Using Respiratory Sinus Arrhythmia to Estimate Inspired Tidal Volume in the Bottlenose Dolphin (*Tursiops truncatus*)

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Man-made environmental change may have significant impact on apex predators, like marine mammals. Thus, it is important to assess the physiological boundaries for survival in these species, and assess how climate change may affect foraging efficiency and the limits for survival. In the current study, we investigated whether the respiratory sinus arrhythmia (RSA) could estimate tidal volume (V_T) in resting bottlenose dolphins (*Tursiops truncatus*). For this purpose, we measured respiratory flow and electrocardiogram (ECG) in five adult bottlenose dolphins at rest while breathing voluntarily. Initially, an exponential decay function, using three parameters (baseline heart rate, the change in heart rate following a breath, and an exponential decay constant) was used to describe the temporal change in instantaneous heart rate following a breath. The three descriptors, in addition to body mass, were used to develop a Generalized Additive Model (GAM) to predict the inspired tidal volume (V_{Tinsp}). The GAM allowed us to predict V_{Tinsp} with an average ($\pm$ SD) overestimate of 3 $\pm$ 2%. A jackknife sensitivity analysis, where 4 of the five dolphins were used to fit the GAM and the 5th dolphin used to make predictions resulted in an average overestimate of 2 $\pm$ 10%. Future studies should be used to assess whether similar relationships exist in active animals, allowing V_T to be studied in free-ranging animals provided that heart rate can be measured.

Keywords: electrocardiogram, spirometry, marine mammals, diving physiology, cardiorespiratory

INTRODUCTION

Marine mammals forage underwater to obtain food and therefore divide their time at the surface to exchange gasses (O_2 and CO_2) with submersions to different depth and of varying durations. Therefore, a better understanding of the metabolic costs associated with underwater foraging, and proxies to assess energy use would help determine how environmental change may alter survival. By increasing the duration underwater, the opportunity to encounter and obtain food, and thereby the foraging efficiency, should be increased. Man-made environmental change such as over-fishing and global warming could cause changes in prey diversity, availability and location

(Perry et al., 2005), which may have detrimental effects on apex marine predators like dolphins. Changes in prey type, abundance, and distribution could result in increases in both foraging duration and distance in order to obtain enough prey for survival. Overfishing will reduce the probability to encounter food, and movement of prey to deeper depths due to ocean warming will increase the transit time and reduce the available time at the prey patch. Longer foraging bouts, and/or deeper dives may reduce the foraging efficiency and thereby cause challenges to obtain sufficient food for survival (Perry et al., 2005). Thus, understanding the cardiorespiratory traits required by marine mammals to manage life in an extreme environment, the physiological constraints imposed on these animals, and how these limitations may affect physiology and survival are crucial.

When studying animals in the wild, measuring the metabolic cost directly is challenging, and a number of proxies have been proposed and tested. One method is to measure the resting metabolic rate (RMR) by measuring the O_2 consumption rate ($\dot{V}O_2$) during rest (Williams et al., 1993; Yazdi et al., 1999; Kastelein et al., 2000; Yeates and Houser, 2008; Noren et al., 2013; Rechsteiner et al., 2013; Worthy et al., 2013; van der Hoop et al., 2014; Fahlman et al., 2015), and a few studies have determined the diving and foraging metabolic rate of marine mammals during quasi-natural conditions (Kooyman et al., 1973; Sparling and Fedak, 2004; Fahlman et al., 2008, 2013). While RMR may not accurately reflect field metabolic rate (FMR), it provides an index about the minimal metabolic requirements of an individual or population against which FMR can be scaled (Bejarano et al., 2017). One method to scale FMR is to estimate FMR by validated metabolic proxies, such as heart rate (f_H) (Young et al., 2011), activity (Enstipp et al., 2011; Fahlman et al., 2013), or respiratory frequency (f_R) (Fahlman et al., 2016, 2017b; Folkow and Blix, 2017). Combining these methods, the metabolic costs for different populations and activities, such as resting, traveling, and foraging, can be defined. The Fick principle states that: $\dot{V}O_2 = f_R \times V_T \times (\Delta O_2)$, where V_T is tidal volume and ΔO_2 the O_2 extracted from the air inhaled with each breath. By assuming that V_T and ΔO_2 are constant at steady state, it should be possible to estimate $\dot{V}O_2$ from f_R (Folkow and Blix, 1992; Christiansen et al., 2014; Fahlman et al., 2016). While marine mammals are at the surface, f_R can be assessed during focal observations. However, this is not practical during long periods at sea. In addition, studies have shown that both V_T and ΔO_2 change for different activities or during recovery from exercise (Fahlman et al., 2016, 2017b; Folkow and Blix, 2017), so the estimated $\dot{V}O_2$ could be improved by also estimating V_T and ΔO_2. Consequently, methods to assess pattern of breathing (f_R, V_T) would provide significant advances to estimate FMR in marine mammals.

