

A Multi-Modal Approach for Investigating the Physiological Responses to Breath-Holding
in Diving Mammals

by

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Dissertation submitted in partial fulfillment of
the requirements for the degree of Doctor
of Philosophy in the Department of
Marine Science and Conservation in the Graduate School
of Duke University

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ABSTRACT

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Abstract

The ocean environment poses several adversities to usual mammalian function. Perhaps most consequential to life is the lack of air underwater. For marine mammals, like whales and dolphins, that are required to perform breath-hold dives to forage for prey, this necessitates a unique set of adaptations to efficiently manage oxygen resources while diving. In an era of global environmental change, this hostile habitat is expected to become increasingly challenging for air-breathing mammals; warming waters will necessitate deeper foraging trips and noisier oceans may compel unplanned dives to evade perceived threats. An understanding of marine mammals' solutions to the physiological challenges of a dually-constrained lifestyle is therefore important not only to reveal how marine mammals are built to thrive where other mammals, particularly humans, falter but also the extent to which these adaptations may scale in a changing ocean environment. In this dissertation, I explore the physiological adaptations, particularly those of the cardiovascular and respiratory systems, that this taxon has evolved to mediate the challenges associated with breath-hold diving. I take a multi-scale approach to investigating these physiological traits, exploring hypotheses at the molecular, tissue-specific, and organismal scales. Accordingly, I leverage both familiar and emerging methodologies in the field of marine mammal physiology to examine adaptations that support the extended dive capacities of whales and dolphins.

Cellular and molecular responses to environmental stimuli influence tissue-specific and organismal physiological responses. Despite the inextricable link between molecular and organismal physiology, studies of the molecular adaptations of marine mammals for diving are limited, in part due to the logistical complexity of obtaining molecular samples from this difficult-to-study group. To fill this gap, my collaborators and

I deployed RNA-seq and enzymatic assays to examine the molecular-level changes that occur in bottlenose dolphins (*Tursiops truncatus*) performing extended breath-holds (Chapter 1). We demonstrated that dolphins exhibit transcriptomic and proteomic changes that occur in a time-dependent fashion during breath-holding that could support their ability to maintain selective perfusion during diving. The upregulation of ALOX5, a gene targeted for the treatment of eosinophilic asthma in humans, and lipoxygenase suggest a mechanism by which differential gene regulation could contribute to sustained vasoconstriction during the dive response. These findings illustrate the importance of responses at the molecular level for supporting the unique physiology of marine mammals.

Coordinated, tissue-specific physiological changes are central to the mammalian dive response. During dives marine mammals drastically reduce their heart rate (f_H) while narrowing the blood vessels that supply their peripheral tissues, thereby slowing oxygen consumption of the heart itself as well as reducing the supply of oxygen-rich blood to non-essential tissues. The factors that modulate f_H and contribute to diving bradycardia are complex, largely because they are numerous and often linked, but are crucial to understanding oxygen consumption patterns and, ultimately, whole-organism physiology and behavior. Using simultaneous electrocardiographic (ECG) recordings and respirometry, I show that whales and dolphins exhibit a strong cardiorespiratory coupling that may support the conservation of blood oxygen for hypoxia-intolerant tissues during a breath-hold. This variation in f_H with breathing, or respiratory sinus arrhythmia (RSA), is modulated by breathing rate (f_R) in bottlenose dolphins such that slow breathing results in larger fluctuations in f_H (Chapter 2). Following a breath, f_H increases rapidly to a maximum and then decreases through the end of the inter-breath

interval (IBI). Notably, some of the minimum f_H 's of the RSA were comparable to reported diving f_H 's for this species suggesting the importance of apnea alone in modulating the f_H of a diving marine mammal. I also demonstrate that this cardiorespiratory coupling scales with body size and f_R across five cetacean species suggesting both physical scaling laws and dynamic physiological needs play a role in determining the magnitude of the RSA (Chapter 3). These studies highlight the complexity of tissue-specific responses and the need to contextualize physiological rates.

Ultimately, it is the interactions of tissues that determine organismal physiology – the fundamental constraint on an organism's behavior. To investigate the connection between organismal physiology and behavior, I developed a novel method for extracting f_R from free-ranging whale biologging tag data (Chapter 4). I found that the high-flow rate and large tidal volume breaths of cetaceans generate movement signals which are captured by the accelerometers of biologging tags, enabling respiration event detection from historical biologging tag datasets. I applied this tool to movement data collected from short-finned pilot whales in Cape Hatteras, North Carolina using digital acoustic recording tags (DTAGs) and examined variation in respiratory patterns associated with diving (Chapter 5). I found that whales vary their pre- and post-dive surface duration and post-dive f_R in proportion to the duration and activity of upcoming dives illustrating the physiological challenge of preparing for and recovering from breath-hold diving and highlighting optimization of surface behavior required to support breath-holds.

Physiological responses are coordinated across multiple levels of biological organization necessitating the use of various tools and techniques to fully elucidate the adaptations that support marine mammals' capacity to dive for minutes to hours without

a breath. The findings of this dissertation underscore that the physiological function of breath-holding whales and dolphins is coordinated across scales, the physiological responses of cardiovascular and respiratory systems are linked, and sensing vital rates can provide insights into the physiological demands of a dive. Future studies should continue to focus on integrating methods across scales to better understand the physiological function of these animals and its plasticity in a changing ocean.

Dedication

To my parents, Dr. Amy and Mike Blawas, your endless love and support have made everything I have, and will, accomplish possible.

To my sisters, Megan and Camryn, who are voices of reason, give the best advice, and have given me endless encouragement.

Co-Authorship Statement

The work presented in this dissertation is part of several collaborative studies. Several colleagues contributed to this research and are (or will be) listed as co-authors on modified versions of each data chapter submitted for peer-review to scientific journals. Jason Somarelli was the principal investigator for Chapter 1 and the work was made possible by a team that also included Kathryn Ware, Emma Schmaltz, Larry Zheng, Jacob Spruance, Austin Allen, Nicole West, Nicolas Devos, David Corcoran, Douglas Nowacek, William Eward, and Andreas Fahlman. Andreas Fahlman was the principal investigator for Chapters 2 and 3 and the work was supported by Douglas Nowacek, Austin Allen, Julie Rocho-Levine, and Todd Robeck. Douglas Nowacek, Andrew Read, and Andreas Fahlman contributed to Chapters 4 and 5.

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1. Introduction

Marine mammals are obligate air-breathers, like humans and other lung-bearing vertebrates, yet their fully-aquatic lifestyle requires that they make excursions from the water's surface to depth to forage upon prey in the water column. This necessitates extended breath-holds during which oxygen (O_2) resources become fixed and the ability to release gaseous by-products of cellular respiration, namely carbon dioxide (CO_2), is eliminated. The use of O_2 should, therefore, in theory, be optimized and directed only to the most critical tissues during diving to maximize dive duration. Yet, dives require exercise to navigate through the water column and chase prey, creating a conflict between exercising muscles and O_2 -conserving responses. These extended submergences, unique among mammals, have long perplexed scientists seeking to understand the adaptations that allow marine mammals to perform extended breath-holds at depth while exercising without any apparent detrimental effects. Understanding the physiological adaptations that enable breath-hold diving, the mechanisms that control these physiological changes, and the plasticity of these physiological adaptations in a changing ocean represent the major challenges in the field of diving physiology today.

In support of the aims of this dissertation, a review of the field of marine mammal cardiorespiratory physiology, the neural drivers of cardiorespiratory adaptations, and the plasticity of the dive response in free-ranging animals is provided. The utility of studying physiology for conservation is discussed and a rationale for considering marine mammal physiological adaptations in the context of comparative medicine is offered. These motivate the research that follows and highlight avenues for further research.

1.1 Cardiorespiratory physiology of diving mammals

1.1.1 The dive response

Though incompletely understood, the factors that influence the variation observed in marine mammals' cardiovascular and respiratory function during a dive are numerous (Table S1). In Irving's review of the respiratory adjustments of diving mammals (and as highlighted in the Blix, 2018 review of phocid adaptations for diving), he claims that "the storage of oxygen is inadequate to provide for its indiscriminate use by all of the tissues. No shift of metabolism can compensate for the lack of oxygen, but differential control of the distribution of oxygen might reasonably serve to maintain the brain, allowing less sensitive tissues or those with fair capacity for anaerobic metabolism to do without oxygen" (Irving, 1939). Per Scholander's 1940 monograph confirmed this suspicion and revealed the physiological changes, including changes in O₂ distribution, that occur with the onset of diving and their reflexive nature (Scholander, 1940). In his experimental dives of a grey seal, a decrease in f_H from 150 beats min⁻¹ to 10 beats min⁻¹ was observed immediately upon forced submersion. This observation agreed with studies in other species, namely ducks, rabbits, and dogs, which asserted that bradycardia, or a reduction in f_H , was important for sustained diving (Artom, 1926; Bert, 1870; Huxley, 1913) and established bradycardia as one of the central components of the "dive reflex." Coincident with bradycardia in diving seals, Scholander also noted a reduction in circulation through the hind flippers and a lack of an appreciable increase in lactic acid in the arterial blood during the dive. During recovery, however, lactic acid increased significantly when f_H was elevated and muscle perfusion restored. These changes provided further support for Irving's theory of controlled perfusion of tissues for selective O₂ distribution. Taken together, the apnea (breath-hold), bradycardia, and

peripheral vasoconstriction comprise what is today regularly referred to as the dive response. Because the dive response provides defense against the threat of asphyxia, Scholander himself spoke of this response more generally as the “master switch of life” (Scholander, 1963).

1.1.1.1 History of the dive response

While Scholander’s initial experiments provided much of the basis for our current understanding of the physiological responses to diving, further forced submersion experiments produced data to support what Scholander had demonstrated in his early studies. In 1941, Irving et al.’s simultaneous recordings of f_H and peripheral blood gases/lactic acid in the hind flipper of harbor seals indicated that indeed f_H is reduced and muscles become isolated from the circulation during diving (Irving et al., 1941a). This pattern was also observed during rebreathing experiments, although in this case, bradycardia developed more slowly. The speed of bradycardia development at the beginning of a dive was correlated with blood O_2 consumption. When bradycardia developed immediately in the first minute of diving, blood O_2 depletion dropped to 20% of its pre-dive levels. In contrast, when bradycardia developed more slowly, diving O_2 consumption remained at above 50% of pre-dive levels. Additionally, this study revealed that although tachycardia during recovery and the release of lactic acid into the circulation are often coincident, these changes do not always occur simultaneously, suggesting that changes in f_H and blood flow can be dissociated.

The following year, another study of harbor seals demonstrated that muscle metabolism is aerobic at the beginning of dives, although reduced from pre-dive levels. As the dive continues past 5-10 minutes, the muscles become dependent on anaerobic processes (Scholander et al., 1942). The researchers noted that free-ranging harbor

seals and those in captivity most often dive for 4-5 minutes, suggesting their muscles depend on aerobic metabolism alone during most dives. A continuation of these experiments was published in 1942 which used a hot wire anemometer to make direct measurements of peripheral blood flow in the harbor seal (Grinnell et al., 1942). This study confirmed that the decrease in f_H during diving was accompanied by an expected reduction in muscular blood flow. This correlation was also shown during respiratory sinus arrhythmia (RSA), where the correlation between f_H and muscular blood flow is maintained even when the animal is at the surface breathing.

The development of more advanced methods to measure blood flow, namely ultrasonic flowmeters and microsphere deposition techniques, enabled later studies of the dive response. In 1965, Van Citters et al. used an ultrasonic flow meter to record blood flow in several arteries along with f_H and blood gases in northern elephant seals (Van Citters et al., 1965). Although these measurements may have been altered by the residual effects of anesthetic drugs used during sensor implantation, the authors found that changes in blood flow through the iliac artery (blood supply to the pelvis and lower limbs) were independent of changes in f_H and at times fell to zero during a dive, while changes in blood flow through the carotid artery (blood supply to the head) closely mirrored changes in f_H and never fell to zero during diving. This trend reinforced the idea of blood redistribution during a dive away from peripheral tissues and towards central tissues like the brain and heart. Using microsphere deposition, Zapol et al. expanded on this conclusion in their 1979 study of Weddell seals. They showed a significant decrease in organ blood flow and percent of cardiac output to many peripheral organs during diving. Notably, blood flow to the brain remained unchanged during diving compared to rest, and the brain, lungs, and adrenals received a larger proportion of the total cardiac

output during diving than during resting conditions (Zapol et al., 1979). A thorough review of experiments in diving physiology up to the mid-1960s, when studies using simulated dives via submersion in a tank or pool comprised much of the published research, is provided by Andersen et al. (Andersen, 1966).

As an understanding of the dive response developed, researchers like Van Citter et al. noted that “dives’ carried out by passive submersion are acknowledged as a poor substitute for natural environmental diving: the animals frequently fought to escape while submerged, squandering their supply of O_2 .” This thinking guided the field towards using trained dives or free dives in both free-ranging and captive animals to better understand how the dive response manifests during natural diving.

Due to the poor response of porpoises to forced submersions (Elsner et al., 1966b; Scholander, 1940), much of our early understanding of the dive response in cetaceans, in addition to further exploration of the response in pinnipeds, was learned from animals trained to perform unrestrained dives while instrumented with physiological sensors. Initial studies of the dive response in bottlenose dolphins, from animals captured in the wild and brought temporarily into an aquarium, made use of an electrocardiogram (ECG) with extended leads to measure f_H during diving (Irving et al., 1941b). In addition to observing diving bradycardia, Irving et al. described an increase in f_H during the ascent of the dive before breathing. In 1964, Elsner et al. and collaborators at the San Diego Zoo trained a California sea lion to submerge its head in a container of water on command (Elsner et al., 1964). Experiments with this animal allowed researchers to measure f_H , stroke volume, and cardiac output during both trained and spontaneous submersions. They observed minimal changes in stroke volume during submersion but did observe the expected diving bradycardia and an associated

reduction in cardiac output. Two years later, Elsner et al. collaborated with Sea World San Diego to study f_H 's of Pacific bottlenose dolphins diving to a 6-7 foot target (Elsner et al., 1966b). In this study, bradycardia was compared during short spontaneous submersions and longer trained submersions. Bradycardia during trained submersions was observed to be greater than that during spontaneous diving. Following the extended trained dive, f_R was increased and the bradycardia that was typically seen between breaths at the surface was not as severe as during resting and, at times, disappeared. These earlier studies established the physiological responses central to the modern understanding of the dive response and began to reveal that the plasticity of this response depends on the context of a dive.

1.1.1.2 The advent of biologgers for diving physiology

The miniaturization of microprocessors, data storage, and batteries has made biologging devices and their use for physiological studies of free-ranging animals widely successful (Block, 2005; Ponganis, 2007). Though some early researchers were able to measure f_H during free dives of bottlenose dolphins, the ECG leads were tethered to a computer system at the surface, limiting the range across which an animal could dive (or otherwise the continued function of the research equipment) (Irving et al., 1941b). Perhaps the most pioneering work concerning the study of diving physiology during free dives was the development of the isolated ice-hole method by Gerry Kooyman. In this paradigm, a seal could be outfitted with instrumentation and released into a hole in the ice, knowing it must return to that particular hole to breathe (Kooyman, 1966). In 1972, Kooyman and Campbell used this method and equipped free-ranging Weddell seals with break-away ECG leads to measure bradycardia during diving. They observed that the onset of bradycardia occurred more quickly as the dive duration increased, suggesting

seals may anticipate the duration of a dive (Kooyman and Campbell, 1972). This method also allowed for the estimation of the aerobic dive limit (ADL) in Weddell seals (20-30 minutes) as researchers could sample blood after natural dives (recorded by a time-depth recorder) of the seal back to the ice hole (Kooyman et al., 1980).

More recent studies of the dive response during trained dives have occurred in collaboration with trained animals at aquaria and with the U.S. Navy Marine Mammal Program. Ridgway et al. demonstrated that bradycardia could be conditioned in a California sea lion in the absence of submersion resulting in a bradycardia of 20 beats min^{-1} by the end of training. This was comparable to a submersion bradycardia of 25-40 beats min^{-1} suggesting that sea lions may have some volitional control over their f_H (Ridgway et al., 1975). Lung collapse, another change known to occur in diving marine mammals, was first observed in a trained Navy bottlenose dolphin, Tuffy, conducting dives to 300 m (Ridgway et al., 1969). Ponganis et al. demonstrated the utility of trained dives in measuring physiological changes during active behaviors. In addition to measuring bradycardia during different activity levels, plasma lactate levels were measured following dives to determine the ADL of 2.3 minutes in California sea lions (Ponganis et al., 1997b). Similar work in bottlenose dolphins trained to dive revealed an ADL close to 3.7 minutes for adult animals (Williams et al., 1999).

Today, studies in the field of diving physiology continue to rely on the collection of physiological data from both marine mammals in aquaria, zoological settings, and those in the wild as we continue to learn about the dive response, its drivers, and understand its plasticity in the face of changing oceans.

1.1.2 Neural drivers of cardiorespiratory rates

An effort to understand the plasticity of the cardiorespiratory system would be incomplete without discussing the neural drivers that play a role in influencing the behavior of the lungs and the heart (Fig. 1). The general form by which a neural mechanism influences the cardiorespiratory response to diving is this: 1) a 'sensor' perceives some stimulus in the body, 2) this signal is transmitted via an afferent pathway to the central nervous system (CNS), 3) the CNS integrates that signal, 4) an outgoing signal is transmitted via an efferent pathway, and finally 5) an effector (typically a muscle) performs the reflex action.

In the case of diving, several 'sensors' monitor various regulatory cues, including changes in blood gases, skin temperature, and pressure (blood pressure and inflation/deflation of the lungs). Central chemoreceptors are located in the medulla and several other areas of the hindbrain and respond to changes primarily in H^+ (pH) in the interstitial fluid (Nattie and Li, 2012). Because H^+ ions do not diffuse across the blood-brain barrier, the central chemoreceptors rely on the diffusion of arterial CO_2 into the interstitial fluid and its combination with water to form carbonic acid, which dissociates into bicarbonate and H^+ ions, thereby decreasing pH and stimulating the chemoreceptors to elicit the respiratory center of the medulla and increase ventilation. Given that cerebral blood flow and cerebral metabolism also influence interstitial fluid pH, it has been proposed that the central chemoreceptors act as indirect brain tissue O_2 sensors (Nattie, 2006). The peripheral chemoreceptors, namely the carotid bodies and aortic bodies, also respond to changes in blood gases (Marshall, 1994). The carotid bodies, located at the carotid bifurcations superior to the carotid sinuses, respond to arterial CO_2 , H^+ , and O_2 . These peripheral chemoreceptors may serve as a rapid

detection mechanism of arterial CO₂ (Dempsey, 2005; Smith et al., 2006), whereas central chemoreceptors provide a steady-state response and a tonic drive for normal CO₂ and pH maintenance (Forster et al., 2000). The aortic bodies, which are located on the superior surface of the aorta, respond to changes in CO₂ and O₂ though generally are less sensitive to changes in arterial blood gases than the carotid bodies.

In addition to sensing changes in blood gas concentration, temperature and pressure also serve as stimuli for changes to cardiorespiratory function. Facial cold receptors transmit afferent signals via the trigeminal nerve and are stimulated by submersion in water, but some evidence suggests they may also be stimulated by inhaled irritants (Panneton, 1991; Panneton and Gan, 2020). Several studies in both humans and marine mammals have noted that bradycardia can be induced by facial submersion (Andersen, 1966; Elsner et al., 1966a) and even, in the case of the Weddell seal, by covering the face with a wet towel (Elsner et al., 1970). Pulmonary stretch receptors are mechanoreceptors located in the lungs that respond to increases in lung volume during inhalation (Schelegle, 2003). Studies in the muskrat demonstrate that lung deflation resulted in bradycardia independently of arterial baroreceptors and carotid body chemoreceptors (Drummond and Jones, 1979). In the anesthetized harbor seal, lung inflation causes reflex tachycardia, while deflation results in bradycardia (Angell-James et al., 1981). This study also suggested that a decrease in pulmonary stretch receptor activity during apnea would lead to eventual bradycardia. Finally, the baroreceptors are mechanoreceptors located in the carotid sinus and the aortic arch. The baroreceptors in the carotid sinus are stimulated by an increase or decrease in arterial blood pressure (ABP), while those in the aortic arch respond only to an increase in ABP (Armstrong et al., 2021). Previous work in the harbor seal has suggested that the

arterial baroreflex is 'reset' towards bradycardia during diving and plays a role in cardiac slowing dependent on the degree of vasoconstriction (Angell-James et al., 1978).

Stimulation of any of these sensors results in a signal sent via several afferent pathways to the brain. The primary afferent pathways relevant for a response to breath-hold diving include the glossopharyngeal nerve, vagus nerve, and trigeminal nerve (Armstrong et al., 2021; Barrett et al., 2019a; Marshall, 1994; Panneton and Gan, 2020). Once a signal is transmitted to the brainstem, two centers are concerned with cardiorespiratory function: the respiratory center and the cardiovascular center (Barrett et al., 2019b). Signals to the respiratory center can be autonomic and inhibitory (i.e., abating respiration), via stimulation of the facial cold receptors or the pulmonary stretch receptors, or autonomic and excitatory (i.e., inducing respiration), via the central or peripheral chemoreceptors. These autonomic controls contribute to the timing and depth of breathing during normal passive breathing, sleep apnea, and unexpected submersion. However, the higher brain centers in the cerebral cortex can also influence the respiratory center to control voluntary respiration – this control over the respiratory center is essential for planned, voluntary breath-holds.

The cardiovascular center of the brain is comprised of three distinct components: the cardioacceleratory center (CAC), the cardioinhibitory center (CIC), and the vasomotor center (VMC). The CAC is the sympathetic nervous system (SNS) arm of the cardiovascular center and is tasked with increasing cardiac output via the cardiac accelerator nerve, whereas the CIC is the parasympathetic nervous system (PNS) arm of the cardiovascular center and is tasked with decreasing cardiac output via the vagus nerve. The VCM regulates vascular tone, thereby controlling vascular resistance, pressure, and flow via sympathetic stimulation. The CAC and CIC primarily respond to

changes to stimulation of baroreceptors and central and peripheral chemoreceptors. When arterial blood pressure is elevated, baroreceptors are stimulated, resulting in stimulation of the CIC and inhibition of the CAC and VCM, allowing vasodilation and a decrease in f_H and contractility. In contrast, a drop in arterial blood pressure results in the inhibition of the baroreceptors and stimulates the CAC and VCM while inhibiting the CIC, inducing vasoconstriction and increasing f_H and contractility. Pulmonary stretch receptors are also known to inhibit the CIC leading to an elevation of f_H during inhalation (Angell-James et al., 1981). Although the direct effect of pulmonary stretch receptors on f_H in a diving marine mammal is challenging to interpret due to possible differences in diving lung volume and exercise, some variation in f_H has been observed in California sea lions ascending from dives that may be associated with lung re-expansion (McDonald and Ponganis, 2014; Ponganis et al., 2017). Although the chemoreceptor reflex primarily acts to regulate breathing, excitation of the chemoreceptors can affect the cardiovascular center of the brain. Importantly, the chemoreceptors' effect on heart rate and vascular resistance is modulated by the ventilatory response. Relevant to marine mammals is the response of the chemoreceptors when ventilation is inhibited; in this case, excitation of the peripheral chemoreceptors results in maximal sympathetic activation (Schultz et al., 2007). Finally, stimulation of the facial cold receptors is also thought to excite the VCM during submersion serving to maintain vasoconstriction during submersion in humans (Elsner and Gooden, 1983; Stromme et al., 1970). Both the effects of chemoreception and facial cold receptor stimulation appear to be modulated or 'gated' because they require the cessation of the inspiratory drive and decreased activity of the pulmonary stretch receptors to be maximally effective (Angell-James and de Burgh Daly, 1981; Elsner and Gooden, 1983).

1.1.2.1 Neural drives in the field

While an understanding of each neural regulatory pathway that influences the dive response can provide some clues as to the cardiorespiratory patterns we expect to observe underwater, “their study in a re-assembled condition and their interaction with other reflexes provide insights, which are sometimes quite different, into the dynamic reactivity of these controls in their natural system” (Elsner and Gooden, 1983). On a ‘natural’ dive, there are many stimuli that simultaneously influence the physiological response to diving. In addition to the stimulation of chemoreceptors, mechanoreceptors, and thermoreceptors previously mentioned, factors like dive duration, dive depth, exercise, and stress/disturbance may also influence physiological responses during diving (Butler and Jones, 1997; Davis et al., 1983; Hochachka, 1986; Scholander, 1940). Of particular focus in recent literature is how the dive response integrates with the exercise response that may play a role during active foraging and the stress response that may be pertinent in a diving marine mammal’s reaction to anthropogenic disturbance.

The exercise response, which is characterized by increased f_H , hyperventilation, increased cardiac output, and increased perfusion of active muscles, directly contradicts the physiological changes associated with the dive response (Butler, 1988; Davis and Williams, 2012; Williams et al., 2015b). Similarly, a central physiological response to stress in mammals includes the stimulation of the SNS and an increase in f_H (Reeder and Kramer, 2005). The combination of diving and exercise results in increased f_H variability and the incidence of arrhythmias in humans (Shattock and Tipton, 2012) suggesting that this autonomic conflict may be harmful in diving marine mammals (Williams et al., 2017, 2015b, 2015a). Given that anthropogenic disturbances have the

potential to incite both an exercise response (via flight/escape response resulting in increased swim speed and stroke rate) (DeRuiter et al., 2013; Goldbogen et al., 2013; Todd et al., 1996) and stress response (via an increase in f_H that is independent of increased movement as seen in other taxa) (de Villiers et al., 2006; Ropert-Coudert et al., 2009; Viblanc et al., 2012), a better understanding of the integration of these responses with the dive response is warranted. A recent commentary on the potential conflict between diving and exercise suggested that vagal activity dominates over the sympathetic cardiac tone during diving and that, though exercise may influence the dive response due to vagal withdrawal, autonomic control of f_H is fine-tuned in marine mammals, and arrhythmias are generally benign (Ponganis et al., 2017).

1.1.3 Free-ranging studies of f_H

While Kooyman and his colleagues pioneered early techniques for studying free-ranging marine mammals using the isolated ice hole method, it was not until the mid-1980s when Hill introduced a device containing a microprocessor and internal memory to record the ECG of Weddell seals that a research team was able to measure and store real-time physiology continuously during unconstrained free dives in free-ranging animals (Hill, 1986; Hill et al., 1987). Hill's first publication using data from this early bilogger deployed on free-ranging Weddell seals revealed trends in diving f_H that continue to be observed today (Hill et al., 1987; also summarized in Ponganis, 2015). The authors found that f_H during both feeding dives (10-15 minutes, 35-45 beats min^{-1}) and exploratory dives (>20 minutes, 29-36 beats min^{-1}) was lower than resting f_H at the surface (60-78 beats min^{-1}). f_H 's during diving decreased with increasing dive duration but were always substantially elevated compared to the minimum bradycardia observed during forced laboratory diving. Hill et al. also observed ascent tachycardia in one seal

and found that f_H during recovery after diving (66-98 beats min^{-1}) was generally higher than resting f_H . Continued development of biologgers since this study have enabled a drastic increase in the amount of f_H data collected from diving marine mammals across a range of physiological and behavioral conditions (Ponganis, 2007; Ropert-Coudert et al., 2018).

A study of diving ringed seals revealed a profound diving bradycardia (often lower than 10 beats min^{-1}) across a range of dive durations in addition to an anticipatory tachycardia that was observed in non-blindfolded seals, but not in blindfolded seals (Elsner et al., 1989). Free-ranging gray seals showed a large degree of variability in f_H during diving and at the surface (Thompson and Fedak, 1993). Surface f_H 's were high (120 beats min^{-1}), demonstrated a sinus arrhythmia, and were negatively related to body size. The authors suggested that the observed anticipatory tachycardia may serve to perfuse O_2 -depleted tissues before surfacing, thereby reducing blood P_{O_2} and increasing O_2 uptake at the start of surface breathing. During diving, f_H fell immediately and the degree of bradycardia was a function of dive duration. Interestingly, the number of heartbeats during a dive increased with dive duration up to dives of 7 minutes, after which the number of heartbeats during the dive was relatively constant, around 220. During most long dives, gray seals demonstrated significant arrhythmias, although one dive of the gray seal, which was of similar depth and duration to other long dives, showed minimal arrhythmias and a much-reduced f_H . These data demonstrate just how plastic the dive response of a single individual can be – despite similar dive parameters, the cardiac patterns observed in this seal during 'natural' diving are in stark contrast. Perhaps an unknown external stimulus could have contributed to the uniquely, stable f_H pattern during the observed dive.

Some pinniped species exhibit sleep apneas on land, providing a unique opportunity to study f_H during apneas without the influences of diving or volitional control. Patterns of f_H in northern elephant seals during diving and sleep apnea revealed similarities between f_H during apneas with different stimuli. Minimum f_H during sleep apneas was similar to the minimum f_H of the RSA, suggesting possible shared control mechanisms (Castellini et al., 1994a). Additionally, the degree of the sinus arrhythmia was found to increase with eupnea, or regular breathing, duration as recovery tachycardia subsided, as well as age, suggesting the importance of fine-tuned cardiorespiratory regulation for diving (Castellini et al., 1994b). Northern elephant seals diving at sea and on land demonstrated higher f_H 's during eupnea at sea than during eupnea on land (Andrews et al., 1997). At sea, the periods of eupnea were shorter than those on land indicating a drive to minimize surface time at sea. Additionally, at the start of apneas on land, f_H decreased to the minimum f_H observed during the RSA; however, at the start of dives, f_H dropped initially to a similar value but continued to decrease as the animal descended. Stroke rate and f_H decreased in parallel during descent and increase in parallel during ascent, accordingly with the angle of ascent in freely diving Weddell seals (Davis and Williams, 2012). The authors suggested that this indicates the intensity of the dive response is inversely correlated with the degree of muscle metabolism (i.e., the dive response is less pronounced as muscle activity increases) which serves to maximize the use of both the blood and muscle O_2 stores.

Data collected from diving California sea lions equipped with ECG recorders and time-depth recorders has allowed for the investigation of various components of dive f_H in relation to dive parameters like depth and duration (McDonald and Ponganis, 2014). The degree of bradycardia of the dive f_H , the minimum f_H during the dive, and the bottom

of dive f_H demonstrated a graded response that increased with dive duration. The study also characterized the f_H patterns observed during longer dives as follows: 1) a pre- and post-dive tachycardia, 2) a gradual, steady decrease in f_H during the early descent phase, 3) a more gradual decrease in f_H in the later descent phase, 4) a limited increase in f_H during the bottom portion of the dive with some arrhythmia-like variation, 5) a slow increase in f_H during ascent and 6) an anticipatory tachycardia prior to surfacing. In California sea lions, f_H is positively correlated with activity such that bradycardia is less intense during more active dives (McDonald et al., 2020). This correlation was particularly evident during ascent, even on long dives, when there is active stroke movement. An elevated f_H is known to increase blood flow which may support increased muscle metabolism during active swimming (Grinnell et al., 1942; Ponganis et al., 2006).

Deployments of ECG tags on free-ranging cetaceans have allowed researchers to explore the relationship between the dive response and behaviors/stimuli that occur during free dives. Studies of f_H in two species of cetaceans were enabled by their capture in nets (either incidentally as by-catch or intentionally as part of traditional indigenous hunts) and subsequent placement of an ECG tag in a location previously validated to produce a clear ECG signal (Elmegaard, 2020; Williams et al., 2017). A wild harbor porpoise exposed to an acoustic harassment device (AHD) increased its f_H and demonstrated a sudden burst of movement (Elmegaard, 2020). An increase in f_H would provide the skeletal muscles with an increased supply of O_2 to support a potential escape response. In contrast, narwhals demonstrating an escape response displayed “cardiac freeze,” or extreme bradycardia, coincident with an increased fluke stroke rate (Ponganis et al., 2017). To date, only one published study has recorded f_H from a tag attached opportunistically using a tag pole (Goldbogen et al., 2019a). In this study, a

blue whale wearing a Customized Animal Tracking Solutions (CATS) tag with ECG electrodes incorporated into two suction cups demonstrated extreme bradycardia during diving (2 beats min^{-1}) and tachycardia during surfacings (~ 35 beats min^{-1}) compared to its predicted resting f_H of 15 beats min^{-1} . During energetically costly lunge feeding, a transient increase in f_H was observed, which agrees with previous studies in sea lions (McDonald et al., 2020).

