

## RESEARCH ARTICLE

# Respiratory sinus arrhythmia and submersion bradycardia in bottlenose dolphins (*Tursiops truncatus*)

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## ABSTRACT

Among the many factors that influence the cardiovascular adjustments of marine mammals is the act of respiration at the surface, which facilitates rapid gas exchange and tissue re-perfusion between dives. We measured heart rate ( $f_H$ ) in six adult male bottlenose dolphins (*Tursiops truncatus*) spontaneously breathing at the surface to quantify the relationship between respiration and  $f_H$ , and compared this with  $f_H$  during submerged breath-holds. We found that dolphins exhibit a pronounced respiratory sinus arrhythmia (RSA) during surface breathing, resulting in a rapid increase in  $f_H$  after a breath followed by a gradual decrease over the following 15–20 s to a steady  $f_H$  that is maintained until the following breath. RSA resulted in a maximum instantaneous  $f_H$  ( $if_H$ ) of  $87.4 \pm 13.6$  beats  $\text{min}^{-1}$  and a minimum  $if_H$  of  $56.8 \pm 14.8$  beats  $\text{min}^{-1}$ , and the degree of RSA was positively correlated with the inter-breath interval (IBI). The minimum  $if_H$  during 2 min submerged breath-holds where dolphins exhibited submersion bradycardia ( $36.4 \pm 9.0$  beats  $\text{min}^{-1}$ ) was lower than the minimum  $if_H$  observed during an average IBI; however, during IBIs longer than 30 s, the minimum  $if_H$  ( $38.7 \pm 10.6$  beats  $\text{min}^{-1}$ ) was not significantly different from that during 2 min breath-holds. These results demonstrate that the  $f_H$  patterns observed during submerged breath-holds are similar to those resulting from RSA during an extended IBI. Here, we highlight the importance of RSA in influencing  $f_H$  variability and emphasize the need to understand its relationship to submersion bradycardia.

**KEY WORDS:** Cardiorespiratory coupling, Heart rate, Marine mammals

## INTRODUCTION

Bradycardia, or the reduction in heart rate ( $f_H$ ) compared with resting  $f_H$ , is considered a central component of the dive response which enables marine mammals to perform extended breath-holds underwater. By reducing cardiac output, bradycardia, in concert with peripheral vasoconstriction, limits the overall rate of  $\text{O}_2$  depletion by the peripheral tissues and helps to conserve  $\text{O}_2$  in the blood for hypoxia-intolerant tissues like the brain and heart (Scholander, 1940, 1963; Irving, 1939). In contrast, increased  $f_H$  and blood flow facilitate rapid gas exchange and reduce the time needed for recovery at the surface (Fedak et al., 1988; Le Boeuf et al., 2000). These differing

cardiovascular and respiratory requirements underpin the need for fine-scale modulation of  $f_H$  in marine mammals to enable the management of differing physiological demands while diving and at the surface. As technological advancements increase the ease of  $f_H$  measurements in freely moving animals, an increasing number of studies have sought to examine  $f_H$  modulation in marine mammals across a range of behavioral and environmental conditions (Block, 2005; Ponganis, 2007; Wilson et al., 2015). It is becoming clear that the suite of physiological adaptations that support an increased capacity for diving in marine mammals (i.e. dive response) is complex and regulated by many factors both during submersion and at the surface during dive preparation and recovery (Bickett et al., 2019; Elmegaard et al., 2016; Fahlman et al., 2020b, 2019; Kaczmarek et al., 2018; Noren et al., 2012).

Given the importance of  $f_H$  in regulating hemodynamic changes during diving, many researchers have investigated factors that influence  $f_H$  in marine mammals including water temperature, area of facial submersion, dive depth, activity, voluntary control, age and nutritional state. In harbor seals (*Phoca vitulina*) and California sea lions (*Zalophus californianus*), bradycardia has been shown to occur without submersion in water, but colder water and an increased area of facial submersion can increase the degree of  $f_H$  reduction (Kaczmarek et al., 2018). Change in the relative lung volume of diving California sea lions was found to follow a similar shape to that of change in  $f_H$ , particularly during descent and late ascent, suggesting a potential role of pulmonary stretch receptors in determining diving  $f_H$  (McDonald et al., 2020; Ponganis et al., 2017). In bottlenose dolphins, lung compression and expansion associated with pressure changes during a dive did influence changes in diving  $f_H$  (Williams et al., 2015b). Studies in bottlenose dolphins, Weddell seals (*Leptonychotes weddellii*) and narwhals (*Monodon monoceros*) have hypothesized that  $f_H$  is elevated during activity associated with diving and may result in sympathetic and parasympathetic conflict, causing cardiac arrhythmias (Davis and Williams, 2012; Williams et al., 2015b). In contrast, others have concluded that when sympathetic and parasympathetic stimulation are highest,  $f_H$  variability is minimal and that arrhythmias due to autonomic adjustment are benign (Fahlman et al., 2020a; Ponganis et al., 2017). Conditioned cognitive adjustment of  $f_H$  has also been shown to play a role in  $f_H$  modulation as harbor porpoises (*Phocoena phocoena*), bottlenose dolphins and California sea lions trained to perform dives of different durations differentially adjusted  $f_H$  proportionally with dive duration (Elmagaard et al., 2016; Fahlman et al., 2020a, 2019; McDonald et al., 2018; Ridgway et al., 1975). Studies in bottlenose dolphins indicate that a dolphin's ability to exhibit bradycardia increases with age, such that mean diving  $f_H$  decreases with increasing age class (Noren et al., 2004). Similarly, in northern elephant seal pups (*Mirounga angustirostris*) and harbor seal pups performing apneas on land, mean  $f_H$  decreased with increasing age (Andrews et al., 1997; Castellini et al., 1994b; Hicks et al., 2004). In spite of these advancements in understanding the

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factors that influence diving  $f_H$ , little attention has been paid to the factors that influence surface  $f_H$ , despite its importance for dive recovery and preparation, and the importance of designating resting  $f_H$  to estimate the magnitude of the dive response. One aspect of surface  $f_H$  control that warrants further investigation is cardiorespiratory coupling through respiratory sinus arrhythmia (RSA) which drives respiration-associated changes in  $f_H$  (Andrews et al., 1997; Castellini et al. 1994a,b; Cauture et al., 2019; Fahlman et al., 2020b, 2019; Lin et al., 1972).

In terrestrial mammals, RSA is known to result in an increase in instantaneous  $f_H$  ( $if_H$ ) during inspiration and a decrease during expiration (Hirsch and Bishop, 1981). Despite differences in breathing strategies between terrestrial and marine mammals, RSA has also been observed and recorded in several marine mammal species, including the gray whale (*Eschrichtius robustus*), killer whale (*Orcinus orca*), short-finned pilot whale (*Globicephala macrorhynchus*), beluga (*Delphinapterus leucas*), common dolphin (*Delphinus delphis*), bottlenose dolphin, harbor porpoise, northern elephant seal, hooded seal (*Cystophora cristata*), California sea lion and fur seal (*Callorhinus ursinus*) (Andrews et al., 1997; Castellini et al., 1994a,b; Cauture et al., 2019; Elsner et al., 1966; Fahlman et al., 2020a, 2019; Hamlin et al., 1972; Irving et al., 1963; Kanwisher and Ridgway, 1983; Kastelein and Meijler, 1989; Lin et al., 1972; Lyamin et al., 2016; McDonald and Ponganis, 2014; Päsche and Krog, 1980; Ponganis and Kooyman, 1999; Ridgway, 1986, 1972). Results from studies in northern elephant seals, hooded seals, California sea lions and bottlenose dolphins have shown that during the inter-breath interval (IBI), RSA results in dramatic changes in  $if_H$ , with high  $if_H$  values exhibited at the beginning of an IBI followed by a continuous decrease towards a low, stable  $if_H$  at the end of an IBI (Andrews et al., 1997; Castellini et al., 1994a; Cauture et al., 2019; Fahlman et al., 2020b, 2019; Lin et al., 1972; Päsche and Krog, 1980). Interestingly, in the California sea lion, the minimum  $f_H$  observed between breaths on land was comparable to the minimum  $f_H$  observed during dives (Lin et al., 1972). Additionally, previous studies in bottlenose dolphins suggested that, after the RSA was accounted for, the base  $f_H$  of dolphins breathing at the surface was similar to reported dolphin  $f_H$  values during diving bradycardia (Cauture et al., 2019; Fahlman et al., 2020a). Hooded seals performing submerged apneas demonstrated similar  $f_H$  to the minimum  $if_H$  measured during periods of breathing on land (Päsche and Krog, 1980). In emperor penguins (*Aptenodytes forsteri*) and California sea lions, the minimum  $if_H$  associated with RSA was comparable to the minimum  $if_H$  seen in dives shorter than the aerobic dive limit (ADL), suggesting that the same mechanism of cardiorespiratory control governs these patterns (McDonald and Ponganis, 2014; Meir et al., 2008). In cetaceans, however, because of the challenge of measuring cardiorespiratory parameters in fully aquatic animals, studies investigating cardiorespiratory coupling and its relationship to surface and diving  $f_H$  are limited (Bickett et al., 2019; Cauture et al., 2019; Fahlman et al., 2020a,b, 2019).