Proxies to estimate FMR from breaths should accurately predict f_R and V_T during continuous recording from free ranging animals (Fahlman et al., 2016; Rojano-Doñate et al., 2018). Such data would allow an assessment of how changes in foraging effort (duration, activity, etc.) alter respiratory function, and estimated FMR. A number of studies have assessed lung function in marine mammals under human care (Olsen et al., 1969; Kerem et al., 1975; Matthews, 1977; Kooyman and Cornell, 1981; Fahlman

et al., 2015, 2018a,b, 2019; Fahlman and Madigan, 2016), and at least in the bottlenose dolphin (*Tursiops truncatus*) these data are representative of their wild counterparts, in both shallow and deep diving ecotypes (Fahlman et al., 2018a,b). Such data are important to establish baseline lung function from animals with known health under controlled situations, and provide methods that will allow proxies to be validated that can predict respiratory effort in free ranging animals.

Estimating lung function of wild populations remains difficult. One alternative proxy could be to use the changes in f_H associated with each breath, the Respiratory Sinus Arrhythmia (RSA) (de Burgh Daly, 1986). While RSA is universally present in a number of air-breathing vertebrates such as the toad, horse, dog, seal, and dolphin (Scholander, 1940; Hayano et al., 1996; Cooper et al., 2003; Noren et al., 2004; Harms et al., 2013; Zena et al., 2017; McDonald et al., 2018; Yaw et al., 2018; Piccione et al., 2019), and even in air-breathing fish (Grossman and Taylor, 2007), its physiological significance is debated (Hayano et al., 1996; Yasuma and Hayano, 2004). It has been suggested that RSA improves gas exchange by enhancing the ventilation-perfusion matching and reduces cardiac work (Yasuma and Hayano, 2004; Ben-Tal et al., 2012, 2014). The RSA causes f_H acceleration during inspiration, and deceleration during expiration (Mortola et al., 2015). Thus, continuous recordings of f_H could allow detection of f_R, which when appropriately validated provide ways to estimate field metabolic rate (Fahlman et al., 2016; Rojano-Doñate et al., 2018). Considering recent progress in the development of biologging system that allow continuous recording of the electrocardiogram (ECG) in free-ranging cetaceans (Elmegaard et al., 2016; McDonald et al., 2018), we speculated that RSA may provide a novel method to estimate V_T in bottlenose dolphins. Currently, there is limited availability of commercial data loggers that can measure continuous ECG, and custom built devices range from units with implantable electrodes used in pinnipeds or diving birds (Thompson and Fedak, 1993; Woakes et al., 1995; McDonald and Ponganis, 2014), to those that are attached externally using suction cups (Noren et al., 2004; Elmegaard et al., 2016).

In the current study, we tested the hypothesis that RSA can estimate V_{Tinsp} in resting bottlenose dolphins by recording f_H and respiratory flow while resting at the surface. Our results provide evidence that using the RSA as a proxy allows us to estimate the average V_{Tinsp} of individual dolphins with an average ($\pm$SD) overestimation of $2 \pm 10\%$ with the data recorded.

MATERIALS AND METHODS

Animals

The study protocols were approved by the Animal Care and Welfare Committee of the Oceanogràfic Foundation (OCE-17-16 and amendment OCE-29-18). Five adult male bottlenose dolphins (*T. truncatus*), housed at Dolphin Quest – Oahu (Honolulu, HI, United States), were used for all the experiments (**Table 1**). All experiments were conducted in January 2018. The dolphins were not restrained and could end the trial at any point. Prior to initiating the study, the dolphins were desensitized to the equipment and trained for novel research-associated

Dolphin ID	M_b (kg)	N	V_{Tinsp} (l)	V_{Tinsp} range (l)
9FL3	235.4	73	3.6 ± 1.0	1.6 – 6.2
01L5	154.6	91	3.2 ± 0.5	2.1 – 4.2
83H1	139.6	53	3.3 ± 0.8	1.8 – 5.6
9ON6	184.1	53	3.9 ± 0.6	2.7 – 5.6
6JK5	206.8	27	4.9 ± 1.2	2.7 – 6.9

behaviors using operant conditioning. Each trial consisted of the animal staying stationary in the water, allowing placement of the equipment. The animals were breathing while continuous measurements were made. Because of familiarity with these procedures, we assumed that the experimental data collected on lung function (respiratory flow) and f_H were representative of a relaxed physiological state.

