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RESEARCH ARTICLE

Dynamics of blood circulation during diving in the bottlenose dolphin (*Tursiops truncatus*): the role of the retia mirabilia

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ABSTRACT

The retia mirabilia are vascular nets composed of small vessels dispersed among numerous veins, allowing blood storage, regulation of flow and pressure damping effects. Here, we investigated their potential role during the diving phase of the bottlenose dolphin (*Tursiops truncatus*). To this effect, the whole vertebral retia mirabilia of a series of dolphins were removed during post-mortem analysis and examined to assess vessel diameters, and estimate vascular volume and flow rate. We formulated a new hemodynamic model to help clarify vascular dynamics throughout the diving phase, based on the total blood volume of a bottlenose dolphin, and using data available about the perfusion of the main organs and body systems. We computed the minimum blood perfusion necessary to the internal organs, and the stroke volume and cardiac output during the surface state. We then simulated breath-holding conditions and perfusion of the internal organs under the diving-induced bradycardia and reduction of stroke volume and cardiac output, using 10 beats min^{-1} as the limit for the heart rate for an extended dive of over 3 min. Within these simulated conditions, the retia mirabilia play a vital role as reservoirs of oxygenated blood that permit functional performances and survival of the heart and brain. Our theoretical model, based on the actual blood capacity of the retia mirabilia and available data on organ perfusion, considers the dynamic trend of vasoconstriction during the diving phase and may represent a baseline for future studies on the diving physiology of dolphins and especially for the blood supply to their brain.

KEY WORDS: Rete mirabilis, Cetacean, Stroke volume, Cardiac output

INTRODUCTION

Cetacean swimming requires considerable energy consumption. The complex metabolic needs of such a continuous effort have been studied and clarified, at least partially. However, the physiology of deep or prolonged diving in dolphins and whales remains largely unsolved.

Marine Cetartiodactyla belonging to the family Delphinidae include small to medium-sized species with variable locomotion and foraging habits, capable of prolonged dives depending on the prey and environment. The bottlenose dolphin, *Tursiops truncatus* (Montagu 1821), is perhaps the most studied member of the dolphin family, because of its worldwide distribution and the presence of individuals kept under human care. The bottlenose dolphin generally prefers shallow depths of −5 to −50 meters of seawater (msw) for hunting pelagic fish of different species. Nonetheless, the bottlenose dolphin is also a potential deep diver, with a recorded maximum depth of 390 msw and a breath-holding record time of 8 min (Ponganis et al., 2003).

A considerable number of studies have explored the diving response induced by breath-holding dives in humans, with its consequent bradycardia, redistribution of blood flow and effects of increased pressure (e.g. Dujic and Breskovic, 2012; Costalat et al., 2013). Human record breath-holding divers have reached a maximum submerged time of over 11 min during pool competitions (static apnea in the swimming pool involves no elevation of external pressure), and an open-water depth limit of −214 msw (source for both: https://www.aidainternational.org/#recordsMan), obtained with a mechanical device to speed up descent and help in ascent to the surface. Human efforts at depth are single episodes, followed by relatively long surface intervals necessary to recover and avoid breath-holding decompression sickness (Goldman and Solano-Altamirano, 2015). Although the general laws of mammalian physiology obviously apply also to the bottlenose dolphin and other marine mammals, some of the basic facts are different. The potential depth of the dives in the bottlenose dolphin is superior to that of humans, and the dives (including the deep ones) may be continuously repeated with a minimal (if any) surface interval. The phenomenon of lung collapse may take place at a depth between −50 and −70 msw in the bottlenose dolphin (Ridgway, 1972; Ridgway and Howard, 1979; Bostrom et al., 2008; Moore et al., 2011; Fahlman et al., 2017), a depth well below that for humans (Fitz-Clarke, 2007). On the whole, the prolonged and repeated exposure to high-pressure environments reached by dolphins would be fatal for humans. Among life-threatening factors are high and dangerous partial pressure of both oxygen and nitrogen in the blood and tissues, possible saturation of the spinal cord, perils linked to repeated bradycardia, and the impending hazard of low oxygenation of neurons in the central nervous system (Goldman and Solano-Altamirano, 2015; Harmsen et al., 2015).

As a general rule, cerebral blood flow in the human brain (average mass 1400 g) requires a perfusion of 756 ml min^{-1}, i.e. a minimum of 54 ml min^{-1} of blood for every 100 g of brain tissue with a systemic pressure range of 65–140 mmHg (Barrett et al., 2016). Taking for granted that the same applies also to other mammalian species, or at least to species with an equivalent or higher brain volume than the dolphins, here we investigate the dynamics of blood circulation in the bottlenose dolphin during simulated conditions of deep and prolonged dives. To this effect, we considered the mass of circulating blood (approximately 7.4% of body mass; Ridgway and Johnston, 1966; Elsner, 1999) and the specific morphological and

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Table 1. Biological data relative to the bottlenose dolphins used in the present study

Specimen ID	Sex	Length (cm)	Mass (kg)	Age class	Used for volume calculation	Cause of death
192	♀	240	178.5	Adult	No (morphology only)	Unknown
195	♂	270	200*	Adult	No (morphology only)	Unknown
196	♂	300	219	Adult	Yes	Unknown
202	♀	264	195*	Adult	No (morphology only)	Drowning
319	♂	310	226.3*	Adult	Yes	Unknown
344	♂	195	98.5	Young	Used for volume of the rete but not for mean length or body mass	Unknown
Mean		276.8	203.76			

*Mass based on ex post computation.

physiological adaptations of the dolphin body, including the flexible thorax, the absence of patent internal carotid arteries, and the presence of extended retia mirabilia (for general reference, see Cozzi et al., 2017). A rete mirabilis (pl. retia mirabilia) is a vascular structure composed of one or more arterioles placed among a complex of veins or venous sinuses (Ask-Upmark, 1935), typical of terrestrial Cetartiodactyla (Baldwin, 1964; Ghoshal and Khamas, 1984; O'Brien, 2018). Retia mirabilia in the vault of the thorax, the vertebral canal and the base of the brain are extremely developed in dolphins (Breschet, 1836; Nakajima, 1961; Galliano et al., 1966; Nagel et al., 1968; McFarland et al., 1979; for overview, see Ponganis, 2015; Cozzi et al., 2017) and other medium-sized toothed whales (Vogl and Fisher, 1982).

Here, we hypothesize that the distribution of blood flow in the body of the diving dolphin requires special vascular adjustments, calculate the perfusion of the key organs (especially the brain and heart) under changing descent conditions, and evaluate the potential role of the retia mirabilia by calculating their volume and likely output.