We examined differences in patterns of  $f_H$  modulation between surface breathing and submerged breath-holds to investigate how RSA influences surface  $f_H$  during rest and the relationship between RSA at the surface and changes in  $f_H$  observed during short submersions in bottlenose dolphins. We hypothesized that (1) IBI would influence the degree of RSA we observed and that RSA should increase with longer IBIs, as has been shown in humans (Hirsch and Bishop, 1981), (2) that the minimum  $if_H$  of the RSA would decrease with increasing IBI as the effect of lung inflation is diminished (Angell-James et al., 1981), and (3) that, as has been suggested in seals and manatees, the minimum  $if_H$  measured during

long IBIs at the surface would be comparable to the minimum  $f_H$  during submerged breath-holds (Castellini et al., 1994a; Castellini and Zenteno-Savin, 1997; Gallivan et al., 1986). Additionally, we examined differences in RSA under fasted and non-fasted, resting conditions as we hypothesized that the increased metabolic cost of digestion under non-fasted conditions would result in increased resting surface  $f_H$  and reduced RSA compared with fasting conditions, but that rates measured under fasted conditions should reflect resting metabolic demands. Finally, we discuss the potential significance of similarities between  $f_H$  observed during long IBIs at the surface and during submerged breath-holds for our contemporary understanding of the dive response.

## MATERIALS AND METHODS

All experiments were done with voluntary participation from the dolphins and an individual could end a trial at any point. The study protocols were accepted at Dolphin Quest Oahu, and also by the Animal Care and Welfare Committee at the Oceanogràfic (OCE-17-16, amendments OCE-29-18 and OCE-3-19i) and the Institutional Animal Care and Use Committee of Duke University (A045-17-02).

### Animals

Six adult male, bottlenose dolphins, *Tursiops truncatus* (Montagu 1821), housed at Dolphin Quest Oahu (Honolulu, HI, USA) with an average age of  $23.2 \pm 7.0$  years (range 11–31 years) and body mass  $189.3 \pm 36.3$  kg (range 147.0–251.7 kg) participated in the study. Prior to the start of the study, all dolphins were desensitized to the research equipment used. Research trials consisted of stationary 10 min resting trials at the surface under fasted or non-fasted conditions, as well as 2 min breath-hold trials under non-fasted conditions. Fasted trials were only conducted in the morning with approximately 15 h having passed since the last meal on the previous day. All trials were preceded by 2 min of low-activity behavior during which the animal was either resting next to the trainer or swimming slowly.

To prepare for a research trial, the dolphin turned ventral side up to allow placement of the electrocardiogram (ECG) electrodes (Cauture et al., 2019; Fahlman et al., 2020b). Following placement of the ECG electrodes, the animal rolled back, dorsal side up. The trial continued if the ECG signal was visible, otherwise the electrodes were taken off, reattached, and the procedure repeated. Next, the pneumotachometer, a device used to measure changes in airflow, was placed over the blowhole and the trial began. For the 2 min breath-hold trials, the animal was asked to roll over onto its side with its blowhole fully submerged immediately following a breath, as previously detailed (Fahlman et al., 2019). The dolphin remained in this position until the end of the pre-determined breath-hold duration, at which time the animal was asked to roll back dorsal-side up to end the breath-hold and reinitiate spontaneous breathing. All experiments were conducted during a 2 week period in May 2019.

### Respiration

A Fleisch type pneumotachometer (Mellow Design, Valencia, Spain) was used to measure breath-by-breath exhaled and inhaled respiratory flow as previously detailed (Fahlman et al., 2015). The flow signal was used to determine the beginning and end of a respiration, and the breathing frequency ( $f_R$ ) and IBI were determined from the duration between breaths. Further analysis of the respiratory parameters was not performed for this study.

### Heart rate

$f_H$  was determined using a three-lead ECG data recording system. The ECG leads were connected to gold-plated electrodes (Disposable

GoldSelect Cup Electrodes, DE-003710, Rochester Med, LifeSync Neuro, Coral Springs, FL, USA) mounted inside custom-made silicone suction cups (Smooth-Sil 940, Smooth-On, Inc., Macungie, PA, USA) connected to a custom-built bio-amplifier (UUB/1-ECG, UFI, Morro Bay, CA, USA) and with a BNC connector to the data acquisition system (Powerlabs 8/35, ADInstruments, Colorado Springs, CO, USA). The electrodes were placed on the ventral side of the animal with one electrode placed inside the top edge of the left and right pectoral fin, respectively, and the third electrode placed on the left side, 15 cm caudal to the upper left electrode. Each suction cup was filled with conductive paste (Ten20 Conductive Paste, Weaver and Company, Aurora, CO, USA) before being placed on the skin.

### Data acquisition and processing

The respiratory flow and ECG were recorded at 400 Hz by a data acquisition system (Powerlabs 8/35, ADInstruments), and displayed in real-time by a laptop computer running LabChart (v.8.1, ADInstruments). The  $i\dot{f}_H$  was extracted from the ECG signal using the ECG Analysis Module in LabChart. All data were analyzed using MATLAB (version 2018b, ©2018 The MathWorks, Inc.). For each IBI, the RSA was estimated using two different methods. The percent RSA [RSA (%)] was calculated as the difference between the peak and the trough of the  $i\dot{f}_H$  signal in beats  $\text{min}^{-1}$ , normalized by the mean  $i\dot{f}_H$  ( $\bar{i}\dot{f}_H$ ) for that section:  $\text{RSA} (\%) = \Delta i\dot{f}_H / \bar{i}\dot{f}_H$  (Mortola et al., 2016). RSA was also calculated using the peak-to-trough method [RSA (s)] (Lewis et al., 2012). For the calculation of averaged  $i\dot{f}_H$  responses for surface trials,  $f_H$  was resampled at 1 s intervals and averaged at every second for the duration of the 90th percentile IBI using the function *resample*.

### Statistics

All statistical analyses were performed using R (v.3.6.2) (<http://www.R-project.org/>). Differences in mean  $i\dot{f}_H$ , maximum  $i\dot{f}_H$ , minimum  $i\dot{f}_H$ , RSA (%) and RSA (s) between fasted and non-fasted surface trials during short (IBI < 10 s), intermediate (10 s ≤ IBI ≤ 30 s) and long IBIs (IBI > 30 s) were determined using a mixed effects ANOVA, followed by a *post hoc* Tukey test. Differences between long surface IBIs (IBI > 30 s) and submerged breath-hold trials were determined using a mixed effects ANOVA. If there was no significant difference in a  $f_H$  parameter during fasted and non-fasted IBIs, those data were combined to compare with the breath-holds. Linear mixed effect models, using the package *nlme*, were used to determine the relationships between IBI and  $f_H$  and RSA measurements with animal ID as a random effect. All variables were  $\log_{10}$ -transformed prior to model fit. Differences in intercepts ( $B_0$ ) and slopes ( $B_1$ ) between fasted and non-fasted surface trials were evaluated as terms within the linear mixed effect model and if there was no significant difference in either parameter, the fasted and non-fasted data were combined and are described by a single model. Linear mixed effect models were

also used to evaluate the relationship between the duration of the previous IBI and the maximum  $i\dot{f}_H$  of the IBI as well as the relationship between the duration of the previous IBI and the difference between the maximum  $i\dot{f}_H$  of the IBI and the minimum  $i\dot{f}_H$  of the previous IBI. The package *r2glmm* was used to determine  $r^2$  values for each model. All statistical tests were done assuming that  $P < 0.05$  indicated a significant difference. Values are presented as means ± s.d. unless stated otherwise.

### RESULTS

A total of 22 trials were conducted with six male bottlenose dolphins (Table S1). These were composed of 8 fasted ( $n=241$  breaths) and 7 non-fasted ( $n=246$  breaths) surface trials, during which the blow-hole was above the water's surface and the dolphin could breathe spontaneously. Another 7 non-fasted breath-hold ( $n=7$  breaths) trials were conducted with the dolphin rolled ventrally and the blow-hole submerged.

#### Fasted versus non-fasted surface breathing $f_H$ and RSA

Mean  $f_H$  for fasted trials was  $67.2 \pm 11.8$  beats  $\text{min}^{-1}$  and mean  $f_H$  for non-fasted trials was  $78.0 \pm 13.1$  beats  $\text{min}^{-1}$  (Welch two-sample *t*-test,  $t = -9.5$ ,  $P < 0.0001$ ). RSA was observed following respiration as an increase in the  $i\dot{f}_H$  immediately following the breath followed by a decrease in  $i\dot{f}_H$  until the next breath. There was large variation in the IBI within and between dolphins (Table S1). The mean IBI during fasted trials was  $19.7 \pm 14.3$  s as compared with  $14.6 \pm 8.0$  s during non-fasted trials. For all surface trials, fasted and non-fasted, maximum  $i\dot{f}_H$  was  $87.4 \pm 13.6$  beats  $\text{min}^{-1}$  and minimum  $i\dot{f}_H$  was  $56.8 \pm 14.8$  beats  $\text{min}^{-1}$  (range:  $124.4 - 23.9$  beats  $\text{min}^{-1}$ ). Summary statistics for  $f_H$ , RSA and body mass ( $M_b$ ) for all animals are displayed in Tables S1 and S2. The mean  $f_H$  during fasted surface trials ( $67.2 \pm 11.8$  beats  $\text{min}^{-1}$ ) closely agreed with the allometric prediction of  $64.2$  beats  $\text{min}^{-1}$  for the average body mass of the animals that participated in the fasted trials, whereas the non-fasted mean  $f_H$  ( $78.0 \pm 13.1$  beats  $\text{min}^{-1}$ ) was elevated as compared with the allometric prediction (Table S1) (Stahl, 1966).