Data Acquisition

A custom-made Fleisch type pneumotachometer (Mellow Design, Valencia) utilizing a low-resistance laminar flow matrix (Item # Z9A887-2, Merriam Process Technologies, Cleveland, OH, United States) was placed over the blow-hole of the dolphin (Fahlman et al., 2015). Differential pressure across the flow matrix was measured using a differential pressure transducer (ML311 Spirometer Pod, ADInstruments, Colorado Springs, CO, United States), connected to the pneumotachometer with two, 310 cm lengths of 2 mm I.D., firm walled, flexible tubing. The pneumotachometer was calibrated using a 7.0 l calibration syringe (Series 4900, Hans-Rudolph Inc., Shawnee, KS, United States). The signal was integrated and the flow determined assuming a linear response between differential pressure and flow. The linear response of the pneumotachometer was confirmed by calibrating with the 7.0 l syringe immediately before and after each trial, through a series of pump cycles at various flows. The pump cycles allowed the relationship between differential pressure and flows for the expiratory and inspiratory phases to be determined. All gas volumes were converted to standard temperature pressure dry (STPD) (Quanjer et al., 1993). Exhaled air was assumed saturated at 37°C, inhaled air volume was corrected for ambient temperature and relative humidity, and V_T was calculated by integrating the flow as previously detailed (Fahlman et al., 2015).

The electrocardiogram (ECG) was recorded using three gold-plated electrodes mounted inside a silicone suction cup connected to a custom-built data recorder (UUB/1-ECGb, UFI, Morro Bay, CA, United States). The three electrodes were placed on the ventral surface: red on the right side close to the pectoral fin, yellow opposite on the left side, and green on the right side approximately 30 cm more caudally from the red. The suctions cups were filled with conducting gel (Redux Gel, Parker Laboratories) before being placed on the skin. Next, the animal rolled over to ensure the suction cups stayed in place.

The respiratory flow and ECG were recorded at 400 Hz using a data acquisition system (Powerlab 8/35, ADInstruments, Colorado Springs, CO, United States), and displayed on a

computer running LabChart (v. 8.1, ADInstruments, Colorado Springs, CO, United States). Initially, the electrodes were adjusted to assure a clear ECG trace. Next, the pneumotachometer was placed over the blow-hole and the animal allowed to breathe spontaneously for up to 10 min.

We used the ECG analysis routine in LabChart to automatically detect the time between R-R peaks using the following settings; typical QRS width = 80 ms, R-waves = 300 ms, pre-P baseline = 120 ms, maximum PR = 240 ms, maximum RT = 400 ms. The detected R peaks were then manually verified and the instantaneous heart rate (if_H) determined from the time between R-R peaks.

Data Processing, Statistical Analysis and Modeling

All data were analyzed using R (version 3.4.3 – © 2017 The R Foundation for Statistical Computing) through RStudio (version 1.1.383 – © 2009–2017 RStudio, Inc.). Initially the temporal changes in if_H were described for each breath. We used a function that fit the exponential decay with time following the beginning of the inspiration for each breath:

$$if_H = \text{Base Heart Rate} + e^{-\text{Decay rate} \times \text{Time}} \quad (1)$$
$$\times \text{Initial change in heart rate}$$

Equation 1 was fit for each breath using the "L-BFGS-B" method of the "optim" function (Byrd et al., 1995), which optimizes parameters between imposed bounds to restrain parameters to physiologically relevant values. Breaths with fewer than seven beats after the inhalation were excluded (44 breaths).

Next the three parameters from Eq. 1 (Base Heart Rate, Decay rate, and initial change in heart rate) for each breath, and body mass (M_b) were fit against inhaled V_T (V_{Tinsp}) using a loess Generalized Additive Model (GAM) (Cleveland, 1979; Hastie and Tibshirani, 1990), with the span fixed at 0.34.