As ECG tags are not yet widely-available, recent methods have focused on detection of heart beats from standard bilogger movement data. High-resolution movements tags like CATS tags and digital acoustic recording tags (DTAGs) contain inertial measurement units (IMUs), comprised of a 3-axis accelerometer and a 3-axis gyroscope, that record linear and angular motion of a tagged whale or dolphin. A recent study of CATS tag data collected from blue whales demonstrated the first use of ballistocardiography to identify heart beats and calculate a continuous f_H signal (Czapanskiy et al., 2022). This study observed a minimal f_H (~ 4 -5 beats min^{-1}) at the start of the dive and increasing f_H (~ 8 -9 beats min^{-1}) as the dive progressed and into the ascent phase of the dive. This work highlights the potential for extracting novel physiological insights from existing data and demonstrates the feasibility of studying f_H more reliably in free-ranging whales and dolphins in the future.

1.1.4 Free-ranging studies of f_R

Unlike f_H , which can only be measured and recorded in a free-ranging marine mammal using a highly sensitive electronic device, f_R can be measured easily by visual observation. The breath of whale is its unavoidable give-away to observers searching for an interruption in the water's smooth surface. As Philip Hoare puts it in the popular novel *Leviathan or, The Whale*, “[the whale] expels exhausted air with the pneumatic sound of

a finger held over a bicycle pump. It is a profound exhalation, rather than a spout of sea water; a visible condensation, like human breath on a frosty morning.” Because of this, many studies have recorded f_R in observational studies of various marine mammal species.

In migrating gray whales, researchers examined the relationship between f_R and swim speed, concluding that they were related curvilinearly such there is an optimal speed at which the number of breaths min^{-1} is minimized (Sumich, 1983). Humpbacks in the Alaskan feeding grounds respired at higher rates following deeper dives and, on average, when prey was located deeper in the water column (Dolphin, 1987). This observation led the authors to suggest that prey distribution could have a substantial influence on f_R . Ventilation patterns in singing humpback whales agreed with previous data suggesting that more exhalations occur following longer dives (Chu, 1988). These and many other early studies began to establish the idea that there was some metabolic cost to activity and prolonged breath-holds that would have to be offset at the surface.

Notably, measurements of f_R have allowed researchers to make estimations of field metabolic rate (FMR) of minke whales off the coasts of Norway and Svalbard and reported an around-the-clock f_R for all animals of $0.77 \text{ breaths min}^{-1}$, which predicted its relatively low energetic cost of swimming (Blix and Folkow, 1995). Though the ease of measuring f_R in free-ranging marine mammals has, historically, made it an attractive method by which to estimate FMR (Blix and Folkow, 1995; Christiansen et al., 2014; Dolphin, 1987; Williams and Noren, 2009), this method has recently been critiqued for not taking into account variation in tidal volume and O_2 exchange fraction which can vary on a breath-to-breath basis, especially following exercise (Fahlman et al., 2016). At least

one recent study has examined the use of variable O_2 -uptake functions for determining accurate FMR's in free-ranging cetaceans (Roos et al., 2016).

With the development of digital recording tags, more recent work has taken advantage of indirect methods to record f_R in free-ranging marine mammals, thereby removing the presence of the researchers as a potential confounding effect on physiological measurements. Andrews et al. cleverly used the presence of RSA in northern elephant seals to extract f_R indirectly from the power spectra of f_H variability (Andrews et al., 2000). These data revealed that f_R following a dive was 2.4 times greater than that following a similar duration apnea on land (22.0 breaths min^{-1} vs. 9.2 breaths min^{-1}). Notably, the number of breaths during the eupnea in both cases was the same. This suggests that it is beneficial for the seals to reduce the amount of time spent at the surface while at sea, thereby maximizing time spent underwater. Using movement and acoustic tags, researchers have been able to successfully identify breathing events using a combination of methods including observing periods when the animal is at the surface, its body is pitched at the correct angle for breathing, and the hydrophones record either the tag breaking the surface (assuming a 1 surfacing: 1 breath ratio) or the sound of breathing. Goldbogen et al. used this methodology to examine breathing patterns related to humpback whales' lunge feeding (Goldbogen et al., 2008). They observed that f_R was more elevated following dives with more lunges and that the number of post-dive breaths after lunge feeding was triple that observed in singing humpbacks in another study. This observation provided evidence in support of their hypothesis that lunge feeding is highly energetically costly.

In mammal-eating killer whales, f_R 's measured from depth data and assuming a 1 surfacing: 1 breath ratio indicated no significant diurnal differences in respiration

patterns (Miller et al., 2010). The researchers also found no significant difference in f_R between adult males, adult females, and juveniles. This result may suggest “physiological compromise” by individual animals where the bigger animals are not reaching their physiological limits, but the smaller animals are such that all animals in the group dive and recover similarly. To determine the physiological consequences of whale-watching vessels, f_R 's of minke whales were compared in the presence and absence of boats. The average f_R of animals in the absence of vessels was 0.88 breaths min^{-1} compared to 1.12 breaths min^{-1} when vessels were present, resulting in a large increase in estimated energy expenditure when the animals interacted with whale-watching vessels. To further elucidate the physiological effect of disturbances, a 2018 study conducted controlled sound exposure experiments with long-finned pilot whales (Isojunno et al., 2018). In addition to a positive correlation between post-dive f_R 's and dive duration for dives >2 minutes, the researchers observed a 13-16% decrease in f_R during naval sonar exposures and playbacks of killer whale sounds. Clearly f_R is an important physiological rate that is modulated in response to metabolic needs: whether they be the result of a breath-hold, exercise, or a combination of both.

1.2 Physiology for conservation

For diving marine mammals, adaptations to maximize O_2 storage and minimize its metabolic consumption are central to enabling extended breath-holds (Irving, 1939, 1934; Scholander, 1940). Termed the ‘dive response,’ the suite of physiological changes that occur at the onset of diving and are modulated throughout a dive impose the ultimate constraint on an individuals’ diving ability (Costa et al., 2004). Consequently, understanding the full range over which these adaptations can vary in a species, or that species’ physiological plasticity, is vital to determine its potential tolerance to the

environmental change occurring in today's oceans (Chevin et al., 2010; Hoffmann and Sgrò, 2011). Several studies have implicated the importance of understanding marine mammals' physiology in estimating their vulnerability to threats like climate change and increasing ocean temperatures (Laidre et al., 2008; Schumann et al., 2013; Simmonds and Isaac, 2007). Prey species moving into deeper, cooler water as the ocean warms will necessitate deeper dives for individuals to forage (McIntyre et al., 2011). Even if the prey species do not move to depths beyond the physiological capacities of diving marine mammals, deeper dives are expected to be more physiologically costly than shallow dives. Physiology, but more specifically physiological plasticity, plays a critical role in how organisms respond to environmental stressors at multiple levels of biological organization and should be considered when suggesting and implementing conservation actions (Cooke et al., 2014; Wikelski and Cooke, 2006).

1.3 Krogh's principle: marine mammals as model organisms

Oxygen's (O_2) role in the aerobic production of adenosine triphosphate (ATP) makes it a vital resource for organisms that depend on aerobic respiration for energy production. Like other environmental variables, O_2 availability can vary across time and space. For example, one well-known co-variate of atmospheric O_2 is elevation; at higher altitude, atmospheric pressure decreases and, with it, the partial pressure of atmospheric O_2 . At the top of Mount Everest (elev. 8900m), inspired O_2 pressure is approximately 30% of its value at sea level (Peacock, 1998). Similarly, the amount of O_2 dissolved in water varies with depth and temperature, which dictates the distribution of organisms dependent on dissolved O_2 for respiration in freshwater and marine ecosystems. However, changes in O_2 availability are not always the product of an organism's external environment but can also be created as a condition of an organism's

behavior. Burrowing and diving animals create hypoxic conditions by limiting or, in the case of divers, eliminating their access to the ambient air. The prevalence of hypoxia across ecosystems has resulted in species across a range of taxa with adaptations to protect against hypoxic damage and thrive in conditions of variable O₂ availability.

As Krogh's Principle describes, for most biological problems, "there will be some animal of choice... on which it can be conveniently studied" (Krogh, 1929; Lindstedt, 2014). Several studies in comparative physiology have taken advantage of so-called "naturally-occurring" defenses against hypoxia in a wide range of species to investigate adaptations that may be applicable in comparative medicine (Nathaniel et al., 2015). Marine mammals are one such "[taxon] of choice" that provides a unique opportunity to study gas management in addition to other physiological changes expected to occur during a breath-hold dive like ischemia, or a reduction in blood flow, and hypoxia. Therefore, many of the primary research findings in diving physiology may also have relevant applications to the biomedical sciences. For example, by examining the mechanisms that marine mammals have evolved to tolerate repeated events of ischemia/reperfusion and tissue hypoxia, we can better understand how hypoxia and the inflammation response may be uncoupled in humans (Allen and Vázquez-Medina, 2019). Ponganis, 2019 provides an excellent review of several diving physiology topics and their relevance to human medicine.

1.3.1 Humans and hypoxia

Humans can experience acute, or short-term, hypoxia due to environmental conditions, changes in behavior, or medical conditions that limit an individual's supply of O₂. For example, climbers at extreme altitudes are exposed to low atmospheric partial pressures of O₂ (P_{O₂}), although some carry supplemental O₂ to offset this reduction in O₂

availability (West, 1983). Free divers, who descend underwater on a breath-hold without an external breathing apparatus, experience reduced arterial and alveolar partial pressure of O₂ during dives (Ferretti, 2001; Muth et al., 2003). Although humans do display some cardiorespiratory plasticity to adjust to high-altitude and diving conditions and reduce the risk of pathological complications (Brugniaux et al., 2007; Hainsworth and Drinkhill, 2007; Schagatay and Andersson, 1998; Tocco et al., 2012), these activities are associated with significant risk (Bärtsch and Swenson, 2013; DeWitt et al., 2019; Houston, 1960). O₂ is also a significant driver in several pathological conditions. Changes in O₂ supply can influence cell migration in cancer metastasis and cause injury associated with ischemia/reperfusion (Choi, 1996; Eltzschig and Carmeliet, 2011; Gottlieb and Engler, 1999). It is essential here to distinguish acute hypoxia from chronic hypoxia. There are many species, including several populations of humans, that have adapted to chronic, or long-term, hypoxia (Beall, 2007, 2006; Frisancho and Baker, 1970); however, for comparison to diving marine mammals, this thesis will focus on gas management associated with acute hypoxia.

1.4 Conclusion

An extensive history of investigations into the physiological function of cetaceans, seals, and sea lions, among other marine mammals, has provided a wealth of information about how these difficult-to-study animals thrive despite the unique challenges of a fully-aquatic lifestyle. Early work with animals in managed care settings characterized the dive response – the critical set of physiological changes that act to support air-breathers ability to breath-hold while diving underwater. Since the pioneering studies of Per Scholander and Lawrence Irving, the advent of the isolated ice hole method and time-depth recorders, translocation studies at the Año Nuevo and

implantable PO₂ electrodes, and remotely-deployable, high-resolution biologging tags with ECG capability have propelled the field of marine mammal physiology forward. By combining findings about how marine mammals vary their physiological rates, e.g., heart rate and breathing rate, with an understanding of neural drivers of these rates, it is possible to begin to understand the physiological drivers that underpin marine mammal diving behavior. In addition to informing our basic physiological understanding of how marine mammals function, an improved understanding of the cardiorespiratory physiology of cetaceans and other marine mammals has the potential to enhance our conservation of these species and inspire new avenues for therapeutics in human medicine. Importantly, multiple modalities of physiological investigation are required to reveal physiological adaptations across biological scales of organization that are relevant for population-level to cellular-level applications. Marine mammal physiology is a field poised to be transformed by the integration of traditional methods in marine mammal science with non-traditional methods from fields like molecular biology and technological advancements in physiologging and wildlife monitoring. As scientists aim to better understand the function of some of the planet's most extreme animals, utilizing multiple modalities of investigation will be critical for maximizing what can be learned about how marine mammals are uniquely adapted for breath-hold diving.

2. An integrated comparative physiology and molecular approach pinpoints mediators of breath-hold capacity in dolphins*

2.1 Introduction

2.1.1 Ischemic stress and hypoxia are associated with negative clinical outcomes in humans

Maintenance of homeostatic function in mammalian tissues is directly dependent on a continuous supply of oxygenated blood. Interruption of this blood supply, known as ischemia, results in reduction in local oxygenation compared to normal physiologic levels, or hypoxia, and can lead to inflammation and cell/tissue death in humans (Bona et al., 1999; Choi, 1996; Eltzschig and Carmeliet, 2011; Gottlieb and Engler, 1999; Murdoch et al., 2005). In the case of a stroke, disruption of cerebral blood flow can result in cell death at the core of infarction within minutes (Mitsios et al., 2006). Ischemia is the causative factor in multiple clinical settings, and ischemic heart disease is the number one cause of death globally, accounting for over 9 million deaths each year (Nowbar et al., 2019; World Health Organization, 2018).

2.1.2 Marine mammals experience regular ischemic events

While humans have little tolerance for ischemic stress and hypoxia, a number of species have evolved unique physiologies that allow them to seemingly thrive despite regular tissue-level ischemia and low-oxygen environments. Marine mammals are one

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group of animals that undergo repeated daily ischemic events. During a dive, a marine mammal experiences a suite of cardiovascular changes that aid in reducing whole-body oxygen (O_2) demand (Irving et al., 1941b; Scholander, 1940). As part of this response, both f_H and stroke volume decrease, resulting in reduced cardiac output (Fahlman et al., 2019b, 2020c). Increased peripheral resistance, through selective vasoconstriction, helps assure that mean arterial blood pressure is maintained, at least in studies on forced diving in seals (Blix et al., 1976; Zapol et al., 1979). Ultimately, this response conserves oxygen in the blood and lungs for O_2 -sensitive tissues like the brain and the heart, while the skeletal muscles rely on endogenous myoglobin-bound O_2 for aerobic metabolism (Davis and Kanatous, 1999; Fahlman et al., 2009). As the dive continues, however, O_2 stores are consumed. The hypoxemia that develops during a dive can be extreme – blood O_2 concentrations as low as 2.7 ml O_2 /dl have been measured in the diving elephant seal. In comparison, the lowest blood O_2 concentration ever measured in a human of 9 ml O_2 /dl was in a climber near the top of Mount Everest (Grocott et al., 2009; Meir et al., 2009). In vitro studies of the seal brain indicate an increased reliance of cerebral tissue on anaerobic metabolism during hypoxia (Hoff et al., 2017). Similarly, seal neurons demonstrate an ability to continue to discharge four times longer under severely hypoxic conditions compared to mouse neurons (Folkow et al., 2008). While the responses to submersion in water are largely conserved across all vertebrates, it is clear that many of the physiological adaptations that support diving are exaggerated in marine mammals compared to other taxa to match the demands of extreme hypoxia (Kooyman

comparative physiology and molecular approach pinpoints mediators of breath-hold capacity in dolphins. *Evol Med Public Health* 9, 420–430. <https://doi.org/10.1093/emph/eoab036>.

and Ponganis, 1998; Panneton, 2013). These physiological differences highlight the tremendous potential to study marine mammals as model organisms for the investigation of adaptations to ischemic and hypoxic stress tolerance, and the cardiorespiratory plasticity that helps prevent hypertension (Blawas et al., 2021a; Fahlman et al., 2019b, 2020c).

2.1.3 Marine mammals have evolved molecular adaptations to ischemic stress tolerance

Increasing attention has been paid to the defenses marine mammals possess against the oxidant by-products and inflammation associated with ischemic, hypoxia, and reperfusion at the molecular level (Allen and Vázquez-Medina, 2019; Hindle, 2020; Zhu et al., 2018). Elevated levels of heme degradation and concentrations of endogenous carbon monoxide (CO) in northern elephant seals have been suggested to potentially protect against damage from ischemia/reperfusion injury due to the known role of CO in supporting vasodilation and decreasing hypertension (Tift et al., 2014). Several studies have highlighted the importance of highly-adapted antioxidant systems in marine mammals for reducing oxidative stress (Vázquez-Medina et al., 2011; Zenteno-Savín et al., 2002) resulting from ischemia/reperfusion secondary to elevated glutathione (Vázquez-Medina et al., 2007, 2006; Wilhelm Filho et al., 2002), superoxide dismutase (Elsner et al., 1998; Vázquez-Medina et al., 2006) and catalase (Vázquez-Medina et al., 2006). Using phylogenetic and evolutionary convergence approaches, several gene families have been identified that may contribute to the increased ischemic stress tolerance of marine mammals, including hypoxia-inducible factor 1 (HIF-1) (Bi et al., 2015; Johnson et al., 2005, 2004), genes relating to the glutathione system and peroxiredoxins (Bagchi et al., 2018; Tift et al., 2014; Yim et al., 2014; Zhou et al., 2018)

and several genes linked to O₂ storage, particularly hemoglobin and myoglobin (Mirceta et al., 2013; Nery et al., 2013; Tian et al., 2017, 2016). Yet, few studies have examined differential gene expression in marine mammals under conditions of ischemia and hypoxia (i.e., diving conditions).

Here, we investigate the dynamic molecular changes that occur during an apnea in bottlenose dolphins using molecular analysis of peripheral blood mononuclear cells (PBMCs) and serum sampled at regular intervals during breath holds. Dolphins are a particularly tractable and well-studied model for understanding the molecular drivers of diving adaptations. Our integrated analyses pinpoint a gene regulatory network centered around the arachidonate 5-lipoxygenase (ALOX5) gene and its downstream metabolites, leukotrienes, as differentially activated during breath holding. This activation of ALOX5 is consistent with cardiovascular control through a reduction in f_H and peripheral vasoconstriction to efficiently manage O₂ use during diving. Based on our collective results, we propose a model in which the ALOX5 pathway is upregulated by blood cells in response to extended breath holds as a mechanism to sustain vasoconstriction and maintain O₂ stores for critical organs while diving.

2.2 Methods

2.2.1 Data collection and animal information

Four adult male bottlenose dolphins (*Tursiops truncatus*) housed at Dolphin Quest Oahu (Honolulu, HI, USA) with an average (\pm S.D.) age of 22.8 ± 9.9 years (range = 11–35 years) and body mass of 198.1 ± 42.9 kg (range = 147.0–251.7 kg, Table 1) participated in this study. All data were collected under voluntary participation and the animals could end a trial at any time.

Table 1: Animal ID, age (years), body mass (kg) and included analyses for all dolphins in the study.

Animal ID	Age (years)	Body mass (kg)	RNA-Seq	Lipoxygenase assay
6JK5	24	200.9	x	x
9FL3	35	251.7	x	
9ON6	21	192.8	x	x
83H1	11	147.0		x
Mean \pm SD	22.8 \pm 9.9	198.1 \pm 42.9		

Routine veterinary assessments include venous blood sampling, and the dolphins that participated in this study had previously been desensitized to the blood sampling protocol. The study protocols were accepted by Dolphin Quest and the Animal Care and Welfare Committee at the Oceanographic (OCE-17-16, amendments OCE-29-18 and OCE-3-19j).

2.2.2 Experimental trials

Whole blood samples were collected at baseline, 3 min and 4.5–5 min of breath holding on fasted dolphins at Dolphin Quest, Oahu, March 2018 and May 2019. All trials were performed in the morning, when the animals were fasted with at least 12 h having passed since the last meal on the previous day to minimize the potential confounding effect of nutritional state. To ensure that the samples were collected during resting behavior each breath hold was preceded by 2 min of rest or slow swimming at the surface. A trial was initiated when the dolphin rolled into dorsal recumbency with its blowhole submerged and continued for approximately 5 min (Supplementary Movie S1). The breath hold ended when the animal rolled into ventral recumbency and took a breath (Supplementary Movie S1). Prior to this study, the animals had previously

participated in breath-hold experiments of durations up to 5 min (Fahlman et al., 2020c, 2020b).

2.2.3 Blood collection and processing for RNA-seq

Whole blood was collected from tail flukes at baseline (0–30 seconds into the breath hold) and during breath holding for 3 min and 4.5 (2018) or 5 (2019) min while the animals were in dorsal recumbency with their blowholes submerged (Fig. 1A and Supplementary Movie S1). For RNA-Seq blood was collected into PAXgene tubes, and

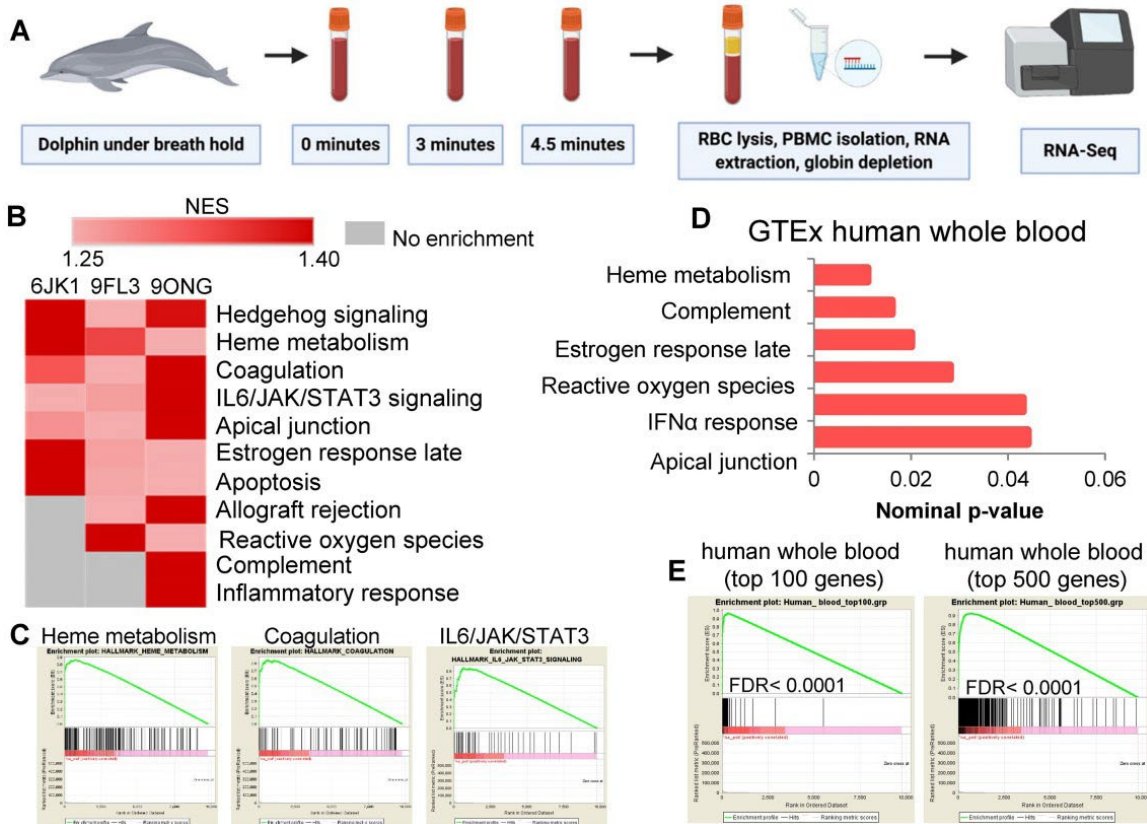


Figure 1. RNA-Seq from dolphin peripheral blood mononuclear cells reveals enrichment of pathways similar to humans. (A) Whole blood from dolphins undergoing fasted breath holds at baseline (0–30 s), 3 min, and 4.5 min was collected from tail flukes and stored in PAXgene tubes for RNA extraction of peripheral blood mononuclear cells and RNA-Seq. (B) Gene set enrichment analysis of baseline RNA-Seq data ranked by total expression pinpoints highly expressed relevant pathways. (C) Enrichment plots for heme metabolism, coagulation and IL6/JAK/STAT3

signaling from baseline dolphin RNA-Seq data. (D) GSEA-based pathway enrichment from GTEx human whole blood RNA-Seq data ranked by total expression. (E) GSEA enrichment plots comparing dolphin RNA-Seq data ranked by total expression with top 100 and top 500 expressed genes in human whole blood.

RNA-Seq was performed after shipping, red blood cell lysis, and RNA extraction (Fig. 1A). All samples were shipped the same day via overnight courier to Duke University for downstream processing. For RNA extraction, tubes were equilibrated to room temperature for 2 h to achieve complete lysis of blood cells. Subsequently, tubes were centrifuged at 4000 x g for 10 min. Pellets were resuspended in 4 ml of RNase-free water and RNA was extracted according to the PAXgene Blood RNA kit (PreAnalytiX #762164). Prior to library prep, RNA quality was evaluated on a Bioanalyzer 2100 (Agilent). Stranded mRNA-seq libraries were prepared using the Nugen Universal Plus mRNA-seq Library preparation kit with Globin AnyDeplete (Tecan #9147-A01). Libraries were sequenced at 150 bp paired-end on one lane of an Illumina NovaSeq 6000 instrument S-Prime flow cell. Library preparation and sequencing were performed in conjunction with the Duke University Sequencing and Genomic Technologies Shared Resource. Samples collected in 2018 were used to conduct RNA-Seq analysis and samples collected in 2019 were used for the lipoxygenase assays.

2.2.4 RNA-seq data analysis

RNA-seq data were processed using the TrimGalore toolkit (Krueger, 2020) which employs Cutadapt (Martin, 2011) to trim low-quality bases and Illumina sequencing adapters from the 3'-end of the reads. Only reads that were 20 nt or longer after trimming were kept for further analysis. Reads were mapped to the turTru1v92 version of the dolphin genome and transcriptome (Kersey et al., 2012) using the STAR RNA-seq alignment tool (Dobin et al., 2013). Reads were kept for subsequent analysis if

they mapped to a single genomic location. Gene counts were compiled using the HTSeq tool (Anders et al., 2015). Only genes that had at least 10 reads in any given library were used in subsequent analysis. Normalization and differential expression across the time points were carried out using the DESeq2 (Love et al., 2014) Bioconductor (Huber et al., 2015) package with the R statistical programming environment (R Core Team, 2020). The false discovery rate was calculated to control for multiple hypothesis testing. To identify relevant molecular features of dolphin breath holding, we first analyzed the RNA-Seq data from all individuals at baseline using gene set enrichment analysis (GSEA) (Mootha et al., 2003; Subramanian et al., 2005). GSEA is a standard pathway analysis tool that calculates enrichment scores for annotated pathways based on the rank order of genes present in the data for each pathway. Pathways with genes that are more upregulated or downregulated are more likely to be enriched in a data set than pathways whose genes are randomly distributed throughout the data. Pathway enrichment in dolphin PBMCs at baseline, with genes ranked on total expression value, were compared with human whole blood pathway enrichments from the Genotype-Tissue Expression (GTEx) project.

2.2.5 Construction of gene regulatory networks

Gene expression networks were created using GeneMANIA (Franz et al., 2018), implemented within the Cytoscape platform (Shannon et al., 2003). For time-dependent gene network construction, all nodes with 0 or 1 connection were trimmed out of the networks. Two additional non-coding RNA genes were eliminated (RF00016 and RF00026). To quantify network connectivity, all genes in the network were individually ranked by the following network parameters: degree, clustering coefficient, closeness, betweenness, neighborhood connectivity and stress. These rankings were summed to

generate a sum rank score for each gene. Pathway enrichments were performed in STRING using the trimmed network of 123 genes. Human whole blood transcriptomics data used for the analyses described in this manuscript were obtained from the Genotype-Tissue Expression (GTEx) Program Portal (<https://gtexportal.org/home/>, accessed on 20 September 2020).

2.2.6 Lipoxygenase assays

Briefly, 5 ml of blood was collected directly into BD Vacutainer® SSTTM Tubes (SST) using a 21 g, ³/₄ in. winged infusion set with a BD Vacutainer adapter and holder. Tubes were gently inverted five times to activate clotting reagent and allowed to clot at room temperature for 30 min in an upright position. Tubes were centrifuged at 1500 X g for 15 min to separate serum fractions, and serum was transferred to 15 ml conical tubes, frozen on dry ice, and shipped to Duke University for downstream analyses. Sera were stored at -80°C until use. Lipoxygenase activity was quantified from 1 µg of total protein using a Fluorometric Lipoxygenase Activity Assay Kit (BioVision Inc; cat. #K978). All lipoxygenase activity assays were performed in triplicate biological replicates from three individual dolphins. Differences in lipoxygenase activity across each time point were analyzed using analysis of variance with Tukey's post-hoc adjustment for multiple testing in Graphpad Prism 8.

2.3 Results

2.3.1 Analysis of baseline RNA-Seq data from dolphins pinpoints enriched gene regulatory networks

All samples produced between 30 and 40 million reads, with no time-dependent changes in read counts across samples (Supplementary Fig. S1A). Principal component analysis and hierarchical clustering of all samples (three individual dolphins x three time

points) revealed both individual and within-individual time-dependent grouping of the data (Supplementary Fig. S1B and C). Analysis of baseline RNA-Seq data by GSEA identified multiple pathways enriched in dolphin PBMCs when ranked by total expression, including hedgehog signaling and several pathways relevant to blood cell metabolism, including heme metabolism, coagulation, IL6/JAK/STAT3 activation, apical junctions, and allograft rejection (Supplementary Fig. 1B and C). GSEA also identified enrichment of pathways related to apical junctions, interferon-alpha response, estrogen response, complement activity and heme metabolism in RNA-Seq data from GTEx human whole blood transcriptomes (Fig. 1D). Comparison of dolphin baseline RNA-Seq data ranked by total expression with the top 100 and 500 most highly expressed genes in human whole blood showed significant enrichment (FDR < 0.0001; Fig. 1E). Together these analyses suggest that significant overlap exists in mRNA expression at both the gene-level and pathway-level between dolphin and human blood at baseline.

2.3.2 Breath holding induces upregulation of multiple regulatory pathways

We next reasoned that patterns of step-wise increases in mRNA expression may pinpoint molecular responses to breath holding common across individuals. We constructed gene regulatory networks for 136 genes with step-wise increases in mRNA expression from baseline to 3 min and again from 3 min to 4.5 min (Fig. 2A). We performed network analysis to identify genes that are upregulated and have the most network interactions. To do this, we analyzed the time-dependent gene regulatory network for the following parameters: degree, clustering coefficient, closeness, betweenness, neighborhood connectivity and stress. We then plotted the sum rank score of these network parameters with gene expression for each gene in the network.