MATERIALS AND METHODS
Animals
We examined six bottlenose dolphins from the Mediterranean Marine Mammal Tissue Bank (MMMTB), located at the Department of Comparative Biomedicine and Food Science of the University of Padova (www.marinemammals.eu). The MMMTB is a CITES recognized institution (IT 020) that receives the whole body, or specific tissues sampled from the body, of whales and dolphins that have stranded along the Italian coastline. The MMMTB preserves the tissues and distributes them for free to scientists worldwide. The MMMTB additionally stores samples recovered from marine mammals sent to the facilities of the Department of Comparative Biomedicine and Food Science from aquaria or marine theme parks for post-mortem diagnosis. A summary of the biological information relative to the dolphins examined is contained in Table 1. The MMMTB works in cooperation with the Italian Ministry of the Environment.

Tissue samples
The whole spinal rete mirabilis and spinal cord, or selected and topographically determined parts of it (cervical, thoracic and lumbar), were sampled in the necropsy room of the Department of Comparative Biomedicine and Food Science during post-mortem examinations of the specimens listed in Table 1. Tissue samples were immediately immersed in buffered formalin for fixation. When the whole rete mirabilis and spinal cord were sampled, sectioning and sampling took place approximately 1 month after immersion fixation. In all cases, the length of the whole vertebral column, the vertebral canal and – whenever available – the whole length of the rete with the spinal cord were recorded.

Formalin-fixed samples were subsequently washed, dehydrated and processed for paraffin embedding. Microtome sections of

4–6 µm were stained with common morphological techniques and photographed using a D-Sight acquisition microscope (Menarini Group, Firenze, Italy). Image analysis was performed using the companion software D-Sight Viewer (Menarini Group).

Volume of the spinal retia mirabilia
The volume of the retia mirabilia was directly calculated in three specimens (Table 2). The volume of the vascular structure was obtained by determination of the area of the vessels in a transverse section of each sector (cervical, thoracic, lumbar and caudal) of the rete and spinal cord, and then multiplied by the length of the sector. When the rete was not continuous in all its length but only as separate cervical, thoracic, lumbar and caudal segments, an approximation of its full extent was made by comparison of the size of the available samples of the spinal cord and rete mirabilis, vertebral column and vertebral canal with the corresponding parts of a whole structure taken from another specimen with complete data. The basic principle is that animals of relatively comparable length of the body and vertebral column have presumably similar extension of the rete mirabilis, if the dimension of the vertebral canal, sectioned spinal cord and rete are also similar. The volume of blood (expressed in liters) stored in the spinal retia was then determined accordingly (see below).

Circulating volume of blood, stroke volume and blood perfusion of the single organs
Circulating blood mass of each specimen was considered as 7.4% of the body mass (Ridgway and Johnston, 1966; Elsner, 1999) and divided by blood density (1.060 kg m^{-3}, at 37°C for blood with a physiologic hematocrit) to obtain the blood volume expressed in liters (Cutnell and Johnson, 1998). For specimens 195, 202 and 319, whose body mass was not recorded (Table 1), a proportion was made with specimen 196 of similar body size and length.

Calculation of the stroke volume (V_S) was obtained using data from specimens 196, 319 and 344 because of the flawless integrity of the whole retia mirabilia. Computation of V_S was based on the research carried out by Miedler et al. (2015) in the bottlenose dolphin. In their paper, Miedler et al. (2015) used echocardiography to determine the percentage variation of the heart rate (f_H) and V_S at rest, during exercise and in the post-exercise phase at different time intervals (1, 3 and 4 min).

Table 2. Volume of the spinal retia mirabilia

Specimen ID	Volume (dm^3)
196	1.72
319	2.06
344	1.34
Mean±s.d.	1.71±0.36

Table 3. Perfusion of single organs

Organ	% Body mass	Mass (kg)	Specific blood perfusion flow rate (ml min^{-1} 100 g^{-1})	Blood perfusion flow rate (l min^{-1})	References
Blood	7.4	15.1	N/A	N/A	Reynolds and Rommel, 1999; Ridgway and Johnston, 1966
Brain	0.8	1.6	50	0.82	Cozzi et al., 2017*; Hall, 2010[‡]
Heart	0.9	1.9	70	1.33	Slijper, 1979; Hall, 2010[‡]
Lungs	3.5	7.1	25[§]	1.78	Slijper, 1979; Hall, 2010[‡]
Kidney	1.1	2.2	360	8.07	Slijper, 1979; Hall, 2010[‡]
Liver	2.2	4.5	30	1.34	Slijper, 1979; Klinke et al., 2012[‡]
Gut	3.4	6.6	70	4.64	Slijper, 1979; Tomilin, 1967; Klinke et al., 2012[‡]
Skeleton	16	32.6	3	0.98	Slijper, 1979; Hall, 2010[‡]
Muscle	36	73.4	14.9	7.34	Ponganis, 2015; Jobsis et al., 2001[¶]
Blubber and skin	30.9	63.0	3	1.89	Tomilin, 1967; Hall, 2010[‡]

*Average value for the mean mass of the brain in the adult bottlenose dolphin.
[‡]Human reference values.
[§]Bronchial circulation only.
[¶]Data derived from research performed on *Phoca vitulina* (Jobsis et al., 2001).

According to their results, average mass-specific V_S was 0.788± 0.135 ml kg^{-1} (Miedler et al., 2015).

The data were plotted in a specific equation (CO=Q_{tot}=f_H×V_S) to calculate the cardiac output (CO), i.e. the flow rate (Q_{tot}) of blood that perfuses the body, depending on the variation of the V_S with the f_h, regardless of the actual depth of the dive.

Blood perfusion of the single organs and pertinent references are reported in Table 3. The values reported are referred either to marine mammals or to human organs when specific data were not available in the literature.

RESULTS
Morphology and volume of the retia mirabilia
The morphology of the retia mirabilia (Fig. 1) of all the examined animals conformed to the model already described in the species (Galliano et al., 1966; Viamonte et al., 1968; McFarland et al., 1979). The retia mirabilia of dolphins are made up by a dense network of vessels wrapped around the spinal cord and contained within the vertebral canal. Their vascular supply comes from dorsal arteries derived from the brachiocephalic trunk, internal thoracic arteries, intercostal arteries and other branches of the descending aorta. The cervical part of the spinal rete mirabilis gives origin to meningeal arteries, which constitute one of the major ways to supply blood to the brain, because the internal carotid arteries are reduced in the adult dolphin and mainly directed to the ophthalmic rete (for review, see Cozzi et al., 2017).