We examined differences in both  $f_H$  and estimated indices of RSA during fasted and non-fasted surface trials binned by IBI (Table 1). Mean  $i\dot{f}_H$  decreased with increasing IBI across the three IBI categories (Table 2, Fig. 1) and mean  $i\dot{f}_H$  was lower during fasted trials as compared with non-fasted trials for short and intermediate IBIs, but not for long IBIs (Fig. 1A,B, Table 1). Minimum  $i\dot{f}_H$  also decreased with increasing IBI (Table 2) and, like mean  $i\dot{f}_H$ , was lower for short and intermediate IBIs during fasted trials than during non-fasted trials, but this difference was absent for long IBIs (Fig. 1C,D, Table 1). Notably, however, the regression describing the relationships between IBI and mean  $i\dot{f}_H$  as well as IBI and minimum  $i\dot{f}_H$  did not differ between fasted and non-fasted trials (mean  $i\dot{f}_H$ :  $B_0$ ,  $P=0.97$ ;  $B_1$ ,  $P=0.22$ , minimum  $i\dot{f}_H$ :  $B_0$ ,  $P=0.45$ ;  $B_1$ ,  $P=0.06$ ) (Fig. 1B,D, Table 2).

**Table 1. Inter-breath interval (IBI), heart rate ( $f_H$ ) and respiratory sinus arrhythmia (RSA) statistics for fasted and non-fasted surface trials binned by IBI**

| IBI (s)           | Fasted state           | IBI (s)           | Mean $i\dot{f}_H$<br>(beats $\text{min}^{-1}$ ) | Maximum $i\dot{f}_H$<br>(beats $\text{min}^{-1}$ ) | Minimum $i\dot{f}_H$<br>(beats $\text{min}^{-1}$ ) | RSA (%)           | RSA (s)               |
|-------------------|------------------------|-------------------|---|--|--|-------------------|-----------------------|
| IBI < 10 s        | Fasted ( $n=53$ )      | $7.9 \pm 1.7^a$   | $74.5 \pm 9.7^c$                                | $83.2 \pm 10.6^{a,b}$                              | $63.8 \pm 11.4^{c,d}$                              | $26.6 \pm 13.7^a$ | $0.25 \pm 0.19^{a,b}$ |
|                   | Non-fasted ( $n=80$ )  | $7.3 \pm 2.1^a$   | $81.5 \pm 12.3^d$                               | $91.1 \pm 13.6^b$                                  | $67.1 \pm 13.9^d$                                  | $29.8 \pm 15.6^a$ | $0.26 \pm 0.19^a$     |
| 10 s ≤ IBI ≤ 30 s | Fasted ( $n=152$ )     | $17.2 \pm 4.7^b$  | $67.6 \pm 10.3^b$                               | $81.6 \pm 11.6^a$                                  | $52.7 \pm 12.1^b$                                  | $44.0 \pm 18.6^b$ | $0.46 \pm 0.26^c$     |
|                   | Non-fasted ( $n=152$ ) | $16.4 \pm 5.0^b$  | $77.3 \pm 13.0^c$                               | $93.3 \pm 13.9^b$                                  | $58.9 \pm 13.3^c$                                  | $45.5 \pm 15.8^b$ | $0.42 \pm 0.20^b$     |
| IBI > 30 s        | Fasted ( $n=36$ )      | $47.9 \pm 15.7^d$ | $55.2 \pm 11.6^a$                               | $84.3 \pm 13.8^{a,b}$                              | $35.7 \pm 9.8^a$                                   | $91.1 \pm 23.8^d$ | $1.1 \pm 0.34^d$      |
|                   | Non-fasted ( $n=14$ )  | $36.0 \pm 4.5^c$  | $65.0 \pm 9.3^{a,b}$                            | $89.7 \pm 10.4^{a,b}$                              | $46.5 \pm 8.5^{a,b}$                               | $67.8 \pm 23.2^c$ | $0.66 \pm 0.26^c$     |

IBI category, trial type and an interaction term were the fixed effects and animal ID was the random effect in a mixed effects ANOVA followed by a *post hoc* Tukey test. Different lowercase letters indicate significant differences (*post hoc* tests).

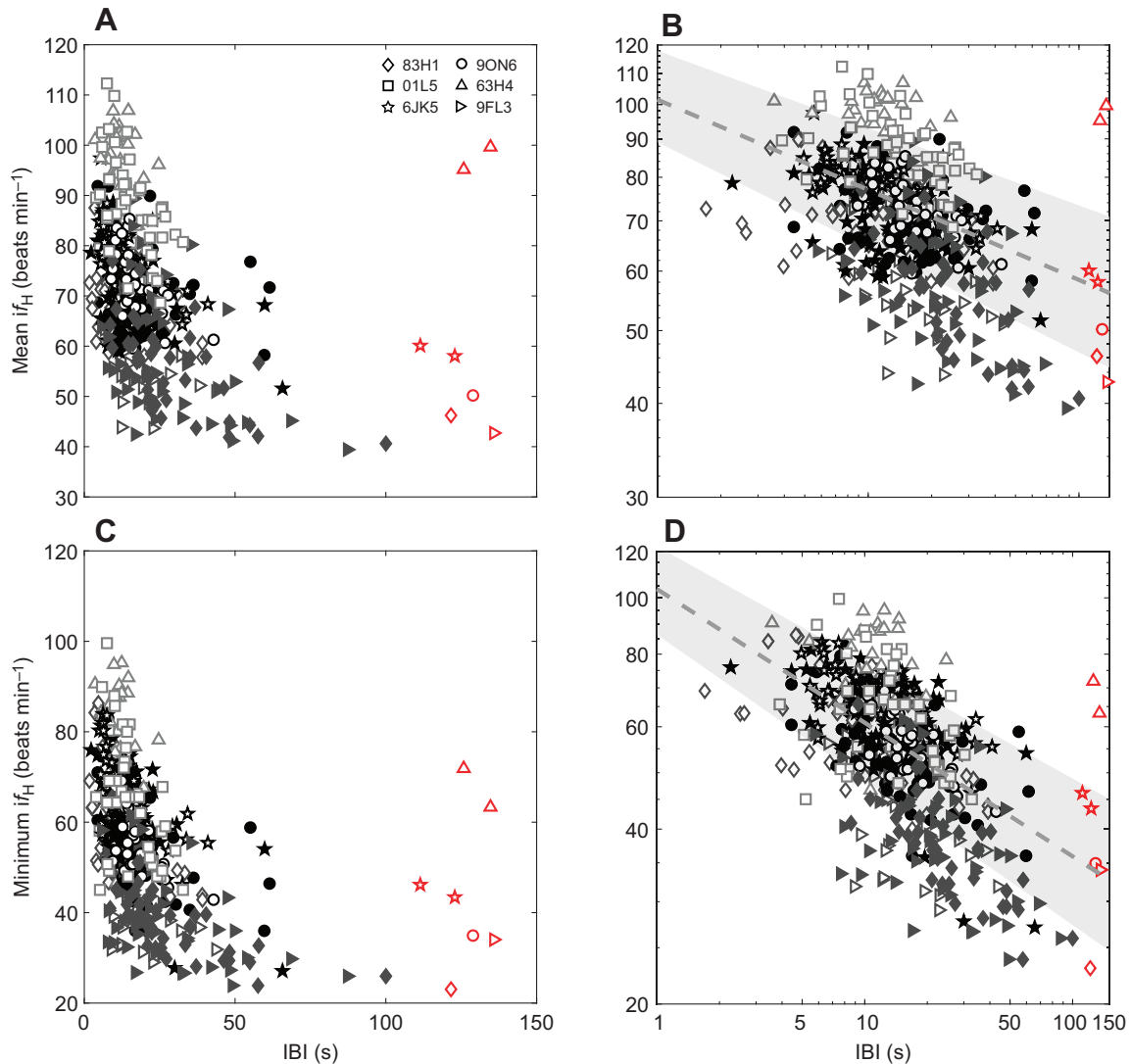
**Table 2. Linear mixed effect model parameters for relationships between  $\log_{10}$ -transformed IBI and  $\log_{10}$ -transformed  $if_H$  during fasted and non-fasted surface trials**

|   | $B_0 \pm s.e.$   | $P$     | $B_1 \pm s.e.$   | $P$     | $r^2$ |
|---|------------------|---------|------------------|---------|-------|
| Mean $if_H$ (beats $\text{min}^{-1}$ )    | $2.01 \pm 0.03$  | <0.0001 | $-0.12 \pm 0.01$ | <0.0001 | 0.25  |
| Minimum $if_H$ (beats $\text{min}^{-1}$ ) | $2.01 \pm 0.04$  | <0.0001 | $-0.23 \pm 0.01$ | <0.0001 | 0.38  |
| Maximum $if_H$ (beats $\text{min}^{-1}$ ) | $1.95 \pm 0.03$  | <0.0001 | $0.003 \pm 0.01$ | 0.77    | 0.01  |
| RSA (%)                                   | $0.95 \pm 0.06$  | <0.0001 | $0.56 \pm 0.02$  | <0.0001 | 0.53  |
| RSA (s)                                   | $-1.22 \pm 0.08$ | <0.0001 | $0.67 \pm 0.03$  | <0.0001 | 0.49  |

