$ (Cauture et al., 2019; Fahlman et al., 2017c).

Conclusions

In the current study, we measured respiratory flow and end-expired O_2 content in bottlenose dolphins before and after surface breath-holds. Our data show a complex relationship between respiratory effort (V_T, f_R, $\dot{V}_E$) and end-expired gas content, suggesting that the dolphins maximized effort following long dives in order to minimize recovery duration. While V_T increased following a breath-hold, most breaths were considerably lower than TLC_{est}. Thus, dolphins may have considerable scope to increase respiratory effort following longer or more strenuous breath-holds to rapidly replenish O_2 stores.

Data availability
The datasets used in this study are freely available at the following link: https://osf.io/wej32. For any questions about the data format, please contact the corresponding author.

Supplementary information
Supplementary information available online at
http://jeb.biologists.org/lookup/doi/10.1242/jeb.192211.supplemental

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