Histological analysis of the blood vessels of the retia showed a structure somewhat resembling that of the aorta, with the walls composed of two-thirds smooth muscle fibers and one-third elastic
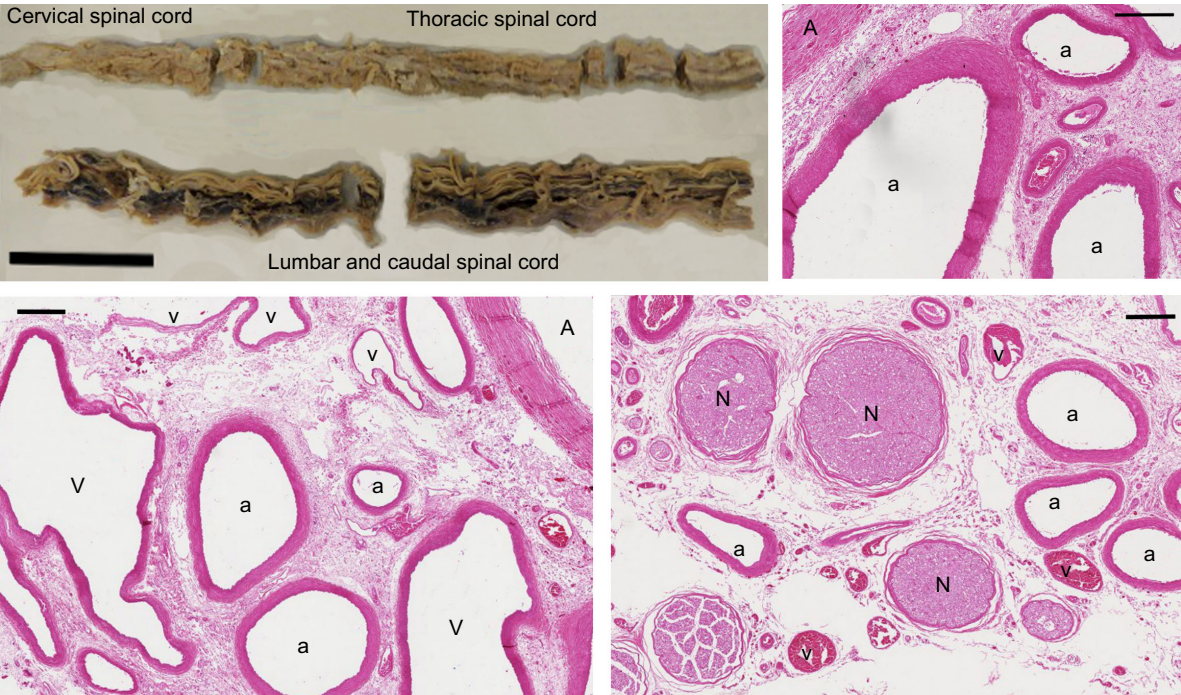

Fig. 1. Spinal cord and associated rete mirabilis of a bottlenose dolphin (specimen 196). A, artery; V, vein; a, arteriole; v, venule; N, nerve fiber. Smaller, non-labeled vessels are difficult to classify. Top left image: scale bar=30 cm. All other images: scale bar=250 μm.

fibers. The volume of the spinal retia mirabilia, calculated as described above, is reported in Table 2.

Stroke volume

For our purposes, we have considered the mean value for the mass of the three specimens (196, 344 and 319) taken from the MMMTB. The V_S for each different f_H, calculated as described above, the cardiac output (CO) and the V_S percentage variation with respect to the rest conditions are reported in Fig. 2. The equation in Fig. 2A was obtained by computing V_S (percentages taken from fig. 3B of Miedler et al., 2015) and f_H (beats min^{-1}) of a dolphin during exercise.

Through this correlation, we computed the following equations to calculate the blood flow rate that perfuses each organ, and then the variation of the V_S at a precise f_H:

$$\text{CO} = Q_{tot} = V_S \times f_H = Q_{brain} + Q_{heart} + Q_{retia} + Q_{other\ organs}. \quad (1)$$

Considering the blood flow rate that perfuses each organ (Table 3) and the variation of the V_S at a precise f_H (Fig. 2), using Eqn 1 and the data reported in Table 4, we obtained the values contained in Table 5. The results show the mean minimal vital blood flow rate of the two vital organs (brain and heart) calculated based on a mean body mass of 203.76 kg (Table 1) and on the relative organ-specific perfusion flow rate values. The minimal perfusion flow rate for the brain and heart was 2.14 l min^{-1}. As noted in Table 5, in the hypothesis of constant diameters of the vessels, at an f_H equal to 20 beats min^{-1}, the blood flow rate reaches the minimum quantity necessary to functionally perfuse the two vital organs listed above. Because the f_H reported in the diving bottlenose dolphin may fall below 10 beats min^{-1} (Elsner et al., 1966; Ridgway, 1976; for review, see Ponganis, 2015), we consequently proceeded to estimate values also for 10 beats min^{-1}.
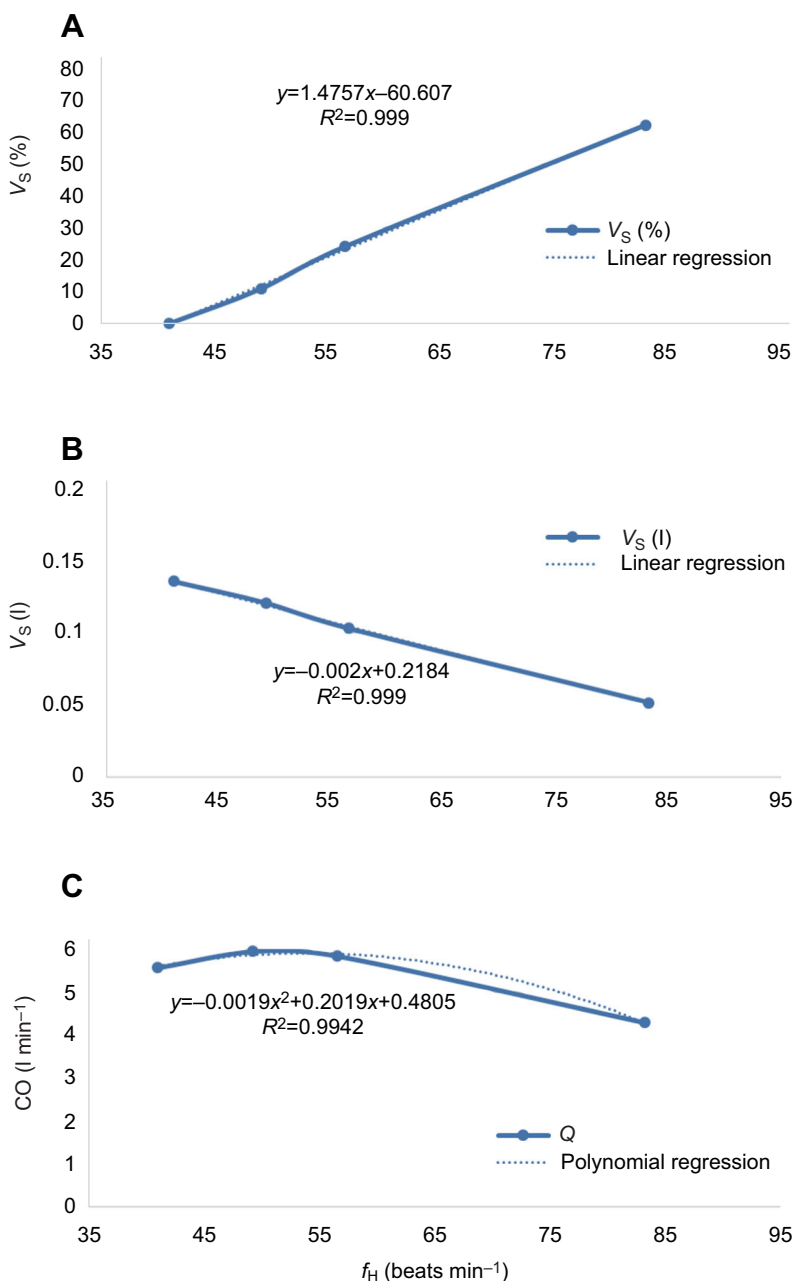

Fig. 2. Vascular dynamics in a bottlenose dolphin during exercise. (A) Percentage stroke volume (V_S; %), (B) stroke volume (V_S; l) and (C) cardiac output (CO; l min^{-1}) versus heart rate (f_H; beats min^{-1}). The best-fitting regression curves and equations are superimposed on the experimental data. The percentage V_S is taken from fig. 3B of Miedler et al. (2015).