These analyses pinpointed arachidonate 5-lipoxygenase (ALOX5) as among the most connected genes with a time-dependent increase in expression (Fig. 2B and C). Additional genes, including EPX, PTGDR2, SIX5, DCN, ADAMTS12, and GLRX2 demonstrated upregulation and/or high network connectivity (Fig. 2B and C). We used GeneMANIA to infer transcription factor and microRNA targets from this time-dependent

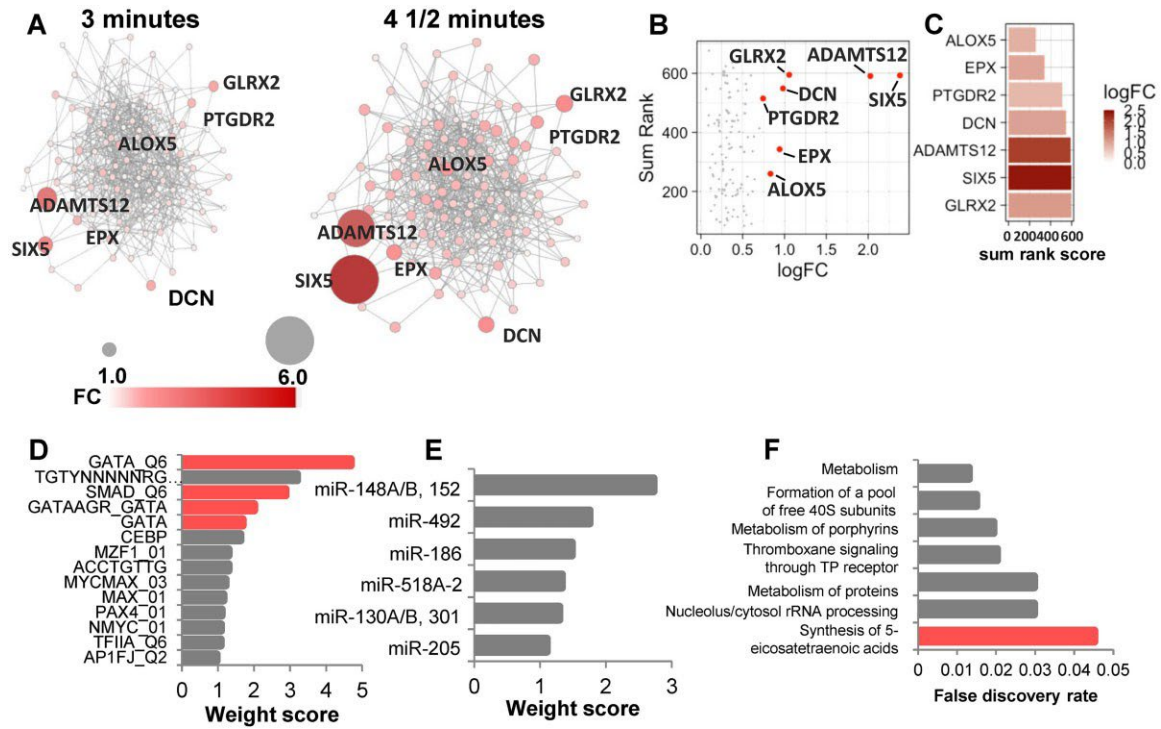


Figure 2. Time-dependent upregulation of gene regulatory pathways during dolphin breath holding. (A) Gene regulatory network formed by the time-dependent increases in mRNAs from baseline to 3 and 4.5 min. Fold changes for each gene over time are indicated by darker red and larger nodes. **(B)** Network analysis of genes within a co-expression network with increased expression over time. **(C)** Top genes with increased expression are sorted by their network analysis parameters. **(D)** GeneMANIA-based transcription factor inference pinpoints GATA and SMAD transcription factor targets within the time-dependent network. **(E)** MicroRNA enrichment inference based on the time-dependent network. **(F)** Functional pathway enrichments for the time-dependent gene regulatory network.

network. The gene regulatory network produced from these genes displayed enrichment in targets from several transcription factor families, including GATA and the small,

mothers against decapentaplegic (SMAD) families (Fig. 2D), both of which have been implicated in hematopoietic development and regulation (Blank and Karlsson, 2011; Lentjes et al., 2016). Network inference also pinpointed enrichment of targets of multiple microRNAs, including the miR148A/B/152 family, miR492, miR186, miR518A-2, the miR130A/B/301 family, and miR205 (Fig. 2E). Consistent with the identification of ALOX5 as a core network node, the network was functionally enriched in the synthesis of 5-eicosatetraenoic acid pathway, which is an initial step in the production of arachidonic acid by ALOX5 (Fig. 2F).

2.3.3 Arachidonate 5-Lipoxygenase (ALOX5) and subsequent lipoxygenase activity is enhanced in breath-holding dolphins

At the gene level, ALOX5 was one of just two genes, along with IL5RA, that was significantly upregulated in all three individuals during breath holding (Fig. 3A and B). Lipoxygenase assays from serum of three individual dolphins collected in 2019 revealed time-dependent increases in lipoxygenase activity during breath holding in all three individuals, consistent with the RNA-Seq analyses (Fig. 3C). Comparison of the timing of these molecular changes with previously-published f_H measurements from the same dolphins demonstrated that changes in gene expression and enzymatic activity were coincident with the expected timing of bradycardia based on the f_H data (Fig. 3D). Overlay of the RNA-Seq data for ALOX5 mRNA expression with the f_H data shows the upregulation of ALOX5 is concomitant with lower heart rate (Fig. 3E).

2.4 Discussion

Dolphins and other cetaceans have evolved exquisite physiological adaptations to deal with the challenges of a fully aquatic lifestyle including having a hydrodynamic shape to reduce drag (Fish, 1993), counter-current heat exchangers for

thermoregulation (Favilla and Costa, 2020; Pabst et al., 1999), and cardiorespiratory plasticity for exquisite management of circulation and respiratory gases (Blawas et al., 2021a; Fahlman et al., 2019b, 2020c, 2020b; Noren et al., 2012). The well-known dive

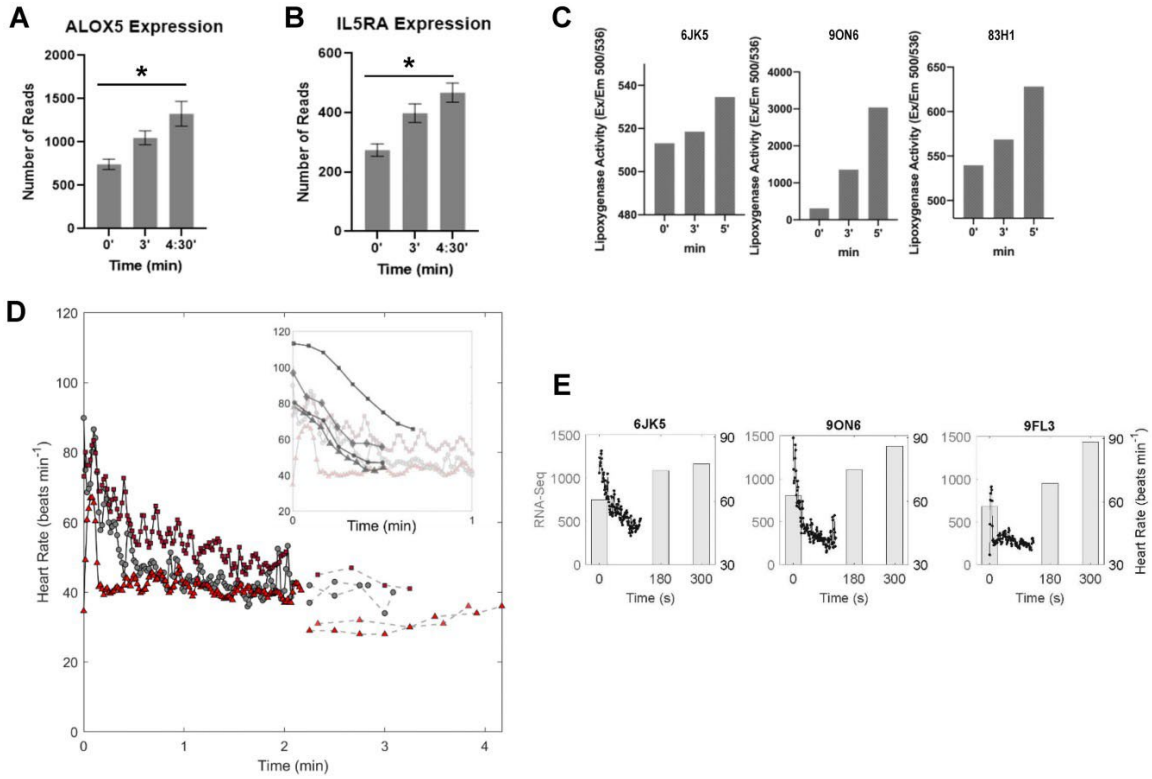


Figure 3. Dolphins induce ALOX5 activity during breath holding. (A) ALOX5 and **(B)** IL5RA mRNA expression is significantly increased over time during breath holding. **(C)** Individual dolphin lipoxigenase activity in whole blood was collected at an independent sampling date. **(D)** Physiological measurements of heart rate for three individual dolphins (black lines from ECG data previously published in Blawas et al., 2021, and dashed lines from echocardiogram data previously published in Fahlman et al., 2020) over time. Inset shows heart rate for humans performing breath holds with facial immersion in water (dark gray in inset) overlaid on dolphin heart rate. Human heart rate traces were digitally extracted from (Andersson et al., 2004; Arnold, 1985; Shattock and Tipton, 2012). **(E)** Overlay of heart rate data with ALOX activity in three individual dolphins.

response, a suite of adaptations that support reduced aerobic metabolism during diving, involves apnea, bradycardia, and peripheral vasoconstriction that assures maintained mean arterial blood pressure as blood flow to peripheral tissues is reduced and allows

regulation of perfusion to conserve O₂-rich blood for the brain and heart. To maintain a constant mean arterial blood pressure and prevent hypertension, these adaptations must work in concert to ensure efficient autoregulation; however, extended dives also result in frequent events of ischemia and hypoxia (Blix et al., 1983; Elsner et al., 1985; Fahlman et al., 2019a; McKnight et al., 2019; Meir et al., 2009; Ridgway et al., 1969). Still, knowledge of the molecular adaptations that contribute to the response to diving, enhanced tolerance to hypoxia and ischemic stress, and prevent reperfusion injury during and following a dive, is rudimentary at best. To address this lack of understanding, we combined analysis of breath-hold responses at the molecular level with existing physiological measurements to define the molecular responses to breath holding in dolphins.

While this study is limited by a small sample size and relatively short breath-hold durations, our analyses provide a proof-of-principle design to study molecular alterations in breath-holding dolphins. Analyses of baseline blood RNA-Seq profiles demonstrates similarity to transcriptional profiles of humans, indicating conserved transcriptional profiles across species. We also identified candidate genes and pathways with time-dependent changes in expression throughout the breath holds that were validated in functional studies using independently collected samples and assays. These molecular changes occurred within the calculated aerobic dive limit (cADL) of bottlenose dolphins—the duration of a dive that can be sustained without requiring anaerobic respiration at the cellular level, which has been estimated to be approximately 6.5 min (Fahlman et al., 2018). It is also worth considering the possibility that changes in gene expression could occur to support specific physiological responses to diving during a dive, and that this gene expression differs when the animal is at the surface. Future

studies will be focused on using novel technologies, such as GRO-Seq (Lopes et al., 2017) and others to measure nascent mRNAs, as well as measuring later time points to understand the changes that occur upon recovery from breath holds.

To provide physiological context for these molecular alterations on the time scales observed, we compared molecular changes to changes in previously published f_H patterns in the same individual dolphins during submerged breath holds (Blawas et al., 2021a; Fahlman et al., 2020c). If we assume that the appearance of vasoconstriction is coincident with bradycardia, our data provide evidence of an increase in the expression of a gene in blood cells, ALOX5, known to promote vasoconstriction coincident with the onset of vasoconstriction (Poeckel and Funk, 2010). As potential first responders to hypoxemia, blood cells may produce signals for extended vasoconstriction to slow O₂ consumption. Indeed, vasoconstriction, or a narrowing of the blood vessels, has been observed during forced dives and suggested as a mechanism by which marine mammals optimize the use of onboard oxygen stores in the blood and muscle (Davis and Kanatous, 1999; Scholander et al., 1942; Zapol et al., 1979). Given the long assumed link between vasoconstriction and bradycardia in marine mammals, the rapid bradycardia we observed suggests that vasoconstriction was occurring in the dolphins in this study during breath holds (Hochachka, 1981; Van Citters et al., 1965). We found that changes in gene expression occurred in all animals during the 5-min breath-hold trials and that the same gene families that were upregulated in the dolphins during breath holds help manage vasoconstriction in mice (Ichinose et al., 2001) and humans (Friedman et al., 1984). It is important to note, however, that these pathways are upregulated during an extended breath hold when the animals are reaching their

maximum breath-hold tolerance. We have no evidence that these pathways are activated during single-breath dives in nature, as many of these dives are 30 s–1 min.

Our integrated approach reveals possible molecular underpinnings that may support and act synergistically with the cardiac response to breath holding in bottlenose dolphins. Specifically, we identified candidate genes that may provide defense against ischemic and hypoxic stress in dolphins, including the GATA and SMAD transcription factors, several microRNAs, a disintegrin and metalloproteinase with thrombospondin motifs 12 (ADAMTS12), mitochondrial glutaredoxin-2 (Glx2) and ALOX5. Interestingly, many of these factors play known roles in regulating hypoxia, hematopoiesis, and ischemic stress responses. For example, the GATA transcription factor family is an important modulator of hematopoietic development of T lymphocytes, mast cells, and erythrocytes (Lentjes et al., 2016). Likewise, the SMAD family regulates hematopoietic stem cells (Blank and Karlsson, 2011). Of the microRNAs identified from our analysis of target enrichments, nearly all have been shown to be protective against ischemia-induced cell death, including miR148A (Zheng et al., 2018), miR492 (Guo et al., 2020), miR186 (Bostjancic et al., 2009; Li et al., 2013; Wang et al., 2018), miR130 (Lu et al., 2015), and miR205 (Chen et al., 2019). At the protein-coding gene level, ADAMTS12 genetic variation is associated with pediatric stroke (Witten et al., 2020), GLRX2 is implicated in neuroprotection during hypoxia and ischemia (Romero et al., 2015), and ALOX5 is known to be induced by hypoxia (Porter et al., 2014) and mediates the production of pro-inflammatory leukotrienes, which induce bronchoconstriction and vasoconstriction (Poeckel and Funk, 2010) as well as the generation of lipid peroxidases secondary to lipoxygenase production (Gaschler and Stockwell, 2017). This suggests that ALOX5 may play a role in an inflammatory cascade in response to changes during

breath holding. In addition, both ALOX5 and IL5RA have been identified as susceptibility genes associated with asthma and asthmatic inflammation in humans (Cheong et al., 2005; Mougey et al., 2013), and a monoclonal antibody to the IL5RA ligand, IL5, is FDA-approved for the treatment of severe eosinophilic asthma (Fala, 2016; Pavord et al., 2012). Given the intricate connection between molecular control and physiologic function to manage ischemia, hypoxia and inflammatory responses in humans and rodent models (Bartels et al., 2013), it is intriguing to speculate as to how dolphins and other marine mammals may uncouple or leverage these interconnected processes for improved tolerance to ischemic/hypoxic stress without the pathological consequences associated with hyper-stimulation of these processes.

Our results reveal upregulation of ALOX5 mRNAs and increased lipoxygenase activity in bottlenose dolphins during breath holds. The changes in gene expression and enzyme activity were measured in unpaired breath-hold trials collected in separate years.

2.5 Conclusion

By examining molecular data through a physiological lens, these data connect the cellular and tissue-level responses of dolphins to apnea to understand whether the bottlenose dolphin may be genetically tuned to dive and withstand the hypoxia and the potential implications of this to translational medicine. Our results uncover potential candidates at the intersection of ischemia, hypoxia, and vasoconstriction that may contribute to the exquisite adaptation of dolphins and other marine mammals to life in the ocean.

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

3.1 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 O_2 depletion by the peripheral tissues and helps to conserve O_2 in the blood for hypoxia-intolerant tissues like the brain and heart (Irving, 1939; Scholander, 1963, 1940). 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

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al., 2019; Elmegaard et al., 2016, 2019; Fahlman et al., 2019b, 2020b; 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., 2020b; 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., 2020b, p. 202; 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 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., 1994b, 1994a; Cature et al., 2019; Fahlman et al., 2019b, 2020c; 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., 1994b, 1994a; Cature et al., 2019; Elsner et al., 1966b; Fahlman et al., 2019b, 2020c; 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; Påsche and Krog, 1980; Ponganis and Kooyman, 1999; Ridgway, 1972, 1986). 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., 2019a, 2020c; Lin et al., 1972; Påsche 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., 2020b). Hooded seals performing submerged apneas demonstrated similar f_H to the minimum if_H measured during periods of breathing on land (Påsche 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., 2019b, 2020c, 2020b).

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.

3.2 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).

3.2.1 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 ± 7.0 years

(range 11–31 years) and body mass 189.3 ± 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 (Cature et al., 2019; Fahlman et al., 2020c). 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., 2019b). 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.

3.2.2 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.

3.2.3 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-ECGb, 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.

3.2.4 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 $i f_H$ 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 $i f_H$ signal in beats min^{-1} ,

normalized by the mean if_H ($if_H^{\bar{}}$) for that section: $RSA (\%) = \Delta if_H / if_H^{\bar{}}$ (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 if_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*.

3.2.5 Statistics

All statistical analyses were performed using R (v.3.6.2) (<http://www.R-project.org/>). Differences in mean if_H , maximum if_H , minimum if_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 if_H of the IBI as well as the 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. 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 \pm S.D. unless stated otherwise.

3.3 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.

3.3.1 Fasted versus non-fasted surface breathing f_H and RSA

Mean f_H for fasted trials was 67.2 ± 11.8 beats min^{-1} and mean f_H for non-fasted trials was 78.0 ± 13.1 beats min^{-1} (Welch two-sample t-test, $t = -9.5$, $P < 0.0001$). RSA was observed following respiration as an increase in the if_H immediately following the breath followed by a decrease in if_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 ± 14.3 s as compared with 14.6 ± 8.0 s during non-fasted trials. For all surface trials, fasted and non-fasted, maximum if_H was 87.4 ± 13.6 beats min^{-1} and minimum if_H was 56.8 ± 14.8 beats min^{-1} (range: $124.4 - 23.9$ beats 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 ± 11.8 beats min^{-1}) closely agreed with the allometric prediction of 64.2 beats 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 ± 13.1 beats min^{-1}) was elevated as compared with the allometric prediction (Table S1) (Stahl, 1966).

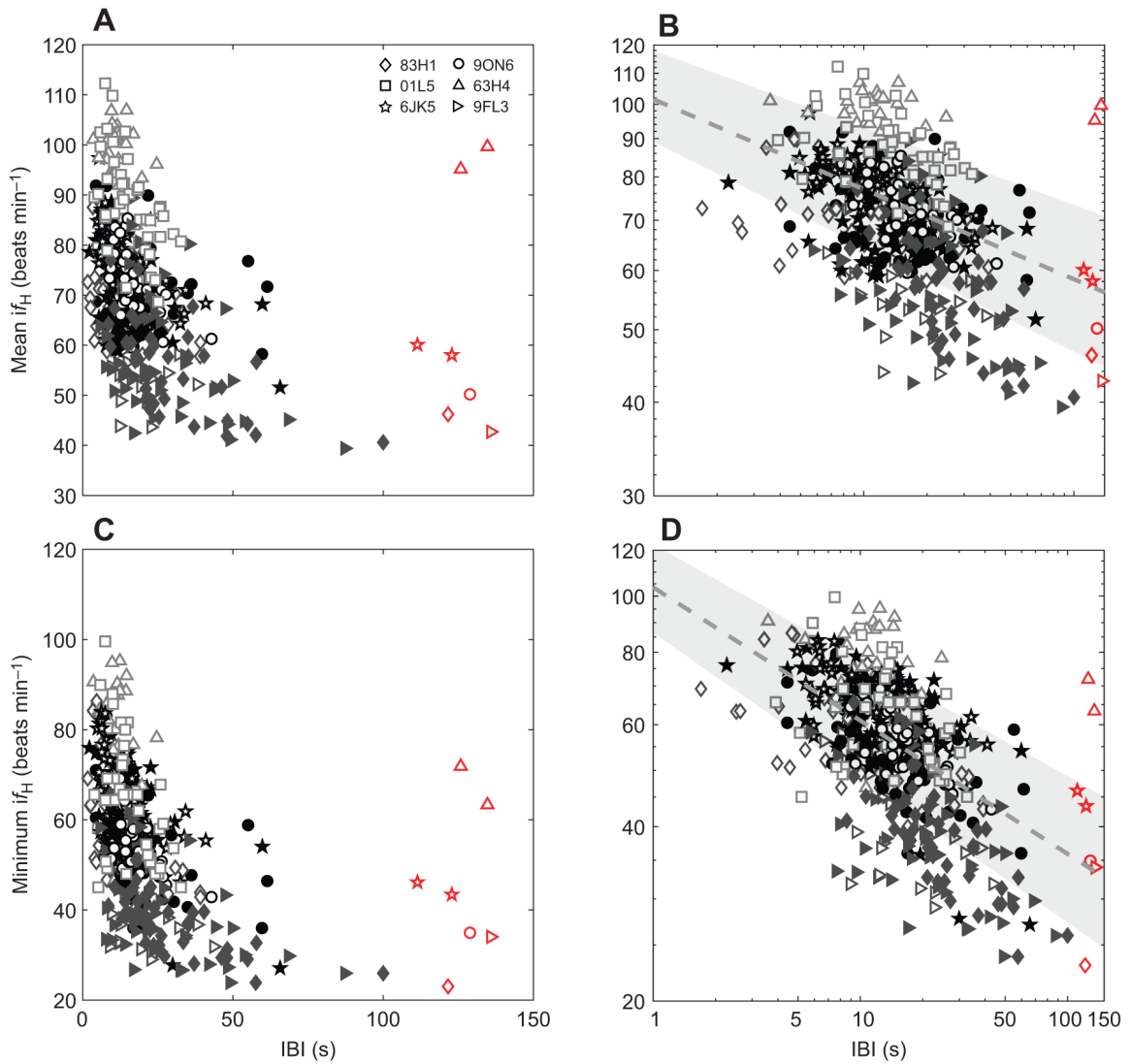


Figure 4. Mean and minimum instantaneous heart rate (if_H) during surface breathing and submerged breath-holds. Mean if_H (A, B) and minimum if_H (C, D) against inter-breath interval (IBI) for fasted surface (filled grayscale symbols, $n=241$), non-fasted surface (open grayscale symbols, $n=246$) and 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.

We examined differences in both f_H and estimated indices of RSA during fasted and non-fasted surface trials binned by IBI (Table 2). Mean if_H decreased with increasing IBI across the three IBI categories (Table 3, Fig. 4) and mean if_H was lower during fasted

Table 2. Inter-breath interval (IBI), heart rate (f_H), and respiratory sinus arrhythmia statistics for surface, fasted and non-fasted trials binned by IBI. 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. Lower case letters indicate the results of the post-hoc tests.

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

trials as compared with non-fasted trials for short and intermediate IBIs, but not for long IBIs (Fig. 4A, B, Table 2). Minimum if_H also decreased with increasing IBI (Table 3) and, like mean if_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. 4C, D, Table 2).

Notably, however, the regression describing the relationships between IBI and mean if_H as well as IBI and minimum if_H did not differ between fasted and non-fasted trials (mean if_H : B_0 , $P=0.97$; B_1 , $P=0.22$, minimum if_H : B_0 , $P=0.45$; B_1 , $P=0.06$) (Fig. 1B, D, Table 3).

The regression analysis showed that maximum if_H did not vary significantly with IBI (Table 3) 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 2). There was no significant difference in the slopes or in the intercepts 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 3). 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. 5, Table 3). 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 2).

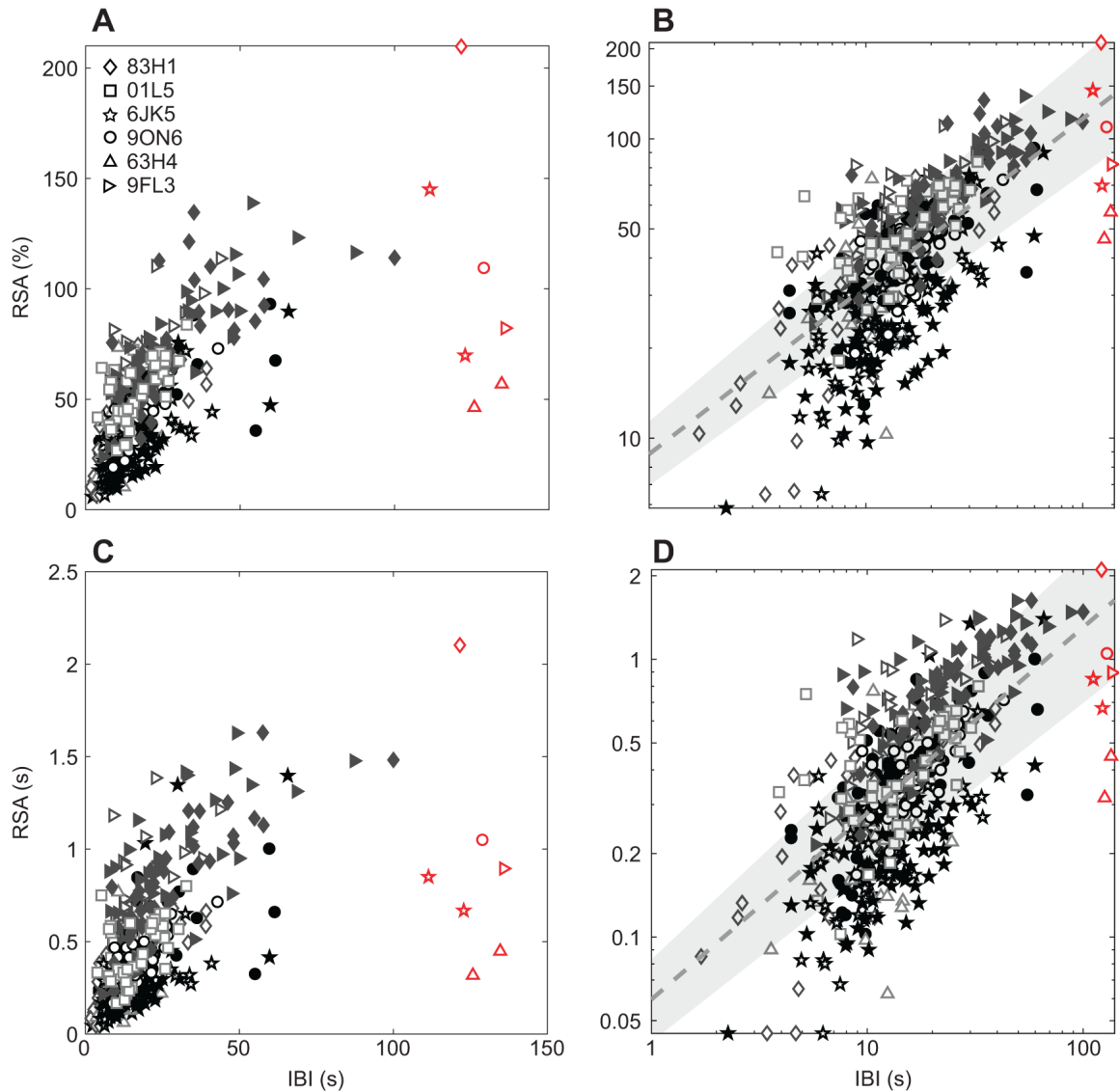


Figure 5. Respiratory sinus arrhythmia (RSA) during surface breathing and submerged breath-holds. Data showing two methods to estimate the RSA as RSA (%) (A, B) and RSA (s) (C, D) against IBI for fasted surface (filled grayscale symbols, n=241) non-fasted surface (open grayscale symbols, n=246) and submerged 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 3.

In addition to RSA, two other distinct arrhythmic patterns were observed in the f_H measured during IBIs at the surface. The first was an oscillatory pattern within the

gradual decrease in if_H of the RSA (Fig. 6). This oscillatory pattern, in addition to the initial peak in if_H , resulted in secondary and tertiary local maxima and minima within the if_H signal. These peaks occurred with a regular period of approximately 10 s (0.1 Hz, see Fig. 6A). The second arrhythmia was a series of paired heart beats that resulted in the oscillation of the if_H signal between approximately 50 beats min^{-1} and 30 beats min^{-1} (Fig. 7). 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).

3.3.2 Surface breathing versus submerged breath-hold f_H and RSA

The mean breath-hold duration was 125.9 ± 8.4 s (range: 121.6– 135.8 s) and the mean f_H during breath-holds was 64.6 ± 23.3 beats min^{-1} . For all breath-hold trials, maximum if_H was 104.6 ± 23.5 beats min^{-1} and minimum if_H was 45.3 ± 17.1 beats min^{-1} (range: 133.3–23.1 beats min^{-1}). In all surface trials (combined fasted and non-fasted) mean IBI and mean f_H were 17.1 ± 11.8 s and 72.7 ± 13.6 beats 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 4). 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.

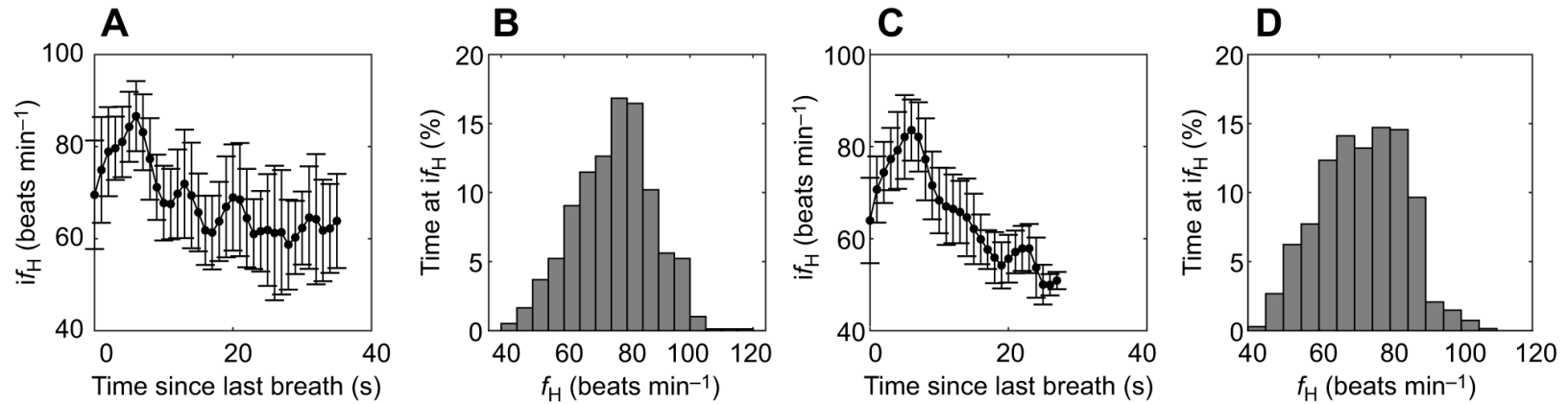


Figure 6. RSA during a fasted and non-fasted surface trial. Mean \pm S.D. if_H responses (A, C) and distribution of if_H (B, D) of 9ON6 for a representative fasted surface trial (A, B) and a representative non-fasted surface trial (C, D) corresponding to the 90th percentile duration IBI for each respective trial.

Table 3. Linear mixed effect model parameters for relationships between \log_{10} -transformed inter-breath interval (IBI) and \log_{10} -transformed f_H during surface, fasted and non-fasted trials. 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.

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

There were three general f_H patterns observed during breath-hold trials (Fig. 8). 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. 8A, B). Interestingly, the minimum if_H during the breath-hold trial for an animal displaying this pattern was approximately 15 beats 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. 8C, D). The third distinct f_H pattern observed was a rapid oscillation with an amplitude of approximately 30 beats min^{-1} around an elevated f_H (see Fig. 8E, F). Notably, this pattern was only observed in one animal (63H4) and was consistent during both breath-hold trials with this individual.

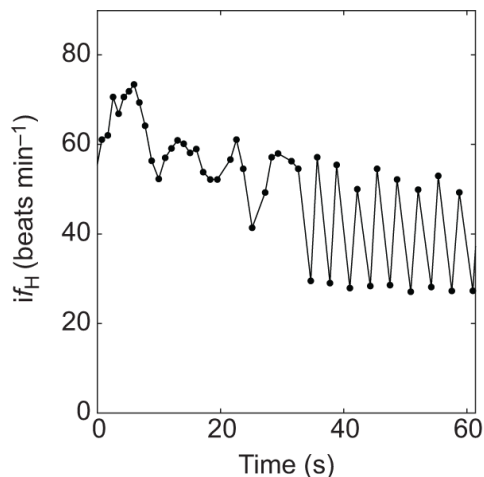


Figure 7. A paired beat arrhythmia. if_H during an IBI shows an example of a paired beat arrhythmia.

