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Endogenous Ouabain in Human and Animal Models of Hypoxia

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Abstract

Endogenous ouabain (EO) is a steroid hormone secreted by the adrenal glands, usually associated with adverse cardiovascular effects. However, recent studies have highlighted its possible role in blood pressure control and in cardio-renal damage, and it seems to be involved in the adaptive response to hypoxia. The aim of this study is to detect the EO in human and animal models of hypoxia. We collected blood samples from seven competitive elite apnea divers, 11 noncompetitive elite apnea divers, and 26 healthy control subjects. Animal blood samples were collected from 16 common bottlenose dolphins (*Tursiops truncatus*), two phocids and two otariids kept under human care, and 11 wild loggerhead sea turtles (*Caretta caretta*) hosted in two rescue centers. We measured EO plasma concentrations with a Scintillation Proximity Assay.

In elite apnea divers and healthy control subjects, EO plasma concentrations were positively correlated to weight ($p < 0.05$). Elite apnea divers showed statistically significant ($p < 10^{-6}$) higher EO plasma concentrations compared to healthy subjects without any diving experience or experience in other sports activities involving breath-holding.

In dolphins, EO plasma concentrations were positively correlated to age, total length, and weight ($p < 0.05$). In loggerhead sea turtles, EO plasma concentrations were negatively correlated to total length and weight ($p < 0.05$). In pinnipeds, correlation analysis was not performed due to the small number of animals. Herein, we demonstrate, for the first time, that different taxa, phylogenetically distant from each other and which perform apneas without reporting neurological damages, express EO.

Our findings, although preliminary, are in line with the recently emerging hypothesis on a possible role of EO in the adaptive response to hypoxia and represent a helpful hint for future investigations aimed to identify novel molecules useful to treat very disabling pathological conditions such as idiopathic pulmonary arterial hypertension or obstructive sleep apnea.

Key Words: hypoxia, breath-holding, divers, ouabain, *Tursiops truncatus*, pinnipeds, *Caretta caretta*

Introduction

Reduced oxygen supply (hypoxia) critically impacts the mammalian brain and causes serious illness or death in millions of humans each year by producing irreversible and fatal damage to neuronal cells (Horner & Gage, 2000). Hypoxia is the main stress factor for the human brain, which is the body's most demanding organ in terms of oxygen consumption. The human brain represents only 2% of total body weight but requires around 20% of the body's oxygen (Sokoloff, 1976). Contrary to most terrestrial mammals, diving mammals and reptiles (e.g., cetaceans, seals, sea turtles) are routinely exposed to severe hypoxia when submerged (Qvist et al., 1986; Hindell et al., 1992; Costa et al., 2004; López-Mendilaharsu et al., 2009; Meir & Ponganis, 2009; Schorr et al., 2014). They overcome this critical condition without any obvious damage and even show remarkable diving capacities during foraging (Butler & Jones, 1997; Butler, 2004; Ramirez et al., 2007; Ponganis, 2015).

Many physiological adaptations to optimize oxygen supply and metabolic demands in relevant tissues of diving mammals have been reported (Butler, 2004; Ramirez et al., 2007; Williams et al., 2007; Wyneken, 2007; Davenport et al., 2009; Mitz et al., 2009; Ponganis, 2015), but abilities vary greatly in different species. In general, seals show better diving performances than dolphins among marine mammals (Schreer et al., 2001; Ponganis, 2011), whereas sea turtles are able to hold their breath for several hours while continuing to maintain intact brain function (Pritchard, 1971; Rebel, 1974). Sea turtles are able to maintain brain ATP levels during long apneas by suppressing the transmembrane ion transport through the Na^+/K^+-ATPase pumps, while depletion of ATP occurs in mammals within a few minutes (Lutz et al., 1984; Lutz & Bentley, 1985; Hochachka, 1986; Lutcavage & Lutz, 1997).

In humans, instead, both acute and chronic exposure to hypoxia induce a range of cognitive and behavioral deficiencies (Rourke & Adams, 1996; Caine & Watson, 2000) with a few exceptions due to either physiological situations (e.g., living at high altitude), clinical conditions (e.g., obstructive sleep apnea, pulmonary arterial hypertension), or sport activities (e.g., mountaineering, apnea diving). The preservation of cognitive functions in the above-mentioned conditions might be attributed either to neuroprotective factors (Ridgway & McFarland, 2006), to hypoxia-inducible factors (HIFs) that are transcriptional activators functioning as master regulators of oxygen homeostasis in all metazoan species (Loenarz et al., 2011), or to physiological adaptive responses to low oxygen availability (LaManna et al., 1992; Boero et al., 1999; Lindholm et al., 1999; Hochachka, 2000; Auer & Sutherland, 2002; Ferretti & Costa, 2003).