To assess the sensitivity of the model, we generated five different GAMs by excluding all observations from one dolphin each time. The data from the excluded dolphin was then used to predict V_{Tinsp}. The error was computed using the formula:

$$\text{Prediction error} = \frac{(\text{Predicted} - \text{Measured})}{\text{Measured}} \times (-100) \quad (2)$$

where a positive value represents an overestimated prediction.

Dolphin ID	Base f_H	Decay	Δf_H	Average V_{Tinsp}
9FL3	34.1	0.0362	37.2	3.6
01L5	37.9	0.0486	46.2	3.2
83H1	39.7	0.0523	46.8	3.3
9ON6	41.2	0.0311	50.5	3.9
6JK5	48.6	0.0280	49.2	4.9
Mean (±SD)	40.3 ± 5.4	0.0393 ± 0.0108	46.0 ± 5.2	3.8 ± 0.7

RESULTS

Data Used for the Analysis

A total of 297 breaths were analyzed following removal of breaths with less than seven heart beats between breaths (**Table 1**). Only spontaneous breaths were used for the analysis, which limited the range of V_T's. In addition, as not all inspired and expired volumes are similar for each breath, we only used the V_{Tinsp} for the analysis.

The average (±SD) V_{Tinsp} was 3.8 ± 0.7 l (range: 1.6–6.9 l, see **Table 1** for individual variation), and the average duration between breaths was 15.3 ± 10.7 s (range: 4–129 s). The average

if_H was 74 ± 24 beats min^{-1} (range: 27–293 beats · min^{-1}). The average fit parameters for Equation 1 for each dolphin are reported in **Table 2**.

Predicting V_T From Instantaneous f_H

Figure 1 shows a representative ECG trace, if_H, and respiratory flow in a dolphin over 3 breaths. The average conditions for estimating V_{Tinsp} are reported in **Table 2**, and the GAMs overestimated V_{Tinsp} by an average 3 ± 2% (range of individual average error: 0.3 to 7.4%, **Figure 2**). A sensitivity analysis was performed to assess how the prediction changed with changes in each variable (**Figure 3**). The decay rate and M_b had less influence
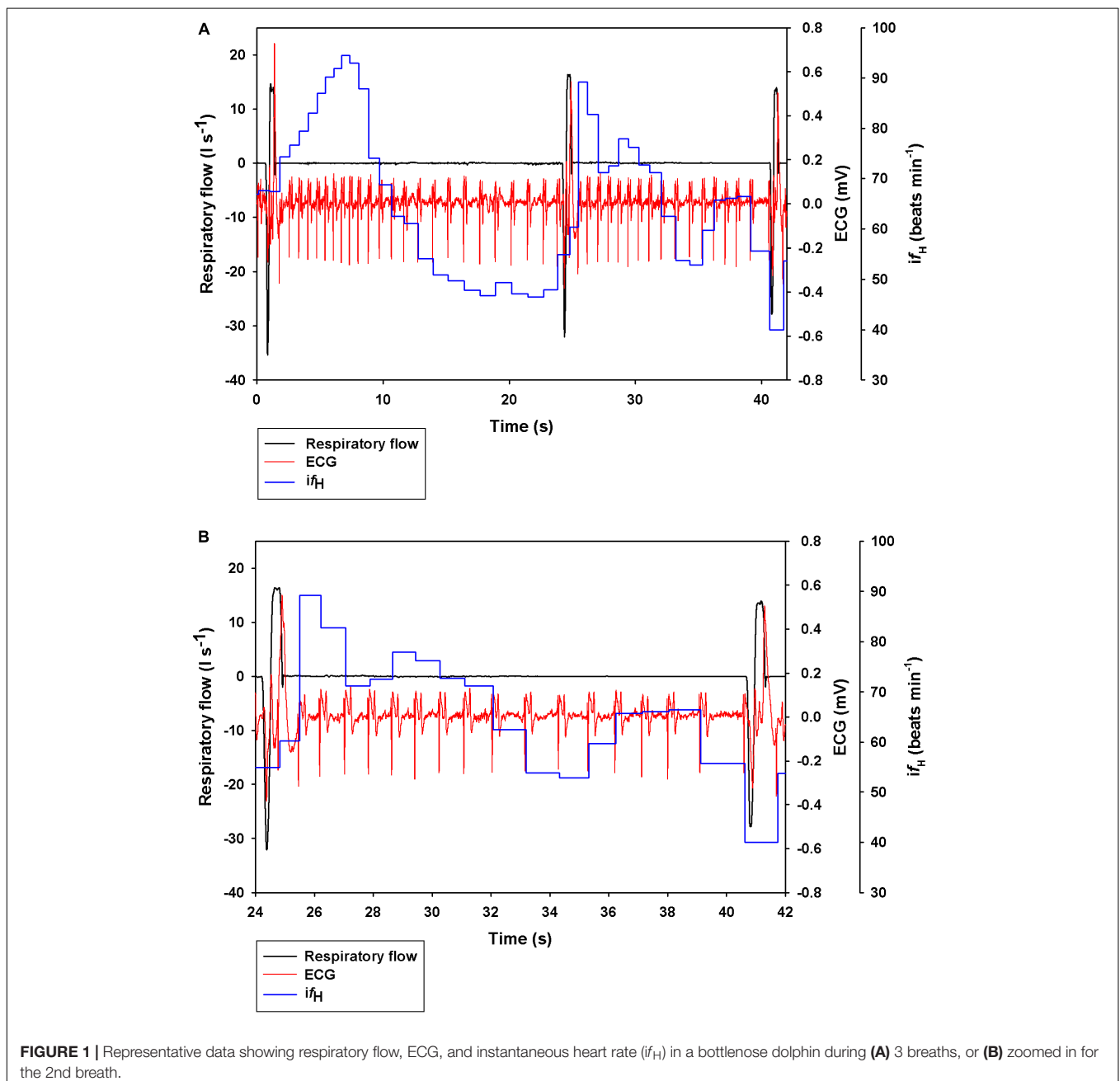