3.4 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, 1967). 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

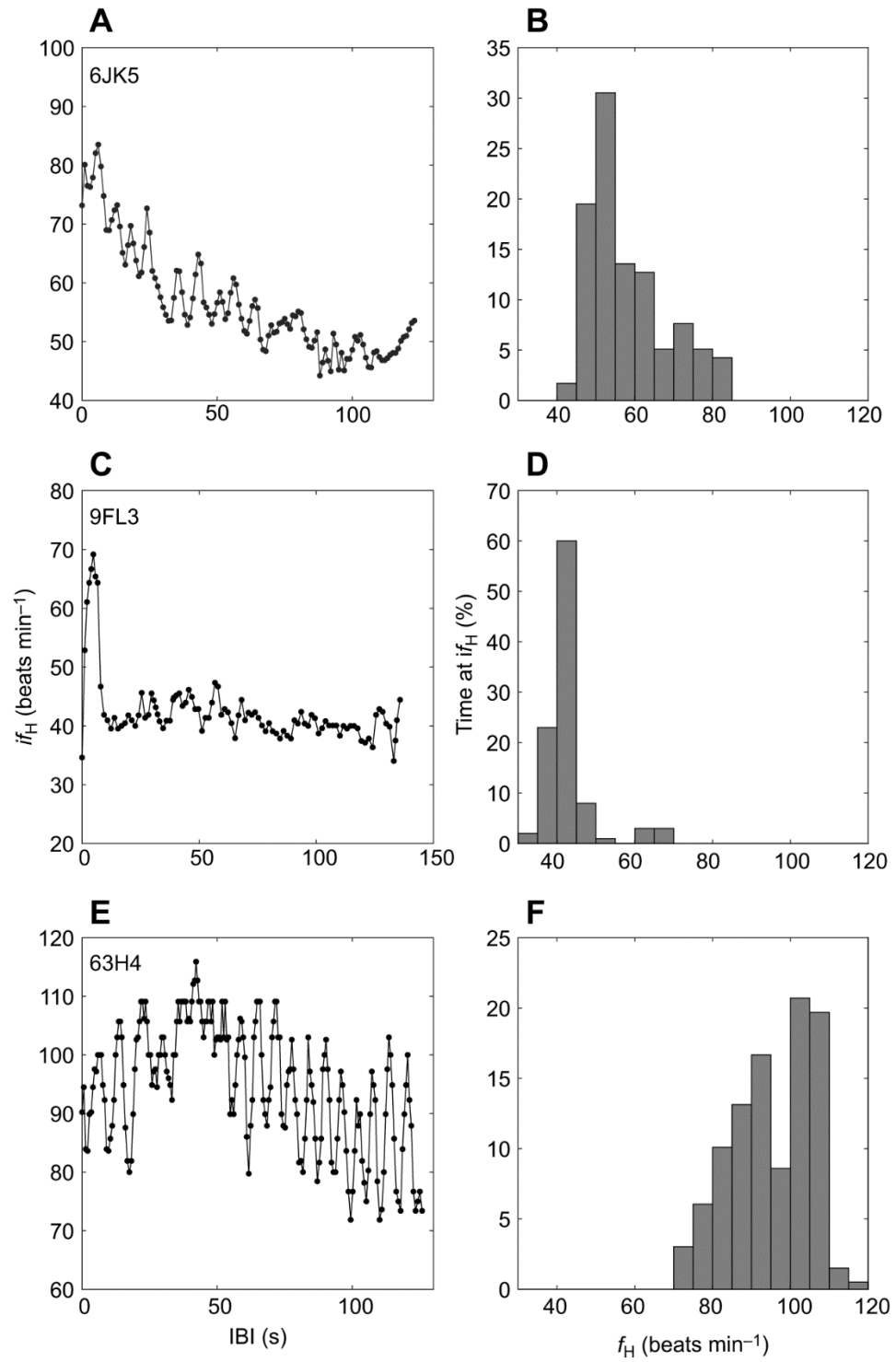


Figure 8. f_H patterns during submerged breath-holds. f_H responses (A, C, E) and distribution of f_H (B, D, F) for three submerged breath-hold trials.

Table 4. f_{H} , and RSA statistics for surface IBIs > 30 s and submerged breath-hold trials during which submersion bradycardia was observed (excludes two trials with 63H4). Trial type (surface vs. 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 2). For parameters that showed a significant difference between fasted and non-fasted trials, only non-fasted trials were compared to the submerged breath-holds.

Trial Type	Mean if_{H} (beats min^{-1})	Maximum if_{H} (beats min^{-1})	Minimum if_{H} (beats min^{-1})	RSA (%)	RSA (s)
Surface (IBI>30 s)	58.0±11.8 (n=50)	85.8±13.1 (n=50)	38.7±10.6 (n=50)	67.8±23.2 (n=14)	0.66±0.26 (n=14)
Submerged (n=5)	50.7±8.3	94.8±24.0	36.4±9.0	116.2±44.5	1.1±0.52
Statistics					
Trial Type	$X^2(1, n=55) = 6.1$ $P=0.01$	$X^2(1, n=55) = 1.5$ $P=0.21$	$X^2(1, n=55) = 2.8$ $P=0.10$	$X^2(1, n=19) = 10.4$ $P<0.001$	$X^2(1, n=19) = 8.3$ $P<0.001$
Intercept	$X^2(1, n=55) = 71.0$ $P<0.0001$	$X^2(1, n=55) = 211.7$ $P<0.0001$	$X^2(1, n=55) = 48.5$ $P<0.0001$	$X^2(1, n=19) = 86.0$ $P<0.0001$	$X^2(1, n=19) = 51.2$ $P<0.0001$

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.

3.4.1 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., 2018, 2020c; 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 (Cature 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. 5). 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 (Påsche 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 if_H of an IBI in two ways: the duration of the previous IBI could determine the 'initial value' from which f_H varies during the next IBI; and the V_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_H . We found that there was a significant negative relationship between the duration of the previous IBI and the maximum if_H of the IBI, suggesting that following a long IBI, maximum if_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 if_H of the IBI and the minimum if_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_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_T associated with longer IBIs may not compensate for the low initial value of the f_H at the beginning of that IBI because of the preceding long IBI. These results suggest that the previous IBI may influence f_H patterns during a given IBI and that changes in f_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_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_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_H seen during IBIs > 30 s reflects the 'normal', intrinsic f_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 apneas in seals that match allometric predictions, as well as measurements of f_H in killer whales during stationary breath-holds that agree with allometric predictions for resting f_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_H of the dolphin, which has previously been assessed during anesthesia with nitrous oxide to be approximately 100–120 beats 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_H is largely regulated by vagal tone (Elliott, 2002). In addition to the lack of RSA at high f_R , in humans RSA has also been observed to be f_R independent at low f_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_H (Hirsch and Bishop, 1981). In this case, the normal rate in a dolphin may be a reduced f_H , or bradycardia, that is maintained in the absence of breathing, which is closer to the average diving f_H . For marine mammals, we propose that the IBI and the corresponding mean f_H where an IBI independence of RSA is observed may be of interest to define resting f_H more clearly in the dolphin for comparison with diving f_H (Fahlman et al., 2019b; Kooyman, 1985).

3.4.2 Minimum if_H of RSA during long IBIs is comparable to bradycardia of submerged breath-holds

The minimum if_H of the RSA during long IBIs did not differ from the minimum if_H observed during submersion bradycardia associated with the breath-hold trials. Several individual dolphins consistently exhibited if_H at or below 35 beats min^{-1} during extended IBIs at the surface. This is comparable to the 24–35 beats min^{-1} measured in previous studies following both active and static breath-holds (Fahlman et al., 2019b, 2020b; Noren et al., 2004, 2012; Ridgway, 1972). Similar observations have been made in California sea lions, hooded seals and emperor penguins, where minimum f_H observed between breaths on land or during resting in water was comparable to the minimum f_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_H associated with RSA was only comparable to that of minimum f_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_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).

3.4.3 Additional arrhythmias present during breathing and submerged breath-holds

In addition to RSA, we identified two other arrhythmic patterns in f_H measurements that may be indicative of additional cardiovascular controls on f_H at the surface. The first was a faster oscillation with an approximate frequency of 0.1 Hz within the gradual decrease in if_H of the RSA (see Fig. 6A). It is worth noting that this pattern was observed during most IBIs, and has also been observed in past studies in bottlenose dolphins (Cature et al., 2019; Fahlman et al., 2020b). 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 if_H followed by an extended R–R interval until the next set of paired beats, resulting in a low if_H (Fig. 7). 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., 2020c), 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_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, 2015b). 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., 2020b, 2020c)

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. 8A), (2) a rapid decrease in f_H , like submersion bradycardia (Fig. 8C), and (3) highly oscillatory and elevated f_H (Fig. 8E). 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 min^{-1} (Fig. 8A). 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. 8C). 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., 2020b; 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. 8E). Across both breath-hold trials this animal displayed a mean if_H of 97.5 ± 11.9 beats min^{-1} and a minimum if_H of 67.6 ± 6.0 beats 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 if_H as has been shown in both harbor porpoises, California sea lions, and bottlenose dolphins (Elmegaard et al., 2016; Fahlman et al., 2020b; 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.

3.4.4 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 2; Table S1). We expect an increase in metabolic overhead, or specific dynamic action (SDA), associated with digestion to result in a higher rate of 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 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 if_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., 2020c). Both would help to increase delivery of 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 if_H and minimum if_H were not significantly different between fasted and non-fasted trials (Table 2), for IBIs < 30 s there

was a significant difference in both mean if_H and minimum if_H for fasted versus non-fasted trials. We found that both mean if_H and minimum if_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 if_H that is associated with a shorter IBI. There was not a clear association between fasted state and maximum if_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 min^{-1} for the average body mass of the animals that participated in the fasted trials (Table S1) (Stahl, 1967). This suggests that the allometric relationship provided by Stahl 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., 2020c). 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 allometric relationship (Stahl, 1967). 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).

3.5 Conclusions

Overall, our work shows that bottlenose dolphins exhibit RSA and that it predictably affects f_H during surface breathing. Minimum if_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 if_H and mean if_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.

4. Scaling of heart rate with breathing frequency and body mass in cetaceans*

4.1 Introduction

Marine mammals are known to demonstrate dramatic changes in heart rate (f_H) associated with diving behavior (Grinnell et al., 1942; Scholander, 1940; Williams et al., 2015b). In the blue whale (*Balaenoptera musculus*), the largest extant animal, regular diving f_H s of 4 beats min^{-1} have been measured (Goldbogen et al., 2019a). The reduction in f_H , or bradycardia, during a dive reduces both the rate of oxygen consumption of the heart and, in conjunction with peripheral vasoconstriction, limits the supply of oxygen-rich blood to peripheral tissues (Irving et al., 1941a; Zapol et al., 1979). By temporarily reducing the oxygen supply to the peripheral tissues and the metabolic needs of the heart, marine mammals prolong the duration of aerobic metabolism and can, in theory, extend dive duration (Irving et al., 1941a; Scholander et al., 1942). The dive response is universal among marine mammals and is known to be modulated in proportion to dive depth and duration. Both harbour porpoises (*Phocoena phocoena*) and bottlenose dolphins (*Tursiops truncatus*) trained to perform dives of varying durations demonstrated a physiological anticipatory preparation in the modulation of their f_H s according to the expected dive duration (Elmegaard et al., 2016; Fahlman et al., 2020b). In Weddell seals (*Leptonychotes weddellii*) forced to dive, the degree of bradycardia was increased compared to spontaneous dives of similar durations, which is

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thought to reflect that the animal responds more conservatively when its ability to plan in advance is removed (Kooyman and Campbell, 1972). The ability to modulate f_H on a fine temporal scale allows a marine mammal to match its cardiovascular changes to the needs associated with its behavioral state, thereby maximizing energetic intake through increased foraging time while minimizing energetic output through reduced metabolic costs (Davis, 2014). Importantly, a marine mammal's ability to modulate its instantaneous heart rate (if_H) has been correlated with increased apnea duration (Castellini et al., 1994b; Fahlman et al., 2019b; Noren et al., 2004). In addition, conditioned variation in if_H has been proposed as an important part of the selective gas exchange hypothesis that explains how cetaceans may exchange metabolic gases while minimizing uptake of N_2 (Fahlman et al., 2021; Garcia Párraga et al., 2018).

Marine mammals also show considerable plasticity in cardiac function during surface intervals. While breathing at the surface marine mammals demonstrate respiratory sinus arrhythmia (RSA), like other terrestrial mammals including humans, where the if_H oscillates in synchrony with the respiratory rate (f_R) (Andrews et al., 1997, 2000; Castellini et al., 1994a; Fahlman et al., 2018, 2019b, 2020c; McDonald and Ponganis, 2014). The mechanism of RSA is understood to be a central phenomenon of respiratory modulation via cardiac vagal stimulation, but its physiological role is less well understood (De Burgh Daly, 2011; Eckberg, 2009; Horner et al., 1995). The dominant hypotheses based on work in humans and dogs suggest that RSA functions to improve cardiorespiratory efficiency by increasing ventilation/perfusion matching and/or reducing cardiac (Arieli and Farhi, 1985; Ben-Tal et al., 2012; Hayano et al., 1996). In cetaceans, which have evolved into a fully aquatic lifestyle, these functions could have downstream effects on an individual's ability to maximize time spent underwater. This is due to the

effect of f_H modulation on perfusion and oxygen supply to the tissues via cardiac output, which are two of the primary factors that determine an individual's aerobic dive limit during diving and affect oxygen transport during surface recovery time (Butler, 2001; Fahlman et al., 2019b, 2020c; Hazen et al., 2015; Murdaugh et al., 1966). RSA has been observed in several cetacean species including the grey whale (*Eschrichtius robustus*), killer whale (*Orcinus orca*), short-finned pilot whale (*Globicephala macrorhynchus*), beluga (*Delphinapterus leucas*), common dolphin (*Delphinus delphis*), bottlenose dolphin and harbour porpoise (Cature et al., 2019; Elsner et al., 1966b; Fahlman et al., 2019b, 2020c; Kanwisher and Ridgway, 1983; Kastelein and Meijler, 1989; Lyamin et al., 2016; Ponganis and Kooyman, 1999; Ridgway, 1972, 1986). In bottlenose dolphins, recent work demonstrated that the resting f_H corrected for f_R was comparable to that during some apneas and underscored the potential utility of using the magnitude of RSA as an index of diving ability, as has previously been supported by studies of pinnipeds (Castellini et al., 1994b; Fahlman et al., 2019b). If the magnitude of RSA does indeed reflect diving ability because of its potential relationship to cardiorespiratory efficiency, quantifying a baseline value and estimating how it could vary in a cetacean in response to changes in f_R during disturbance are necessary for its utility as a conservation tool.

In addition to the known effect of respiration on f_H and RSA, f_H also scales allometrically across vertebrate taxa making body mass (M_b) critical in the interpretation of f_H measurements (Fahlman et al., 2019b, 2020c; West et al., 1997; White and Kearney, 2014). In previous analyses examining the relationship between M_b and f_H in mammals, a scaling exponent of -0.25 has been suggested (Stahl, 1967). Given the relationship of metabolic rate and f_H through Fick's principle (Fick, 1870), differences in f_H are expected to estimate differences in metabolic rate when accounting for M_b (Butler

et al., 2004; Green et al., 2005b; Young et al., 2011). Although this relationship may be more complicated in breath-holding marine mammals (Green et al., 2007; Webb et al., 1998; Young et al., 2011), a lower rate of mass-specific oxygen consumption coupled with an isometric relationship between M_b and body oxygen stores suggests that larger animals should have increased diving capacity (Goldbogen and Madsen, 2018; Halsey et al., 2006; Schreer and Kovacs, 1997). In fact, many studies in marine mammals have shown that M_b does positively correlate with dive duration, but M_b alone does not explain all of the observed inter-species variation in diving patterns nor the variation across taxa (Halsey et al., 2006; Hindell et al., 2000; Le Boeuf, 1994; Noren et al., 2002). In cetaceans, other ecological and physiological factors have been suggested to play a role in determining diving behavior including diel patterns (Miller et al., 2010), foraging ecology (Goldbogen et al., 2019b, 2012), and the biochemical structure of respiratory pigments (Helbo and Fago, 2012; Mirceta et al., 2013). Given the potential importance of RSA in contributing to efficient cardiorespiratory patterns of cetaceans, we aimed to investigate the scaling of RSA and f_H across cetacean species to examine inter-species variation in the degree of RSA. Our specific objectives were to collect simultaneous f_H and f_R data from five species of cetaceans in human care across a range of M_b s and determine how f_H and the degree of RSA vary allometrically with f_R and M_b .

4.2 Methods

4.2.1 Animals

We collected data from six bottlenose dolphins, a beluga whale, a false killer whale (*Pseudorca crassidens*), four killer whales, and three short-finned pilot whales in human care for inclusion in this study. All procedures described were performed in strict accordance with the US Animal Welfare Act for the care of Marine Mammals (US-based

facilities) or the European Association for Aquatic Mammals (EAAM) (Europe-based facilities). Additionally, animals were housed in outdoor facilities with access to shade whose size met or exceeded the standards set by the Alliance of Marine Mammal Parks & Aquariums (AMMPA) and the EAAM. All animals were adults and considered non-reproductive during the period of data collection. A summary of the animals that participated in the study can be found in electronic supplementary material, Table S1.

4.2.2 Data collection

All data were collected during stationary, non-fasted, resting trials with cetaceans in human care (Table 5). Prior to the study, animals were desensitized to the research equipment using operant conditioning. All research trials began with a 2 min period of slow swimming or resting poolside prior to the start of data collection. To start a trial, the animal was positioned next to the trainer and rolled into dorsal recumbency to allow placement of three electrocardiogram (ECG) electrodes. The three-lead ECG used gold-plated electrodes (Disposable GoldSelect Cup Electrodes, DE-003710, Rochester Med, FL, USA), which were mounted inside custom silicone suction cups (Smooth-Sil 940, Smooth-On, Inc., PA, USA). The ECG electrodes were connected to a custom-built bio-amplifier (UUB/1-ECGb, UFI, Morro Bay, CA, United States), and then, with a BNC connector, to the data acquisition system (Powerlabs 8/35, ADInstruments, Colorado Springs, CO, USA). Conductive paste (Ten20 Conductive Paste, Weaver and Company, Aurora, Colorado, USA) was applied to each suction cup before being placed on the skin (see electronic supplementary material, Fig. S1). With the electrodes placed, the animal then rolled back, dorsal side up while the electrodes continued to stay attached and were submerged on the ventral side. A trial began when the animal was in ventral recumbency and positioned next to the trainer in a resting position. One trial consisted of

5– 10 min of stationary rest at the surface while the ECG and breaths were recorded. From the ECG, the R–R interval was used to determine the if_H during post-processing.

For trials with bottlenose dolphins, the false killer whale and the beluga whale, f_R was recorded using a Fleisch-type pneumotachometer (Mellow Design, Valencia, Spain) developed to measure breath-to-breath exhaled and inhaled respiratory flow in small- and medium-sized cetaceans (Fahlman et al., 2020a). The start of a breath was determined from the flow signal and the f_R was determined from the duration between successive breaths. Further analysis of the respiratory flow was not performed for this study. For the larger species (short-finned pilot whales and killer whales), the breaths were recorded manually and verified by a change in the baseline ECG signal, which was indicative of both movement and muscle electrical activity associated with exhalation. All data were collected during the day when the feeding state of the animals was considered non-fasted. All experiments were conducted across a 2-year period between autumn 2017 and autumn 2019.

4.2.3 Data processing

The respiratory flow and ECG were sampled at 400 Hz by a data acquisition system (Powerlabs 8/35, ADInstruments, Colorado Springs, CO, USA), which displayed all data streams in real-time on a laptop computer running LabChart (v.8.1, ADInstruments, Colorado Springs, CO, USA). The ECG Analysis Module in LabChart was used to extract if_H from the ECG signal. All further data analysis and processing were done using MATLAB (version 2018b, © 2018 The MathWorks, Inc., Natick, MA, USA). Maximum if_H and minimum if_H were the maximum or minimum f_H measured during a given inter-breath interval (IBI), respectively. Mean if_H was the average if_H measured during an IBI, whereas mean f_H (or \bar{f}_H) was the average of all if_H s measured for an

individual. The degree of RSA was estimated using $\text{RSA} (\%) = \Delta f_H / \bar{f}_H$, where $\Delta f_H =$ maximum f_H of the IBI – minimum f_H of the IBI as has previously been described (Mortola et al., 2016).

4.2.4 Statistical analysis

All statistical analyses were conducted using R version 3.6.2 (R Core Team, 2020). Linear mixed-effects models (*nlme* package) were used to evaluate the effects of f_R and M_b simultaneously and individually on f_H and the degree of RSA across all individuals, with species and animal ID included as random effects. Individual relationships between predictor and outcome variables were evaluated, with the predictor variables, f_R and M_b , \log_{10} -transformed to improve the normality of both variables. The outcome variables, RSA and all f_H variables were normally distributed without being \log_{10} -transformed and therefore were not transformed for the initial models. However, to be able to compare these data with previously published allometric relationships, the outcome variables were \log_{10} -transformed in subsequent models to model power law relationships between individual predictor and outcome variables. The Akaike information criterion (AIC) was used to determine the most parsimonious model for each dependent variable by selecting the model with the lowest associated AIC. For nested models, a likelihood ratio test was used to evaluate whether the most parsimonious model variable was significantly better than the nested model with the next-lowest AIC and one less predictor variable. In determining the most parsimonious model for each outcome variable, predictor variables were \log_{10} -transformed to improve the normality of both variables, but outcome variables were not transformed given that they were normally distributed without transformation. A model that included the interaction of f_R and M_b was considered for all dependent variables to account for the

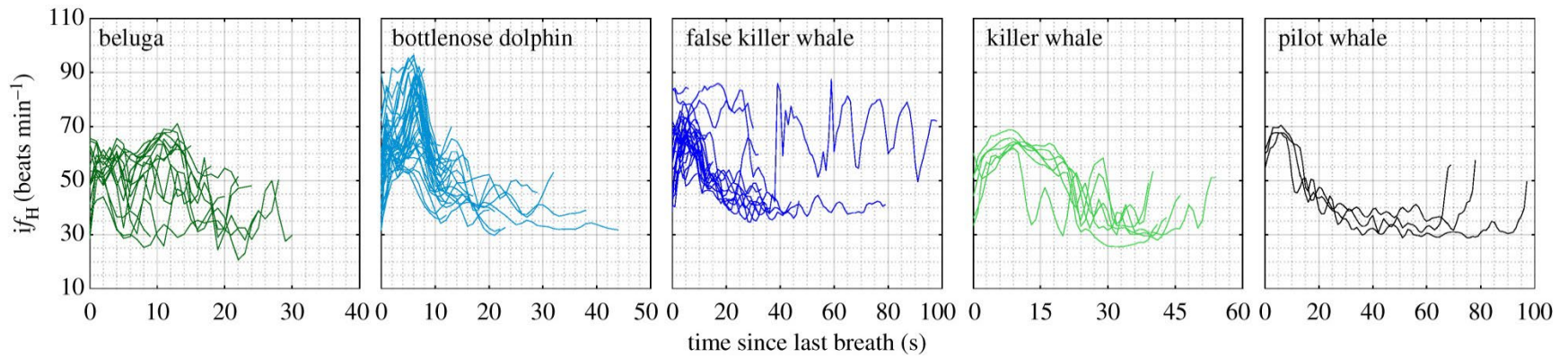


Figure 9. A representative trial for one individual of each species showing changes in heart rate (f_H) during the IBI.

known mass dependence of f_R (Gehr et al., 1981). All statistical tests were done assuming that a p-value < 0.05 indicated a significant difference. Values are presented as average \pm S.D. unless stated otherwise.

4.3 Results

Average values for age, M_b , f_R , f_H , and RSA (%) are reported for each species in Table 5. The killer whales were the largest cetaceans in the study and approximately 13 times larger than the average mass of the bottlenose dolphins studied (Table 2). In total, 18 trials with 15 individuals were conducted, which contained a total of 324 breaths. All species and all individuals displayed a clear RSA, although there was some variation in the shape of the f_H patterns throughout the IBI (Fig. 9). The average f_R was lowest in the short-finned pilot whales and highest in the bottlenose dolphins (Table 5). Short-finned pilot whales also displayed the lowest mean f_H (38.3 ± 12.1 beats min^{-1}) and the greatest degree of RSA, while bottlenose dolphins displayed the highest mean f_H (77.8 ± 16.9 beats min^{-1}) and the lowest degree of RSA of the five species studied (Table 5).

Possible allometric relationships were evaluated by examining the relationship between individual predictor variables and outcome variables with both variables \log_{10} -transformed (Table 6). RSA was significantly related to f_R with a scaling coefficient of -0.56 ± 0.03 (coefficient \pm S.E.) and to M_b with a scaling coefficient of 0.35 ± 0.11 (Fig. 10A, B). Similarly, both mean if_H and minimum if_H of the RSA were significantly

Table 5. Average age, body mass (M_b), breathing frequency (f_R), heart rate (f_H), degree of RSA, maximum if_H of the RSA, and minimum if_H of the RSA across all individuals of each species. The number of breaths is indicated by n and the number of individuals is indicated in parentheses.

Species	Age (years)	M_b (kg)	f_R (breaths min ⁻¹)	f_H (beats min ⁻¹)	RSA (%)	Max. if_H (beats min ⁻¹)	Min. if_H (beats min ⁻¹)
Bottlenose dolphin (n=246 breaths from 6 animals)	23.8±7.9	189.3±36.3	5.6±3.8	77.8±16.9	41.7±18.9	92.3±13.6	60.9±14.2
False killer whale (n=27 breaths)	30	520.0	4.1±2.7	59.4±13.7	43.4±23.5	73.5±9.4	48.3±12.7
Beluga whale (n=18 breaths)	24	800.0	4.3±2.7	54.6±14.1	58.4±23.3	69.0±14.4	37.8±12.6
Short-finned pilot whale (n=8 breaths from 3 animals)	8	843.3±242.1	0.7±0.2	38.3±12.1	90.9±14.2	62.1±10.2	27.1±3.1
Killer whale (n=25 breaths from 4 animals)	20.0±15.3	2217.4±344.1	1.3±0.6	46.7±14.0	77.0±17.3	64.2±4.3	28.6±5.1

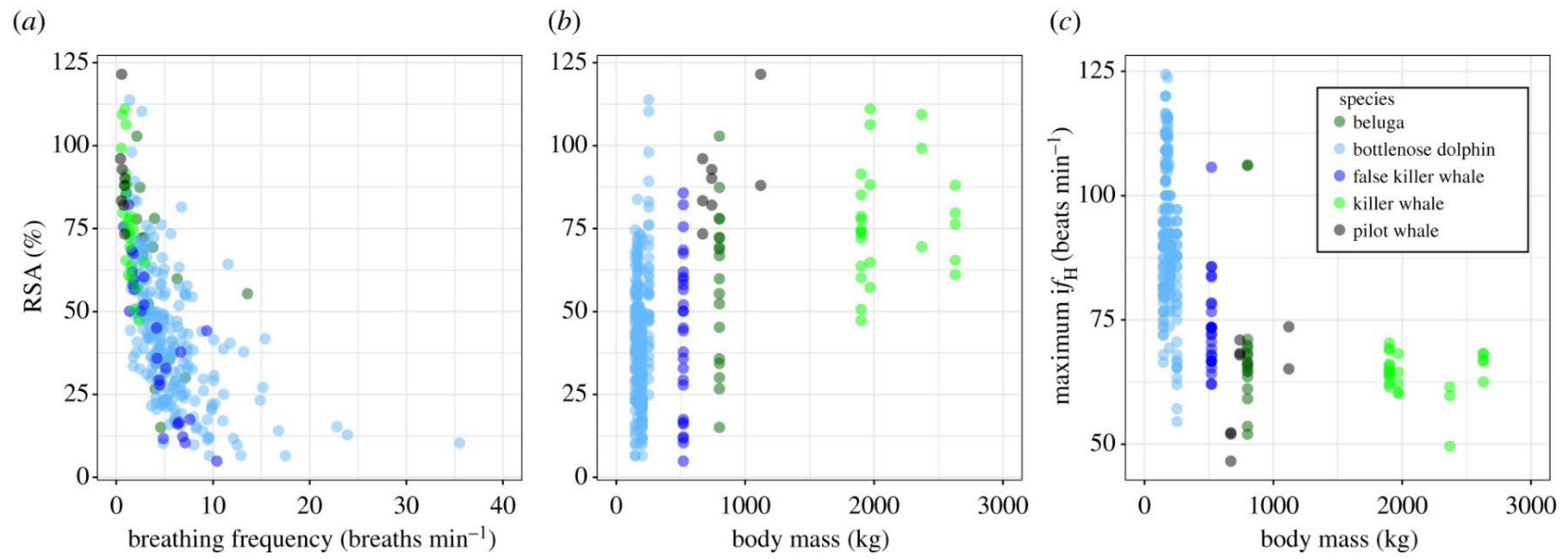


Figure 10. (A) RSA versus breathing frequency (f_R) and (B) RSA versus body mass (M_b). (C) Maximum if_H of the RSA versus M_b (324 IBIs from 15 individuals).

related to both f_R and M_b . Both mean if_H and minimum if_H were positively related to f_R and scaled with coefficients of 0.11 ± 0.01 and 0.21 ± 0.02 , respectively (Fig. 11A, C).

Maximum if_H of the RSA was not significantly related to f_R . There was a significant negative relationship between f_H and M_b and maximum if_H , mean if_H , and minimum if_H of the RSA were related to M_b by the scaling coefficients -0.16 ± 0.03 , -0.24 ± 0.07 and -0.34 ± 0.05 , respectively (Fig. 10C and 7B, D).

Linear mixed models revealed the most parsimonious model for each outcome variable that best described the variation in the outcome variable with the fewest predictor variables (Table 7). Before models were constructed, we examined the individual relationships between each predictor variable and outcome variable. There was a significant relationship between f_R and all outcome variables except for the maximum if_H of the RSA. f_R showed a negative relationship with RSA ($p < 0.0001$) and a positive relationship with mean if_H ($p < 0.0001$) and minimum if_H ($p < 0.0001$) of the RSA. M_b was significantly related to all outcome variables and showed a positive relationship with RSA ($p = 0.01$) and a negative relationship with maximum if_H ($p < 0.001$), mean if_H ($p < 0.01$) and minimum if_H ($p = 0.0001$) of the RSA. The most parsimonious model of the degree of RSA included both f_R , M_b and their interaction, such that the relationship between f_R and RSA varied with M_b (Table 7). In the most parsimonious models for both mean if_H and minimum if_H of the RSA, f_H increased with increasing f_R (Fig. 11A, C) and decreased with increasing M_b (Fig. 11B, D). Minimum if_H increased more rapidly with increasing f_R as compared with mean if_H but decreased less rapidly with increasing M_b than mean if_H and maximum if_H (Table 7). M_b was the only predictor variable included in the most parsimonious model of maximum if_H of the RSA (Table 7).

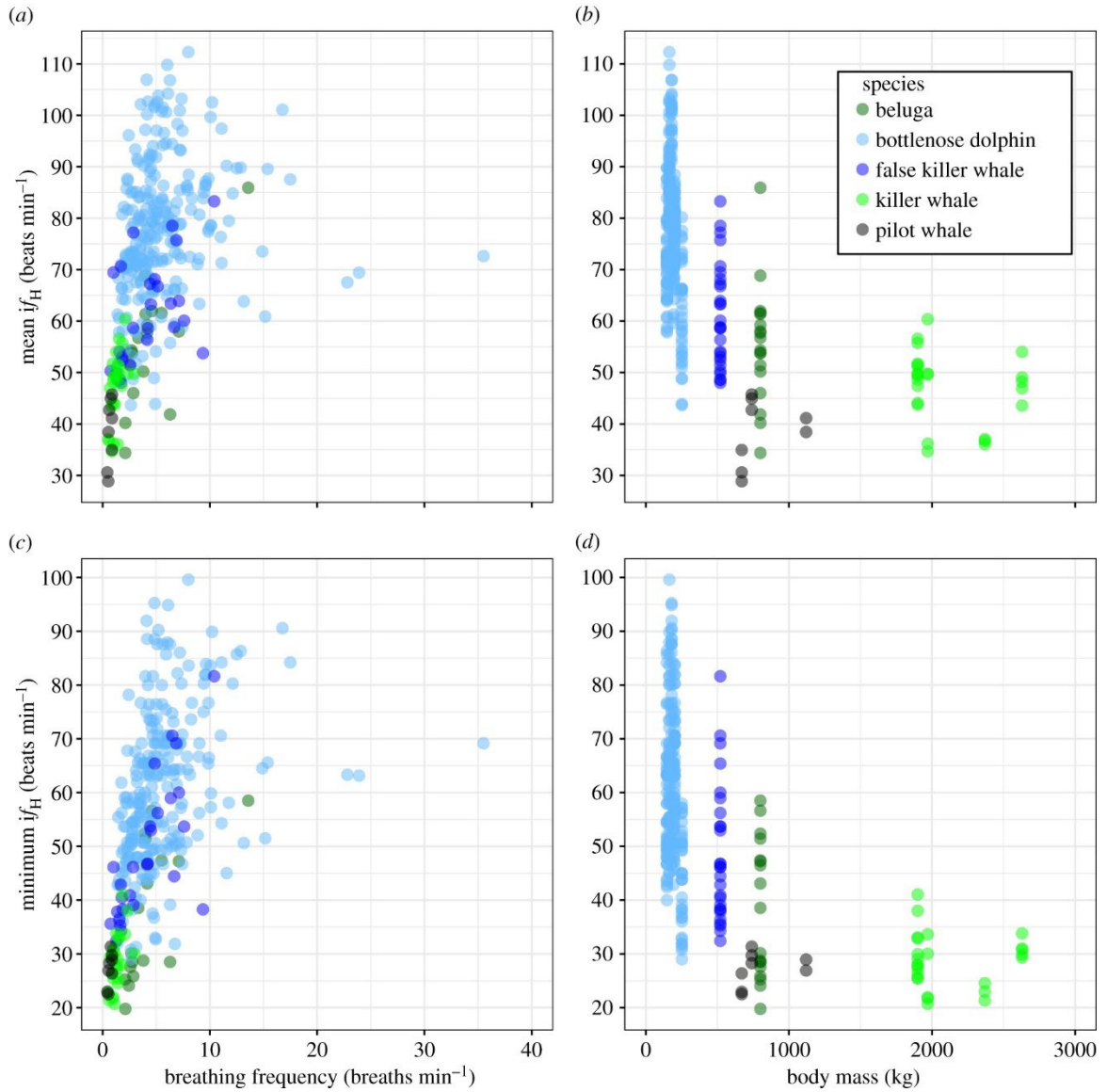


Figure 11. (A) Mean i_{f_H} versus breathing frequency (f_R) and (B) mean i_{f_H} versus body mass (M_b). (C) Minimum i_{f_H} versus f_R and (D) minimum i_{f_H} versus M_b (324 IBIs from 15 individuals).