Table 4. Blood perfusion flow rate for single organs for a bottlenose dolphin with a mass of 203.76 kg swimming at the surface with heart rate (f_H)=100 beats min^{-1}

Perfusion	Brain	Gut	Liver	Heart	Kidney	Skeleton	Blubber and skin	Muscle	Lung	Retia mirabilia
Mass (%)	0.8	3.3	2.2	0.9	1.1	16	30.9	36	3.5	5
Mass (kg)	1.6	6.6	4.5	1.9	2.2	32.6	63	73.4	7.1	10.2
Minimal specific perfusion flow rate (ml min^{-1} 100 g^{-1})	50	70	30	70	360	3	3	10	25	N/A
Flow rate (l min^{-1})	0.82	4.6	1.34	1.33	8.07	0.98	1.89	7.34	1.78	1.84*

*Estimation of the volume of blood flow rate through retia mirabilia.

We thus obtained the following values: at 41 beats min^{-1}, Q_{tot} is 6.48 l min^{-1}; at 20 beats min^{-1}, Q_{tot} is 2.21 l min^{-1}; and at 10 beats min^{-1}, Q_{tot} is 0.87 l min^{-1}.

Blood flow variation has been calculated from the value of 100 beats min^{-1} (therefore considering the f_H under exercise condition) to the minimum value reported in the literature, which is 10 beats min^{-1} (Elsner et al., 1966; Ridgway, 1976). The other values reported in Table 5 [V_S (%), reduction of blood flow (%), blood flow rate (l min^{-1}), perfusion (flow rate; l min^{-1})] are calculated from the f_H variation.

The value of blood flow rate (l min^{-1}) drops with the reduction of f_H; paradoxically, with the hypothetical stopping of the heart and consequent absence of beat, there will be 100% reduction of blood flow and the blood flow deficit will be equal to the blood flow rate at 100 beats min^{-1}. Here, we emphasize that the narwhal and other potentially other deep-diving cetaceans may lower their f_H to 4 beats min^{-1} or less (Williams et al., 2017).

DISCUSSION

The diving response takes place when a mammal enters the water and holds its breath. The response summarizes several physiological consequences, including, among others, bradycardia, afflux of blood to the thorax and consequent increase of intrathoracic blood pressure (for marine mammals in general, see Elsner, 1999; for dolphins in specific, see Cozzi et al., 2017). The changes undergone by the respiratory tract include lung compliance and recoiling of the flexible thorax (for review, see Fitz-Clarke, 2009; Pendergast and Lundgren, 2009; Piscitelli et al., 2013; Fahlman et al., 2017), owing to negative intrathoracic pressure caused by lung compression. The presence of bronchiolar sphincters (Goudappel and Slijper, 1958; Piscitelli et al., 2013) and the puzzling vascular structure of the dolphin trachea (Cozzi et al., 2005; Bagnoli et al., 2011a,b; Ballarin et al., 2018) may also indicate an evolutionary adaptation to the liquid environment and diving. However, all of the above-mentioned studies consider the physiology, adaptations and plasticity of the respiratory tract. Here, we decided to study the distribution of blood in a diving dolphin based on the available data and the results of our experimental calculations. Specifically, here we pose a simple question: what about the blood supply to the brain and heart?

It is well known that adult dolphins and other cetacean species have no patent internal carotid arteries and no complete arterial cerebral circle (circle of Willis) (see Cozzi et al., 2017 for review). The absence of patent internal carotid arteries in adult mammals is a feature present in several terrestrial Cetartiodactyla (Ask-Upmark, 1935). The common carotid arteries are not essential to ensure proper cerebral functions even in other mammals, including the carnivores (Whisnant et al., 1956). However, dolphins possess a uniquely developed system of sub-vertebral and spinal retia mirabilia, a feature partially shared by larger cetaceans (Pfeiffer and Kinkead, 1990; Ekdale and Kienle, 2015; Costidis and Rommel, 2012, 2016). Pioneering anatomical and angiographic studies demonstrated that the spinal meningeal arteries, derived from the retia mirabilia of the vertebral canal, are the key vessels that vascularize the brain of the bottlenose dolphin (Galliano et al., 1966; Viamonte et al., 1968; McFarland et al., 1979). The same anatomical disposition has been described in the beluga and narwhal brain (Vogl and Fisher, 1981a,b). Such changes in the vascular anatomy must be functional to marine life. To date, a quantification of the volume/capacity of the retia mirabilia has been proposed only in the cervical spine of the bowhead whale (Pfeiffer and Kinkead, 1990) and in the beluga and narwhal (Vogl and Fisher, 1982).

The reduction of brain oxygen supply and the increase of carbon dioxide in the blood consequent to prolonged breath-holding dives may lead to severe brain injury and eventually drowning induced by black-out, at least in animal models (Toklu et al., 2006; Fahlman, 2017) and humans (Modell, 2010; Dujic and Breskovic, 2012). The complex physiological conditions that take place during

Table 5. Stroke volume (V_S) percentage variation related to different f_H, blood flow rate for each f_H and total blood flow rate for the minimum vital perfusion of brain and heart

f_H (beats min^{-1})	V_S (%)	Variation of blood flow (l min^{-1})	Blood flow rate (l min^{-1})	Minimum vital perfusion (l min^{-1})*
100	86.96	23.6	30.03	2.14
90	72.21	18.0	24.48	2.14
80	57.45	13.8	20.23	2.14
70	42.69	9.6	16.04	2.14
60	27.94	5.9	12.33	2.14
50	13.18	2.6	9.09	2.14
41±9	0	0	6.48	2.14
30	−16.34	−2.5	4.03	2.14
20	−31.09	−4.3	2.21	2.14
10	−45.85	−5.6	0.87	2.14

Upper and lower V_S relative to the 'resting' condition reported in Miedler et al. (2015) (V_S=136±19 ml; f_H=41±9 beats min^{-1}; cardiac output=5.514±1.182 l min^{-1}).
*Minimum perfusion (flow rate) required for survival of the brain and heart in a specimen of 203.76 kg (see Table 1).