Among these protective factors, there appears to be the endogenous ouabain (EO), a stress-related hormone secreted by the adrenal glands (Schoner & Scheiner-Bobis, 2007). EO is a cardiac glycoside structurally similar to digoxin (El-Masri et al., 2002). Newborns affected by transient tachypnea and patients with obstructive sleep apnea have a digoxin-like immunoreactive substance (Paci et al., 2000; Yalaz et al., 2013). It increases the cardiac contraction (Eisner & Smith, 1992) and is associated, in humans, with adverse cardiovascular outcomes (Bignami et al., 2013). EO is also a specific inhibitor of the Na^+/K^+-ATPase pump (Pierre et al., 2007), which ac tachypnea counts for 50 to 80% of neuronal energy consumption (Falkowska et al., 2015). A role in preserving from adverse developmental programming (Li et al., 2010) and in protecting the brain from traumatic injury (Dvela-Levitt et al., 2014) has been already described for this hormone. The binding of ouabain to Na^+/K^+-ATPase at nanomolar concentrations also induces the activation of mitogen-activated protein kinases (MAPK) and the Akt (protein kinase B) signaling cascades (Li et al., 2010), thus stimulating the viability and proliferation of various cell types, including neuronal cells. EO has also been shown to be a valuable biomarker of heart failure (Simonini et al., 2015) in patients who underwent elective cardiac surgery or with acute kidney injury (Bignami et al., 2013; Simonini et al., 2014). Recent studies, both *in vivo* and *in vitro*, have highlighted a possible role for EO in both acute and chronic kidney damage (Iatrino et al., 2018).

This hormone has been isolated not only from human plasma (Ferrandi et al., 1993; Komiyama et al., 2001) and adrenal glands (El-Masri et al., 2002), but also from rat adrenomedullary cells (Di Bartolo et al., 1995) and biological fluids (Tymiak et al., 1993; Schneider et al., 1998), and from bovine adrenal glands (Kawamura et al., 1999) and hypothalamus (Irwin et al., 1991; Graur & Higgins, 1994). As Bovidae belong to Artiodactyla, and the intra-artiodactyl affinity of Cetacea has been accepted since the early 1990s (Laredo et al., 1994; Antolovic et al., 2000; Asher & Helgen, 2010), in this study, we investigated, for the first time, whether EO is detectable in the plasma of common bottlenose dolphins (*Tursiops truncatus*; Linnaeus, 1758). In addition, we investigated seals, sea lions, and sea turtles as they are

greater diving species than bottlenose dolphins. Last, we enrolled elite apnea divers to apply a translational approach from animal models of hypoxia to diving humans and back.

Methods

Human Study

Forty-four Caucasian males were involved in this study. For each subject, we collected age, height, weight, and endogenous ouabain plasma concentration. Among the participants, 18 were elite apnea divers—seven competitive divers, including two world-renowned record-holders, and 11 noncompetitive divers (Table 1). The eligibility of elite apnea divers was assessed by a specific medical questionnaire for selecting the best performers (this questionnaire is available in the "Supplementary Material" section of the *Aquatic Mammals* website: https://www.aquaticmammalsjournal.org/index.php?option=com_content&view=article&id=10&Itemid=147). Among the competitive divers, five belonged to the Italian National Apnea Team of the Federazione Italiana Pesca Sportiva e Attività Subacquee (Italian Federation of Sports Fishing and Underwater Activities). They represented 55.55% of the total competitive divers potentially available from the Apnea Team, while 11 noncompetitive divers belonged to three Italian associations (Apnea Free, Water Instinct, and Free Divers Italia) based in Rome. They had never participated in international games, but their performances were comparable to those of the Apnea Team divers. Indeed, they were all able to perform a static breath-hold higher than 4 min, a constant weight breath-hold of 30 m, and a dynamic breath-hold greater than 75 m. In addition, Apnea Team divers and two world-renowned record-holders showed even

Table 1. Variables collected in competitive elite apnea divers (EAD), noncompetitive elite apnea divers (EADn), and control subjects (CTRL). H = height, W = weight, EO = endogenous ouabain, and pM = picomolar.