Table 6. Results of linear mixed effects models with species and animal ID included as random effects determine the allometric relationships between f_R , M_b , f_H , and RSA. Value of the coefficient of the predictor variable, standard error of the coefficient, t-value, and P-value are presented for each model. P-value indicates significance of the linear relationship between the predictor and outcome variable.

Model	Coeff.±S.E.	t-value	P-value
$\log_{10}(\text{RSA}) \sim \log_{10}(f_R)$	-0.56±0.03	-20.3	<0.0001
$\log_{10}(\text{RSA}) \sim \log_{10}(M_b)$	0.35±0.11	3.3	<0.01
$\log_{10}(\text{Mean } if_H) \sim \log_{10}(f_R)$	0.11±0.01	9.6	<0.0001
$\log_{10}(\text{Mean } if_H) \sim \log_{10}(M_b)$	-0.24±0.07	-3.2	0.01
$\log_{10}(\text{Max. } if_H) \sim \log_{10}(f_R)$	-0.0±0.01	-0.7	0.49
$\log_{10}(\text{Max. } if_H) \sim \log_{10}(M_b)$	-0.16±0.03	-4.1	<0.001
$\log_{10}(\text{Min. } if_H) \sim \log_{10}(f_R)$	0.21±0.02	12.8	<0.0001
$\log_{10}(\text{Min. } if_H) \sim \log_{10}(M_b)$	-0.34±0.05	-6.3	<0.0001

Table 7. Results of linear mixed effects models with species and animal ID included as random effects reveal the most parsimonious model of f_H and RSA. Predictor variables were \log_{10} -transformed prior to model fit. Each most parsimonious model was evaluated against the nested model with the next smallest AIC using a log-likelihood ratio test.

Model	AIC	Parameter	Coeff±S.E.	t-value	P-value
RSA ~ $\log_{10}(f_R)$	2426.2	$\log_{10}(f_R)$	60.4±24.5	2.5	0.01
RSA ~ $\log_{10}(M_b)$	2629.4	$\log_{10}(M_b)$	12.6±6.3	2.0	0.08
RSA ~ $\log_{10}(f_R) + \log_{10}(M_b)$	2426.3	$\log_{10}(f_R)^*$	-47.0±10.3	-4.6	<0.0001
		$\log_{10}(M_b)$			
RSA ~ $\log_{10}(f_R)^*\log_{10}(M_b)$	2410.9	Intercept	43.8±17.5	2.5	0.01
Mean $if_H \sim \log_{10}(f_R)$	2059.2	$\log_{10}(f_R)$	20.0±1.7	11.6	<0.0001
Mean $if_H \sim \log_{10}(M_b)$	2165.1	$\log_{10}(M_b)$	-21.7±5.3	-4.1	<0.0001
Mean $if_H \sim \log_{10}(f_R) + \log_{10}(M_b)$	2054.4	Intercept	112.6±14.9	7.6	<0.01
Mean $if_H \sim \log_{10}(f_R)^*\log_{10}(M_b)$	2055.3				
Max. $if_H \sim \log_{10}(f_R)$	2355.0	$\log_{10}(M_b)$	-29.4±5.8	-5.1	<0.001
Max. $if_H \sim \log_{10}(M_b)$	2347.8	Intercept	157.1±15.9	9.9	<0.0001
Max. $if_H \sim \log_{10}(f_R) + \log_{10}(M_b)$	2349.3				
Max $if_H \sim \log_{10}(f_R)^*\log_{10}(M_b)$	2349.4				
Min. $if_H \sim \log_{10}(f_R)$	2367.0	$\log_{10}(f_R)$	25.2±2.1	12.3	<0.0001
Min. $if_H \sim \log_{10}(M_b)$	2484.3	$\log_{10}(M_b)$	-17.9±4.7	-3.8	<0.01
Min. $if_H \sim \log_{10}(f_R) + \log_{10}(M_b)$	2361.4	Intercept	84.3±13.3	6.4	<0.0001
Min. $if_H \sim \log_{10}(f_R)^*\log_{10}(M_b)$	2363.0				

4.4 Discussion

Cardiorespiratory coupling has been suggested to help cetaceans maximize gas exchange during short surfacing intervals by producing a large cardiac response, or a brief tachycardia followed by a gradual decrease in f_H , following respiration (Fahlman et al., 2020c). Studies in humans and dogs have suggested that RSA reduces cardiac work and/or increases ventilation/ perfusion matching, both of which could benefit cetaceans that are dually constrained by their need to conduct gas exchange at the surface and forage underwater (Ben-Tal et al., 2012; Hayano et al., 1996). Here, we quantify RSA and make the first comparisons of the degree of RSA across several species of cetaceans. The data not only demonstrate that all five cetacean species studied exhibit RSA and that there are large differences in the degree of RSA across species, but also that these differences scale with M_b and f_R .

While this study is constrained by a limited sample size, particularly with one individual beluga and false killer whale, analyses were conducted on a breath-by-breath basis and therefore multiple data points were used in calculating variables both within and between individuals and species. All data used in this study were collected under resting conditions when the animals were stationary at the surface and breathing spontaneously, so the patterns of cardiorespiratory coupling observed may not be extended to diving conditions. Additionally, variation in f_R likely does not capture the overall variation in gas exchange and therefore an examination of f_R alone in an analysis of cardiorespiratory coupling is incomplete. Both tidal volume (V_T) and O_2 extraction (E_{O_2}) are known to vary in cetaceans during resting and active behaviors in addition to f_R (Fahlman et al., 2020c, 2016; Reed et al., 2000; Ridgway et al., 1969; Sumich, 2000). However, given that f_R is a common metric used to evaluate respiratory physiology in

free-ranging cetaceans because it can be determined from visual observation and/or tag data (Goldbogen et al., 2008; Isojunno et al., 2018; Miller et al., 2010; Roos et al., 2016), we decided to use f_R in this preliminary study.

All animals studied exhibited RSA, which was observed as an increase in if_H directly following a breath and a gradual decrease in if_H until the next breath, although there was some variation in the degree of RSA between species (Fig. 9). The peak in if_H consistently occurred between 5–10 s in all species. If the brief tachycardia in cetaceans is driven by lung inflation, as has been suggested in seals (Angell-James et al., 1981), this result could suggest that the post-respiration tachycardia is secondary to an absolute change in intrathoracic pressure during breathing. Given that intrapleural pressure and air velocity in the trachea are known to be size-independent in terrestrial mammals (West et al., 1997) and that changes in intrathoracic pressure are known to result in changes in f_H during breath holds in humans (Craig, 1963), the brief post-respiration tachycardia may be related to changes in local pressure gradients surrounding the heart.

The mixed effects analyses allowed us to determine that both f_R , M_b and their interaction are included in the most parsimonious model of RSA (Table 7 and Fig. 10A, B). Thus, RSA is affected by f_R but this relationship varies depending on M_b . The effect of f_R on the degree of RSA was expected given that RSA is driven by breathing; however, the effect of M_b on this relationship provides valuable information about how RSA varies, particularly at low f_R s. Given the variation in f_R s in free-ranging cetaceans (Blix and Folkow, 1995; Goldbogen et al., 2008; Isojunno et al., 2018; Sumich, 1983), especially following a dive, this model suggests that the magnitude of the RSA varies

during regular diving behavior and that following a dive, when f_R is elevated, the benefits of a high f_R may dominate over the potential cardiorespiratory benefits of a large RSA.

For both mean if_H and minimum if_H of the RSA, f_R and M_b were included in the most parsimonious model (Table 7). Because mean if_H was largely determined by the duration over which f_H decreased during the IBI, the effect of these predictors on mean if_H was likely secondary to their effect on minimum if_H . Like maximum if_H , mean if_H and minimum if_H scaled negatively with M_b , such that bigger animals displayed lower maximum if_H , mean if_H and minimum if_H (Table 7 and Fig. 10C, 7B, D). f_R was positively related to both mean if_H and minimum if_H , but minimum if_H increased more quickly with increasing f_R . The results of this model suggest that even when differences in f_R are accounted for, M_b describes additional variation in mean if_H and minimum if_H . The most parsimonious model of maximum if_H included M_b , but not f_R (Table 7 and Fig. 10B). This result agrees with our previous work in bottlenose dolphins where the maximum if_H did not vary across f_{RS} for a given individual (Blawas et al., 2021a). This could reflect that the maximum if_H of the RSA is determined largely by physical constraints of body size, and that any if_H lower than the maximum if_H is a result of changes to the ‘default’ neural controls on f_H . Interestingly, a previous study noted that an adult Pacific white-sided dolphin (*Lagenorhynchus obliquidens*) and two adult bottlenose dolphins under anaesthesia with nitrous oxide displayed steady f_{HS} averaging 80–120 beats min^{-1} , with larger animals displaying lower f_{HS} (Ridgway and McCormick, 1967). Although the M_b s of these animals were not reported, this range is comparable to the measured maximum if_{HS} measured in the bottlenose dolphins in this study. It is important to note that it is also possible that the maximum if_H of the RSA may be affected by other respiratory parameters that were not included in our analyses, like V_T .

RSA scaled allometrically with f_R with a scaling exponent of -0.56 (Table 6). This pattern can be understood by examining Figure 5. Given that the maximum if_H of the RSA appears to occur at approximately the same time (5–10 s) regardless of the IBI, this suggests that the degree of RSA, or oscillation in if_H , is largely dependent on the time over which f_H can decrease before the next breath is taken. M_b was positively related to RSA by the mass exponent of 0.35, such that bigger animals showed a higher degree of RSA. Interestingly, this is comparable to the exponent of 0.33 reported for many diving variables (Halsey et al., 2006). This perhaps suggests that the degree of RSA reflects a relationship between cardiorespiratory function and diving. Mean if_H and minimum if_H were related to f_R by the scaling exponents 0.11 and 0.21, respectively (Table 6). These exponents are, to our knowledge, the first reported coefficients relating f_R and if_H in cetaceans. This relationship may be driven by the greater effect of lung inflation over the IBI as f_R increases, therefore resulting in higher if_H s during more rapid breathing (Angell-James et al., 1981). We suggest that the influence of both f_R and M_b , which is negatively related to f_H , on mean if_H and minimum if_H of the RSA may be explained as follows: f_R determines the degree to which the if_H decays to a stable, low f_H following a respiration, but M_b ultimately determines the value of this low f_H . In this case, the effect of M_b could reflect a physical scaling constraint on the minimum if_H during an IBI.

Kleiber originally proposed an allometric mass exponent of 0.75 for metabolic rate, although there is considerable controversy as to whether this value is 0.67 or 0.75, or whether a universal mass exponent is valid at all (Butler and Jones, 1982; Kleiber, 1932; White et al., 2007; White and Kearney, 2014). Because the action of the heart controls the supply of oxygen to the tissues, we would expect the cardiovascular variables to follow this allometry. White and Kearney suggested that the sum of the

mass exponents of f_H and stroke volume should equal the mass exponent of metabolic rate. Because stroke volume scales allometrically by 1.03, this implies that f_H should scale with an allometric mass exponent in the range of -0.36 to -0.28 , depending on the value assumed for the allometric exponent of metabolic rate (White and Kearney, 2014). The allometric analyses in this study allow us to compare these expected values with the exponents we obtained. Both the mass exponents for mean if_H , -0.24 ± 0.07 , and minimum if_H , -0.34 ± 0.05 , determined in this study make this equality true; however, the mass exponent for maximum if_H , -0.16 ± 0.03 , does not. While it is important to consider the standard errors associated with the coefficients, this could indicate that, at least in cetaceans, the periods of reduced f_H during an IBI better reflect metabolic rate than the tachycardia of the respiration. In addition, the mass exponent for mean if_H is comparable to the -0.25 proposed by Stahl for scaling of f_H in terrestrial mammals and the mass exponents calculated for pinnipeds, which ranged from -0.20 to -0.24 (Castellini and Zenteno-Savin, 1997; Stahl, 1967). However, when the predicted f_H for each species is calculated using Stahl's equation based on the average M_b of individuals of that species in this study, we found that our measured f_H s were 17%, 15%, 17% and 4% higher than predicted for the bottlenose dolphins, false killer whale, beluga, and killer whales, respectively and 17% lower than predicted for the short-finned pilot whales. Because all of the data included in this study were recorded under non-fasted conditions, the f_H s measured could be affected by metabolic changes associated with digestion (Blawas et al., 2021a; Blaxter, 1989). We should also consider the possibility that species-specific differences likely result in natural variation around the predicted values. Though exploration of RSA in free-ranging cetaceans is critical to further determine the role of RSA during active, diving behaviors, this study suggests that cardiorespiratory coupling

plays a role in determining f_{Hs} across species of different sizes as it scales both with body size, M_b , and with f_R . We show that RSA scales with M_b similarly to that of other diving-related parameters and suggest the potential for RSA to reflect a relationship between cardiorespiratory function and diving capacity. We also compare allometric exponents with previously published mass exponents in terrestrial mammals and pinnipeds and identify similarities and differences in predicted values of f_H . Because the various components of the RSA are differentially affected by f_R and M_b , we suggest that there may be multiple controls that determine the modulation of f_H that cetaceans exhibit. Given the importance of f_H in determining oxygen consumption during diving, the known variation in f_{RS} in cetaceans and the coupling of f_H and f_R through RSA, we recommend that M_b and f_R should be accounted for when attempting to understand f_H measurements in relation to the diving capacities of cetaceans.

5. Automated detection of breathing events from whale movement tags

5.1 Introduction

Physiological investigations of free-swimming cetaceans have, historically, been limited because of the logistical complexities of studying large, fully-aquatic animals (Block, 2005; Hooker et al., 2007; Ponganis, 2007). Recent developments in digital biologging devices have enabled direct measurements of some physiological parameters in free-swimming cetaceans (Czapanskiy et al., 2022; Goldbogen et al., 2019a; Williams et al., 2022, 2017), but such investigations remain challenging due to constraints on the design, deployment, and functionality of these devices across environments, species, and behavioral states. Yet, the ability to measure physiological parameters and ultimately vital rates in free-swimming cetaceans is critical to understanding how these species respond to disturbance and environmental change. The development of novel methods to extract physiological information from existing biologging tools, therefore, has great potential to advance the fields of vertebrate physiology and conservation biology.

Respiratory parameters, including respiratory rate (f_R), are of particular interest in understanding the diving physiology of cetaceans because of their role in determining gas exchange and, therefore, the oxygen (O_2) supply and carbon dioxide (CO_2) offload that supports breath-hold diving. Previous studies have determined the f_{RS} of free-swimming cetaceans by 1) direct observation (Blix and Folkow, 1995; Christiansen et al., 2014; Sumich, 1983; van der Hoop et al., 2014; Williams and Noren, 2009), 2) identifying acoustic signals in tags equipped with hydrophones (Goldbogen et al., 2008; Isojunno et al., 2018; Roos et al., 2016), 3) using patterns in the pitch and depth signals in

accelerometry tags (Goldbogen et al., 2008; Miller et al., 2010; Roos et al., 2016), and, most recently, 4) examining electrocardiogram (ECG) records where respiratory movements are captured by the chest electrodes (Williams et al., 2022). The first three methods are viable in some species, particularly those for which a single breath is taken during each surfacing event, but they are not applicable and/or become less reliable for others that exhibit long periods at the surface (i.e., logging) with multiple breathing events, such as short-finned pilot whales. The lack of a widely-available ECG suction cup tag also limits the use of an ECG-based method for breath detection. A method for detecting respiratory events in a species like short-finned pilot whales using data that can be collected with widely-available biologging tags could revolutionize the measurement of f_R as a vital rate in free-ranging cetaceans. Continuous f_R data combined with the typical DTAG datastreams will enable investigations into how f_R is modulated to support diving behavior and can be applied to understand how our planet's largest animals and most extreme divers are responding to a changing ocean environment.

Here, I present *respdetect*, a set of MATLAB tools to detect breathing events from high-resolution movement data recorded by digital acoustic recording tags (DTAGs). This novel method takes advantage of the high flow rates and large tidal volumes that are typical of cetacean breaths and detects the movement artifacts resulting from a breathing event during logging. The method employs several signal processing steps followed by peak detection based on the Czapanskiy et al. method for ballistocardiography in large cetaceans (Czapanskiy et al., 2022). Derived movement parameters from acceleration and pitch data are used to identify movement artifacts associated with respirations during logging and combined with breaths detected from

single-surfacing events, *i.e.*, short excursions to the surface where a 1 breath:1 surfacing ratio is assumed. The library of functions associated with *respdetect* allows users to identify all breaths in the tag record and export the timing of each breath. *Respdetect* also provides users with tools to visualize breath detections with other dive parameters and validate breath detections made during logging with acoustic records from the DTAG.

Respdetect is intended to be used with existing DTAG processing tools developed by Mark Johnson. These tools require the same folder structure necessary for DTAG data processing. A DTAG record should be processed by exporting the *prh* file which contains the accelerometer and magnetometer data transformed into the whale frame, as well as the animal's pitch, roll, and heading. To use *respdetect* tools, the user is asked to specify the deployment *prh* files to analyze. After creating a metadata file for each deployment, *respdetect* identifies dives and calculates movement parameters before executing breath detection. The output of *respdetect* is a *.mat* file containing the timing of each breath the breath type, *i.e.*, single-surfacing or logging, and metadata and well as dive information for each tag deployment analyzed.

5.2 Materials and Methods

Respdetect was developed and tested on 64-bit Windows 10 using MATLAB version 2020a and has not yet been tested on other platforms or with other versions of MATLAB. The functions contained in *respdetect* require prior installation of several MATLAB toolbox dependencies: the Audio Toolbox, Signal Processing Toolbox, Image Processing Toolbox, and Statistic and Machine Learning Toolbox.

The functionality of *respdetect* is dependent on several DTAG tools written by Mark Johnson. A stable copy of those DTAG tools made available on GitHub by Stacy

DeRuiter is linked in the *README.md* file. These tools can also be installed either by directly downloading the entire zipped repository or by cloning the repository to the user's local machine.

Before using *respdetect*, users should ensure that their DTAG data meets several requirements. First, it is assumed that DTAG files have been calibrated, *prhpredictor.m* has been run, and data has been transformed into the whale frame. Importantly, *respdetect* requires an accurate pressure calibration with the temperature data. This can be particularly challenging to achieve using DTAG version 3's because of the placement of the temperature sensor in the tag. If at the start of a surfacing following a dive, the user's calibrated pressure is skewed above 0 m *respdetect* will not work well. Additionally, if the DTAG was deployed (or slid) far away from the blowhole or thoracic cavity, the movement signatures generated by breathing may be too weak to detect during logging periods. In this case, it may be not feasible to use *respdetect*.

Finally, the use of *respdetect* only requires a *prh.m* file, however, if the user intends to perform acoustic validation of breaths during logging, the *.wav* files for each tag deployment will also need to be saved in the typical DTAG data file structure.

The following sections will follow the setup of *main.m*. This is the only MATLAB file that the user will interface with to execute the functionality of *respdetect*.

5.3 Results

Respdetect is available as a GitHub repository located at <https://github.com/ashleyblawas/respdetect/> for use by any researcher interested in detecting f_R from DTAG data. Its functionality is described below and is summarized in the "Wiki" page on GitHub for users to follow step-by-step as they deploy *respdetect* on their datasets. A *README.md* file, which is displayed on the repository's main webpage,

includes a summary of the use of *respdetect* as well as information about installation, usage, contributions, and the repository's license (GNU General Public License). The repository also includes multiple test *prh* files to allow users to test the functionality of the tools on validated datasets before applying it to new data.

5.3.1 Installation and requirements

Users can install *respdetect* in two ways: 1) the repository can be downloaded directly in a ZIP file which can then be unzipped in the user's chosen file location or 2) the repository can be cloned via the command line using Terminal in macOS or Command Prompt or PowerShell in Windows with the command *git clone https://github.com/ashleyblawas/respdetect.git*.

5.3.2 Step 1: Workspace setup

First, the user must identify three different paths to direct MATLAB to the tools and data on their local machine. These three paths are where the user is storing the:

1. *respdetect* tools (from the *respdetect* repository),
2. DTAG tools (*dtagtools* can be cloned from: <https://github.com/stacyderuiter/dtagtools.git>), and
3. DTAG data (ending in the two-letter species name, e.g., *gm* for short-finned pilot whale, where the *prh* folder lives).

The user must first open the *paths.txt* file in the repository and edit the three path lines to be the respective paths identified above. Below is an example of what these paths may look like on a user's local machine (Fig. 12). If the user is working with the test DTAG data provided in the *respdetect* repository, the DTAG data path will be *tests* + the two letter species name of interest (either *gm* or *mn*).

```

## respdetect path (i.e. path to full repo, likely ending in respdetect or
respdetect-main)
C:\Users\ashle\Dropbox\Ashley\Graduate\Manuscripts\Gm_BreathingPatterns\respdetect-
main

## dtag tools path (i.e. path to full repo, likely ending in dtagtools)
C:\Users\ashle\Dropbox\Ashley\Graduate\Tools\dtagtools

## data path (i.e. path to species level folder, likely ending in two letter
species name)
C:\Users\ashle\Dropbox\Ashley\Graduate\Manuscripts\Gm_BreathingPatterns\respdetect-
main\data

```

Figure 12. Example of paths.txt file.

With a filled-in *paths.txt* file the user is ready for Step 1 of *main.m*. Upon running this section, the user is first prompted to navigate to and select the *paths.txt* file. This file should be living in the main repository folder. Next, this section opens a window in the *prh* folder in the DTAG data path and allows the user to choose one or multiple *prh.m* files (hold down *Ctrl* to select multiple files). In addition to a *prh* folder containing the *prh* files, *respdetect* expects several other folders to exist where it will save outputs. When the user runs Step 1 of *main.m* folders named *breaths*, *diving*, *figs*, *metadata*, and *movement* are created in the DTAG data path, if they don't already exist, to store data outputs and relevant figures.

```

data_path (e.g.
C:\Users\ashle\Dropbox\Ashley\Graduate\Manuscripts\respdetect\tests\gm)
├──breaths
├──diving
├──figs
├──metadata
├──movement
├──prh
│   └── gm08_143bprh.mat

```

Figure 13. Example of folder structure for the data path.

After running Step 1, the data path, as specified in *paths.txt*, should direct to a folder that looks like Fig. 13.

5.3.3 Step 2: Creating metadata file

Step 2 of *main.m* loops through each selected tag *prh* file and prompts the user to enter the tag on and tag off time (in seconds). A display of the depth profile appears such that the user can use the zoom function to zoom in on the start/end of the dive profile to determine where the dive starts/ends. The purpose of this step is two-fold. First, it is to ensure that the breath detection step only receives portions of the data where the tag is on the animal, and therefore doesn't attempt to detect respiration events when the tag is off the animal. Second, it ensures that the tagging event is removed from the portion of tag data to analyze. The actual event of tagging results in a jerk signal whose magnitude is much greater than peaks in jerk resulting from breathing such that, if the tagging event is not removed, *respdetect* will perform poorly when detecting breaths in the initial surfacing. As a safety factor, if the animal is at the surface at the tag on time (the alternative being the tag doesn't switch on until the animal is submerged) the breath detection step will redefine tag on time as the first instance when the depth is ≥ 5 m to remove the surfacing when tagging occurred.

During this step, the user is asked to specify the DTAG version. A file ending in *-md.mat* is saved with the metadata information including tag name, tag version, tag on time, tag off time, tag deployment duration, sampling rate (f_s) of the accelerometer and gyroscope, and tag filenames and paths, in the *metadata* folder on the data path.

5.3.4 Step 3: Finding dives

This step allows the user to set a dive threshold for finding dives using the *finddives.m* script by Mark Johnson. The user is prompted to enter a dive threshold (in meters [m]). The default dive threshold is 5 m. Note that dive information is not used any further for the *respdetect* tools, but the user can choose to export the breath data for

further analysis in R and the dive information is included in this exported data. This step outputs two files containing the same information into the *dive* folder on the data path. One is a mat file containing each dive variable saved as a vector (*-dives.mat*) and the other is the same information in table format (*-divetable.mat*). A figure of the dive profile, depths, and durations of all dives will also be exported to the *figs* folder (Figure 14).

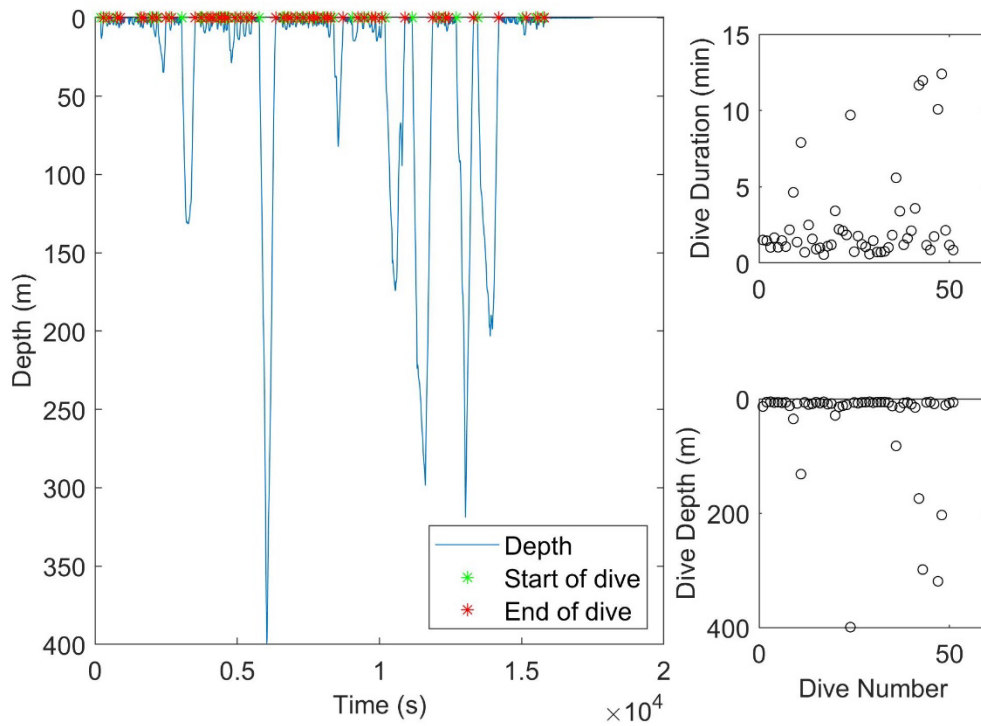


Figure 14. Dive profile and dive characteristics for test DTAG deployment gm08_143b.

5.3.5 Step 4: Processing movement data

In Step 4 the acceleration and pitch data are processed through a series of steps in *calc_move.m* to amplify the movement signatures associated with breathing. Several of these steps are based on the Czapanskiy et al. method for ballistocardiography (Czapanskiy et al., 2022).

First, acceleration in all three axes as well as pitch, roll, and heading are filtered using a 5th order Butterworth filter with a passband of 2 – 15 Hz unless the sampling frequency is less than 30 Hz ($f_s/2$), then a 5th-order highpass Butterworth filter with a cutoff frequency of 2 Hz is applied. Next, *calc_move.m* takes the derivative of each acceleration vector, pitch, roll, and heading and calculates Shannon entropy (SE) to emphasize changes in movement. Finally, a centered moving average of the SE is calculated using a window length of 5 seconds (s). The filtered acceleration in all three axes is used to compute the norm-jerk from which the SE of jerk is calculated and smoothed. The movement data is saved in the *movement* folder on the data path in a file ending in *-movement.mat*.

5.3.6 Step 5: Detecting breaths

To begin breath detection, Step 5 identifies the start and end of the tag on time as specified in the metadata. If the tag turns on at the surface, *i.e.*, when the depth is less than 5 m, then the start of the tag on time will be redefined as the first instance when the depth is ≥ 5 m to remove the tag deployment surfacing. The depth signal is smoothed using a moving average with a 1 s window length to reduce the high-frequency noise that could result in erroneous surface detections. Surfacing are then detected using a 0.5 m threshold. From here, surfacings are filtered by duration. If a surfacing is < 1 s in duration, it is assumed to be not a true surfacing but a brief deviation of the depth signal above 0.5 m and is removed. To account for instances when an animal may foray to the surface but not break the surface and breathe, if the depth minima of a surfacing is more than 0.15 m deeper than the mean depth minima of the two shallowest in a neighborhood of four surfacings around this detection, this event was not considered a surfacing. All surfacings that remain after this filtering are divided into

either single-breath surfacings (≤ 10 s) or logging surfacings (> 10 seconds). This distinction was made to allow for the use of a 1 breath:1 surfacing assumption during short-excursions to the surface but allow for multiple breath detections when a pilot whale is logging. The 10 s threshold was selected based on a qualitative examination of surfacing durations. One limitation of setting a threshold for dividing surfacings into two types is that an f_R above 6 breaths min^{-1} cannot be achieved with the single-breath surfacing strategy. For single-breath surfacings, a breath is defined to occur at the depth minima of the surfacing. A figure that plots both types of surfacing interval is exported into the *fig* folder (Fig. 15).

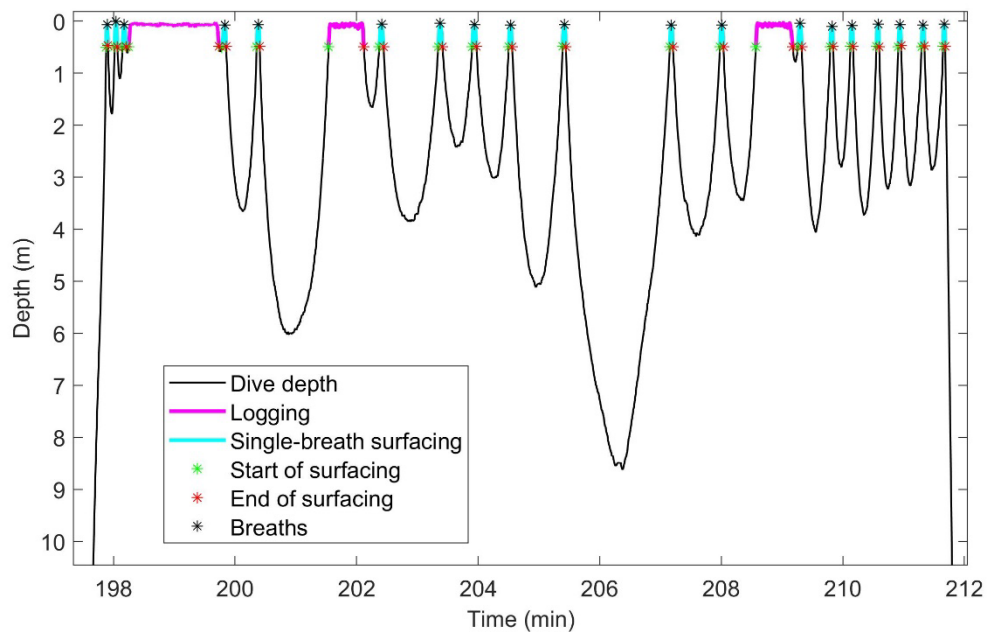


Figure 15. Surfacing detections for a 14-minute surface period in gm08_143b. Breaths are marked for single-breath surfacings with black asterisks.