FIGURE 1 | Representative data showing respiratory flow, ECG, and instantaneous heart rate (if_H) in a bottlenose dolphin during **(A)** 3 breaths, or **(B)** zoomed in for the 2nd breath.
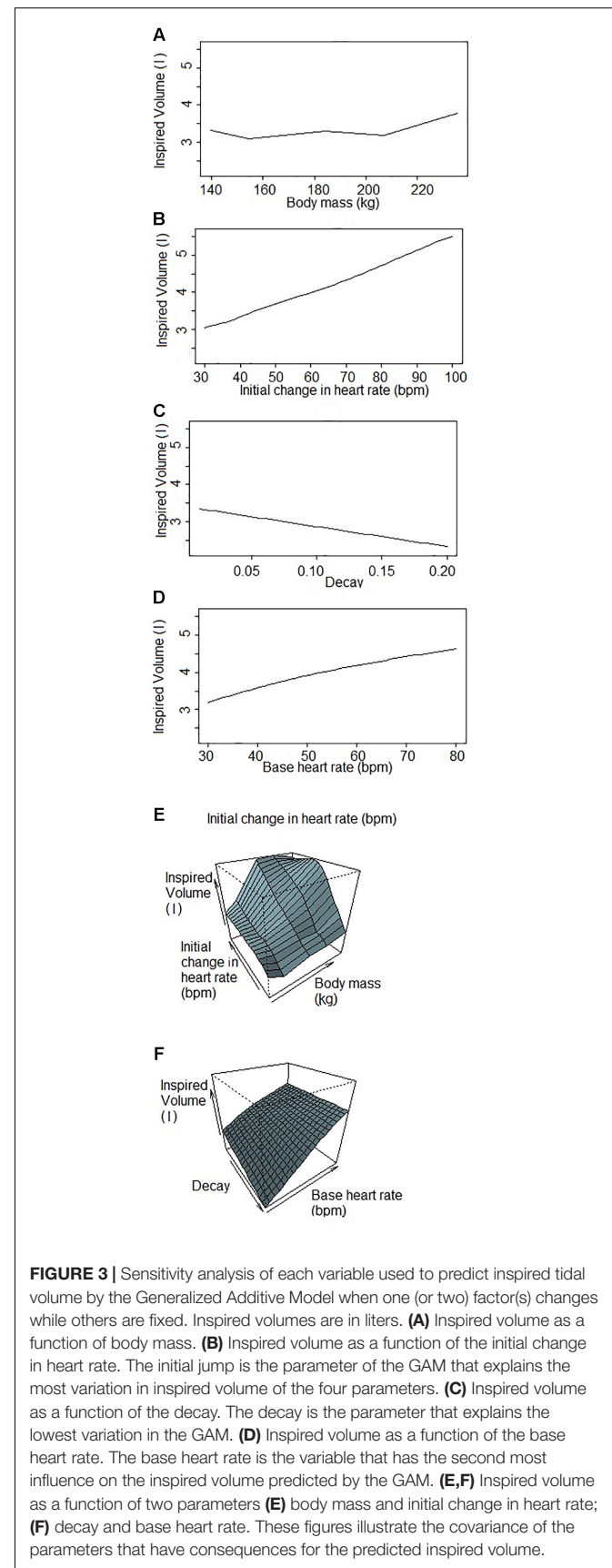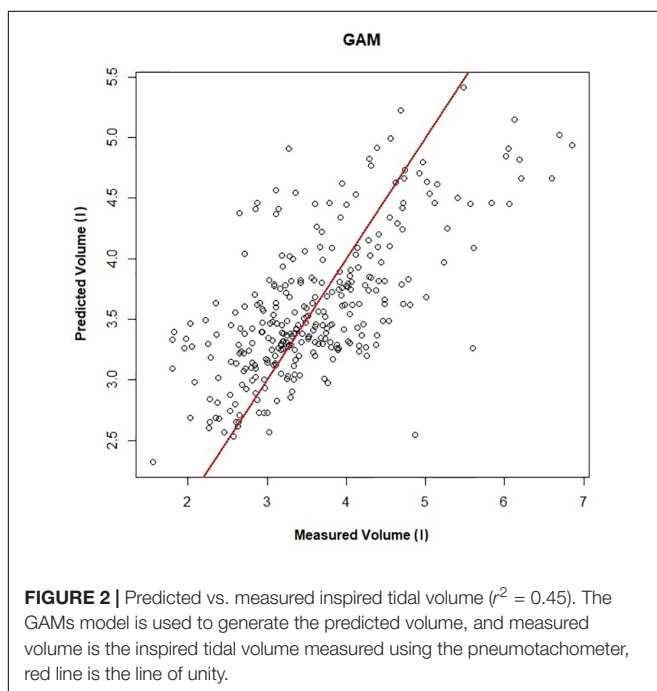
on the model output as compared with base f_H and the initial change in f_H.
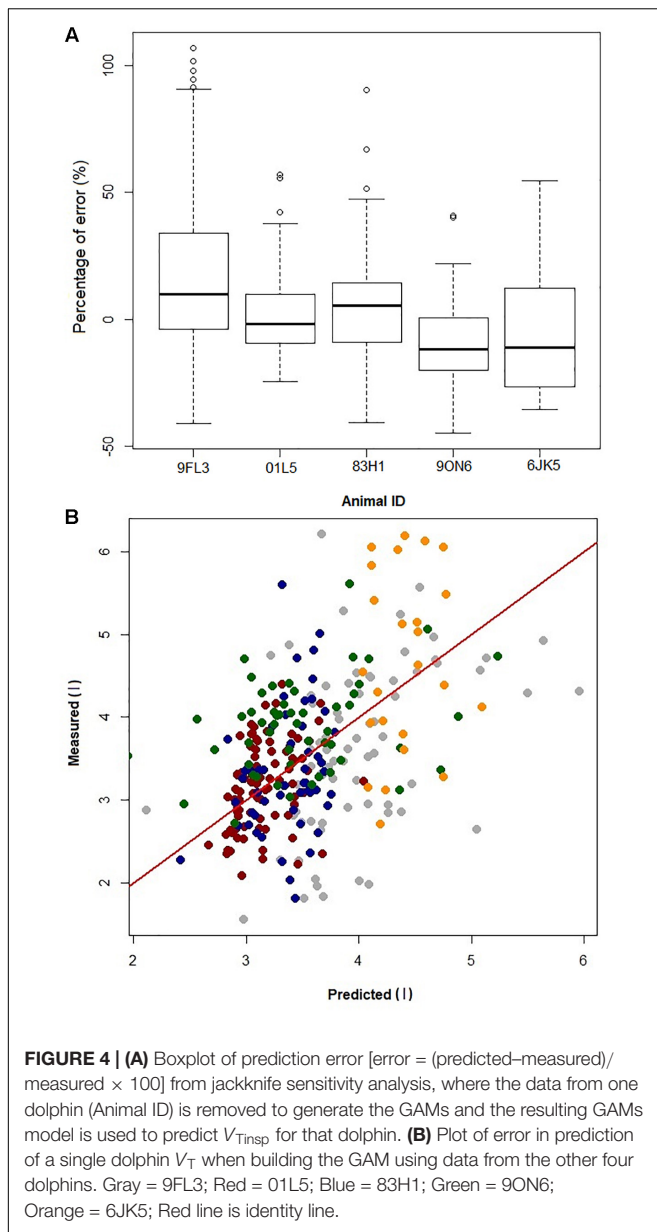
By removing one dolphin, fitting the GAMs with the four dolphins, and then predicting V_{Tinsp} for the 5th dolphin resulted in an average ($\pm$SD) overestimation of $2 \pm 10\%$, (range of individual average error: -10 to 18%, **Figures 4A,B**). The error for individual breaths ranged from 107 to -45%, with 95% confidence limits ranging between 12 to -7% (median: 12 to -10%, **Figure 4B**).