mammalian apnea in the water, including the effect of thermo-dispersion, have been reviewed by Fahlman et al. (2006), Pendergast and Lundgren (2009) and Fahlman and Schagatay (2014). In the present study, we hypothesized a specific role for the retia mirabilia in the blood supply to the brain during the dive, based on the distribution of flow under the changing conditions that take place during descent. Tables 4 and 5 summarize the results of our calculations on the changes of the blood circulation in the diving bottlenose dolphin. If we assume that the perfusion requirements of the main organs of the dolphins remain in the range considered normal for humans (Fox, 2016), with the notable exception of the muscle (Noren and Williams, 2000), Table 5 shows that, for f_H values below 20 beats min^{-1}, the vascular supply to the brain could be insufficient to ensure proper oxygenation of neural cells. The consequences of the dolphin breath-holding phase have been discussed before, and anaerobic metabolism, based on alveolar gas tensions, has been hypothesized for short periods during the dive in the bottlenose dolphin (Ridgway et al., 1969). However, anaerobic metabolism is presently difficult to associate with normal cerebral activities, and any explanation that considers alternative supply of oxygen and glucose and maintain aerobic metabolism requires careful consideration and scrutiny.

In an elegant study performed on Weddell seals, *Leptonychotes weddellii*, the application of Fick's principle, which regulates gas transport phenomena through the tissues, demonstrated that convective oxygen transport to the brain during routine aerobic dives was more than sufficient, and that the calculated oxygen extraction coefficient never exceeded 50%, even when the arterial $\dot{P}_{O_2}$ fell to 20 mmHg (Davis and Kanatous, 1999). If applied to the bottlenose dolphin, and without taking into account the difference in diving modalities between the two species, these data obtained in a pinniped carnivore would explain the complex diving phenomena without reserving a special role for the retia mirabilia. We have no way to determine the oxygen content of the blood in the spinal retia mirabilia, an essential factor to verify this latter hypothesis in *T. truncatus*. However, several factors suggest that the situation in the dolphin may be different, and requires additional, perhaps dissimilar, explanations. The phylogenetic distance between seals and dolphins would be scarcely relevant per se, but the anatomical differences indicate other evolutionary pathways for Cetacea. Seals contract their spleen to redistribute blood during the dive (Hurford et al., 1996), and possess an extradural venous system (Harrison and Tomlinson, 1956), but have no retia mirabilia comparable to those of dolphins for position and capacity. Furthermore, dolphins (but not pinnipeds) are characterized by the lack of coagulation factor XII (Lewis et al., 1969; Robinson et al., 1969; Semba et al., 1998; Tibbs et al., 2005). In contrast, the absolute breath-holding capacity of the Weddell seal (82 min; Castellini et al., 1992) is 10-fold that of the bottlenose dolphin (8 min; Ponganis et al., 2003). The extent of vasoconstriction cannot be precisely ascertained in diving dolphins, and we cannot exclude that sufficient arterial oxygen capacity could be maintained to supply their brain and heart similarly to what happens in seals even when arterial pressure and blood flow rate drop during the dive. Nevertheless, the anatomical conformation of the vascular system supports a role for the retia mirabilia in breath-holding diving, possibly, according to our theoretical model, as a supplementary source of blood. Their role as an oxygen reserve requires further experimental work.

Elegant functional investigations performed in the laboratory on live specimens indicated that blood supply to the dolphin brain may be specifically regulated to ensure preferential flow to auditory and navigation centers (Houser et al., 2010b), and support differential supply during unihemispheric sleep at the surface (Ridgway et al., 2006). To this effect, the absence of a complete cerebral arterial circle at the base of the brain, substituted by a mesh of retia mirabilia connected to the ophthalmic retia (Galliano et al., 1966), may be an additional indication of a specific and potentially differential supply of brain districts. During the deep part of the dive, oxygen and glucose are essential to maintain navigation abilities and functions of the auditory cortex. The ability to selectively increase vascular supply to the latter centers, and, simultaneously, reduce temperature and glucose consumption in other districts, may be essential to successful hunting, echolocation performance and survival (Ridgway et al., 2006).

Nevertheless, Table 5 shows that, within our theoretical simulation, below 20 beats min^{-1} the brain of the bottlenose dolphin does not receive enough blood to maintain functions. Comparable f_H values were reported in a field study on the bottlenose dolphin (Williams et al., 1999), and in a seminal investigation on venous gas formation, in which the f_H of repetitively diving bottlenose dolphins consistently declined below 30 beats min^{-1} during the initial 15 s and remained at 20–40 beats min^{-1} until ascent (Houser et al., 2010a).

A mechanism of differential vascularization (or differential regulation) of blood flow to key districts of the central nervous system may partially explain the extension of the breath-holding periods in dolphins, but still leaves open the question of how the remaining parts of the brain do not became damaged or die. The situation changes if we consider the retia mirabilia as 'reservoirs' of oxygenized blood during the diving phase. If we hypothesize that blood contained in the retia mirabilia may be actively mobilized during the diving phase, an equilibrium is reached with a high enough blood flow rate to maintain neural metabolism. The rete mirabilis has been considered to receive considerable adrenergic control (Diéguez et al., 1983) and exert a dampening effect on the brain circle of ruminants (Lluch et al., 1985). But a specific study on the innervation of the retia mirabilia (in the narwhal) identified only a few nerve fibers (Vogl et al., 1981). Although we have shown that nerve fibers are present within the retia (Fig. 1), the question that must be answered is why should the rete mirabilis become especially active during the descent? In other words, what is the physiological mechanism behind any eventual role for these structures only during a specific phase of the dive and not during all phases? To this effect, we emphasize that the rete mirabilis has been considered a non-pulsatile source of blood, with a dampening effect on cerebral circulation (Nagel et al., 1968). However, dolphin dives require periodical strokes of the flukes, potentially minimal but present also during the gliding descent (Williams et al., 2018), and energetic in the subsequent ascent phase. The downstroke movements of the flukes are generated in great part by the contraction of the powerful m. rectus abdominis, and one consequence is an increase in the intra-abdominal pressure. Given the anatomical characteristics of the abdominal cavity of dolphins, and especially its reduced volume and the presence of powerful muscle layers surrounding it (for reference, see Cotten et al., 2008; Cozzi et al., 2017), the obvious effect of an increase in intra-abdominal pressure is a forward movement of the diaphragmatic cupola. This latter action brings further pressure into the thorax (Lillie et al., 2017). But because the external walls of the thorax have little to no way to expand under the effects of environmental pressure, blood in the thorax and heart is squeezed into the upper thoracic arteries that furnish the retia mirabilia (see also Hui, 1975). The whole cycle that starts with the downstroke may be the motor that changes the output of the retia mirabilia from non-pulsatile to temporarily rhythmic during the dive. In fact, the histological

structure of the spinal rete mirabilis supports a passive–active elastic mechanism of blood progression. We have no basis to assume that the retia mirabilia become suddenly active under conditions of prolonged breath-holding and bradycardia, following a hypothetical on/off mechanism activated by impending hypoxia. On the contrary, our data show that the retia gradually contribute to the oxygenation of the brain during the descent phase of the dive, and became essential only under extreme conditions (below 20 beats min^{-1}). Dolphins may not always dive very deep, but they dive and face some degree of bradycardia throughout their entire life. Here, we also note that different studies have described an increase in the heart rate of submerged bottlenose dolphin in response to an intensification of stroke frequency (e.g. Davis and Williams, 2012). Yet bradycardia in response to diving appears not only in bottlenose dolphins (Elsner et al., 1966), but also in killer whales, pilot whales and belugas (Bickett et al., 2019). The voluntary modulation of bradycardia has been described in harbor porpoises (Elmegaard et al., 2016), and extreme bradycardia has also been reported in narwhals (Williams et al., 2017; for review, see Ponganis, 2015).