To detect respiration events during logging, further processing of the smoothed SE of surge acceleration, jerk, and pitch is required. First, the pitch signal is shifted to be entirely positive for peak detection. Next, the smoothed SE signals associated with each logging surfacing period (± 5 seconds on each end) are identified and normalized on a surface interval-to-surface interval basis to improve peak detection.

Initial peak detection of the smoothed SE surge, jerk, and pitch signals is performed using the built-in MATLAB function *findpeaks* with a minimum peak distance of 3 s, allowing for a maximal f_R during logging of 20 breaths min^{-1} . Then, a distance metric is calculated which describes the difference in height, width, and prominence between each peak at the highest, widest, and most prominent peak in the surfacing, respectively. If there are enough peaks detected that the distribution of distances displays a clear multimodal distribution, the first local minimum of the distribution is identified and only peaks with distance values less than this value are kept as true peaks. If the distribution of distance is not multimodal, then peak width and peak height are used in hierarchical clustering with Ward's linkage to discriminate large peaks from small peaks and only larger peaks are kept as true peaks. This step results in a set of peaks from all three movement signals which are plotted along with the distance distributions used to select these peaks and saved in a figure in the *figs* folder.

Because peaks in each of the movement signals, smoothed SE of surge, jerk, and pitch, may not all occur at the same time, a windowing method is used to robustly detect breaths by identifying instances where peaks in at least two of the movement signals occur within a 5-second window of each other and identify where these windows overlap as periods where it is likely a breath has occurred (Fig. 16). Further filtering occurred as detailed in Table 8.

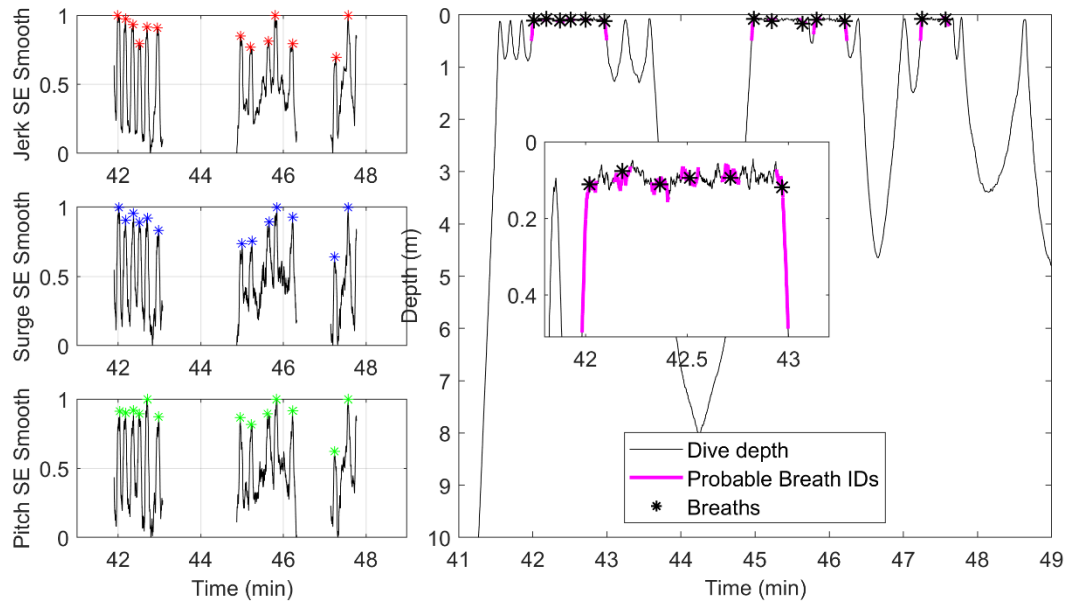


Figure 16. Peak detection for three logging surfacings in a ~6-minute period, overlapping peak windows resulting from those peaks, and breath detections in peak windows from gm08_143b.

For a logging surfacing, a breath is defined to occur at the midpoint point of each overlapping peak window (shown in pink in Fig. 16). This section will produce several figures (Fig. 17, Fig. 18) which will be saved in the *figs* folder in the data path and will save detected breaths and their timestamps (relative to the full tag duration) in a file ending with *-breaths.mat* in the *breaths* folder.

5.3.7 Step 6: Saving detections

Step 6 saves diving and breathing variables to a *.mat* file which can be brought into R for analysis and acoustic validation.

5.3.8 Step 7: Acoustic validation

The R script *respdetect_workup.Rmd* includes two sections for validating breath detections during logging with acoustic audits. The first section of this R script randomly

Table 8. Filtering for overlapping peak windows.

Condition	Purpose
The overlapping peak window segment is > 1 second	To eliminate short segments that are unlikely to contain a breath
The overlapping peak window and the following overlapping peak window were not derived from the same single peak window for any of the movement signals	To maintains only the first incidence of overlap in the case that a single peak window in one signal (indicative of only one breath) overlaps with two peak windows in another signal
The overlapping peak window did not occur within 0.1 seconds of another overlapping peak window where neither of these windows reached a maximum depth of >0.5 m	To eliminate the second of two overlapping peak windows where there is an extremely brief interruption (<0.1 s) in the conditions being met yet retain instances where two overlapping peak window occur closely in time, but the animal clearly descends away from surface in between
The logging breath event must be at least 3 seconds away from any single-breath event	To ensure that a maximum f_R of 20 breaths/min is maintained in the case of a single-breath surfacing occurring closely in time to a logging event (e.g., with depth calibration artifacts)

samples logging intervals that sum to a user-selected percentage of the total logging time in the dataset for acoustic auditing. This will produce a dataframe called *logs_to_audit*. Once this dataframe has been created the user can then use either Step S2a (for DTAG version 2's) or S2b (for DTAG version 3's) in *main.m* to conduct an acoustic audit for respirations of each of the logging periods. Not all breaths during each logging interval will likely be audible depending on the position of the tag and/or splashing noise – the user should only audit breaths that can be clearly distinguished.

Once acoustic audits have been conducted, the user will have many files ending in *_acousticaud.txt* in the *audit* folder in the data path. If any of these are empty

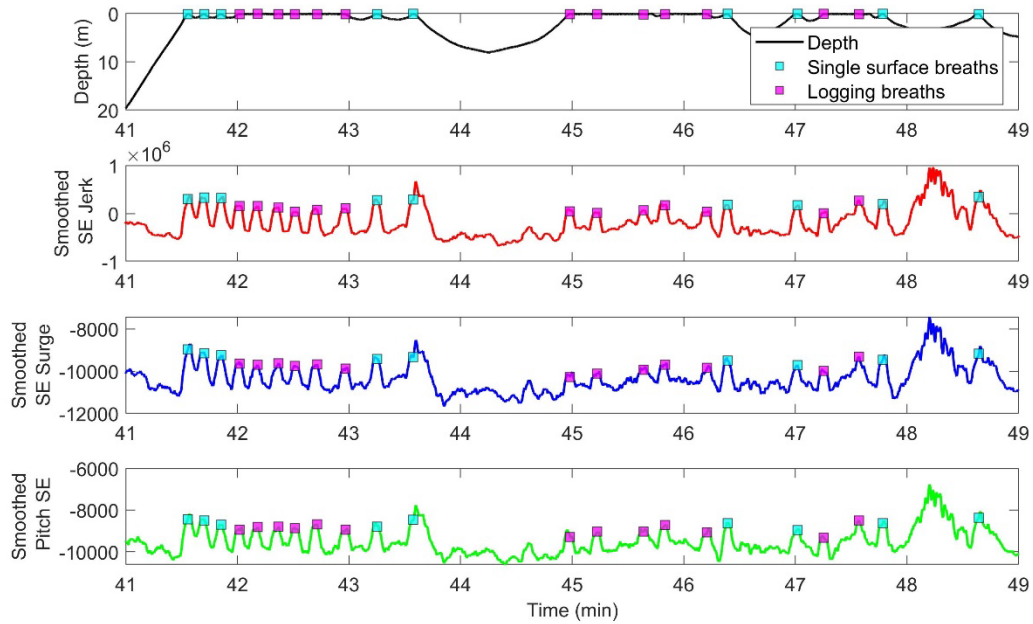


Figure 17. All breaths detected for an 8-minute period in gm08_143b.

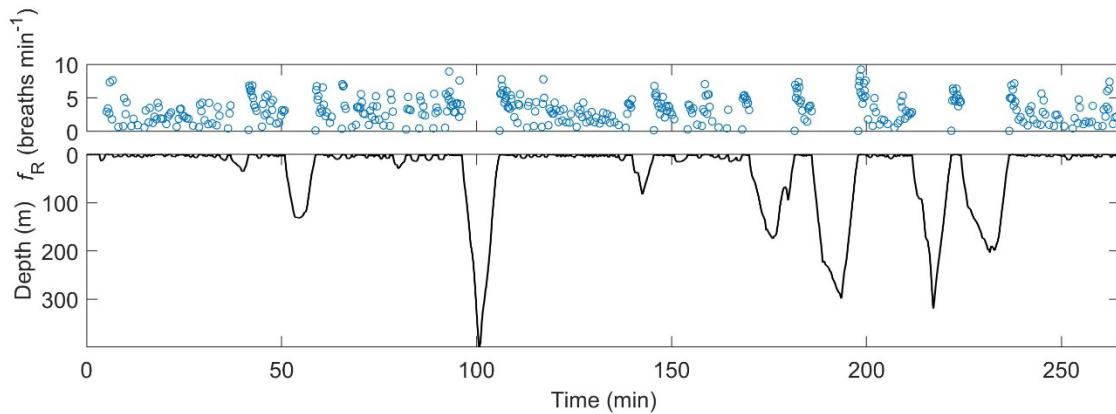


Figure 18. Instantaneous f_R for the entire tag deployment for gm08_143b.

(i.e., there were no audited breaths for that tag), the user should remove them from that folder or delete them as the R scripts will not function correctly if there are empty files.

Now the user can run the second section in R for the acoustic auditing which will match up acoustic audits with the closest detected breath by *respdetect*. It will output a plot with the time differences between the acoustic audits and detections which can be

used to determine the accuracy of the detections for this random sample (Fig. 19). A movement detection within 5 seconds of the start or end of an acoustic detection is considered a “hit” and the converse a “miss” so the performance of *respdetect* can be determined as the percentage of acoustic breaths that were hits of all acoustic breaths.

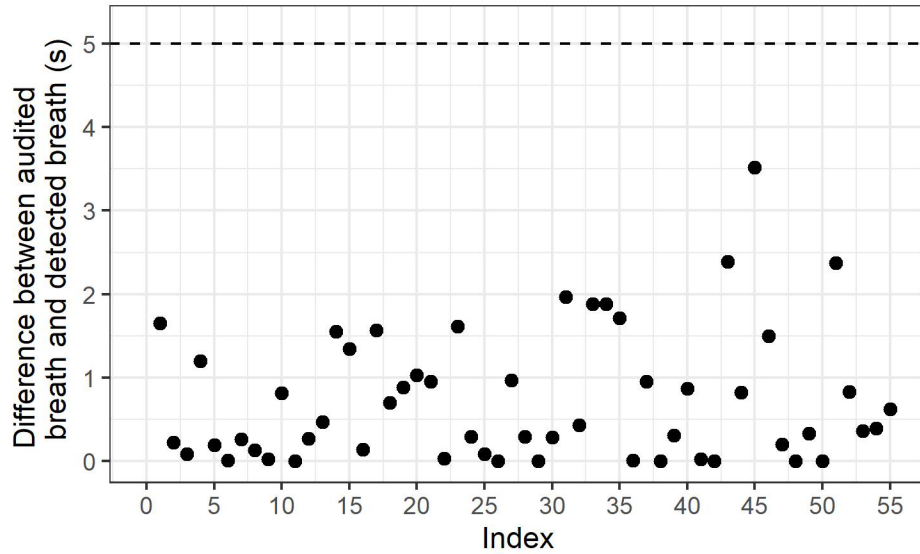


Figure 19. Comparison of timing of 55 logging breaths from gm081_143b acoustically audited with breath detections by *respdetect*.

5.4 Discussion

The application of *respdetect* to DTAG data collected from a variety of cetacean species across environmental contexts has the potential to greatly expand our understanding of f_R and its use for measuring physiological state and ultimately health.

5.4.1 Vital rate measurement for tracking acute wellbeing and chronic health

f_R is an important vital rate commonly monitored in human and veterinary medicine context to assess an individual’s health state. There are multiple scenarios in

which f_R is already or has been used as an indicator of wellbeing in cetaceans including during capture-release studies (Fair et al., 2006; Gulland et al., 2018), live stranding events (Bloodgood et al., 2022; Kastelein et al., 1990), the successful return of a rehabilitated gray whale calf into the open ocean (Stewart et al., 2001), and even monitoring of free-ranging animals during sedation given to improve disentanglement success (Moore et al., 2010). Behavioral clearance for designating a rehabilitated cetacean as released requires normal breathing, “including rate, pattern, quality, and absence of respiratory noise” (Whaley and Borkowski, 2009). If early identification of an individual or group that may be at risk for injury or illness occurs and deployment of a biologging tag is possible, *respdetect* could help to generate data that may support intervention or management actions to protect an individual or group of cetaceans.

5.4.2 Understanding physiological drivers, and limits, of diving

The ability to rapidly exchange metabolic gases at the surface underpins the unique diving ability of cetaceans. By manipulating their f_R , cetaceans can increase minute ventilation and the rate at which they replenish O_2 stores and offload CO_2 , making it important to study how the f_R of cetaceans varies during surface intervals. Several previous studies have focused on estimating how the duration of diving affects f_R . In bottlenose dolphins, each additional minute of diving increased f_R by 0.02 breaths min^{-1} in the first minute following the dive (Williams et al., 1999). A study of breathing patterns in killer whales revealed a distinct lower bound on the number of post-dive breaths related to the duration of the previous dive suggesting a minimum number of breaths may be required to recover to a degree that permits initiating the next dive (Miller et al., 2010). Isojunno et al. examined the relationship between instantaneous f_R and dive duration during pre- and post-dive intervals for dives ≥ 31 m by long-finned pilot

whales and found that f_R increased from a recovered level before dives that were ≥ 2 min and were nearly doubled from recovered levels after a 10-minute dive (Isojunno et al., 2018). In contrast to the relatively low respiration cost of diving in dolphins, the authors estimated that each additional minute of diving would incur 0.52 breaths min^{-1} in the initial post-dive f_R for long-finned pilot whales. Examination of the number of breaths in the post-dive interval suggested a cost of 1.7 breaths per minute of diving (Isojunno et al., 2018). By measuring baseline breathing patterns, as is made possible by the application of *respdetect* to DTAG data, it is possible to estimate preparation and recovery thresholds that may need to be met to support cetacean diving.

5.4.3 Indicator of disturbance/stress

The aforementioned Isojunno et al. study was also able to examine differences in breathing costs associated with dives with sound exposure and those without exposure and revealed that breathing costs varied depending on whether a sound exposure was the first in a series or whether it was a subsequent exposure (Isojunno et al., 2018). Initial exposures increased f_R while subsequent exposures resulted in a decreased f_R that could reflect 1) a stable minute ventilation if associated with increased tidal volumes, 2) a hypometabolic state as part of a stress response, or 3) a flexible breathing pattern that allows for lower-than-expected breathing at the cost of non-optimized breathing pattern for minimizing surface time. Narwhals exposed to seismic airgun pulses demonstrated elevated f_{RS} and a limited f_R recovery over the first 4 minutes following the dive after exposed dives compared to non-exposed dives (Williams et al., 2022). Vessel traffic, which is a consistent stressor particularly in popular recreation and/or shipping areas is correlated with changes in f_R in bottlenose dolphins in Sarasota Bay, FL where the presence of a vessel nearby resulted in a reduction in f_R that the authors proposed

could reduce time at the surface and therefore minimize the potential for vessel strikes (Nowacek et al., 2001). The application of f_R as a tool to indicate a stress response is particularly useful as it can directly reflect changes in metabolic costs due to disturbance. The use of f_R measurements provides a valuable method for examining how a changing ocean may require diving cetaceans to modulate their breathing strategies in a variety of disturbance regimes.

5.4.4 Energetic costs across life history stages (migration, feeding, reproduction, etc.)

The use of f_R to estimate metabolic rate has been applied to cetaceans in a variety of life history stages to determine the metabolic cost associated with activities like long-distance migration, foraging, and reproduction. Examination of f_{RS} of migrating gray whales revealed a lower f_R per km for animals migrating north than animals migrating south but this difference was absent when animals migrating north were mothers with calves (Rodríguez de la Gala-Hernández et al., 2008). f_R data has also been used to inform bioenergetic models to estimate thresholds of energy loss and their effect on gray whale reproduction (Villegas-Amtmann et al., 2015). By monitoring f_R of a minke whale for 24 hours, Blix and Folkow were able to make estimates of daily energetic needs and the associated prey abundance that would be required to meet this estimate, an important parameter to quantify the role of top consumers in their ecosystems (Blix and Folkow, 1995). Importantly this method often, though not in all aforementioned studies, assumes a consistent tidal volume and oxygen extraction fraction for all breaths which has been shown to lead to a significant overestimation of O_2 consumption rate in resting bottlenose dolphins compared to O_2 consumption calculated by combining f_R with breath-by-breath tidal volume and oxygen extraction fractions (Fahlman et al., 2016).

However, these methods, which tend to assume high tidal volumes and oxygen extraction fractions, may be more accurate for high-activity behaviors though this is difficult to validate with current technology.

5.5 Conclusion

Fully automated detection of f_R from DTAG data represents a significant opportunity to extract new information from historical datasets. The seminal paper describing the design and functionality of the digital acoustic recording tag (DTAG) has been cited ~1,000 times to date, indicative of the widespread use of this biologging tool (Johnson and Tyack, 2003). Applying *respdetect* to DTAGs deployed on species in different environmental contexts across a range of life history stages provides an exciting opportunity to learn more about these difficult-to-study species. However, *respdetect* has several limitations that constrain its use. First, the tag should be deployed, and remain, as close to the blowhole as possible on the dorsal side of the animal to maximize the likelihood of capturing movement due to breathing. For smaller cetaceans, like short-finned pilot whales, this is an easier task than for a large baleen whale. It might be the case that *respdetect* cannot be applied to many baleen whale DTAGs depending on the tag's placement. Additionally, without acoustic recordings of breaths, these detections are difficult to validate. If there is significant splashing noise at the surface due to a high sea-state, for example, it may not be possible to audibly distinguish breaths. While it is possible to quantify true positives (detected, audible breaths) and false negatives (not detected, audible breaths), detecting true negatives (not detected, not audible breaths) and false negatives (detected, not audible breaths) is difficult due to the possibility that acoustic breaths can easily be masked by splashing noise. Future studies could employ drones to capture surface behavior and validate

both the presence and absence of breathing with *respdetect* breath detections. Despite these limitations, *respdetect* has the potential to drastically increase the feasibility and ease of physiological studies of free-ranging cetaceans and positively impact the fields of marine mammal energetics and conservation physiology.

6. Fine-scale respiratory and surface interval patterns of short-finned pilot whales in Cape Hatteras, North Carolina

6.1 Introduction

Aquatic mammals must dive to forage at depths that are often 100s of meters away from the air they breathe at the surface. As breathing and diving are mutually exclusive activities, a diver's behavior is constrained, requiring prioritization of these two behaviors on short timescales (Rosen et al., 2007). The question of how diving mammals make decisions about apportioning their time at the surface and depth has therefore been the focus of many theoretical and empirical studies. Models of optimal diving suggest that surfacing and diving patterns should maximize the proportion of time spent foraging, net energetic gain, and energetic efficiency (Houston and Carbone, 1992; Kramer, 1988; Stephens et al., 2008). Many empirical studies of diving mammals and birds provide evidence to support this theory in certain circumstances (Acevedo-Gutiérrez et al., 2002; Doniol-Valcroze et al., 2011; Halsey et al., 2003b; Halsey and Butler, 2006; Heaslip et al., 2014; Tyson et al., 2016). However, time allocation models are limited in their ability to make quantitative predictions about diving (Halsey et al., 2003b; Halsey and Butler, 2006; Houston, 2011). This is in part due to the complexity of several physiological parameters that are not entirely accounted for in typical optimal diving models including variation in oxygen (O_2) uptake at the surface (Halsey et al., 2003b; Houston, 2011; Parkes et al., 2002), the importance of carbon dioxide (CO_2) versus O_2 for influencing diving behavior (Boutilier et al., 2001; Fahlman et al., 2008; Halsey et al., 2003a; Halsey and Butler, 2006; Houston, 2011), variation in diving metabolic rate due to factors like swim speed, dive duration, and body condition, (Aoki et

al., 2017; Castellini et al., 1992; Green et al., 2005a; Hindell et al., 2000; Houston, 2011; Sparling and Fedak, 2004), and the possibility of a diver utilizing both aerobic and anaerobic cellular respiration during a dive (Houston, 2011; Mori, 2002; Purdy, 2019; Ydenberg and Clark, 1989). For understanding the relationship between diving and surfacing patterns, more fine-scale physiological information is needed.

Gas exchange during a surfacing interval is influenced by the number of respirations, their timing (i.e., respiratory frequency [f_R]), their depth (i.e., tidal volume), and the partial pressure difference of the blood gas interface in the lung, e.g., the Fick equation. Though these first three factors can be controlled voluntarily, they are largely driven by central and peripheral chemoreceptors that monitor local changes in blood gases. In mammals, CO_2 is the driver of ventilation, not O_2 (Barrett et al., 2019b). Although some evidence indicates that marine mammals have a higher capacity to buffer increased P_{CO_2} (Boutilier et al., 1993; Castellini et al., 1981; Lenfant et al., 1970), CO_2 still may play a role in determining when a diver must surface to breathe (Boutilier et al., 2001; Craig and Päsche, 1980; Päsche, 1976, 1976). While much of the literature concerning surface duration has focused on the recovery of O_2 as a major driver, more recent work has highlighted that CO_2 elimination takes longer than O_2 uptake following a dive (Boutilier et al., 2001; Fahlman et al., 2008; Gerlinsky et al., 2014; Purdy, 2019). In one study of free-diving Weddell seals, three breath cycles were required following a dive for CO_2 levels to be elevated in the exhalate and the rate of CO_2 elimination did not reach a maximum until 2-3 minutes after surfacing (Falke et al., 2008). While no technology currently exists to measure CO_2 elimination and O_2 uptake in free-ranging cetaceans, if the respiratory stimulus of increased P_{CO_2} and/or decreased P_{O_2} is the primary cause for an elevated breathing rate and increased ventilation depth, then we

may indirectly understand changes in metabolic demands by examining changes in the number, frequency, and tidal volume of breaths following a dive.

One framework within which the relationship between surface intervals and diving can be evaluated is that of anticipatory versus reactive breathing, which is commonly applied to understand the behavior of diving birds (Cosolo et al., 2010; Elliott et al., 2008; Jodice and Collopy, 1999; Lea et al., 1996). A positive correlation between dive duration and the duration of the surface interval before the dive indicates *anticipatory breathing* – as though the animal is anticipating the duration of the upcoming dive. In contrast, *reactive breathers* show a strong correlation between dive duration and the duration of the surface interval after the dive. An anticipatory and/or reactive response to diving may also be reflected in an instantaneous change in breath number, f_R , or tidal volume and can inform how intermittent breathers may modulate these variables to maximize gas exchange at the surface while minimizing surface duration (Wilson, 2003). Work in free-diving Magellanic penguins showed that both f_R and tidal volume are elevated following a dive, fall gradually during the surface interval, but rise again before the next dive potentially suggesting a linkage to both pre- and post-dive O_2 and CO_2 levels although these were not measured (Wilson et al., 2003). The authors hypothesized that this type of breathing may act to efficiently renew O_2 and eliminate CO_2 . Increases in the number of pre-dive breaths in Magellanic penguins were also correlated with the likelihood of foraging success, suggesting anticipation of foraging effort (Wilson, 2003).

Several studies in marine mammals have looked at changes in f_R before and after dives, providing insight into the physiological nature of surface intervals. Trained dives of belugas and bottlenose dolphins to a target revealed patterns of pre-dive

hyperventilation (Ridgway et al., 1969) and increasing post-dive f_R with increasing dive duration (Fahlman et al., 2019a; Shaffer et al., 1997; Williams et al., 1999). A method for detecting f_R from heart rate (f_H) data showed a positive correlation between the post-dive f_R of free-ranging elephant seals during the first minute of the surface interval and dive duration (Andrews et al., 2000). More recently, the f_R of long-finned pilot whales, detected using acoustics, was found to increase directly before and after a dive (Isojunno et al., 2018). The increase in f_R after a dive was positively correlated with dive duration as was the duration it took for the f_R to recover to normal levels. These patterns suggest the use of both anticipatory and reactive strategies by marine mammals which can inform an understanding of their ability to predict dives and the physiological drivers of preparation and recovery at the surface. A robust investigation of changes in breathing patterns in the context of dive preparation and recovery can shed light on how divers make decisions about the allocation of time at the surface and depth. Additionally, improving our understanding of how aquatic mammals make decisions about diving will inform how their physiology may be influenced by behavioral changes in response to unexpected disturbances such as predation attempts or anthropogenic disturbances.

Using a novel method to detect breathing events from archival acoustic and movement tags, we aimed to investigate the relationship between f_R , inter-deep dive intervals (IDDI), and diving behavior in short-finned pilot whales. We hypothesized that (1) pilot whales will display both an anticipatory and reactive breathing strategy with respect to IDDI and f_R but that dive duration will have a larger effect on pre- and post-dive f_R s than IDDI as pilot whales should modulate f_R to minimize overall surface duration; (2) short-finned pilot whales will anticipate and react to dive ODBA, in addition to dive duration, and therefore dive ODBA will affect IDDI and f_R ; and that (3) fine-scale

changes in f_R before and after dives will reflect varying requirements to anticipate and react to dives of different durations and activity levels.

6.2 Methods

6.2.1 Tag deployments

42 digital acoustic recording tags (DTAGs) (Johnson and Tyack, 2003) were deployed on short-finned pilot whales between 2010-2018 in Cape Hatteras, North Carolina. DTAG version 2's were used for 39 deployments between 2010-2014 and in 2016 and DTAG version 3's were used for 3 deployments in 2015 and 2018. The suction cup-attached tags were deployed onto the dorsal fin or dorsal surface of the animal using a carbon fiber pole from a small (<10 m) rigid inflatable boat (RIB). DTAGs contain a suite of sensors including a pressure sensor to determine depth as well as a 3-axis accelerometer and 3-axis magnetometer to infer depth and animal orientation, all of which sampled at 50 Hz.

6.2.2 Depth analysis

Data collected by the DTAGs were calibrated using previously collected bench calibrations. Depth data were corrected for variations in temperature at the pressure sensor. Dives were identified as any submergence greater than 5 m. The depth of each dive is reported as the maximum depth reached during that dive.

6.2.3 Movement analysis

The accelerometer and magnetometer data were transformed into an animal-fixed reference frame and used to calculate pitch, roll, and heading throughout the dive record. Acceleration information was used to calculate the magnitude of the three-dimensional jerk vector and overall dynamic body acceleration (ODBA). To do so,

acceleration was filtered using a zero-phase, 5th-order Butterworth bandpass filter with cutoff frequencies of 2 Hz and 15 Hz. The norm-jerk was determined from filtered acceleration. The Shannon entropy (SE) of differentiated acceleration in the surge direction and differentiated jerk were calculated and then smoothed with a moving mean filter using a window length of 5 seconds. Pitch was similarly filtered using a zero-phase, 5th-order Butterworth bandpass filter with cutoff frequencies of 2 Hz and 15 Hz to remove noise and then differentiated. The SE of the differentiated pitch was calculated and then smoothed with a moving mean filter using a window length of 5 seconds. Smoothed SE of differentiated acceleration in the surge direction, differentiated three-dimensional jerk, and differentiated pitch were used for later breath detections. Movement data were analyzed at their full sampling frequency of 50 Hz.

6.2.4 Breath detections

Breathing events were detected using *respdetect*, a set of custom MATLAB tools to identify signatures of respiratory events in DTAG movement data. The development and functionality of these tools are detailed in Chapter 4. Because previous studies have assumed a 1 surfacing: 1 breath ratio, breaths during brief surfacing events (< 10 seconds) were not validated against acoustic detections. To validate breath detections during periods of logging (surfacing \geq 10 seconds), we randomly sampled 10% of the total period of logging across all tags. We then conducted manual acoustic audits, using *tagaudit* and *d3tagaudit* for D2's and D3's respectively, of each of these logging periods and noted the start and end of all audible breaths. If more than one breath was audible within 3 seconds, we only included the louder breath in the audit based on the assumption that the quieter breath was from nearby a conspecific. If neither breath was louder than the other both were excluded from the audit. We then compared all acoustic

detections during the 10% of logging periods to the breath detections from *respdetect*. A movement detection within 5 seconds of the start or end of an acoustic detection was considered a “hit” and the converse a “miss.” We assessed the performance of *respdetect* as the percentage of acoustic breaths successfully detected by *respdetect* during logging.

6.2.5 Statistical analysis

All statistical analyses were conducted using R version 4.1.0 (R Core Team, 2020). Dives were further divided into shallow (< 20 m) and deep (\geq 20 m) dive types using a depth threshold of 20 m that has been used in previous studies of short-finned pilot whales (Aguilar Soto et al., 2008). This depth is approximately 4 – 6 body lengths and discounts shallow submersions between respirations as dives. All further analyses were conducted only on deep dives and shallow dives were considered to be part of the surface interval between two deep dives or IDDI.

IDDI were divided into “regular” and “extended” surfacing types by identifying the local minima of the bimodal distribution calculated from the kernel density estimate using *density* (*stats* package) with a bandwidth calculated using the rule of thumb approach. Analyses of IDDI and f_R were conducted separately for regular surface intervals and extended surface intervals, as we made the *a priori* assumption that physiological drivers would be different between these two types of surfacings. Because it is not possible to know the start of an IDDI directly following tag deployment and the end of an IDDI at the end of a tag record, these periods, and their associated breaths, were not included in analyses. Overall species f_R is reported as the mean f_R for pooled data from all individuals and the mean f_R for each individual was determined from all breaths during all surface intervals in the respective tag deployment.

Wilcoxon rank-sum tests (*wilcox.test*, *stats* package) were used to test differences in IDDI characteristics of regular and extended IDDIs. Linear mixed effects models (*lme*, *nlme* package) with individual as a random intercept were used to evaluate the effect of dive duration and dive ODBA on pre- and post-dive IDDI duration for both surface interval types. We also constructed models to determine the effect of dive duration, dive ODBA, the interaction of dive duration and dive ODBA, and time until dive/time since dive on pre- and post- dive f_R during both regular and extended IDDIs. IDDI number nested in individual was included as a random intercept in all models of f_R . A 1st order auto-regressive serial correlation structure of IDDI number within a tag deployment (i.e., individual) and breath number within IDDI number within a tag deployment was included to account for temporal autocorrelation of dives for models of IDDI and f_R , respectively, during regular IDDIs. Residual plots were visually examined to ensure all fixed effects met assumptions of linearity, normality, and homoscedasticity. Q-Q plots showed that the distribution of IDDIs was right-skewed, so we applied a \log_{10} -transformation to meet the assumption of normality. To increase the interpretability of model coefficients, we also \log_{10} -transformed predictor variables in models of IDDI. Log-likelihood ratio tests were used to compare the full model for each outcome variable to a model with each predictor variable excluded to determine whether the predictor had a significant effect on IDDI or f_R when controlling for the other predictor variables. For example, to evaluate the contribution of dive duration to the model of pre-dive IDDI, a null model with dive ODBA as the only fixed effect was compared to a model with dive duration, dive ODBA, and their interaction as fixed effects.

To examine fine-scale changes in pre- and post-dive f_R during regular IDDIs for dives of different durations, dives were binned into five 5-minute intervals and f_R was

binned into 2-minute intervals for the 10 minutes before and after dives within each bin. A Wilcoxon rank-sum test with a Bonferroni correction for multiple comparisons was used for pairwise comparisons of mean f_R during each pre- and post-dive interval across dive duration bins. This analysis was not conducted on pre- and post-dive f_R during extended IDDI due to a small sample size. A significance of $\alpha = 0.05$ was used for all statistical tests.