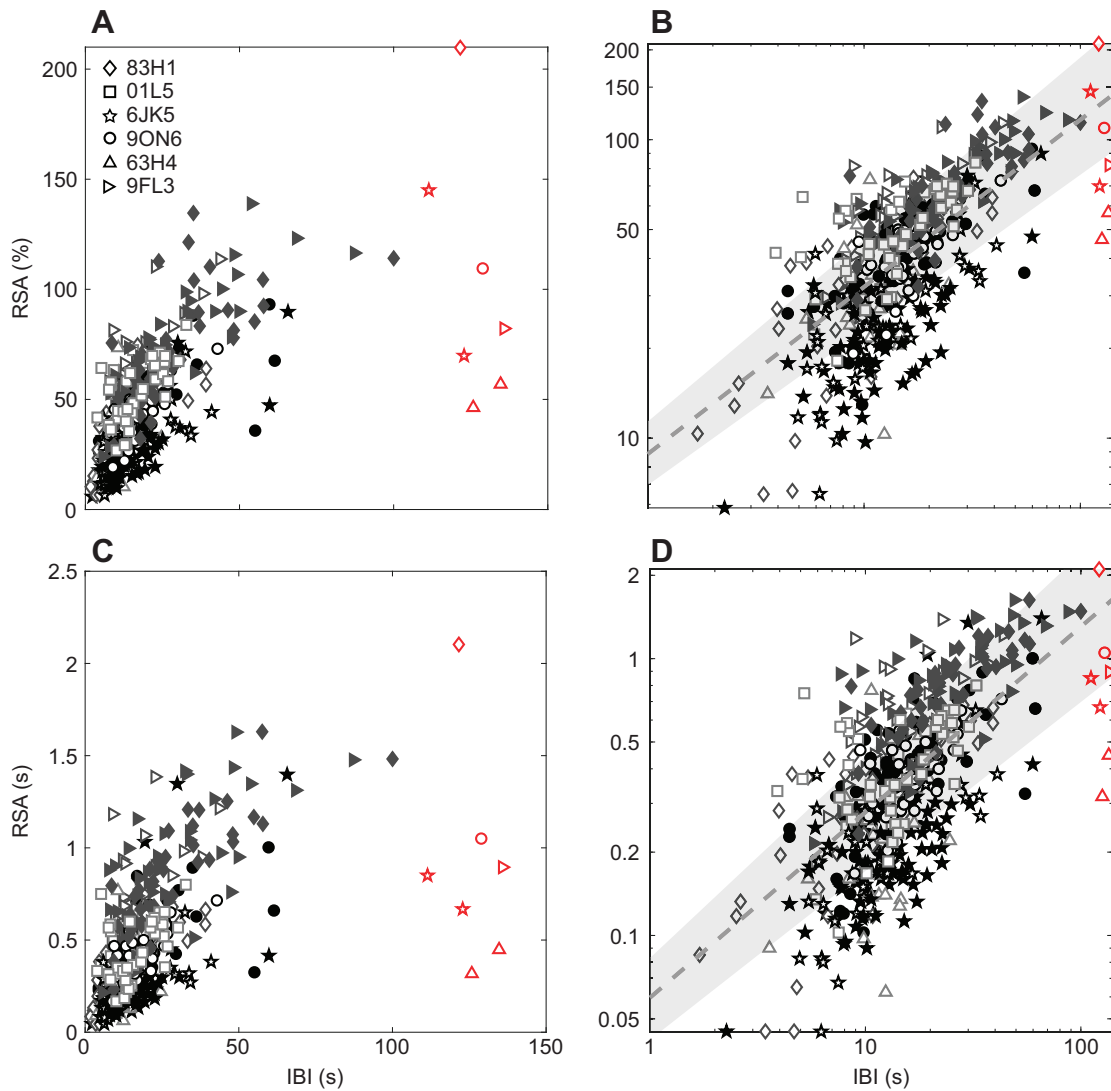
Regressions are in the form  $\log_{10}(Y) = B_0 + B_1 \cdot \log_{10}(X)$ , where IBI is the independent variable. Animal ID was included as a random variable.

The regression analysis showed that maximum  $if_H$  did not vary significantly with IBI (Table 2) and pair-wise comparison across IBI categories revealed a significant difference in maximum  $if_H$  between fasted and non-fasted trials only for intermediate IBIs (Table 1). There was no significant difference in the slopes or in the exponents of the regression of IBI and maximum  $if_H$  for fasted as compared with non-fasted trials ( $B_0, P=0.18$ ;  $B_1, P=0.68$ ) and therefore they are described by a single equation (Table 2). There was a significant negative relationship between the duration of the previous IBI and the maximum  $if_H$  of the IBI, and a significant positive relationship

between the duration of the previous IBI and the difference between the maximum  $if_H$  of the IBI and the minimum  $if_H$  of the previous IBI, a potential proxy for the effect of lung inflation (Table S3, Fig. S1). Both metrics of RSA, RSA (%) and RSA (s), increased with increasing IBI and there was no difference in the coefficients of the regression between fasted and non-fasted trials [RSA (%):  $B_0, P=0.77$ ;  $B_1, P=0.92$ ; RSA (s):  $B_0, P=0.75$ ;  $B_1, P=0.83$ ] (Fig. 2, Table 2). There was a significant interaction between IBI and fasted state for both metrics of RSA. Both metrics of RSA only differed for fasted as compared with non-fasted IBIs that were longer than 30 s (Table 1).



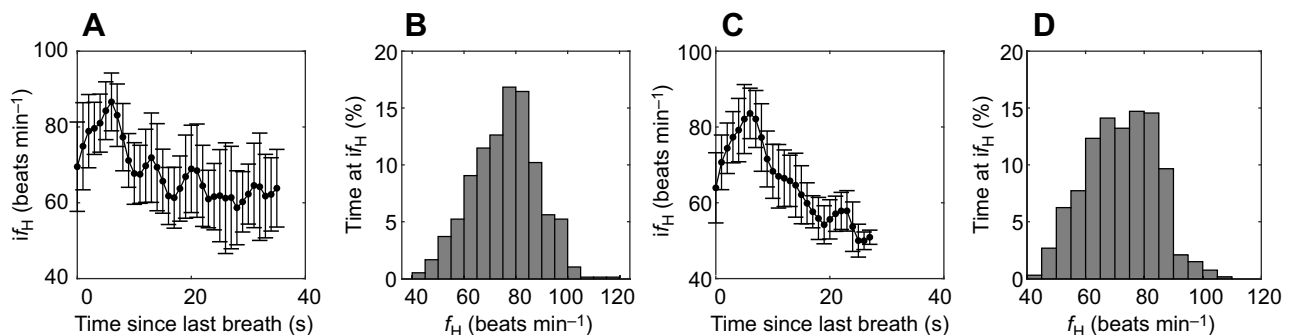
**Fig. 1. Mean and minimum instantaneous heart rate ( $if_H$ ) during surface breathing and submerged breath-holds.** Mean  $if_H$  (A,B) and minimum  $if_H$  (C,D) against inter-breath interval (IBI) for fasted surface (filled grayscale symbols,  $n=241$ ), non-fasted surface (open grayscale symbols,  $n=246$ ) and breath-hold trials (open red symbols,  $n=7$ ). A and C are plotted on a linear scale and B and D are plotted on a log–log scale with regressions and 95% confidence intervals for the surface data as detailed in Table 2.



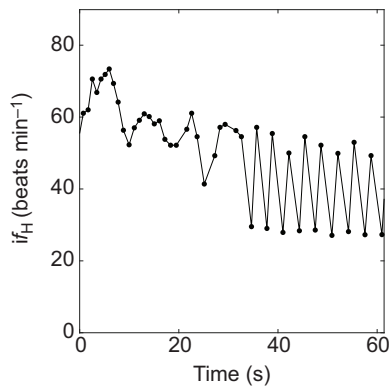
**Fig. 2. Respiratory sinus arrhythmia (RSA) during surface breathing and submerged breath-holds.** Data showing two methods to estimate the RSA as RSA (%) (A,B) and RSA (s) (C,D) against IBI for fasted surface (filled grayscale symbols,  $n=241$ ) non-fasted surface (open grayscale symbols,  $n=246$ ) and submerged breath-hold trials (open red symbols,  $n=7$ ). A and C are plotted on a linear scale and B and D are plotted on a log–log scale with regressions and 95% confidence intervals for the surface data as detailed in Table 2.

In addition to RSA, two other distinct arrhythmic patterns were observed in the  $f_{\text{H}}$  measured during IBIs at the surface. The first was an oscillatory pattern within the gradual decrease in  $if_{\text{H}}$  of the RSA (Fig. 3). This oscillatory pattern, in addition to the initial peak in  $if_{\text{H}}$ ,

resulted in secondary and tertiary local maxima and minima within the  $if_{\text{H}}$  signal. These peaks occurred with a regular period of approximately 10 s (0.1 Hz, see Fig. 3A). The second arrhythmia was a series of paired heart beats that resulted in the oscillation of the  $if_{\text{H}}$  signal



**Fig. 3. RSA during a fasted and non-fasted surface trial.** Mean  $\pm$  s.d.  $if_{\text{H}}$  responses (A,C) and distribution of  $if_{\text{H}}$  (B,D) of 9ON6 for a representative fasted surface trial (A,B) and a representative non-fasted surface trial (C,D) corresponding to the 90th percentile duration IBI for each respective trial.