We are aware that our data are partially based on values derived from non-dolphin species and extrapolated from different sources, and may therefore contain hidden confounding factors. However, we attempted to design a novel model and an experimental setting that answers some of the puzzling facts previously discussed regarding dolphin dive metabolism. Our hypothesis may contribute to solve, at least in part, the question of oxygen needs of the cerebral tissue. The involvement of the retia would also explain why dolphins and other cetacean species have a relatively small spleen (Cozzi et al., 2017), as the retia would supply the reservoir of blood given by the spleen to breath-holding seals and humans during prolonged dives (Hurford et al., 1996; Inoue et al., 2013). Here, we add that dolphins lack certain components of the coagulation cascade (Semba et al., 1998; Tibbs et al., 2005), a protective factor against decompression sickness. To this effect, the presence of extensive, sponge-like spinal retia mirabilia composed of myriad small vessels may trap bubbles, because of their position interposed between the heart and the arteries directed to the brain. Finally, the structure of the retia, and their potential role in the general balance of the arterial system during diving, may prevent brain damage induced by excessive and prolonged bradycardia (Noren et al., 2004, 2012; Williams et al., 2015).

References

Ask-Upmark, E. (1935). The carotid sinus and the cerebral circulation. An anatomical, experimental and clinical investigation, including some observations on rete mirabile caroticum. *Acta Psychiatr. Neurol.* **6**, 1-374.

Bagnoli, P., Peruffo, A., Costantino, M. L. and Cozzi, B. (2011a). The upper respiratory tract of dolphins. *Ital. J. Anat. Embryol.* **116**, 159-164.

Bagnoli, P., Cozzi, B., Zaffora, A., Acocella, F., Fumero, R. and Costantino, M. L. (2011b). Experimental and computational biomechanical characterisation of the tracheo-bronchial tree of the bottlenose dolphin (*Tursiops truncatus*) during diving. *J. Biomech.* **44**, 1040-1045.

Baldwin, B. A. (1964). The anatomy of the arterial supply to the cranial regions of the sheep and ox. *Am. J. Anat.* **115**, 101-118.

Ballarin, C., Bagnoli, P., Peruffo, A. and Cozzi, B. (2018). Vascularization of the trachea in the bottlenose dolphin: Comparison with bovine and evidence for evolutionary adaptations to diving. *R. Soc. Open Sci.* **5**, 171645.

Barrett, K. E., Barman, S. M., Boitano, S. and Brooks, H. L. (2016). *Ganong's Review of Medical Physiology*, 25th edn. New York: Lange Mc Graw Hill.

Bickett, N. J., Tift, M. S., Leger, J. St. and Ponganis, P. J. (2019). Heart rates, heart rate profiles, and electrocardiograms in three killer whales, a beluga, and a pilot whale: an exploratory investigation. *Marine Mammal Sci*.

Bostrom, B. L., Fahlman, A. and Jones, D. R. (2008). Tracheal compression delays alveolar collapse during deep diving in marine mammals. *Respir. Physiol. Neurobiol.* **161**, 298-305.

Breschet, G. (1836). *Histoire Anatomique et Physiologique d'un Organe de Nature Vasculaire Découvert Dans Les Cétacés*. Paris: Bechet Jeune.

Castellini, M. A., Kooyman, G. L. and Ponganis, P. J. (1992). Metabolic rates of freely diving Weddell seals: correlations with oxygen stores, swim velocity and diving duration. *J. Exp. Biol.* **165**, 181-194.

Costalat, G., Coquart, J., Castres, I., Tourny, C. and Lemaitre, F. (2013). Hemodynamic adjustments during breath-holding in trained divers. *Eur. J. Appl. Physiol.* **113**, 2523-2529.

Costidis, A. M. and Rommel, S. A. (2012). Vascularization of air sinuses and fat bodies in the head of the Bottlenose dolphin (*Tursiops truncatus*): morphological implications on physiology. *Front. Physiol.* **3**, 243.

Costidis, A. M. and Rommel, S. A. (2016). The extracranial arterial system in the heads of beaked whales, with implications on diving physiology and pathogenesis. *J. Morphol.* **277**, 5-33.

Cotten, P. B., Piscitelli, M. A., McLellan, W. A., Rommel, S. A., Dearolf, J. L. and Pabst, D. A. (2008). The gross morphology and histochemistry of respiratory muscles in bottlenose dolphins *Tursiops truncatus*. *J. Morphol.* **269**, 1520-1538.

Cozzi, B., Bagnoli, P., Acocella, F. and Costantino, M. L. (2005). Structure and biomechanical properties of the trachea of the striped dolphin *Stenella coeruleoalba*: evidence for evolutionary adaptations to diving. *Anat. Rec. A* **284**, 500-510.

Cozzi, B., Huggenberger, S. and Oelschläger, H. A. (2017). *The Anatomy of Dolphins. An Insight into Body Structure and Function*. London: Academic Press.

Cutnell, J. D. and Johnson, K. W. (1998). *Physics*, 4th edn. Wiley.

Davis, R. W. and Kanatous, S. B. (1999). Convective oxygen transport and tissue oxygen consumption in Weddell seals during aerobic dives. *J. Exp. Biol.* **202**, 1091-1113.

Davis, R. W. and Williams, T. M. (2012). The marine mammal dive response is exercise modulated to maximize aerobic dive duration. *J Comp Physiol. A* **198**, 583-591.

Diéguez, G., Conde, M. V., Gómez, B., Iglesias, J. R., Marín, J. and Lluch, S. (1983). Rete mirabile of goat: *in vitro* effects of adrenergic stimulation. *Brain Res.* **289**, 281-284.

Dujic, Z. and Breskovic, T. (2012). Impact of breath holding on cardiovascular respiratory and cerebrovascular health. *Sports Med.* **42**, 459-472.

Ekdale, E. G. and Kienle, S. S. (2015). Passive restriction of blood flow and counter-current heat exchange via lingual retia in the tongue of a neonatal gray whale *Eschrichtius robustus* (Cetacea, Mysticeti). *Anat. Rec.* **298**, 675-679.