DISCUSSION

The main objective with the current study was to determine if the changes in f_H associated with RSA can be used to predict the V_{Tinsp} in the bottlenose dolphin. For this purpose, we collected continuous ECG and respiratory flow in bottlenose dolphins. A jackknife method to resample the data showed that RSA, in addition to M_b, can be used to predict the average V_{Tinsp} of an individual dolphin to within $2 \pm 10\%$ of the measured value, and all individual average prediction errors were less than 20%. This shows that the GAMs should be able to predict the average V_{Tinsp} of individual dolphins using data from another population of bottlenose dolphins. If future studies can verify a similar relationship in active animals, RSA could be a useful proxy to estimate V_{Tinsp} from free-ranging marine mammals as methods to continuously measure f_H are developed.

The average if_H reported in the current study was similar to those reported in previous studies in the bottlenose dolphin (ranging from 60 to 105 beats min^{-1}) (Noren et al., 2004, 2012; Houser et al., 2010), when the f_H is calculated without accounting for the RSA. However, in our past study, using trans-thoracic echocardiography to measure f_H and stroke volume, it was

FIGURE 2 | Predicted vs. measured inspired tidal volume ($r^2 = 0.45$). The GAMs model is used to generate the predicted volume, and measured volume is the inspired tidal volume measured using the pneumotachometer, red line is the line of unity.

FIGURE 3 | Sensitivity analysis of each variable used to predict inspired tidal volume by the Generalized Additive Model when one (or two) factor(s) changes while others are fixed. Inspired volumes are in liters. **(A)** Inspired volume as a function of body mass. **(B)** Inspired volume as a function of the initial change in heart rate. The initial jump is the parameter of the GAM that explains the most variation in inspired volume of the four parameters. **(C)** Inspired volume as a function of the decay. The decay is the parameter that explains the lowest variation in the GAM. **(D)** Inspired volume as a function of the base heart rate. The base heart rate is the variable that has the second most influence on the inspired volume predicted by the GAM. **(E,F)** Inspired volume as a function of two parameters **(E)** body mass and initial change in heart rate; **(F)** decay and base heart rate. These figures illustrate the covariance of the parameters that have consequences for the predicted inspired volume.

FIGURE 4 | **(A)** Boxplot of prediction error [error = (predicted–measured)/measured × 100] from jackknife sensitivity analysis, where the data from one dolphin (Animal ID) is removed to generate the GAMs and the resulting GAMs model is used to predict V_{Tinsp} for that dolphin. **(B)** Plot of error in prediction of a single dolphin V_T when building the GAM using data from the other four dolphins. Gray = 9FL3; Red = 01L5; Blue = 83H1; Green = 9ON6; Orange = 6JK5; Red line is identity line.

f_H in the bottlenose dolphin. If this value is used, it provides an interesting perspective as that value is similar to the diving bradycardia reported in previous studies (Noren et al., 2012). Thus, we propose that future studies should evaluate the resting f_H in voluntary diving animals after correcting for the RSA.

While the current method clearly shows that RSA is useful to estimate V_{Tinsp}, there are a number of limitations with the current method. First of all, due to the limited data set, we aimed to reduce the number of parameters used in the model. To simplify the analysis the current method did not include the duration between breaths. We analyzed each breath separately, which was both time consuming and does not account for the dependence between breaths. However, breathing and f_H are continuous data. Future studies could assess time-series methods to predict V_{Tinsp}, which allows the dependence between breaths to be considered. For Equation 1, there was considerable variation in the fitted values for the base f_H. When accounting for the changes in f_H associated with a breath there is usually minimal variation in the base f_H within one dolphin (Miedler et al., 2015). The large variation could be related to varying duration between breaths, which may alter the base f_H. Thus, for breaths close together the f_H may not have reached the base f_H before the next breath, and may have influenced the f_H variation for the next breath. Based on the current analysis, this method cannot accurately predict the V_{Tinsp} of individual breaths, but was able to provide reliable average estimated V_{Tinsp} for each animal based on the GAMs fitted to the other animals. This is similar to the method using f_H to estimate field metabolic rate, where the there are limitations to estimate the metabolic cost for each dive, but where large data sets are able to estimate the energy requirements for different activities (Fahlman et al., 2004; Halsey et al., 2007; Young et al., 2011). Given the limitations with the current data, we propose that further development of this method may provide an interesting approach to study cardiorespiratory physiology in free-ranging marine mammals.