**Fig. 4. A paired beat arrhythmia.**  $if_H$  during an IBI shows an example of a paired beat arrhythmia.

between approximately 50 beats  $\text{min}^{-1}$  and 30 beats  $\text{min}^{-1}$  (Fig. 4). This pattern resulted from a short R–R interval between paired beats followed by a longer R–R interval until the next set of paired beats and was only observed during one surface IBI in a single dolphin (63H4).

#### Surface breathing versus submerged breath-hold $f_H$ and RSA

The mean breath-hold duration was  $125.9 \pm 8.4$  s (range: 121.6–135.8 s) and the mean  $f_H$  during breath-holds was  $64.6 \pm 23.3$  beats  $\text{min}^{-1}$ . For all breath-hold trials, maximum  $if_H$  was  $104.6 \pm 23.5$  beats  $\text{min}^{-1}$  and minimum  $if_H$  was  $45.3 \pm 17.1$  beats  $\text{min}^{-1}$  (range: 133.3–23.1 beats  $\text{min}^{-1}$ ). In all surface trials (combined fasted and non-fasted) mean IBI and mean  $f_H$  were  $17.1 \pm 11.8$  s and  $72.7 \pm 13.6$  beats  $\text{min}^{-1}$ , respectively. One dolphin (63H4) did not demonstrate submersion bradycardia during the submerged breath-holds.

There was no significant difference between the maximum  $if_H$  or minimum  $if_H$  of the RSA during long IBIs at the surface compared with breath-hold trials when dolphins demonstrated some degree of submersion bradycardia (excludes two trials with 63H4, Table 3). There was a significant difference in mean  $if_H$ , with lower mean  $if_H$  during the breath-holds as compared with the surface IBIs, but this was largely driven by the extended period of low  $if_H$  following the first 15–20 s of the breath-hold. In addition, there was a significant difference in both metrics of RSA, with higher RSA during breath-hold as compared with surface IBIs.

There were three general  $f_H$  patterns observed during breath-hold trials (Fig. 5). The first was a pattern similar in shape to that observed between breaths during surface trials. This pattern began with a brief increase in  $if_H$  directly following the breath and as the animal submerged followed by a gradual, oscillating decrease in  $if_H$  (see Fig. 5A,B). Interestingly, the minimum  $if_H$  during the breath-

hold trial for an animal displaying this pattern was approximately 15 beats  $\text{min}^{-1}$  lower as compared with the minimum  $if_H$  during the same animal's non-fasted surface trials. The second pattern seen during the breath-hold trials was a rapid increase in  $if_H$  after the breath followed by a rapid decrease in  $if_H$  to a stable, reduced  $f_H$  (see Fig. 5C,D). The third distinct  $f_H$  pattern observed was a rapid oscillation with an amplitude of approximately 30 beats  $\text{min}^{-1}$  around an elevated  $f_H$  (see Fig. 5E,F). Notably, this pattern was only observed in one animal (63H4) and was consistent during both breath-hold trials with this individual.

#### DISCUSSION

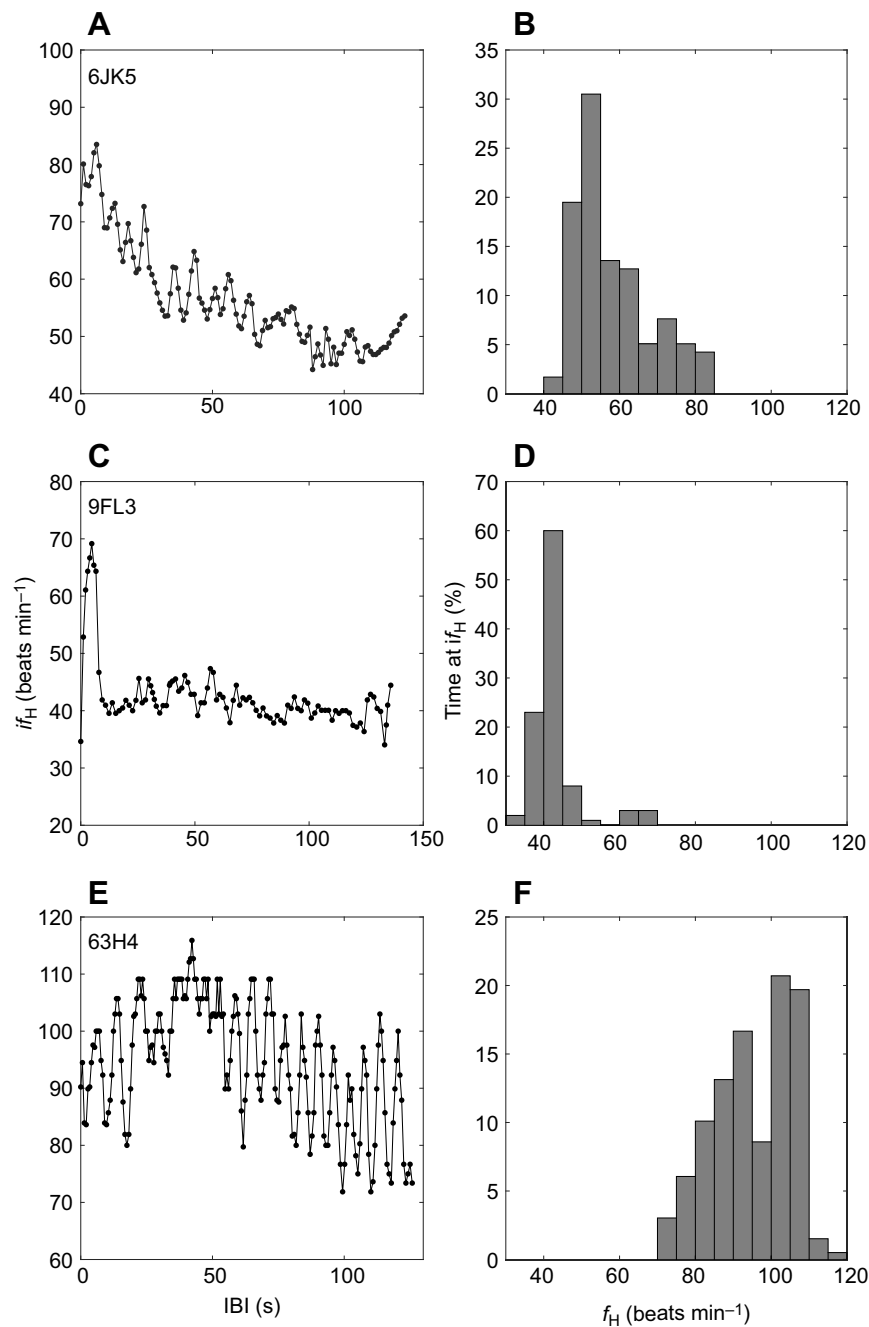
We measured  $f_H$  and IBI in bottlenose dolphins and found that they exhibit large RSA during surface breathing and that, for long IBIs, the minimum  $if_H$  of the RSA was not significantly different from the minimum  $if_H$  observed during a 2 min, static submersion. In addition to RSA, we observed several other arrhythmias while the dolphins were breathing at the surface and during submerged breath-holds. We examined cardiorespiratory parameters during fasted and non-fasted surface trials and found that average  $f_H$  during fasted trials agreed with the previously published allometric relationship for resting  $f_H$ , whereas the average  $f_H$  during non-fasted trials was comparatively higher, and suggest that these differences are secondary to differences in average IBI (Stahl, 1966). The regression analysis revealed no differences between any  $if_H$  or RSA parameters during fasted and non-fasted trials when accounting for variation in IBI, but when data were binned by IBI, there were significant differences in the same parameters.

Importantly, this study does not account for other factors that may affect RSA and surface  $f_H$  including tidal volume ( $V_T$ ) or exercise. We would expect to observe a stronger relationship between RSA and IBI duration if we had accounted for the variation in  $V_T$ , which is known to affect RSA (Cauture et al., 2019; Hirsch and Bishop, 1981). Similarly, because this study was only conducted under resting conditions, we cannot predict the impact of exercise or extended diving behavior on surface RSA or its relationship to submerged breath-holds. After an extended dive, where the dolphin remains actively stroking during the bottom portion and during the ascent of the dive, RSA may be disrupted by the requirements to recover quickly at the surface, as in elephant seals (Andrews et al., 1997). However, this study aimed only to establish resting surface  $f_H$  patterns in comparison to submersion bradycardia, considering the importance of surface  $f_H$  in underlying  $O_2$  management requirements during gas exchange and the possibility of overlapping mechanisms regulating surface and diving  $f_H$ . The results of this work contribute to our understanding of the factors that influence fine-scale changes in  $f_H$  in marine mammals, which is critical for estimating the physiological thresholds on diving behavior.