Elmegaard, S. L., Johnson, M., Madsen, P. T. and McDonald, B. I. (2016). Cognitive control of heart rate in diving harbor porpoises. *Current Biol.* **26**, R1167-R1176.

Elsner, R. (1999). Living in water. Solutions to physiological problems. In *Biology of Marine Mammals* (ed. J. E. Reynolds, , III and S. A. Rommel), pp. 73-116. Washington, DC: Smithsonian Institution Press.

Elsner, R., Kenney, D. W. and Burges, K. (1966). Diving bradycardia in the trained dolphin. *Nature.* **212**, 407-408.

Fahlman, A. (2017). Allometric scaling of decompression sickness risk in terrestrial mammals; cardiac output explains risk of decompression sickness. *Sci. Rep.* **7**, 40918.

Fahlman, A. and Schagatay, E. (2014). Man's place among the diving mammals. *Hum. Evol.* **29**, 47-66.

Fahlman, A., Olszowka, A., Bostrom, B. and Jones, D. R. (2006). Deep diving mammals: dive behaviour and circulatory adjustments contribute to bends avoidance. *Resp. Physiol. Neurobiol.* **153**, 66-77.

Fahlman, A., Moore, M. J. and Garcia-Parraga, D. (2017). Respiratory function and mechanics in pinnipeds and cetaceans. *J. Exp. Biol.* **220**, 1761-1773.

Fitz-Clarke, J. R. (2007). Mechanism of airway and alveolar collapse in human breath-holding diving. *Resp. Physiol. Neurobiol.* **159**, 202-210.

Fitz-Clarke, J. R. (2009). Lung compression effects on gas exchange in human breath-hold diving. *Resp. Physiol. Neurobiol.* **165**, 221-228.

Fox, S. I. (2016). *Human Physiology*, 14th edn. McGraw-Hill Education.

Galliano, R. E., Morgane, P. J., McFarland, W. L., Nagel, E. L. and Catherman, R. L. (1966). The anatomy of the cervicothoracic arterial system in the bottlenose

dolphin (*Tursiops truncatus*) with a surgical approach suitable for guided angiography. *Anat. Rec.* **155**, 325-338.

Ghoshal, N. G. and Khamas, W. A. H. (1984). Gross and histomorphological study on the rostral epidural rete mirabile of the pig. *Indian J. Anim. Sci.* **55**, 304-310.

Goldman, S. and Solano-Altamirano, J. M. (2015). Decompression sickness in breath-hold diving, and its probable connection to the growth and dissolution of small arterial emboli. *Math. Biosci.* **262**, 1-9.

Goudappel, J. R. and Slijper, E. J. (1958). Microscopic structure of the lungs of the bottlenose whale. *Nature* **182**, 479.

Hall, J. E. (2010). *Guyton and Hall Textbook of Medical Physiology*, 12th edn. Saunders.

Harmsen, S., Schramm, D., Karenfort, M., Christaras, A., Euler, M., Mayatepek, E. and Tibussek, D. (2015). Presumed arterial gas embolism after breath-hold diving in shallow water. *Pediatrics* **136**, e687-e690.

Harrison, R. J. and Tomlinson, J. D. (1956). Observations on the venous system in certain Pinnipedia and Cetacea. *Proc. Zool. Soc. London.* **126**, 205-233.

Houser, D. S., Dankiewicz-Talmadge, L. A., Stockard, T. K. and Ponganis, P. J. (2010a). Investigation of the potential for vascular bubble formation in a repetitively diving dolphin. *J. Exp. Biol.* **213**, 52-62.

Houser, D. S., Moore, P. W., Johnson, S., Lutmerding, B., Branstetter, B., Ridgway, S. H., Trickey, J., Finneran, J. J., Jensen, E. and Hoh, C. (2010b). Relationship of blood flow and metabolism to acoustic processing centers of the dolphin brain. *J. Acoust. Soc. Am.* **128**, 1460-1466.

Hui, C. A. (1975). Thoracic collapse as affected by the retia thoracica in the dolphin. *Respir. Physiol.* **25**, 63-70.

Hurford, W. E., Hochachka, P. W., Schneider, R. C., Guyton, G. P., Stanek, K. S., Zapol, D. G., Liggins, G. C. and Zapol, W. M. (1996). Splenic contraction, catecholamine release, and blood volume redistribution during diving in the Weddell seal. *J. Appl. Physiol.* **80**, 298-306.

Inoue, Y., Nakajima, A., Mizukami, S. and Hata, H. (2013). Effect of breath holding on spleen volume measured by magnetic resonance imaging. *PLoS ONE* **8**, e68670.

Jobsis, P. D., Ponganis, P. J. and Kooyman, G. L. (2001). Effects of training on forced submersion responses in harbour seals. *J. Exp. Biol.* **204**, 3877-3885.

Klinke, R., Pape, H. C., Kurtz, A. and Silbernagl, S. (2012). *Fisiologia*, 3rd edn. Città di Castello: EdiSES-Napoli.

Lewis, J. H., Bayer, W. L. and Szeto, I. L. F. (1969). Coagulation factor XII deficiency in the porpoise, *Tursiops truncatus*. *Comp. Biochem. Physiol.* **31**, 667-670.

Lillie, M. A., Vogl, A. W., Raverty, S., Haulena, M. H., McLellan, W. A., Stenson, G. B. and Shadwick, R. E. (2017). Controlling thoracic pressures in cetaceans during a breath-hold dive: importance of the diaphragm. *J. Exp. Biol.* **220**, 3464-3477.

Lluch, S., Diéguez, G., Garcia, A. L. and Gòmez, B. (1985). Rete mirabile of goat: its flow-damping effect on cerebral circulation. *Am. J. Physiol.* **249**, R482-R489.

McFarland, W. L., Jacobs, M. S. and Morgane, P. J. (1979). Blood supply to the brain of the dolphin, *Tursiops truncatus*, with comparative observations on special aspects of the cerebrovascular supply of other vertebrates. *Neurosci. Biobehav. Rev.* **3**, 1-82.

Miedler, S., Fahlman, A., Valls Torres, M., Álvarez, T. Á. and Garcia-Parraga, D. (2015). Evaluating cardiac physiology through echocardiography in bottlenose dolphins: using stroke volume and cardiac output to estimate systolic left ventricular function during rest and following exercise. *J. Exp. Biol.* **218**, 3604-3610.

Modell, J. H. (2010). Prevention of needless deaths from drowning. *Southern Med. J.* **103**, 650-653.

Moore, M. J., Hammar, T., Arruda, J., Cramer, S., Dennison, S., Montie, E. and Fahlman, A. (2011). Hyperbaric computed tomographic measurement of lung compression in seals and dolphins. *J. Exp. Biol.* **217**, 1154-1166.

Nagel, E. L., Morgane, P. J., McFarland, W. L. and Galliano, R. E. (1968). Rete mirabile of dolphin: its pressure-damping effect on cerebral circulation. *Science.* **161**, 898-900.