6.3 Results

1,934 dives to depths of > 5 m from 42 short-finned pilot whales were analyzed for this study. 76% of dives were of the short/shallow dive type and 24% were of the deep/long dive type (Table 9, Fig. 20). One animal did not perform any deep dives and one individual only displayed one deep dive, therefore 40 tags in total are represented in further analyses unless otherwise stated.

Table 9. Dive characteristics for 42 short-finned pilot whales tagged in Cape Hatteras, NC.

Dive Type	# of dives	Mean duration (min)	Min. – Max. duration (min)	Mean depth (m)	Min. – Max. depth (m)
Shallow	1463	1.5±0.9	0.17-8.5	8.8±3.5	5.0-20.0
Deep	471	9.9±5.1	1.0-22.5	316±277	20.1-1077.0

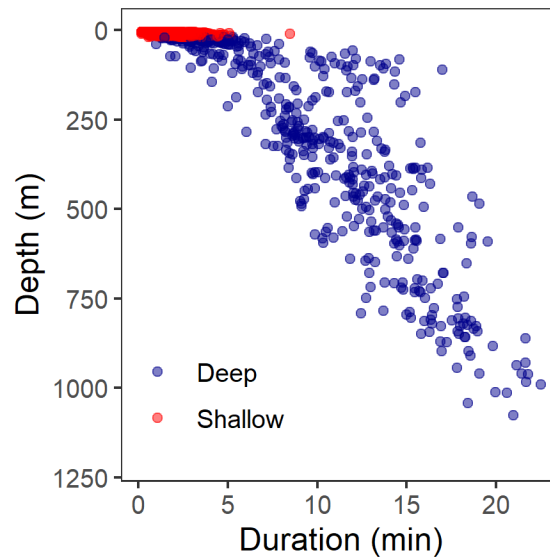


Figure 20. Dive duration versus depth for 1,934 dives by 42 short-finned pilot whales. Red points indicate shallow (< 20 m) dives and blue points indicate deep (≥ 20 m) dives.

IDDIs displayed a right-skewed, bimodal distribution (Fig. 21A). The local minima of the bimodal distribution occurred at 31.4 minutes which was used to distinguish regular from extended IDDIs. 93% of IDDIs were considered regular (< 31.4 min) and 7% of IDDIs were considered extended (≥ 31.4 min) (Table 10). *A priori*, we assumed that different drivers for surfacing (e.g., not necessarily physiological drivers) may exist during hour to multi-hour surface intervals and therefore decided to analyze regular and extended IDDIs separately. There were observable differences in the relationship between the duration of the dive succeeding (Fig. 21B) and preceding (Fig. 21C) the IDDI and the duration of the IDDI for regular and extended IDDIs. There was no difference in the duration of dives following extended IDDIs compared to regular IDDIs (Wilcoxon rank sum test $W = 6,515$, $p = 0.27$), but the duration of dives immediately preceding extended IDDIs was shorter than those before regular IDDIs (Wilcoxon rank sum test $W = 6,921$, $p = 0.04$). We examined whether extended IDDIs tended to occur

more closely in time to the longest dives, calculated as the 95th percentile of dive durations. The 95th percentile of all dive durations was 18.4 minutes and the time since the last dive ≥ 18.4 min was lower for extended IDDI than regular IDDI ($W = 398, p = 0.02$) as was the time until the next dive ≥ 18.4 min ($W = 398, p = 0.03$).

20,046 breaths were detected across all 40 tag deployments. Acoustic validation resulted in audits of 157 audible breaths and a 93% true positive rate for *respdetect* breath detections for these breaths (Figure S1). 15,626 breaths occurred during IDDI; 11,114 occurred during regular IDDI and 4,512 occurred during extended IDDI. The overall mean f_R for all f_{RS} from all animals during IDDI was 4.7 ± 2.2 breaths min^{-1} and the range of individual mean f_{RS} was 2.7 – 5.8 breaths min^{-1} (Figure S2). The total number of breaths taken during regular IDDI was significantly lower than that during extended IDDI (Wilcoxon rank sum $W = 134, p < 0.0001$), while the number of breaths taken per minute of the IDDI during regular IDDI was significantly higher than during extended IDDI (Wilcoxon rank sum $W = 11,189, p < 0.0001$) (Table 10).

Results of the linear mixed effects models with individual as a random intercept showed a relationship between pre- and post-dive IDDI and dive duration when the IDDI was regular, and this relationship was modulated by dive ODBA (Table 11). Increasing dive duration resulted in an increase in pre- and post-dive IDDI and this effect was greater with higher dive ODBA. With dive ODBA held at its mean value, a doubling of dive duration resulted in a 24% increase in pre-dive IDDI and a 17% increase in post-dive IDDI. The increased cost of a 25 versus 5-minute dive was 4.0 minutes in pre-dive IDDI and 2.6 minutes in post-dive IDDI. Increasing dive duration, therefore, has a greater impact on the surface interval before the dive than after, implying a greater reliance on an anticipatory response to increasing dive duration. In contrast, a doubling of dive

ODBA resulted in a 17% increase in pre-dive IDDI and a 23% increase in post-dive IDDI. The increased cost of a dive with the maximum dive ODBA observed in this dataset compared to the minimum ODBA was 5.1 minutes in pre-dive IDDI and 7.7 minutes in post-dive IDDI. The f_R response to increased activity level during a dive is, therefore, more reactive in nature than anticipatory. When the IDDI was extended, dive duration and dive ODBA did not affect pre- or post-dive IDDI. However, log-likelihood ratio tests suggested dive ODBA did improve the model of extended, pre-dive IDDI.

Linear mixed effects models identified the effects of dive duration, dive ODBA, and time since dive/time until dive on both pre- and post-dive f_R s during regular and extended IDDI (Table 11). For pre-dive f_R during regular IDDI, dive duration, dive ODBA, time until dive, and the interaction of dive duration and ODBA were significant predictors in the full model. Time until dive had the greatest effect on pre-dive f_R . A 1-minute decrease in time until the dive resulted in a decrease of 0.14 breaths min^{-1} in pre-dive f_R . When dive duration and dive ODBA are held at their mean value, pre-dive f_R was predicted to decrease from 5.9 to 4.5 breaths min^{-1} in the 10 minutes leading up to a dive. A 1-minute increase in dive duration was predicted to result in a 0.01 breaths min^{-1} increase in pre-dive f_R . This means that the pre-dive f_R cost of a 25-minute dive versus a 5-minute dive is an increase of 0.20 breaths min^{-1} or 2 more breaths across the 10 minutes before the dive. Similarly, the difference in pre-dive f_R for the maximum and minimum dive ODBA recorded in this dataset results in a predicted 0.03 breaths min^{-1} decrease in pre-dive f_R or approximately 0.3 fewer breaths before a maximally active dive.

Post-dive f_R during regular IDDI was affected by dive duration, time since dive, and the interaction of dive duration and ODBA. Generally, the effect of each predictor on

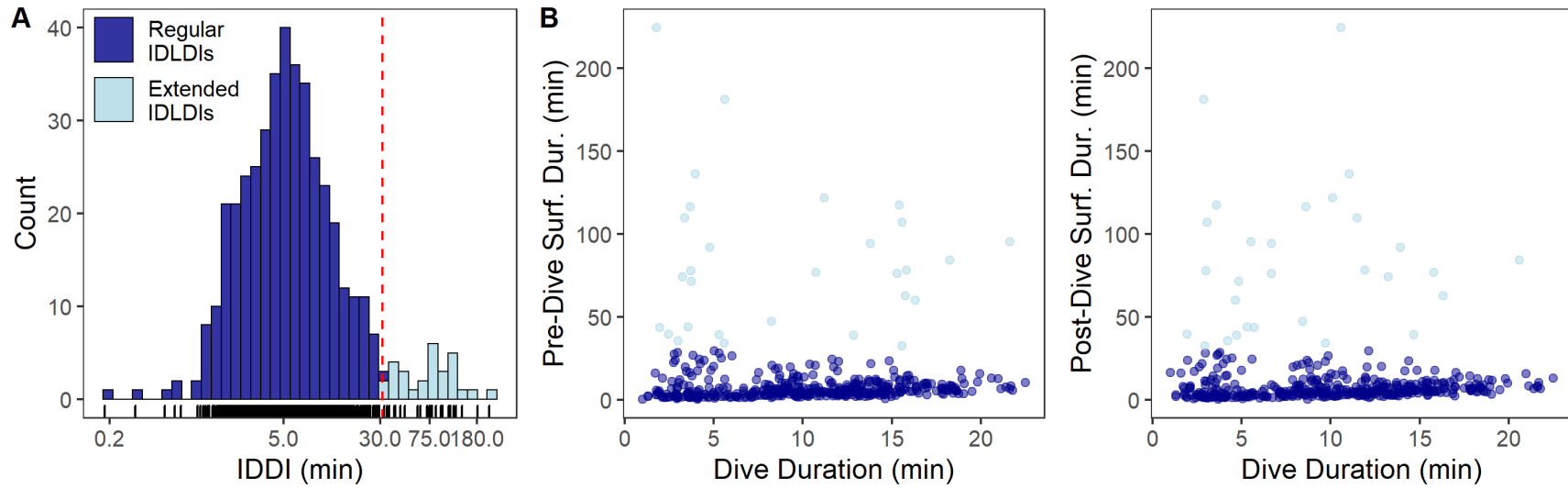


Figure 21. (A) Distribution of the duration of IDDIs and the relationship between dive duration and (B) pre- and (C) post-dive surface interval for both regular IDDIs and extended IDDIs.

Table 10. Characteristics of 429 inter-deep dive intervals (IDDIs) by short-finned pilot whales.

IDDI Type	# of IDDIs	Mean duration (min)	Min. – Max. duration (min)	Time since last dive \geq 18.4 min (min)	Time until next dive \geq 18.4 min (min)	Mean # of breaths	Mean # breaths per minute of IDDI (breaths min^{-1})
Regular (< 31.4 min)	400	6.9 \pm 5.7	0.2-29.4	196.6 \pm 166.1	105.5 \pm 89.7	27.8 \pm 15.9	4.8 \pm 1.5
Extended (\geq 31.4 min)	29	83.1 \pm 44.9	32.3-224	55.4 \pm 62.2	49.9 \pm 37.0	156.0 \pm 93.1	1.9 \pm 0.5

the full model of post-dive f_R was greater than that for pre-dive f_R . A 1-minute increase in time since dive resulted in a 0.33 breath min^{-1} decrease in post-dive f_R . During the 10 minutes following a dive, this results in a decrease in f_R from 6.2 to 3.0 breaths min^{-1} when dive duration and ODBA are held at their mean values. Increasing dive duration by 1 minute resulted in a 0.09 breaths min^{-1} increase in post-dive f_R . For a 25-minute vs 5-minute dive of average ODBA, the model predicts an additional cost of 1.9 breath min^{-1} or approximately 19 extra breaths in the 10 minutes following the dive. An average dive of the maximum ODBA versus minimum ODBA observed in this dataset results in an additional 2.4 breaths min^{-1} during the 10 minutes following a maximally active dive or approximately 24 additional breaths. Similarly to pre-dive f_R , log-likelihood ratios indicated that time since dive had the greatest effect on post-dive f_R .

Pre- and post-dive f_{RS} during extended IDDI were predicted by time until dive/time since dive, but not by dive duration, dive ODBA, or the interaction between dive duration and ODBA. For f_R during extended pre-dive IDDI, a 1-minute decrease in time until the dive resulted in a 0.18 breaths min^{-1} increase in pre-dive f_R . This means pre-dive f_R is predicted to increase from 2.5 to 4.3 breaths min^{-1} before a dive of average duration and ODBA. For f_R during extended post-dive IDDI, a 1-minute increase in time since dive resulted in a 0.31 breaths min^{-1} decrease in post-dive f_R during the 10 minutes before a dive. For a dive of average duration and ODBA, this predicts a decrease in f_R from 5.3 to 2.2 breaths min^{-1} in the 10 minutes following the dive. Predicted f_{RS} during extended IDDI were generally lower than those during regular IDDI.

Pairwise comparisons of pre-dive f_R during 2-minute intervals revealed significant changes in f_R in the 10 minutes leading up to a dive that varied both with the duration of

Table 11. Linear mixed effects models for pre- and post-dive IDDIs and f_{R} s during the 10 minutes before and after dives. Outcome variables are indicated in bold on the left and predictors included as fixed effects are listed below each outcome variable. Coefficients for all fixed effects and their associated P-values are shown. Individual ID was included as a random intercept in all models. Log-likelihood ratios and associated P-values indicate statistical significance of inclusion of each fixed effect in the full model compared to a null model without that predictor.

	Regular IDDIs				Extended IDDIs			
	Coeff. \pm S.E.	P-Value	LRT	P-Value	Coeff. \pm S.E.	P-Value	LRT	P-Value
log₁₀(Pre-dive IDDI) (min)								
Intercept	-0.28 \pm 0.41	0.49			0.4 \pm 0.51	0.41		
log ₁₀ (Dive Duration)	1.45\pm0.48	<0.01	27.5	<0.001	1.17 \pm 0.91	0.27	3.81	0.15
log ₁₀ (Dive ODBA)	-0.46 \pm 0.26	0.08	5.78	0.06	-0.84 \pm 0.32	0.06	9.14	0.01
log ₁₀ (Dive Dur.)* log ₁₀ (Dive ODBA)	0.73\pm0.32	0.02	5.05	0.02	0.68 \pm 0.63	0.34	1.30	0.25
log₁₀(Post-dive IDDI) (min)								
Intercept	0.30 \pm 0.39	0.43			1.29 \pm 0.87	0.15		
log ₁₀ (Dive Duration)	1.02\pm0.48	0.03	14.3	<0.001	-0.005 \pm 0.9	1.0	0.98	0.61
log ₁₀ (Dive ODBA)	-0.14 \pm 0.25	0.56	7.88	0.02	-0.29 \pm 0.55	0.62	4.29	0.12
log ₁₀ (Dive Dur.)* log ₁₀ (Dive ODBA)	0.52 \pm 0.32	0.11	2.60	0.11	-0.10 \pm 0.64	0.88	0.03	0.86
Pre-dive f_R (breaths min⁻¹)								
Intercept	3.48\pm0.30	<0.001			3.82\pm0.60	<0.001		
Time Until Dive (min)	0.14\pm0.009	<0.001	44.6	<0.001	-0.18\pm0.02	<0.001	63.6	<0.001
Dive Duration (min)	0.09\pm0.03	<0.01	21.1	<0.001	0.05 \pm 0.09	0.59	2.37	0.31
Dive ODBA	23.5\pm7.7	<0.01	21.7	<0.001	4.56 \pm 18.59	0.81	0.03	0.99
Dive Dur.* Dive ODBA	-2.00\pm0.83	0.02	15.6	<0.001	-0.45 \pm 2.26	0.84	0.01	0.91
Post-dive f_R (breaths min⁻¹)								
Intercept	5.00\pm0.16	<0.001			5.18\pm0.57	<0.001		
Time Since Dive (min)	-0.33\pm0.008	<0.01	1293.1	<0.001	-0.31\pm0.02	<0.001	168.6	<0.001
Dive Duration (min)	0.05\pm0.02	<0.001	283.0	<0.001	-16.4 \pm 14.8	0.86	19.1	<0.001
Dive ODBA	2.82 \pm 3.67	0.44	53.6	<0.001	-0.31 \pm 0.02	0.26	1.7	0.43
Dive Dur.* Dive ODBA	1.10\pm0.45	0.02	13.1	<0.001	1.83 \pm 1.40	0.19	1.7	0.20

the upcoming dive (Fig. 22A) and with the time until the dive occurred (Fig. 23A). Preceding dives 0 – 5 minutes long, f_R was stable at a mean of 3.4 ± 1.4 breaths min^{-1} in the 10 minutes leading up to the dive (Fig. 22A, Table 11) and was generally lower than f_{RS} preceding longer dives (Fig. 23A). For the 5 – 10 minutes dive duration bin, pre-dive f_R was stable at approximately 3.8 ± 1.5 breaths min^{-1} until the final 2 minutes of the pre-dive IDDI during which it increased to 4.7 ± 1.1 breaths min^{-1} (Fig. 22A, Table 11). f_{RS} before 5 – 10 minutes dives were typically lower compared to those before longer dives except in the final 2 minutes before diving (Fig. 23A). For dives 10 – 15 minutes in duration there was no change in pre-dive f_R in the 10 minutes before a dive (Fig. 22A), but the mean f_R during this pre-dive IDDI of 4.9 ± 1.5 breaths min^{-1} was elevated above that before shorter dives, especially in the last 6 minutes before diving (Fig. 23A). In contrast, for dives longer than 15 minutes, pre-dive f_R generally appeared to decrease leading up to a dive, though this decrease was only significant in the final 2 minutes before diving (Fig. 22A). Despite the decrease in f_R leading up to longer dives, pre-dive f_R in the final 2 minutes of the pre-dive IDDI for dives longer than 15 minutes was 4.6 ± 0.7 breaths min^{-1} which was elevated compared to f_R before shorter dives (e.g., 3.7 ± 1.2 breaths min^{-1} in the final 2 minutes before 0 – 5 minute dives) (Table 11). Notably, there were no differences in pre-dive f_R across all time intervals between the two longest dive duration bins: 15 – 20 minutes and ≥ 20 minutes (Fig. 23A).

From 8 – 10 minutes before a dive, there were no differences in pre-dives f_R except that f_R for the shortest dives (0 – 5 minutes) was lower than before the longest dives (≥ 20 min) (2.9 ± 1.2 breaths min^{-1} and 6.0 ± 1.6 breaths min^{-1} , respectively) (Fig. 23A, Table 11). As time until dive decreased, there were more differences in pre-dive f_{RS} between the shortest and longest dives (Fig. 23A). From 2 – 8 minutes before the

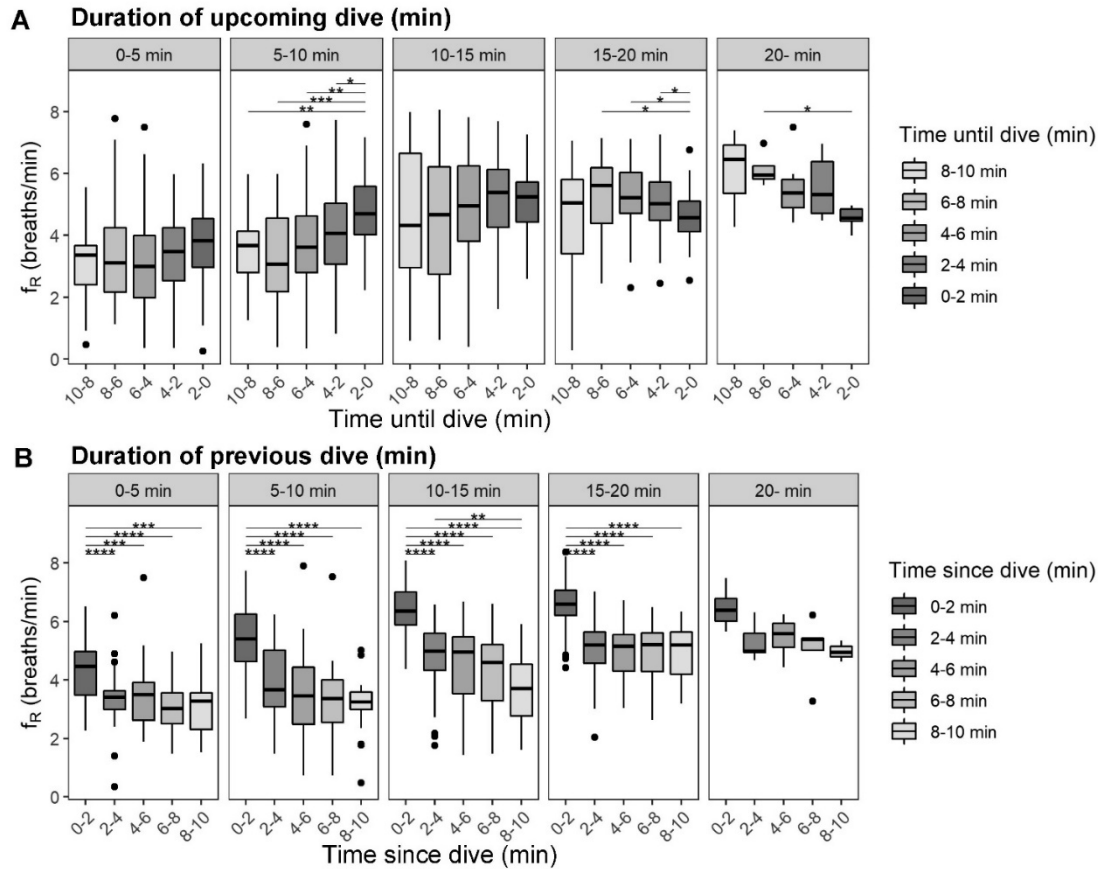


Figure 22. (A) Pre-dive f_R leading up dives of varying durations in 2-minute intervals. (B) Post-dive f_R s following dives of varying durations in 2-minute intervals. Asterisks indicate the significance of a two-tailed Wilcoxon rank sum test to compare sample means of paired boxplots with a Bonferroni correction for multiple comparisons. * $p < 0.001$, ** $p < 0.01$, * $p < 0.05$**

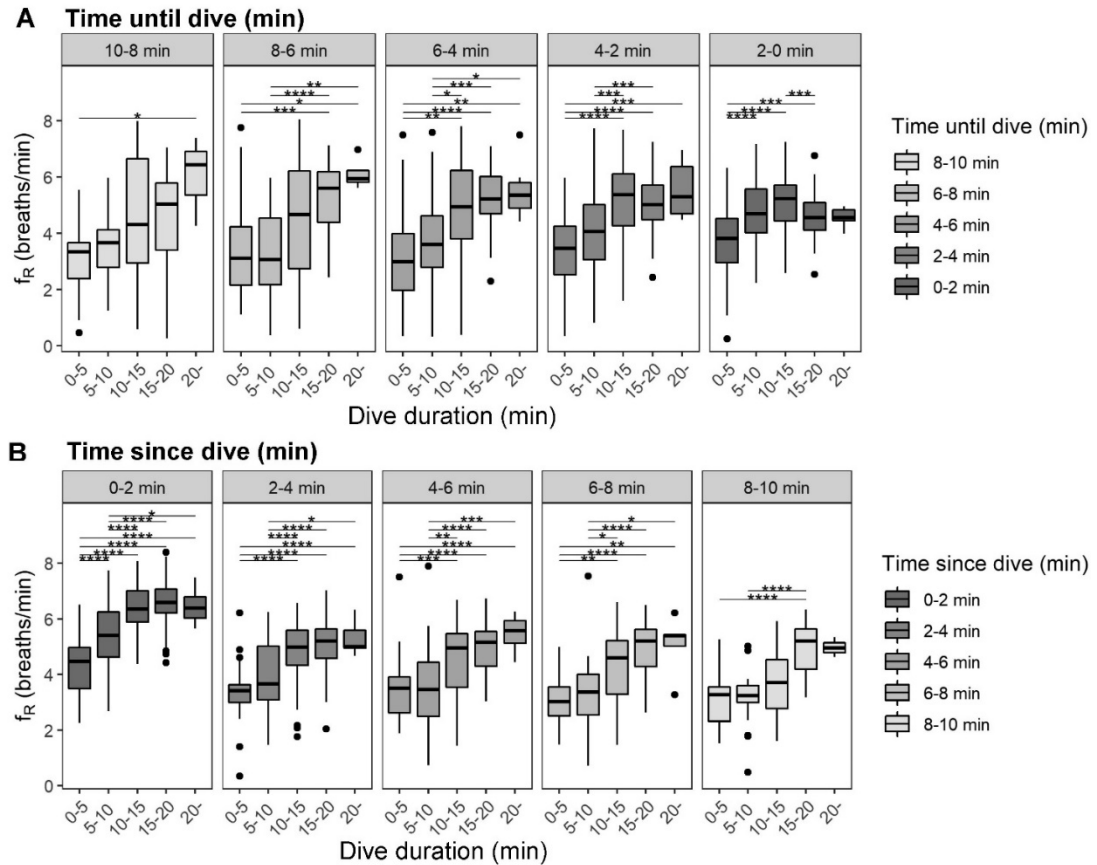


Figure 23. (A) Pre-dive f_R in 2-minute intervals leading up dives of varying durations. (B) Post-dive f_R s in 2-minute intervals following dives of varying durations. Asterisks indicate the significance of a two-tailed Wilcoxon rank sum test to compare sample means of paired boxplots with a Bonferroni correction for multiple comparisons. * $p < 0.001$, ** $p < 0.01$, * $p < 0.05$**

Table 12. f_{RS} during 2-minute intervals before and after dives of varying durations. Each f_R value is presented as the mean $f_R \pm S.D.$

Pre-dive f_R	Time until dive (min)				
Dive Duration (min)	8 – 10	6 – 8	4 – 6	2 – 4	0 – 2
0-5	3.0±1.2	3.4±1.8	3.1±1.6	3.4±1.5	3.7±1.2
5-10	3.6±1.4	3.2±1.5	3.8±1.6	4.1±1.5	4.8±1.1
10-15	4.6±2.3	4.6±2.1	4.7±1.9	5.1±1.4	5.1±0.9
15-20	4.5±2.1	5.3±1.3	5.2±1.2	5.1±1.0	4.6±0.8
≥20	6.0±1.6	6.1±0.6	5.5±1.0	5.5±1.0	4.6±0.3

Post-dive f_R	Time since dive (min)				
Dive Duration (min)	0 – 2	2 – 4	4 – 6	6 – 8	8 – 10
0-5	4.4±1.0	3.4±0.9	3.5±1.1	3.1±0.9	3.1±1.0
5-10	5.5±1.1	3.9±1.2	3.5±1.5	3.4±1.3	3.1±1.0
10-15	6.4±0.8	4.9±1.1	4.5±1.4	4.4±1.4	3.7±1.3
15-20	6.6±0.8	5.1±1.0	5.0±0.9	4.9±1.0	5.0±0.9
≥20	6.4±0.6	5.3±0.6	5.5±0.7	5.1±1.1	5.0±0.4

dive, dives 0 – 10 minutes in duration were preceded by lower f_{RS} , generally, than dives greater than 15 minutes. In the final 2 minutes before diving, f_R for dives 0 – 5 minutes was lower than for many longer dive duration bins (Fig. 23A). Notably, the three longer dive bins were not significantly different from one another during the pre-dive IDDI (5.0±1.6 breaths min⁻¹ for 2 – 10 minutes before the dive) except f_R was lower in the last 2 minutes for dives 15 – 20 minutes long than for dives 10 – 15 minutes long (Fig. 23A).

Pairwise comparisons of post-dive f_{RS} revealed significant changes in post-dive f_R in the 10 minutes following dives both by dive duration (Fig. 22B) and with time since dive (Fig. 23B). Generally, for all dive durations except dives greater than 20 minutes, post-dive f_R was higher in the first 2 minutes following a dive than the rest of the first 10 minutes of the post-dive IDDI (Fig. 22B). For dives ≥ 20 min, post-dive f_R was stable in the 10 minutes following a dive around (5.6±0.9 breaths min⁻¹). In the first 2 minutes following dives, post-dive f_R following dives 0 – 5 minutes (4.4±1.0 breaths min⁻¹) and 5 – 10 minutes (5.5±1.1 breaths min⁻¹) in duration were different from each other and lower

than all other dive bins, but the three longest dive bins were not different from one another in the first 2 minutes at a mean f_R of 6.5 ± 0.8 breaths min^{-1} (Fig. 23B, Table 11). In the next several 2-minute intervals following dives, post-dive f_R after shorter dives (0 – 10 minutes) remained lower than longer dives (≥ 10 minutes), but there were no differences within these groups. At 8 – 10 minutes following a dive, post-dive f_R generally remained lower for 0 – 10 minute dives (3.1 ± 1.0 breaths min^{-1}) than for dives longer than 10 minutes (4.3 ± 1.3 breaths min^{-1}) (Fig. 23B, Table 11).

6.4 Discussion

Short-finned pilot whales exhibit variation in time spent at the surface between dives, including demonstrating extended IDDI (≥ 31.4 minutes) that represent long breaks in otherwise regular diving beyond 20 m. The overall f_{RS} during regular IDDI (< 31.4 minutes) and extended IDDI differed, indicating the physiological function of these two types of surface intervals is likely different (Table 8). The lower overall f_R during extended IDDI suggests that pilot whales are in a more recovered physiological state during these surface intervals compared to regular IDDI during continual diving. The duration of dives that occurred directly before an extended IDDI was shorter than those directly before regular IDDI, counter to what would be expected if extended IDDI played a role in a reactive response to diving. There was no difference in the duration of dives directly following regular and extended IDDI suggesting these extended periods at the surface do not function to support an anticipatory response to diving, at least for the most recent dive. However, extended IDDI occurred closer in time to dives beyond the 95th percentile of dive duration (≥ 18.4 min) so these especially long surface intervals may serve a role in preparation or recovery from particularly physiologically-taxing dives on longer timescales.

Short- and long-finned pilot whales are also known to use surface periods to rest, socialize, and travel (Baird et al., 2002; Isojunno et al., 2017; Jensen et al., 2011) and display synchronicity of breathing, implying the importance of social cohesion when pilot whales are at the surface (Senigaglia and Whitehead, 2012). Therefore, extended IDDIs may alternatively be secondary to social drivers not accounted for in this study, rather than physiological needs. If groups of pilot whales travel together, which is supported by their known long-term social associations (Heimlich-Boran, 1993), and because short-finned pilot whale groups are not thought to dive together (Aguilar Soto, 2006), these long surface intervals may be required to coordinate with an individual's group to initiate transit to a new foraging area.

6.4.1 Pre-dive and post-dive IDDIs

For regular IDDIs, the duration of the pre-dive IDDI was affected by the duration of the upcoming dive and the interaction of the upcoming dive duration and ODBA (Table 3). This suggests that short-finned pilots do display an anticipatory response to diving with respect to surface duration, and also anticipate dive ODBA. Lea et al. suggest that Kramer's model of optimal diving necessitates an anticipatory breathing strategy as time spent at the surface should be limited to what is required for the upcoming dive and not necessarily to fully recover from the previous dive (Kramer, 1988; Lea et al., 1996). This is argued because O_2 uptake at the surface diminishes as stores recover, and therefore the rate of O_2 gain can be maximized and surface time reduced by only replenishing O_2 to the level needed for the upcoming dive (Kramer, 1988). Optimal divers are expected not to dive at a maximal physiological limit continuously, but instead, dive until blood O_2 falls to a certain level. Given that short-finned pilot whales also appear to modulate surface time before a dive based on the ODBA of the upcoming

dive suggests that they anticipate both dive duration and dive activity. In birds, anticipatory strategies have been associated with species that forage benthically, consume their prey underwater, and require high capture effort (Cosolo et al., 2010; Jodice and Collopy, 1999; Lea et al., 1996) as these foraging characteristics allow for predictable diving patterns.

For regular post-dive IDDI, dive duration and the interaction between duration and ODBA significantly affected IDDI duration. Thus, short-finned pilot whales appear to demonstrate both anticipatory and reactive breathing strategies. The influence of dive duration on post-dive IDDI was slightly less than that on pre-IDDI (a 17% increase vs. a 24% increase in IDDI for a doubling of dive duration, respectively), but the effect of ODBA was greater on the post-IDDI than pre-dive IDDI (a 23% increase vs. a 17% increase in IDDI for a doubling of dive ODBA, respectively). Thus, it may be more possible to anticipate dive duration while activity level during a dive is more difficult to predict. Foraging characteristics of reactive divers are thought to include foraging in the water column, consuming prey at the surface, and low capture effort all of which contribute to a dive being less predictable (Cosolo et al., 2010; Jodice and Collopy, 1999; Lea et al., 1996). Short-finned pilot whales in Cape Hatteras are known to use a combination of benthic and pelagic foraging (Shearer, 2022), and therefore may display a combination strategy that reflects more predictable, consistent-depth benthic dives and less predictable, variable-depth pelagic dives.