**Table 3.  $f_H$  and RSA statistics for surface IBIs >30 s and submerged breath-hold trials during which submersion bradycardia was observed**

| Trial type          | Mean $if_H$ (beats $\text{min}^{-1}$ )       | Maximum $if_H$ (beats $\text{min}^{-1}$ )     | Minimum $if_H$ (beats $\text{min}^{-1}$ )    | RSA (%)                                      | RSA (s)                                      |
|---------------------|--|---|--|--|--|
| Surface (IBI >30 s) | $58.0 \pm 11.8$ (n=50)                       | $85.8 \pm 13.1$ (n=50)                        | $38.7 \pm 10.6$ (n=50)                       | $67.8 \pm 23.2$ (n=14)                       | $0.66 \pm 0.26$ (n=14)                       |
| Submerged (n=5)     | $50.7 \pm 8.3$                               | $94.8 \pm 24.0$                               | $36.4 \pm 9.0$                               | $116.2 \pm 44.5$                             | $1.1 \pm 0.52$                               |
| Statistics          |  |   |  |  |  |
| Trial type          | $\chi^2(1, n=55)=6.1$<br><b>P=0.01</b>       | $\chi^2(1, n=55)=1.5$<br>P=0.21               | $\chi^2(1, n=55)=2.8$<br>P=0.10              | $\chi^2(1, n=19)=10.4$<br><b>P&lt;0.001</b>  | $\chi^2(1, n=19)=8.3$<br><b>P&lt;0.001</b>   |
| Intercept           | $\chi^2(1, n=55)=71.0$<br><b>P&lt;0.0001</b> | $\chi^2(1, n=55)=211.7$<br><b>P&lt;0.0001</b> | $\chi^2(1, n=55)=48.5$<br><b>P&lt;0.0001</b> | $\chi^2(1, n=19)=86.0$<br><b>P&lt;0.0001</b> | $\chi^2(1, n=19)=51.2$<br><b>P&lt;0.0001</b> |

Submerged data exclude two trials with 63H4. Trial type (surface versus submerged) was the fixed effect and animal ID was the random effect in a mixed effects ANOVA. For parameters that showed no significant difference between fasted and non-fasted surface trials for IBIs >30 s, fasted and non-fasted data were pooled (based on Table 1). For parameters that showed a significant difference between fasted and non-fasted trials, only non-fasted trials were compared with the submerged breath-holds. Bold indicates significance.



**Fig. 5.**  $f_H$  patterns during submerged breath-holds.  $if_H$  responses (A,C,E) and distribution of  $if_H$  (B,D,F) for three submerged breath-hold trials.

### RSA and $f_H$ are correlated with IBI

Although the patterns we observed in this study have previously been observed in many marine mammal species, the potential relationship between RSA and apnea has been studied in pinnipeds, but not cetaceans, which are fully aquatic, obligate divers (Castellini et al., 1994b). In contrast to those during diving, circulatory adjustments during surface intervals should increase perfusion to maximize gas exchange and minimize time spent during an otherwise unproductive state (Fahlman et al., 2020b, 2018; Fedak et al., 1988). For example, the rapid increase in  $if_H$  of the RSA may be important in generating an increase in peripheral perfusion to quickly replenish muscle and blood  $O_2$  stores as a clustering of heart beats has been shown to improve  $O_2$  uptake and reduce the physiological dead space to  $V_T$  ratio in humans (Arieli and Farhi, 1985; Yasuma and Hayano, 2004). We found that the maximum  $if_H$  following inhalation did not vary with IBI, although it

was variable and could have been influenced by  $V_T$  (Cauture et al., 2019). This result indicates that, under resting conditions at the surface, the increase in  $f_H$  during the RSA is not modulated in relation to the upcoming IBI duration and could instead be a function of other respiratory variables (i.e.  $V_T$ ). The data did, however, show a positive correlation between RSA and IBI, which is likely secondary to a negative correlation between mean and minimum  $if_H$  and IBI (Fig. 2). This result agrees with work in harbor seals breathing spontaneously on land where there was an observed oscillation in  $f_H$  between 120 and 70 beats  $min^{-1}$  for long IBIs, and high  $V_T$ , but for short IBIs, this oscillation was reduced (Päsche and Krog, 1980).

We also investigated the potential effect of the duration of the previous IBI on maximum  $if_H$  of the IBI, given that IBI was not a significant predictor of maximum  $if_H$ . Based on the observed  $f_H$  patterns, we expect that the previous IBI could have an effect on

maximum  $f_{\text{H}}$  of an IBI in two ways: the duration of the previous IBI could determine the ‘initial value’ from which  $f_{\text{H}}$  varies during the next IBI; and the  $V_{\text{T}}$  of a breath, which may be correlated with the duration of the previous IBI and the corresponding degree of lung inflation, could result in differential changes in  $f_{\text{H}}$ . We found that there was a significant negative relationship between the duration of the previous IBI and the maximum  $f_{\text{H}}$  of the IBI, suggesting that following a long IBI, maximum  $f_{\text{H}}$  of the next IBI is lower and therefore may start from a lower initial value than that following short IBIs (Table S3, Fig. S1). Additionally, we found that there was a significant positive relationship between the duration of the previous IBI and the difference between maximum  $f_{\text{H}}$  of the IBI and the minimum  $f_{\text{H}}$  of the previous IBI. A possible explanation for this pattern is that following a long IBI there may be a greater need for gas exchange such that the animal compensates with a larger  $V_{\text{T}}$  of the breath and therefore the effect of lung inflation may be greater following long IBIs than short IBIs. Together, however, these patterns indicate that a higher degree of tachycardia that may result from a larger  $V_{\text{T}}$  associated with longer IBIs may not compensate for the low initial value of the  $f_{\text{H}}$  at the beginning of that IBI because of the preceding long IBI. These results suggest that the previous IBI may influence  $f_{\text{H}}$  patterns during a given IBI and that changes in  $f_{\text{H}}$  during breathing may be useful to estimate lung function, as previous studies have suggested (Cauture et al., 2019).

Given the relationships between RSA,  $f_{\text{H}}$  and IBI, we suggest that RSA in bottlenose dolphins is driven by a brief period of tachycardia associated with lung inflation whose effect is slowed and eventually eliminated as the IBI increases. Past studies in harbor seals have indicated an association between lung inflation and intermittent tachycardia followed by an exponential decay in  $f_{\text{H}}$  (Angell-James et al., 1981). If this is the case in bottlenose dolphins, an interesting question arises as to whether the tachycardia of breathing or the lower, stable  $f_{\text{H}}$  seen during IBIs > 30 s reflects the ‘normal’, intrinsic  $f_{\text{H}}$  of the dolphin, the latter having been suggested for seals and manatees (Castellini et al., 1994a; Castellini and Zenteno-Savin, 1997; Gallivan et al., 1986). There is further support for this suggestion from cardiac output measurements during apnea in seals that match allometric predictions, as well as measurements of  $f_{\text{H}}$  in killer whales during stationary breath-holds that agree with allometric predictions for resting  $f_{\text{H}}$  (Bickett et al., 2019; Ponganis et al., 2006). Then, the tachycardia associated with breathing may be a response to improve gas exchange and not a ‘normal’ rate, an idea that has previously been proposed in studies in other taxa (Castellini et al., 1994a; Castellini and Zenteno-Savin, 1997; Fedak et al., 1988; Gallivan et al., 1986). Alternatively, this pattern could suggest that the tachycardia associated with breathing is the intrinsic  $f_{\text{H}}$  of the dolphin, which has previously been assessed during anesthesia with nitrous oxide to be approximately 100–120 beats  $\text{min}^{-1}$ , and that the response to apnea overrides this normal rate (Ridgway and McCormick, 1967). This would be similar to the accentuated antagonism proposed in other diving marine mammals where the diving  $f_{\text{H}}$  is largely regulated by vagal tone (Elliott, 2002). In addition to the lack of RSA at high  $f_{\text{R}}$ , in humans RSA has also been observed to be  $f_{\text{R}}$  independent at low  $f_{\text{R}}$  (long IBIs), where the degree of RSA does not continue to increase during an extended breath-hold or IBI, and this ‘corner’ frequency is correlated with resting  $f_{\text{H}}$  (Hirsch and Bishop, 1981). In this case, the normal rate in a dolphin may be a reduced  $f_{\text{H}}$ , or bradycardia, that is maintained in the absence of breathing, which is closer to the average diving  $f_{\text{H}}$ . For marine mammals, we propose that the IBI and the corresponding mean  $f_{\text{H}}$  where an IBI independence of RSA is observed may be of interest to define resting  $f_{\text{H}}$  more clearly in the dolphin for comparison with diving  $f_{\text{H}}$  (Fahlman et al., 2019; Kooyman, 1985).

### Minimum $f_{\text{H}}$ of RSA during long IBIs is comparable to bradycardia of submerged breath-holds

The minimum  $f_{\text{H}}$  of the RSA during long IBIs did not differ from the minimum  $f_{\text{H}}$  observed during submersion bradycardia associated with the breath-hold trials. Several individual dolphins consistently exhibited  $f_{\text{H}}$  at or below 35 beats  $\text{min}^{-1}$  during extended IBIs at the surface. This is comparable to the 24–35 beats  $\text{min}^{-1}$  measured in previous studies following both active and static breath-holds (Fahlman et al., 2020a, 2019; Noren et al., 2012, 2004; Ridgway, 1972). Similar observations have been made in California sea lions, hooded seals and emperor penguins, where minimum  $f_{\text{H}}$  observed between breaths on land or during resting in water was comparable to the minimum  $f_{\text{H}}$  observed during some periods of diving, particularly during dives below the ADL in studies of emperor penguins and California sea lions (Castellini et al., 1994a; Lin et al., 1972; McDonald and Ponganis, 2014; Meir et al., 2008; Päsche and Krog, 1980). In emperor penguins and sea lions, it was found that minimum  $f_{\text{H}}$  associated with RSA was only comparable to that of minimum  $f_{\text{H}}$  during diving for dives shorter than the species ADL (McDonald and Ponganis, 2014; Meir et al., 2008). The researchers suggested that for dives shorter than the ADL, bradycardia during diving could be regulated by a similar mechanism of cardiorespiratory control to that which drives RSA and that further reduction in  $f_{\text{H}}$  only occurs during dives past the ADL. Our data agree with this finding given that the 2 min breath-holds should be well within the calculated ADL of 6.5 min and the measured ADL of approximately 4 min of bottlenose dolphins (Fahlman et al., 2018; Williams et al., 1999).