The V_{Tinsp} in the current study were of limited range (average ± SD = 3.8 ± 0.7 l, range: 1.6–6.9 l). While variation in the V_{Tinsp} during voluntary breaths is difficult to control, animals under managed care can be trained to perform maximal respiratory effort, which allows V_T to vary over a much greater range (Kooyman and Cornell, 1981; Fahlman et al., 2015). For example, maximal respiratory efforts in dolphins in the weight range of the current study would increase the V_T range to around 20 l (Kooyman and Cornell, 1981; Fahlman et al., 2015). It would also be useful to include females and individuals from other age classes to increase the range of variation and allow the model to be used for wild dolphin populations.

In addition, future studies should also assess whether this method is robust enough to study active or free-ranging dolphins. During natural dives f_R and V_T may both be highly variable and irregular, and alterations in the relationship between RSA, vagal tone, f_R, and V_T (de Burgh Daly, 1986; Ben-Tal et al., 2014; Guillén-Mandujano and Carrasco-Sosa, 2014; Mortola et al., 2016) may limit the accuracy of a model developed for dolphins at rest. Thus, changes in activity state, e.g., exercise, rest, travel, diving, may significantly influence autonomic tone and alter the relationship.

pointed out that estimating f_H without accounting for the RSA will result in average surface f_H values that are confounded by the f_R (Miedler et al., 2015). This is particularly problematic in marine mammals, with an f_R ranging from 1 to 5 breaths · min^{-1} (Piscitelli et al., 2013; Fahlman et al., 2017a). Consequently, estimating resting f_H without accounting for the RSA will overestimate the resting f_H. As these resting f_H's have been used to assess the magnitude of the cardiovascular changes associated with diving they would erroneously overestimate the magnitude of the dive response (Fahlman et al., unpublished). The average base f_H in the current study (**Table 2**, 40 ± 5 beats min^{-1}) was similar to those reported in our past study in the bottlenose dolphin (f_H = 41 ± 9 beats min^{-1}) (Miedler et al., 2015), where the RSA was accounted for. Consequently, the base f_H reported in the current study is a more appropriate value for the resting

Finally, the GAM model does not provide an estimate of uncertainty around the predicted values with new data (data that were not in the dataset used to fit the model), nor does it provide a prediction equation for the non-parametric part of the model. To avoid these drawbacks, additional measurements with a larger range of V_{Tinsp}, f_R, and activity states would help define a prediction equation that could be used in free-ranging dolphins. In addition, we propose that this method could be used for other cetaceans and marine mammal species that exhibit significant RSA. If future studies are able to verify that this method is able to estimate f_R and V_{Tinsp} in actively swimming or diving dolphins this method may provide a predictive procedure for free-ranging mammals that may significantly enhance our knowledge of how marine mammals partition energy use during diving, and how the environment may limit foraging efficiency.

In summary, we show that that RSA can be used to accurately predict the average V_{Tinsp} of individual resting bottlenose dolphins with an average overestimated error of $2 \pm 10\%$. While a number of factors appear to alter RSA (de Burgh Daly, 1986), the universal existence of RSA in vertebrates, and the suggestions that it is independent of body size (Piccione et al., 2019), could provide a method to study cardiorespiratory physiology in free ranging marine vertebrates, from marine mammals, to birds and reptiles, unraveling important mechanisms to understand the ecophysiology of these species.

DATA AVAILABILITY STATEMENT

The data used in this study are freely available at the following link: osf.io/buwdp.

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AUTHOR CONTRIBUTIONS

FC performed the data and statistical analysis and drafted the first draft of the manuscript. BS-B provided statistical advice and edited the manuscript. JR-L supervised all animal training and helped with all research trials. CH helped conceive the study and helped consult with the methods. SM helped with the trials and the analysis. AF conceived the study, designed the experiments, collected the data, provided funding, helped with the data analysis, and helped to draft the manuscript. All authors helped to revise the various drafts and gave final permission to publish the study.

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