Notably, the costs of long, active dives are high in terms of the extra time required at the surface during the pre- and post-dive IDDI. Independent of changes in dive ODBA, an additional cost of 4 minutes of pre-dive IDDI and 2.6 minutes in post-dive IDDI for a 20-minute increase in dive time is a 33% surface time cost of added time

underwater. Similarly, high ODBA dives accrue a high IDDI cost. The difference between the maximum and minimum dive ODBA in this dataset during a dive of average duration cost an additional 5.1 minutes during the pre-dive IDDI and 7.7 minutes during the post-dive IDDI which indicates a 128% surface time cost increased activity underwater. This reflects the importance of limiting activity underwater and is likely an important reason for the short-finned pilot whale's use of short sprints and extended gliding (Aguilar Soto et al., 2008).

The absence of an effect of dive duration or dive ODBA on pre- or post-dive IDDI when the IDDI was extended further supports that these extended IDDI do not occur, at least immediately, before the longest dives. Extended IDDI did occur closer in time to dives ≥ 18.4 minutes suggesting the potential for extended IDDI to serve a role in preparation or recovery from long dives, or perhaps series of long dives. The models did not include an auto-regressive correlation structure as the sample size was small and extended IDDI were infrequent, however, more complex correlation structures could reveal a potential physiological relevance for these extended IDDI on longer time scales. Perhaps it is a series of long, deep dives that relates to the occurrence of extended IDDI due to the accumulation of lactate.

Traditionally, it has been assumed that dives beyond the aerobic dive limit (ADL) (Kooyman et al., 1980), require extra time at the surface to replenish O_2 stores and to process accumulated lactate (Horning, 2012). For a Weddell seal with an ADL of 20 minutes, a dive of 35 minutes required nearly 80 minutes for lactate to reach baseline levels (Kooyman et al., 1980). However, lactate levels in diving seals can decrease during short, shallow dives (Kooyman et al., 1980; Ponganis et al., 1997a) and this has been proposed as a functional role for the shallow "bounce" dives performed by beaked

whales between deep dives that can last for multiple hours (Tyack et al., 2006). If short, shallow dives support a similar function in short-finned pilot whales, this could suggest that they have decoupled the need to process lactate with the short-term need to remain at the surface for an extended period (i.e., ≥ 31.4 min). The association between the occurrence of extended IDDI and the longest dives could mean that, though the clearing lactate may be staved off in the short term, across a series of dives lactate accumulation requires extended periods of recovery at the surface.

6.4.2 Pre-dive f_R s

Pre-dive f_R in the 10 minutes before a dive is modulated by the duration of the upcoming dive, the ODBA of the upcoming dive, and time until dive in short-finned pilot whales (Table 9). For pre-dive IDDI that were regular, time until dive, dive duration, and dive ODBA all had a positive effect on the model of pre-dive f_R . Therefore, when the IDDI occurs in a regular series of dives pilot whales reduce their f_R as a dive approaches (i.e., as time until dive decreases, f_R decreases) when accounting for the effects of dive duration and dive ODBA, but both increases in dive duration and ODBA increase pre-dive IDDI. Importantly, the effect sizes of this relationship are small. A decrease in pre-dive f_R of 1.4 breaths min^{-1} as the dive approaches is unlikely to reflect significant differences in gas exchange dynamics in the 10 minutes before diving. Similarly, an additional cost of 2 breaths and 0.3 breaths within the 10-minute pre-dive interval for an added 20 minutes of diving or a high-activity dive, respectively, is unlikely to have a large biological effect. The model results, therefore, do not suggest a biologically relevant modulation of pre-dive f_R in an anticipatory fashion.

Examining pre-dive f_R s by dive duration and in 2-minute pre-dive intervals allowed for finer-scale analysis of changes in pre-dive f_R before dives. Before the

shortest dives, pre-dive f_R was stable leading up to the dive suggesting no preparation for the upcoming dive. In contrast, for 5 – 10 minute dives, pre-dive f_R increased in the final 2 minutes before the dive (Fig. 22A). This increase in f_R could reflect a pre-dive hyperventilation response occurs shortly before initiating a 5 – 10 minute dive, though these pre-dive f_{RS} were still lower than most pre-dive f_{RS} for longer dives across the entire 10-minute pre-dive interval (Fig. 23A, Table 12).

For dives 10 – 15 min in duration, a stable pre-dive f_R in the 10 minutes before a dive (Fig. 22A) that was higher than most f_{RS} preceding 0 – 10 minute dives (Table 12) suggests several possibilities: 1) pre-dive hyperventilation for 10 – 15 minute dive begins more than 10 minutes before the dive begins and remains at a constant level for 0 – 10 minutes before the dive, 2) pre-dive f_{RS} before these dives remain elevated from a reactive breathing strategy used following the previous dive and do not demonstrate recovery towards a baseline f_R during this interval, or 3) both an anticipation of the upcoming dive and recovery from the previous dive are occurring resulting in constant pre-dive f_R from 0-10 minutes before the dive. Previous studies have shown that short-finned pilot whales in Cape Hatteras exhibit temporal clustering in dive states classified using dive duration, maximum depth, and the number of buzzes (Quick et al., 2017). Therefore, it is likely that long dives tend to be preceded and succeeded by long dives, and the physiological requirement to recover from a previous dive increases with the increasing duration of the upcoming dive. This supports the possibility that anticipatory and reactive breathing strategies are occurring simultaneously before longer (10 – 15 minute) dives. Patterns in pre-dive f_R before the longest dives (≥ 15 minutes) provide further evidence for this interpretation. In the 10 minutes leading up to dives ≥ 15 minutes, pre-dive f_R appeared to decrease especially in the final 2 minutes (Fig. 22A).

Still, pre-dive $f_{R,S}$ for these longest dives were elevated across most pre-dive intervals compared to the shortest dives (Fig. 23A, Table 12). We suggest that this reflects a reactive breathing response to the dive that occurred before this IDDI, where f_R continues to recover towards a basal level in the 10 minutes preceding the next dive, but, unlike for 10 – 15 minute dives, f_R remains elevated enough that a distinct anticipatory effort is not required to maintain an elevated f_R . This suggests that pilot whales may use an anticipatory breathing strategy before short-duration dives, but that pre-dive hyperventilation is not utilized when f_R remains elevated from a reactive response to the previous dive.

For extended pre-dive IDDI, time until dive negatively affected pre-dive f_R such that f_R increased leading up to a dive. There was no effect of either dive duration or ODBA on f_R during extended pre-dive IDDI. Therefore, when the pre-dive IDDI is especially long, this suggests there is a dive duration and ODBA-independent increase in pre-dive f_R as the dive approaches (2.9 to 4.1 breaths min^{-1} for a dive of average duration and ODBA). Because overall f_R was significantly lower during extended IDDI, this “ramp up” in f_R could serve two potential functions. Firstly, pre-dive hyperventilation could serve to reduce alveolar and arterial CO_2 and delay the urge to breathe. This is a common goal of pre-dive hyperventilation “purges” by free divers and theoretical work suggests hyperventilation could add as much as 10 minutes to maximal apneas in humans (Fitz-Clarke, 2018). Pre-dive hyperventilation has been observed to result in a lower end-apneic arterial CO_2 value after long dives compared to end-apneic arterial CO_2 after shorter surface apneas in Weddell seals (Kooyman et al., 1973). Alternatively, pre-dive hyperventilation could serve to increase perfusion before a dive, perhaps to facilitate maximal loading of O_2 in the blood and muscle if the extended IDDI was used

for travel, for example, and therefore resulted in some minor accumulated O₂ debt. The lack of association between pre-dive f_R during extended IDDI and dive duration as well as dive ODBA could indicate that the dive immediately following an extended IDDI is “unpredictable” and possibly exploratory in nature.

Thus, by examining pre-dive f_R s it appears that short-finned pilot whales do demonstrate a slight anticipatory breathing strategy with respect to f_R that is evident between the shortest and longest dives, particularly in the final minutes before diving (Fig. 23A), though the predicted effect of dive duration on pre-dive f_R in models of all dives together is not likely biologically significant. Pilot whales demonstrate pre-dive hyperventilation before diving when pre-dive f_R is otherwise low (e.g., before short dives [Fig. 22A] when the previous dive may also have been short or at the end of extended IDDI), but generally they do not appear to rely on the modulation of pre-dive f_R in the 10 minutes before a dive to prepare for dives of varying durations or activity levels.

Given the observed relationship between pre-dive IDDI and dive duration in this study, the overall number of breaths in the pre-dive IDDI is likely to be higher from longer, more active dives though the timing of these breaths is not necessarily more rapid. Magellanic penguins appear to modulate their number of pre-dive breaths with the number of prey captured on the previous dive implying anticipation of foraging effort (Wilson, 2003) and agreeing with predictions of optimal diving. Therefore, while short-finned pilot whales may not modulate f_R in an anticipatory fashion, they still display anticipatory breathing secondary to the positive effect of dive duration of pre-dive IDDI.

Leading up to longer dives it seems likely that a reactive breathing strategy following the previous dive persists into the 10 minutes before the next dive and dominates over any distinct pre-dive hyperventilation. The combination of pre-dive

hyperventilation before short dives and reactive breathing could result in higher pre-dive f_{RS} before longer dives (Fig. 23A) but would not necessitate concerted hyperventilation before long dives. The effect of f_R on f_H (Blawas et al., 2021a, 2021b) and perfusion suggests that pilot whales maintain a higher degree of perfusion in advance of longer dives which should maximize the pre-dive loading and unloading of O_2 and CO_2 , respectively.

6.4.3 Post-dive f_{RS}

Post-dive f_R during regular IDDI was modulated by time since dive, dive duration, and the interaction of dive duration and dive ODBA (Table 3). As time since diving increased, post-dive f_R decreased reflecting a recovery of f_R towards baseline in a time-dependent fashion. Long, active dives resulted in higher post-dive f_{RS} than short, less active dives. Unlike pre-dive f_{RS} , the effects of changes in time since diving, dive duration, and dive ODBA predicted by the model are likely large enough to be biologically relevant. Independent of changes in dive duration or ODBA, the effect of time since dive alone predicts a decrease in f_R from 6.2 to 3.0 breaths min^{-1} , a more than 50% reduction in f_R in the 10 minutes following a dive. The cost of an additional 20 minutes of diving was an extra 19 breaths in the first 10 minutes following the dive and the cost of a high-activity dive was an extra 24 breaths in the first 10 minutes following the dive. At an average f_R of 4.7 breaths min^{-1} during the 10 minutes following a dive of mean dive duration and ODBA, this could alternatively be interpreted to approximate a 4.0 and 5.1-minute increase in post-dive IDDI, respectively, which is higher than the effect of an increase in dive duration on post-dive IDDI (2.6 minutes) but lower than the effect of an increase in ODBA on post-dive IDDI (7.7 minutes). Therefore, post-dive f_R seems to be more important for responding to longer dives while post-dive IDDI is more

important for responding to active dives. This could reflect differences in the amount of time required for gas exchange and that required to process lactate. A study of respiratory timing in killer whales suggests that O₂ stores in diving killer whales are likely renewed after only six respirations (Roos et al., 2016). In bottlenose dolphins, recovery of end-tidal O₂ to pre-breath hold level requires 10 breaths and occurs in 1.2 minutes (Fahlman et al., 2019a). If short-finned pilot whales require a similar number of breaths to recover O₂, then these additional breaths may be more important for CO₂ recovery and/or lactate processing.

Post-dive f_R during regular IDDI decreased over time in the 10 minutes following a dive (Table 12), but the reduction in f_R was only significant for the first 2 minutes post-dive compared to the rest of the post-dive interval for dives 0 – 20 minutes long (Fig. 22B). This pattern is indicative of not only an immediate physiological response upon surfacing from a dive but also a rapid recovery of f_R in 2 minutes upon surfacing. Though recovery of f_R to baseline may still be occurring throughout later phases of the post-dive interval (Table 12), it occurs more slowly from 4 – 10 minutes than from 0 – 2 minutes post-dive.

Post-dive f_R was higher after longer dives than shorter dives supporting a reactive f_R response to diving (Fig. 23B). Generally, however, there were no differences in post-dive f_R for dives ≥ 10 minutes (Fig. 23B) which could suggest that these post-dive f_{RS} represent an optimal respiratory timing for maximizing O₂ uptake when O₂ stores have been depleted beyond a threshold. Because killer whales and bottlenose dolphins demonstrate O₂ recovery on much shorter timescales (Fahlman et al., 2019a; Roos et al., 2016) and the recovery of O₂ stores is more efficient following dives of increasing duration (Fahlman et al., 2008; Parkes et al., 2002; Sumich, 2000, 1983; Wilson et al.,

2003), it is unlikely that O_2 dynamics are the primary driver of f_R patterns 10 minutes after diving. The driver of an f_R that is elevated from basal levels after at least 10 minutes since the previous dive could instead be related to CO_2 offload which has been shown to require longer recovery times in harbor porpoises, grey seals, and Steller sea lions (Boutilier et al., 2001; Purdy, 2019; Reed et al., 1994). Given the similarity of f_{RS} following longer dives, these f_{RS} could represent a maximal $f_R - f_H$ relationship at which cardiac output is maximized and more rapid breathing would not improve ventilation but would incur the cost of respiration as has been suggested in juvenile elephant seals (Andrews et al., 2000, 1997). f_{RS} during the later time intervals as the pilot whale is recovering could represent an f_R that maximizes gas exchange and simultaneously minimizes unnecessary breaths as CO_2 is removed from the blood.

The lack of differences in post-dive f_R in the 8 – 10 minute post-dive interval for dives 0 – 15 minutes, which suggests a stable f_R late in the post-dive interval, combined with the lack of difference in post-dive f_R after 2 minutes following 0 – 5 minute dives, indicative of a recovered f_R after the shortest dives, suggests that pilot whales do recover their f_R within 10 minutes for dives of these durations.

For extended IDDs, post-dive f_R was affected by time since diving but not by dive duration or dive ODBA. Thus, pilot whales do not exhibit a reactive breathing strategy when they are about to spend an extended period at the surface. This could indicate that there is a cost of rapid breathing at the surface and if it is not necessary to rapidly recover from a dive then it is preferable to utilize an optimal respiratory timing that is independent of O_2 store depletion. This result emphasizes the importance of recovery in preparing for the next dive in that, if the next dive will not occur within a regular time interval, there is less need to recover quickly. That short-finned pilot whales demonstrate

a reactive f_R response during regular IDDIs, but not extended IDDIs suggests possible anticipation of extended surface intervals. If long surface intervals are used to coordinate with an individual's group to initiate travel, perhaps these could be vocally coordinated in advance.

These results suggest that pilot whales utilize a reactive breathing strategy upon immediate surfacing from a dive, but post-dive f_R s do not increase further with increasing dive duration ≥ 10 minutes. This may represent an optimal timing of breaths during recovery that maximizes O_2 uptake or CO_2 offload. The lack of a reactive f_R response during extended IDDIs provides further support for an optimal f_R pattern that could maximize gas exchange while minimizing respiratory effort. Post-dive recovery of f_R occurs within 10 minutes for shorter dives but remains elevated at the end of 10 minutes for longer dives. Therefore a reactive response to diving may not enable full recovery in 10 minutes for longer dives. This agrees with the patterns in pre-dive f_R which suggested that a reactive f_R response may still dominate during the 10 minutes leading up to longer dives.

6.5 Conclusion

Short-finned pilot whales exhibit two distinct types of surfacing behavior that can be categorized as regular IDDIs during continual diving and extended IDDIs that represent 30-minute to multi-hour periods at the surface. Pilot whales appear to be in a more basal physiological state during extended IDDIs, though these extended surface periods do not serve a role in immediate anticipation or recovery from a dive. Instead, extended IDDIs may support anticipation and recovery from a series of long dives which supports the possibility that marine mammals can delay the requirement to process lactate. Ultimately, a larger sample size and more complex auto-regressive correlation

structure are required to explore surfacing and diving patterns on longer time scales. Alternatively, extended IDDIs may occur secondary to social drivers and serve a role in social coordination with an individual's group before group travel.

f_{RS} during extended IDDIs indicate that these extended periods at the surface may be anticipated, as post-dive f_{RS} during extended IDDIs are not modulated by the duration or ODBA of the previous dive which is the case during regular IDDIs. We suggest this could reflect an optimal timing of breaths that maximizes gas exchange while minimizing the effort of additional breaths is prioritized over rapid recovery when an upcoming dive is not imminent. Hyperventilation at the end of extended IDDIs that is independent of the duration or ODBA of the upcoming dive suggests that an f_R elevated from baseline may be important for O_2 loading or CO_2 offloading before diving, but that in general dives following extended IDDIs are unpredictable, and possibly exploratory, in nature.

Pre- and post-dive IDDIs during continual diving, i.e., regular IDDIs, are both affected by dive duration and dive ODBA such that longer and more active dives are associated with longer pre- and post-dive IDDIs. Thus, short-finned pilot whales demonstrate both substantial anticipatory and reactive breathing strategies with respect to surface duration. We suggest that this could reflect the combined benthic and pelagic foraging strategy of short-finned pilot whales. Benthic diving could be anticipated as the duration required to reach the seafloor is predictable, whereas when in a pelagic dive prey items are likely to be captured is likely to be more variable.

Pre-dive f_{RS} do not indicate a strong anticipatory modulation of f_R although fine-scale investigation of f_R did reveal a general pattern of higher f_{RS} before longer dives. This pattern potentially reflects that a reactive increase in f_R persists from the previous

dive before longer dives, given that long dives are expected to be temporally clustered. Therefore, the observation of higher f_{R} s before longer dives on a fine scale likely reflects the large role of a reactive increase in post-dive f_{R} . Post-dive f_{R} , in contrast, does reflect a clear reactive modulation of f_{R} that is strongest in the first 2 minutes after a dive but is not necessarily reactive to the exact duration of a dive but whether it is more generally short or long. This provides further evidence for the importance of respiratory timing and perhaps supports an optimized breathing pattern that facilitates recovery if O_2 stores have been depleted past a certain threshold and are decoupled from the exact dive duration, at least in the first 10 minutes following diving. The predicted effect of increasing duration and activity during diving on modeled post-dive f_{R} is large, therefore we can conclude that pilot whales demonstrate a much stronger reactive f_{R} response to diving than an anticipatory f_{R} response.

Taken together, these results demonstrate anticipatory and reactive breathing concerning the duration of regular IDDI, but only demonstrate a strong reactive, rather than anticipatory, modulation of f_{R} with dive duration. The effect of increasing dive duration is greater on post-dive f_{R} than IDDI, whereas the effect of increasing activity is greater on post-dive IDDI than f_{R} . This suggests duration and activity affect O_2 , CO_2 , and lactate dynamics differently and therefore the particular metabolic requirements of a given surface interval. Our findings reveal important information about the connection between vital rates, like f_{R} , and diving behavior in free-ranging cetaceans and ultimately help to understand how marine mammals efficiently divide their time between the surface and depth.

7. Conclusion

Cetaceans and other marine mammals possess adaptations at the cellular and molecular, tissue-specific, and organismal scales that enable their unique breath-hold capacity. The findings of this dissertation are informative about the complexity of the physiological adaptations that enable breath-hold diving, the mechanisms that control these physiological changes, and the plasticity of these physiological adaptations. Additionally, these results highlight the importance of using multiple tools and technologies to investigate the physiology of these difficult-to-study species.

Genomic analyses have enabled significant exploration of DNA-level mechanisms of adaptation in marine mammals (Cammen et al., 2016; Foote et al., 2015; Huelsmann et al., 2019), but less research has focused on expression-level mechanisms that may support their unique diving abilities. Compared to genetic changes, changes in the RNA and protein-related responses to environmental stimuli could act on timescales relevant to diving and therefore represent an important avenue for research into molecular and cellular-level adaptations. The findings in Chapter 1 suggest that the upregulation of two genes, ALOX5 and IL5RA, occurs in a time-dependent fashion in breath-holding bottlenose dolphins. Changes in ALOX5, which is known to induce vasoconstriction in humans and other species, were mirrored by an increase in lipoygenase activity during breath-holds when dolphins are expected to exhibit dive response. These changes were consistent across individuals and time points studied and highlight the potential for an expression-level mechanism that supports known the neurally-modulated vasomotor response to diving, perhaps on longer timescales. Importantly, the breath holds in this study likely represent near-maximal voluntary breath holds for this species so it is difficult to surmise how expression-level mechanisms may

play a role in free-ranging dives. Despite this limitation, it is clear that the application of tools from molecular biology to investigate molecular-level influences on the dive response will continue to reveal new insights into the physiological function of diving mammals.

There is a growing body of literature utilizing tissue samples and cultured cells to investigate transcriptomic responses to the hypoxia and ischemia that are known to occur during diving *in vitro* (summarized in Allen and Vázquez-Medina, 2019; Fabrizio et al., 2016; Hoff et al., 2017; Krüger et al., 2020; Lam et al., 2020; Penso-Dolfin et al., 2020; Pujade Busqueta et al., 2020; Torres-Velarde et al., 2021). Though *in vitro* studies may not recapitulate whole-organism responses *in vivo*, they reveal potential genotype-phenotype relationships measured under controlled conditions that are difficult, if not impossible, to obtain on the whole-organism level. Marine mammal cell culture models will enable the examination of responses across species, individuals, and even tissue and cell types to a range of environmental stimuli (e.g., hypoxia, ischemia, pressure, hormones that mimic a stress response, and environmental contaminants). By identifying genetic and molecular targets, this research will inform functional studies that can link molecular adaptations to physiological traits.

The dive response and factors that influence the heart rate (f_H) of diving mammals have been rigorously explored, yet how these factors combine and interact is complex and incompletely understood. The respiratory influence on f_H is one potential driver of f_H patterns that has not been fully explored in marine mammals. Previous studies have highlighted the respiratory sinus arrhythmia (RSA) and observed that the minimum f_H of the RSA was similar in magnitude to that of the bradycardia that occurs during diving, particularly during dives less than the ADL (Castellini et al., 1994a; Lin et

al., 1972; McDonald and Ponganis, 2014; Meir et al., 2008; Päsche and Krog, 1980).

The results of Chapter 2 and Chapter 3 suggest that respiration has a significant influence on changes in f_H at the surface and that the modulation associated with the RSA in bottlenose dolphins is similar to that seen during short, submerged breath-holds. This could indicate that RSA and changes in f_H , at least during short dives, are under similar mechanisms of control. It also highlights the potential importance of f_R at the surface for determining f_H and ultimately influencing cardiac output and tissue perfusion during dive preparation and recovery. The results of Chapter 3 further indicate the importance of f_R on f_H and suggest that f_H associated with RSA scales allometrically with both body size and f_R . This indicates differences in the dependence of f_H on f_R across species and a role for both physical scaling laws and dynamic physiological needs in modulating the relationship between f_R and f_H . The role of physical scaling laws is especially important for understanding thresholds on physiological function. Extensions of this work should seek to investigate the role of RSA during surface intervals of free-ranging whales and dolphins. Though it is expected that high f_H s dominate the RSA upon surfacing from a dive, RSA could play a role in pre-dive preparation if f_H recovers during a surface interval and pilot whales hyperventilate before diving.

Given the influence of f_R on f_H and their importance for gas exchange, Chapters 4 and 5 aimed to develop and subsequently apply a method to detect breaths from whale and dolphin biologging data to study surface interval and f_R patterns of short-finned pilot whales. Chapter 4 shows that movement data recorded using a biologging tag, like the digital acoustic recording tag (DTAG), can be used to detect breathing events, provided the tag is deployed in the vicinity of the thoracic cavity/blowhole. The ability to detect vital rates like f_R from tag data is an important step in the remote monitoring of health

and energetics in free-ranging cetaceans and ultimately, other marine mammal species. Further, methods for vital rate detection using existing data types that are commonly collected by multiple types of biologging tags provide a new use for historical datasets and a new variable that can indicate physiological status which has not previously been possible with DTAG and other biologging data.

As researchers seek to understand the impacts of anthropogenic activities and climate change on marine mammals, establishing an understanding of baseline physiological function is critical. By employing *respdetect*, a novel breath detection method from DTAG movement data, the results of Chapter 5 show that short-finned pilot whales in Cape Hatteras, North Carolina demonstrate variable surfacing and breathing behavior. Pilot whales modulate time spent at the surface before and after dives according to the duration of the dive, supporting both an anticipatory and reactive response to diving. While post-dive f_R s are modulated with dive duration and activity, such that f_R is higher after longer and more active dives, there does not appear to be much of an anticipatory modulation of f_R before diving. We, therefore, found evidence for both an anticipatory and reactive breathing strategy in terms of surface duration and a reactive strategy in f_R patterns. Examinations of anticipatory and reactive breathing (these studies focus on surface interval duration only) in birds have suggested different species utilize different surfacing strategies based on prey handling, depth of foraging, and prey capture effort (Jodice and Collopy, 1999). Extending these concepts to understand the surfacing and breathing strategies of cetaceans and other marine mammals provides a new framework for understanding the conflict between gas exchange and foraging in diving mammals. The combination of breathing strategies used by pilot whales indicates that they may take advantage of multiple foraging

strategies where some dives can be anticipated (e.g., benthic diving to a predictable depth), and others are less predictable in their duration and activity level (e.g., pelagic foraging) and therefore a reactive response is required. Short-finned pilot whales in Cape Hatteras demonstrate both benthic and pelagic feeding, so a combined breathing strategy could support the use of different types of foraging dives. A combination of anticipatory and reactive breathing strategies has previously been shown in long-finned pilot whales (Isojunno et al., 2018). Notably, the data in this study were compared to that during sonar exposure demonstrating changes in f_R associated with exposure. The use of f_R as an indicator of stress during exposure to anthropogenic activities like military sonar, seismic surveying, and offshore energy installation is an important and exciting avenue for future research and the application of *respdetect* to DTAG datasets.

These studies contribute to a large body of research that outlines the features of a “good” diver. Ultimately, it is the ability of cetaceans to survive for extended periods without breathing that most readily distinguishes their physiology from that of humans and other terrestrial mammals. As Lawrence Irving remarked in his paper “On the Ability of Warm-Blooded Animals to Survive Without Breathing”:

“The need for oxygen is the most insistent requirement for human life. It is quite different from the requirements of food and water, which need satisfaction only at intervals which may be quite prolonged without discomfort... The brevity of duration of life without oxygen leaves only a narrow margin of oxygen reserve to separate life from death” (Irving, 1934).

The solution as to how exactly marine mammals lengthen this “narrow” margin to such a great degree compared to humans remains incomplete, especially for our planet’s deepest divers. By combining cutting-edge molecular techniques with novel perspectives on long-standing theories and innovative explorations of existing data we will continue to discover how marine mammals have been shaped, over evolutionary time, to thrive underwater without access to life’s most basic requirement.

Appendix A: Supplementary figures for Chapter 6

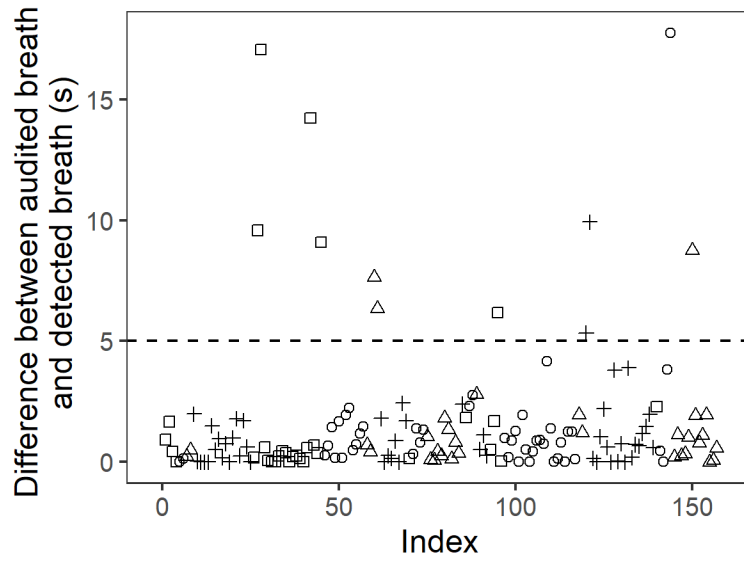


Figure S1. Results of acoustic validation of 157 breaths during logging. 11 breaths were missed resulting in a 93% accuracy of *respdetect* for detecting true positives.

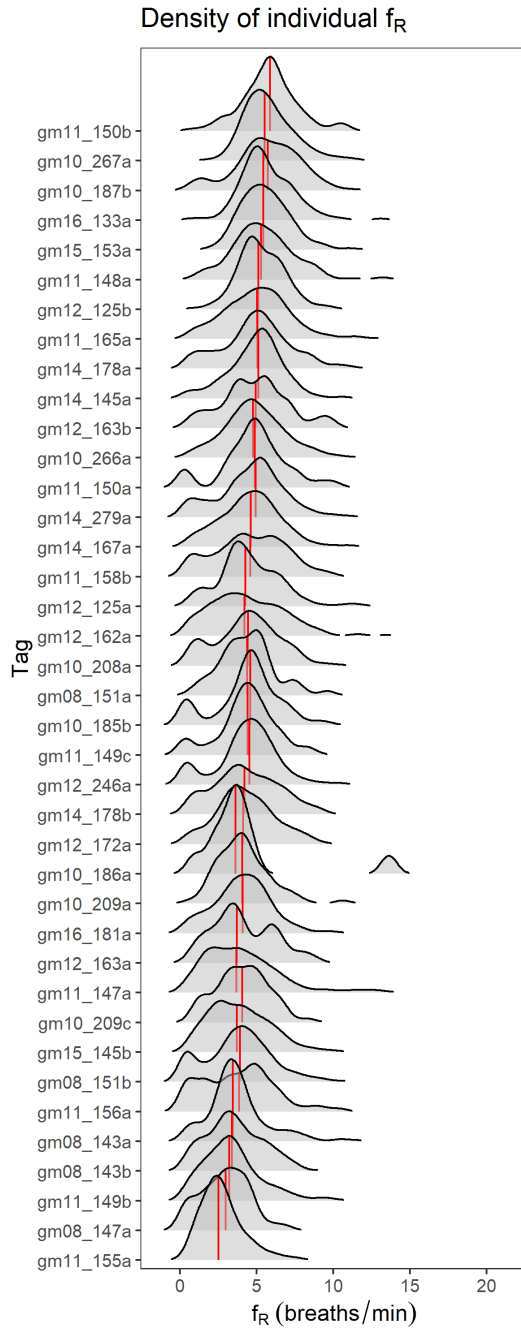


Figure S2. Distributions of individual f_{RS} for all individuals.

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Biography

Ashley Blawas received a Bachelor of Science in Engineering from Duke University in 2018 where she studied Biomedical Engineering and earned a certificate in Marine Science and Conservation Leadership. As a Ph.D. student, she was awarded both the E. Bayard Halsted Scholarship and the Katherine Goodman Stern Fellowship from the Duke Graduate School. Her research was supported by multiple funding sources including an International Dissertation Travel Award from the Duke Graduate School, a Graduate Student Award from the Triangle Center for Evolutionary Medicine, a Summer Research Fellowship from the Duke Graduate School, and multiple co-authored grants from Dolphin Quest. Ashley served as a co-Principal investigator on the inaugural student-led R/V Shearwater cruise at the Duke University Marine Lab. She is a member of the Society for Marine Mammalogy and has reviewed for the journals *Marine Ecology Progress Series* and *Veterinary Research Communications*. She has authored or co-authored eight publications including “Dynamic body acceleration as a proxy to predict the cost of locomotion in bottlenose dolphins” in the *Journal of Experimental Biology* in 2022; “An integrated comparative physiology and molecular approach pinpoints mediators of breath-hold capacity in dolphins” in *Evolution, Medicine, and Public Health* in 2021; “Scaling of heart rate with breathing frequency and body mass in cetaceans” in *Philosophical Transactions of the Royal Society B* in 2021; “Respiratory sinus arrhythmia and submersion bradycardia in bottlenose dolphins (*Tursiops truncatus*)” in the *Journal of Experimental Biology* in 2021; “Cardiorespiratory coupling in cetaceans; a physiological strategy to improve gas exchange?” in the *Journal of Experimental Biology* in 2020; “Conditioned variation in heart rate during static breath-holds in the bottlenose dolphin (*Tursiops truncatus*)” in *Frontiers in Physiology* in 2020; “Effects of methyl and

inorganic mercury exposure on genome homeostasis and mitochondrial function in *Caenorhabditis elegans*” in *DNA Repair* in 2017; and “Elucidating the links between endocrine disruptors and neurodevelopment” in *Endocrinology* in 2015.