### Additional arrhythmias present during breathing and submerged breath-holds

In addition to RSA, we identified two other arrhythmic patterns in  $f_{\text{H}}$  measurements that may be indicative of additional cardiovascular controls on  $f_{\text{H}}$  at the surface. The first was a faster oscillation with an approximate frequency of 0.1 Hz within the gradual decrease in  $f_{\text{H}}$  of the RSA (see Fig. 3A). It is worth noting that this pattern was observed during most IBIs, and has also been observed in past studies in bottlenose dolphins (Cauture et al., 2019; Fahlman et al., 2020a). Although the mechanism for these oscillations is not immediately clear, possibilities may include local fluctuations in blood pressure or the influence of respiratory drive. The second arrhythmic pattern we observed during a single IBI was a series of paired heart beats that resulted in a higher frequency oscillation at approximately 0.3 Hz. The R–R interval between paired beats was reduced, resulting in a high  $f_{\text{H}}$  followed by an extended R–R interval until the next set of paired beats, resulting in a low  $f_{\text{H}}$  (Fig. 4). This pattern was only observed during one IBI, but a similar arrhythmia has been observed in some instances in resting killer whales, pilot whales and belugas (Bickett et al., 2019; Fahlman et al., 2020b), in wild gray seals at a frequency of approximately 0.1 Hz (Thompson and Fedak, 1993), and in elite free divers (Ferrigno et al., 1991). High-frequency changes in the R–R interval in pinnipeds have been reported across multiple species, during routine diving, resting and sleeping behaviors (Andrews et al., 1997; Ponganis et al., 2017). The pathological potential of these arrhythmias is debated. Some researchers have suggested that the sympathetic stimulation associated with activity, which acts to elevate  $f_{\text{H}}$ , conflicts with parasympathetic stimulation that regulates diving bradycardia, leading to cardiac anomalies and making diving marine mammals such as bottlenose dolphins, Weddell seals and narwhals more susceptible to injury (Davis and Williams, 2012; Williams et al., 2017, 2015a,b). In contrast, some studies have suggested that when both sympathetic and parasympathetic



stimulation are highest, the variability in  $f_H$  is minimal and that benign arrhythmias are common in marine mammals (Ponganis et al., 2017). In the current study and from past observations, a range of  $f_H$  changes both while breathing spontaneously and while performing surface breath-holds are common in dolphins, killer whale, pilot whale and beluga (Fahlman et al., 2020a,b).

Compared with the surface  $f_H$  values, the patterns observed during breath-holds were more variable. This variation may be indicative of the presence of multiple factors contributing to  $f_H$  control during submersion. There were three distinct  $f_H$  modulation patterns seen during breath-hold trials: (1) a gradual decrease in  $f_H$ , like an extended RSA (Fig. 5A), (2) a rapid decrease in  $f_H$ , like submersion bradycardia (Fig. 5C), and (3) highly oscillatory and elevated  $f_H$  (Fig. 5E). The first pattern, an extended RSA-like modulation, was observed in two individual breath-hold trials, with minimum  $f_H$  around 35 and 45 beats  $\text{min}^{-1}$  (Fig. 5A). This result could suggest that  $f_H$  control during these breath-holds was under a similar mechanism to that which governs RSA at the surface and may also indicate that RSA becomes  $f_R$  independent at low breathing frequencies, as has been shown in humans (Hirsch and Bishop, 1981). The second, more rapid reduction in  $f_H$  was seen during three of the breath-hold trials (Fig. 5C). In these trials,  $f_H$  increased following the breath and then decreased rapidly before leveling off for the remainder of the breath-hold. This rapid bradycardia-like pattern could indicate a conservative response to performing a submerged breath-hold of an unknown duration where anticipation, or conditioning, alters the physiological response (Blix, 2018; Fahlman et al., 2020a; Thompson and Fedak, 1993). The final pattern, a  $f_H$  that was highly oscillatory and elevated during the entire breath-hold, was observed in one animal during two breath-holds on separate days (Fig. 5E). Across both breath-hold trials this animal displayed a mean  $f_H$  of  $97.5 \pm 11.9$  beats  $\text{min}^{-1}$  and a minimum  $f_H$  of  $67.6 \pm 6.0$  beats  $\text{min}^{-1}$ , both which are elevated compared to all other animals. For this dolphin, minimal  $f_H$  changes, e.g. RSA, were also seen during surface trials. It is possible that these differences indicate an animal that is not fully relaxed during the trials, resulting in a comparatively higher  $f_H$  during both the surface IBI and while submerged. Alternatively, it may be additional evidence of conditioned control of  $f_H$  as has been shown in both harbor porpoises, California sea lions, and bottlenose dolphins (Elmegaard et al., 2016; Fahlman et al., 2020a; Kaczmarek et al., 2018), where the dolphin is aware that it can terminate the breath-hold at any time and, for a relatively short apnea, does not require cardiovascular adjustment. These three distinct  $f_H$  responses during breath-holds are further evidence that the dive response is not a reflex and that dolphins, like other marine mammals, exhibit extensive cardiovascular plasticity.

### IBI is shorter and $f_H$ is elevated under non-fasted conditions

These data also provide an opportunity to investigate whether there are differences in cardiorespiratory control during fasted versus non-fasted surface trials that could relate to expected differences in metabolic requirements associated with feeding. We examined the relationship between surface  $f_H$  parameters and IBI duration and found several significant differences between data collected under fasted and non-fasted conditions. Generally, non-fasted trials showed increased  $f_H$ , and had a greater proportion of short IBIs and a smaller proportion of long IBIs compared with fasted trials (Table 1; Table S1). We expect an increase in metabolic overhead, or specific dynamic action (SDA), associated with digestion to result in a higher rate of  $\text{O}_2$  consumption when an animal is non-fasted (Rosen et al., 2007). This increased metabolic demand could result in a number of cardiorespiratory changes to increase  $\text{O}_2$  delivery, such as increased alveolar respiration, e.g. increased

breathing frequency, or  $V_T$ , and/or increased cardiac output or arterial/venous difference. Given that shorter IBIs are associated with higher mean  $f_H$  and the dolphins demonstrated an increased  $f_R$  during non-fasted trials, our results support the possibility of dolphins increasing their alveolar ventilation through an increase in  $f_R$ . Increased  $f_R$  would also result in increased cardiac output due to the effect of  $f_R$  on both  $f_H$  and stroke volume (Cauture et al., 2019; Fahlman et al., 2020b). Both would help to increase delivery of  $\text{O}_2$  to support the increased metabolic requirements of digestion, and thus these data suggest that cardiorespiratory function of bottlenose dolphins is responsive to metabolic changes. In gray seals it has been shown that rapid breathing and high  $f_H$  are utilized during periods associated with food processing and it was suggested that these changes may increase blood flow to splanchnic organs involved in digestion (Sparling et al., 2007).

Despite the regression analyses revealing no differences between fasted and non-fasted  $f_H$  and RSA when controlling for IBI, binned analyses suggested significant differences between some fasted and non-fasted  $f_H$  and RSA metrics within an IBI category. Although for long IBIs (IBIs > 30 s), mean  $f_H$  and minimum  $f_H$  were not significantly different between fasted and non-fasted trials (Table 1), for IBIs < 30 s there was a significant difference in both mean  $f_H$  and minimum  $f_H$  for fasted versus non-fasted trials. We found that both mean  $f_H$  and minimum  $f_H$  were higher during non-fasted as compared with fasted IBIs < 30 s, likely reflecting differences in average IBI and a shorter duration of the stabilized minimum  $f_H$  that is associated with a shorter IBI. There was not a clear association between fasted state and maximum  $f_H$ , or either index of RSA; however, for long IBIs, RSA was greater for fasted than non-fasted trials. These results further suggest the importance of considering  $f_R$  when interpreting  $f_H$  as even when  $f_H$  and RSA metrics were compared within selective bins, we still observed significant differences due to the relative difference in IBIs within each bin.

Interestingly, the mean  $f_H$  of all combined fasted surface trials closely agreed with the allometric prediction of 64.2 beats  $\text{min}^{-1}$  for the average body mass of the animals that participated in the fasted trials (Table S1) (Stahl, 1966). This suggests that the allometric relationship provided by Stahl (1966) may be extrapolated to bottlenose dolphins under fasted, resting conditions. In contrast, the mean  $f_H$  for all combined non-fasted trials was 22% greater than the predicted resting  $f_H$ , highlighting the importance of specifying the conditions under which any  $f_H$  data were collected, as has previously been suggested (Fahlman et al., 2020b). Similar findings have resulted from  $f_H$  measurements in killer whales and pilot whales (Bickett, 2017), where the resting  $f_H$  under non-fasted conditions was 42% and 39% higher, respectively, than would be predicted by Stahl's (1966) allometric relationship. However, this same study (Bickett, 2017) found that the resting  $f_H$  of a beluga under non-fasted conditions was 18% lower than that predicted allometrically. Clearly, there are important differences in cardiovascular patterns in fasted versus non-fasted animals and, like metabolic rate, it may be the case that  $f_H$  is only comparable if measured under standardized conditions (Kleiber, 1932). Then, given that data collection conditions are standardized, the elevated  $f_H$  during non-fasted compared with fasted trials may indicate the potential for  $f_H$  to be used as a proxy for changes in metabolic rate in marine mammals (Fahlman et al., 2004; Green et al., 2007; Henderson and Prince, 1914).