Nakajima, M. (1961). In regard to the rete mirabile of the Cetacea: with emphasis especially on *Grampidelphis griseus* and *Tursiops truncatus*. *J. Med. Acad. Toho.* **8**, 1-23.

Noren, S. R., Cuccurullo, V. and Williams, T. M. (2004). The development of diving bradycardia in bottlenose dolphin (*Tursiops truncatus*). *J. Comp. Physiol. B* **174**, 139-147.

Noren, S. R. and Williams, T. M. (2000). Body size and skeletal muscle myoglobin of cetaceans: adaptations for maximizing dive duration. *Comp. Biochem. Physiol. Part A* **126**, 181-191.

Noren, S. R., Kendall, T., Cuccurullo, V. and Williams, T. M. (2012). The dive response redefined: underwater behaviour influences cardiac variability in freely diving dolphins. *J. Exp. Biol.* **215**, 2735-2741.

O'Brien, H. D. (2018). From anomalous arteries to selective brain cooling: parallel evolution of the artiodactyl carotid rete. *Anat. Rec.*

Pendergast, D. R. and Lundgren, C. E. G. (2009). The underwater environment: cardiopulmonary, thermal, and energetic demands. *J. Appl. Physiol.* **106**, 276-283.

Pfeiffer, C. J. and Kinkead, T. P. (1990). Microanatomy of retia mirabilia of bowhead whale foramen magnum and mandibular foramen. *Acta Anat.* **139**, 141-150.

Piscitelli, M. A., Raverty, S. A., Lillie, M. A. and Shadwick, R. E. (2013). A review of cetacean lung morphology and mechanics. *J. Morphol.* **274**, 1425-1440.

Ponganis, P. J. (2015). *Diving Physiology of Marine Mammals And Seabirds*. Cambridge: Cambridge University Press.

Ponganis, P. J., Kooyman, G. L. and Ridgway, S. H. (2003). Comparative diving physiology. In *Bennett and Elliott's Physiology and Medicine of Diving* (ed. A. Brubakk and T. S. Neuman), pp. 211-226. Edinburgh: Saunders Ltd.

Reynolds, J. E. and Rommel, S. A. (1999). *Biology of Marine Mammals*. Melbourne University Press.

Ridgway, S. H. (1972). Homeostasis in the aquatic environment. In *Mammals of the Sea: Biology and Medicine* (ed. S. H. Ridgway), pp. 690-747. Springfield: C. C. Thomas Co.

Ridgway, S. H. (1976). Diving mammals and biomedical research. *Oceanus* **19**, 49-55.

Ridgway, S. H. and Howard, R. (1979). Dolphin lung collapse and intramuscular circulation during free diving: evidence from nitrogen washout. *Science* **203**, 1182-1183.

Ridgway, S. H. and Johnston, D. G. (1966). Blood oxygen and ecology of porpoises of three genera. *Science* **151**, 456-458.

Ridgway, S. H., Scronce, B. L. and Kanwisher, J. (1969). Respiration and deep diving in the bottlenose porpoise. *Science* **166**, 1651-1654.

Ridgway, S., Houser, D., Finneran, J., Carder, D., Keogh, M., Van Bonn, W., Smith, C., Scadeng, M., Dubowitz, D., Mattrey, R. et al. (2006). Functional imaging of dolphin brain metabolism and blood flow. *J. Exp. Biol.* **209**, 2902-2910.

Robinson, A. J., Kropatkin, M. and Aggeler, P. M. (1969). Hageman factor (factor XII) deficiency in marine mammals. *Science* **166**, 1420-1422.

Semba, U., Shibuya, Y., Okabe, H. and Yamamoto, T. (1998). Whale Hageman factor (factor XII): prevented production due to pseudogene conversion. *Thromb. Res.* **90**, 31-37.

Slijper, E. J. (1979). *Whales*, 2nd edn. London: Hutchinson.

Tibbs, R., Elghetany, M. T., Tran, L., Van Bonn, W., Romano, T. and Cowan, D. F. (2005). Characterization of the coagulation system in healthy dolphins: the coagulation factors, natural anticoagulants, and fibrinolytic products. *Comp. Clin. Path.* **14**, 95-98.

Toklu, A. S., Alkan, N., Gürel, A., Cimsit, M., Haktanır, D., Körpınar, S. and Purisa, S. (2006). Comparison of pulmonary autopsy findings of the rats drowned at surface and 50 ft depth. *Forensic Sci. Int.* **164**, 122-125.

Tomilin, A. G. (1967). *Mammals of the U.S.S.R. and Adjacent Countries*. Israel program for Scientific Translations.

Viamonte, M., Morgane, P. J., Galliano, R. E., Nagel, E. L. and McFarland, W. L. (1968). Angiography in the living dolphin and observations on blood supply to the brain. *Am. J. Physiol.* **214**, 1225-1249.

Vogl, A. W. and Fisher, H. D. (1981a). Arterial circulation of the spinal cord and brain in the Monodontidae (order Cetacea). *J. Morphol.* **170**, 171-180.

Vogl, A. W. and Fisher, H. D. (1981b). The internal carotid artery does not directly supply the brain in the Monodontidae (order Cetacea). *J. Morphol.* **170**, 207-214.

Vogl, A. W. and Fisher, H. D. (1982). Arterial retia related to supply of the central nervous system in two small toothed whales–narwhal (*Monodon monoceros*) and beluga (*Delphinapterus leucas*). *J. Morphol.* **174**, 41-56.

Vogl, A. W., Todd, M. E. and Fischer, H. D. (1981). An ultrastructural and fluorescence histochemical investigation of the innervation of retial arteries in *Monodon monoceros*. *J. Morphol.* **168**, 109-119.

Whisnant, J. P., Millikan, C. H., Wakim, K. G. and Sayre, G. P. (1956). Collateral circulation to the brain of the dog following bilateral ligation of the carotid and vertebral arteries. *Am. J. Physiol.* **186**, 275-277.

Williams, T. M., Haun, J. E. and Friedl, W. A. (1999). The diving physiology of bottlenose dolphins (*Tursiops truncatus*) I. Balancing the demands of exercise for energy conservation at depth. *J. Exp. Biol.* **202**, 2793-2748.

Williams, T. M., Fuiman, L. A., Kendall, T., Berry, P., Richter, B., Noren, S. R., Thometz, N., Shattock, M. J., Farrell, E., Stamper, A. M. et al. (2015). Exercise at depth alters bradycardia and incidence of cardiac anomalies in deep-diving marine mammals. *Nat. Commun.* **6**, 6055.

Williams, T. M., Blackwell, S. B., Richter, B., Sinding, M. H. S. and Heide-Jørgensen, M. P. (2017). Paradoxical escape responses by narwhals (*Monodon monoceros*). *Science* **358**, 1328-1331.

Williams, T. M., Davis, R. W., Fuiman, L. A., Francis, J., Le Boeuf, B. J., Horning, M., Calambokidis, J. and Croll, D. A. (2018). Sink or swim: strategies for cost-efficient diving by marine mammals. *Science* **288**, 133-136.