### Conclusions

Overall, our work shows that bottlenose dolphins exhibit RSA and that it predictably affects  $f_H$  during surface breathing. Minimum  $f_H$  during long IBIs is comparable to diving  $f_H$  reported for bottlenose

dolphins in the literature and in our submerged breath-hold trials. We suggest that the bradycardia observed during diving, particularly in dives below the ADL, may partly be an extension of normal RSA to a long duration IBI. We observed that RSA was positively correlated with IBI and suggest that this relationship is secondary to a decrease in both minimum  $f_{H}$  and mean  $f_{H}$  during the IBI. The mean  $f_{H}$  during fasted trials agreed with the allometric prediction for resting  $f_{H}$  of a bottlenose dolphin, while mean  $f_{H}$  during non-fasted trials was higher. This suggests that  $f_{H}$  may fluctuate in relation to short-term metabolic requirements and highlights the importance of reporting the conditions under which any  $f_{H}$  data are collected such that they can be accurately compared with other datasets. Although there are other factors known to influence the progression of bradycardia, we believe that a component of the change in  $f_{H}$  associated with diving is not an adaptation specifically for diving but instead is a response to apnea in an intermittently breathing mammal. Additionally, RSA may provide a new method for determining resting  $f_{H}$  of an intermittent breather. These results demonstrate the importance of RSA in influencing  $f_{H}$  variability and emphasize the need to understand differing controls on  $f_{H}$  during surface and diving behavior in bottlenose dolphins. Investigations of  $f_{H}$  in free-ranging animals to understand how diving behavior alters surface  $f_{H}$  will be informative in further understanding the controls on fine-scale changes in  $f_{H}$  in cetaceans.

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#### Competing interests

The authors declare no competing or financial interests.

#### Author contributions

Conceptualization: A.M.B., A.S.A., A.F.; Formal analysis: A.M.B.; Investigation: A.M.B., A.S.A., J.R.-L.; Resources: J.R.-L., A.F.; Writing - original draft: A.M.B., A.F.; Writing - review & editing: D.P.N., A.S.A., J.R.-L.; Supervision: D.P.N., A.F.

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#### Data availability

Data are available from GitHub: [https://github.com/ashleyblawas/Blawas\\_RSAindolphins](https://github.com/ashleyblawas/Blawas_RSAindolphins)

#### Supplementary information

Supplementary information available online at <https://jeb.biologists.org/lookup/doi/10.1242/jeb.234096.supplemental>

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Supplementary Information

**Table S1. Animal ID (ID), body mass, ( $M_b$ ), average interbreath interval (IBI) and average heart rate ( $f_H$ ) during fasted (F) and non-fasted trials (NF) at the surface where the dolphin breathes spontaneously, and during 2-minute breath-holds (BH).**

| ID             | $M_b$ (kg)        | IBI <sub>F</sub> (s) | IBI <sub>NF</sub> (s) | IBI <sub>BH</sub> (s) | $f_{H, F}$ (beats min <sup>-1</sup> ) | $f_{H, NF}$ (beats min <sup>-1</sup> ) | $f_{H, BH}$ (beats min <sup>-1</sup> ) |
|----------------|-------------------|----------------------|-----------------------|-----------------------|---------------------------------------|--|--|
| 83H1           | 147.0             | 28.3±17.6            | 13.6±9.2              | 121.6                 | 56.3±8.8                              | 72.1±8.1                               | 46.2±23.3                              |
| 01L5           | 163.7             | -                    | 15.1±6.9              | -                     | -                                     | 86.7±9.2                               | -                                      |
| 6JK5           | 200.9             | 14.6±9.6             | 13.6±9.3              | 117.1±8.1             | 73.1±8.5                              | 78.5±5.5                               | 59.1±1.5                               |
| 9ON6           | 192.8             | 16.4±10.9            | 17.2±6.8              | 128.9                 | 72.2±8.2                              | 72.4±6.4                               | 50.2±12.1                              |
| 63H4           | 179.6             | -                    | 12.0±4.4              | 130.3±6.3             | -                                     | 98.6±5.3                               | 97.4±3.2                               |
| 9FL3           | 251.7             | 26.5±17.0            | 15.6±8.4              | 135.8                 | 58.6±12.4                             | 61.7±9.8                               | 42.7±6.3                               |
| <b>Average</b> | <b>189.3±36.3</b> | <b>19.7±14.3</b>     | <b>14.6±8.0</b>       | <b>125.9±8.4</b>      | <b>67.2±11.8</b>                      | <b>78.0±13.1</b>                       | <b>64.6±23.3</b>                       |

**Table S2. Characteristics of respiratory sinus arrhythmia (RSA) during surface, fasted trials and non-fasted trials, and submerged breath-hold trials.**

| ID (n = # of breaths) | RSA (%)    | RSA (s)   | Maximum $\dot{f}_H$ (beats min <sup>-1</sup> ) | Minimum $\dot{f}_H$ (beats min <sup>-1</sup> ) |
|-----------------------|------------|-----------|--|--|
| <b>83H1</b>           |            |           |  |  |
| Fasted (n = 41)       | 70.2±25.9  | 0.82±0.31 | 77.4±14.8                                      | 38.5±8.1                                       |
| Non-fasted (n = 48)   | 37.2±17.1  | 0.36±0.20 | 83.6±7.6                                       | 57.5±11.5                                      |
| Breath-hold (n = 1)   | 209.7      | 0.21      | 120.0  | 23.0   |
| <b>01L5</b>           |            |           |  |  |
| Fasted                | -          | -         | -  | -  |
| Non-fasted (n = 58)   | 49.5±14.1  | 0.41±0.16 | 105.4±8.6                                      | 63.3±12.5                                      |
| Breath-hold           | -          | -         | -  | -  |
| <b>6JK5</b>           |            |           |  |  |
| Fasted (n = 81)       | 25.5±13.1  | 0.24±0.22 | 82.0±9.2                                       | 63.7±10.1                                      |
| Non-fasted (n = 46)   | 26.9±13.4  | 0.22±0.13 | 88.2±4.2                                       | 67.6±8.9                                       |
| Breath-hold (n = 2)   | 107.4±53.2 | 0.76±0.13 | 108.6±34.9                                     | 44.8±1.9                                       |
| <b>9ON6</b>           |            |           |  |  |
| Fasted (n = 74)       | 43.1±15.9  | 0.40±0.18 | 86.8±9.7                                       | 56.1±9.7                                       |
| Non-fasted (n = 33)   | 42.6±11.7  | 0.39±0.13 | 86.8±7.9                                       | 56.1±7.0                                       |
| Breath-hold (n = 1)   | 109.5      | 0.11      | 89.9   | 34.9   |
| <b>63H4</b>           |            |           |  |  |
| Fasted                | -          | -         | -  | -  |
| Non-fasted (n = 25)   | 32.8±15.2  | 0.23±0.15 | 111.7±4.3                                      | 79.9±12.0                                      |
| Breath-hold (n = 2)   | 51.6±7.5   | 0.38±0.09 | 118.0±2.9                                      | 67.6±6.0                                       |
| <b>9FL3</b>           |            |           |  |  |
| Fasted (n = 45)       | 72.2±24.6  | 0.84±0.35 | 80.4±13.4                                      | 39.7±10.8                                      |
| Non-fasted (n = 36)   | 59.2±21.4  | 0.65±0.28 | 80.1±12.3                                      | 44.1±8.9                                       |
| Breath-hold (n = 1)   | 82.2       | 0.90      | 69.2   | 34.0   |

**Table S3. Results of linear mixed effects models of the effect of the previous inter-breath interval (IBI) duration on the maximum  $if_H$  of the IBI and the difference between the maximum  $if_H$  of the IBI and the minimum  $if_H$  of the previous IBI during surface breathing trials.** Data were  $\log_{10}$ -transformed for analysis. Individual ID was included as a random effect and nutritional state (fasted vs. non-fasted) was included as a fixed effect.

| <b>Response variable</b>   | <b>Independent variables</b>       | <b>Value</b> | <b>S.E.</b> | <b>P-value</b>    |
|--|------------------------------------|--------------|-------------|-------------------|
| Maximum $if_H$ (beats $\text{min}^{-1}$ )                              | nutritional state                  | -0.07        | 0.02        | <b>&lt;0.01</b>   |
|  | previous IBI                       | -0.07        | 0.01        | <b>&lt;0.0001</b> |
|  | nutritional state*<br>previous IBI | 0.05         | 0.02        | <b>0.02</b>       |
| Maximum $if_H$ – previous<br>Minimum $if_H$ (beats $\text{min}^{-1}$ ) | nutritional state                  | -0.12        | 0.07        | 0.09              |
|  | previous IBI                       | 0.23         | 0.04        | <b>&lt;0.0001</b> |
|  | nutritional state*<br>previous IBI | 0.10         | 0.06        | 0.08              |

Figure S1. Effect of duration of the previous inter-breath interval (IBI) on A) maximum  $if_H$  and B) the difference between the maximum  $if_H$  of the IBI and the minimum  $if_H$  of the previous IBI during surface breathing trials. Data were  $\log_{10}$ -transformed for plotting.

Fasted data points are plotted in red and non-fasted data points are plotted in blue.