n	Code	Age (y)	H (m)	W (kg)	EO (pM)	*n*	Code	Age (y)	H (m)	W (kg)	EO (pM)
1	EAD01	49	1.92	88	275	1	CTRL1828	51	1.82	77	233
2	EAD02	49	1.83	79	192	2	CTRL1840	35	1.93	102	174
3	EAD03	39	1.73	63	213	3	CTRL1942	26	1.75	80	299
4	EAD04	43	1.78	74	147	4	CTRL3087	42	1.75	81	91
5	EAD05	39	1.78	72	165	5	CTRL3103	48	1.70	69	85
6	EAD06	44	1.75	80	185	6	CTRL3105	45	1.75	80	81
7	EAD07	46	1.77	76	168	7	CTRL3109	58	1.68	73	123
8	EADn11	41	1.80	77	194	8	CTRL3111	49	1.78	82	202
9	EADn12	41	1.78	68	228	9	CTRL3112	28	1.75	80	73
10	EADn13	41	1.70	55	181	10	CTRL3151	48	1.83	81	207
11	EADn14	32	1.90	93	245	11	CTRL3152	29	1.74	71	235
12	EADn15	58	1.80	76	234	12	CTRL3176	41	1.76	80	113
13	EADn16	33	1.70	67	264	13	CTRL3191	42	1.68	73	148
14	EADn17	41	1.74	80	245	14	CTRL3201	40	1.84	92	129
15	EADn18	32	1.80	73	233	15	CTRL3210	40	1.75	92	179
16	EADn19	42	1.74	70	232	16	CTRL3218	36	1.77	75	226
17	EADn20	46	1.84	94	245	17	CTRL3281	48	1.79	70	87
18	EADn21	47	1.80	75	241	18	CTRL3552	34	1.78	76	68
						19	CTRL3585	50	1.82	82	185
						20	CTRL3589	56	1.78	93	132
						21	CTRL3659	46	1.78	78	210
						22	CTRL3666	34	1.74	74	215
						23	CTRL3670	52	1.71	63	180
						24	CTRL3707	54	1.91	96	237
						25	CTRL3709	47	1.80	80	185
						26	CTRL3753	32	1.75	85	198

greater performances, including static breath-hold > 7 min, 85 to 100 m constant weight breath-hold, and a dynamic breath-hold of > 230 m. Finally, 26 healthy control subjects were recruited from the clinic database of the Genomics of Renal Diseases and Hypertension Laboratory of University "Vita-Salute" San Raffaele of Milan, Italy. They were matched to apnea divers by gender, age, and ethnicity. Other inclusion criteria were as follows: no diving experience or participation in any other sports activity involving breath-holding, nonsmokers, nonsnorers, non-obstructive sleep apnea sufferers, and not being under specific therapies. Human study was approved by the Ethics Committee of Sapienza University (Policlinico Umberto I) in Rome, Italy (Ref. No. 4468; Protocol No. 303/17). Each participant provided written informed consent before being enrolled in the study. This study adhered to the Declaration of Helsinki and the International Conference on Harmonisation Good Clinical Practice (ICH GCP) guidelines and was conducted in compliance with the protocol, data protection regulations, and all other regulatory requirements, as appropriate.

Animal Study

Thirty-one marine animals were involved in the study (Table 2). Specifically, we sampled 16 common bottlenose dolphins (hereafter "dolphins"); one common seal (*Phoca vitulina*; Linnaeus, 1758), one gray seal (*Halichoerus grypus*; Fabricius, 1791), one South African fur seal (*Arctocephalus pusillus pusillus*; Schreber, 1775), and one California sea lion (*Zalophus californianus*; Lesson, 1828) (hereafter "pinnipeds"); and 11 loggerhead sea turtles (*Caretta caretta*; Linnaeus, 1758) (hereafter "sea turtles"). Marine mammals were born under human care or kept for many years in three Italian marine mammal facilities (Zoomarine Italia, Oltremare, and Acquario di Genova). They were trained to participate voluntarily in blood sampling. The dolphins involved in this study represented over 64% of those potentially available and trained in voluntary blood sampling in the above facilities (Ceta-Base, https://www.cetabase.org) when the study was conducted. The sea turtles were housed in two Italian sea turtle rescue centers (Rescue Center "Luigi Cagnolaro" of Centro Studi Cetacei Onlus [nonprofit association; the first Italian National Stranding Network] in Pescara, Italy, and Rescue Center of Zoomarine Trust Onlus in Pomezia-Rome, Italy). All sea turtles were incidentally caught in fishing gear (bycatch). Nine were rescued in the Adriatic Sea, and two were rescued in the Tyrrhenian Sea (GeoCetus, https://www.geocetus.it).

Only one blood sample was collected from each animal, from July 2016 to June 2017, by expert veterinarians during regular health monitoring of the animals. No sampling was performed exclusively for the current study but was always part of a routine medical procedure required by the European/Italian laws and regulations (see below) aimed to guarantee the health and well-being of dolphins and sea turtles kept under human care.

As mentioned above, dolphins and pinnipeds were born in a controlled environment or had been kept under human care for decades. They were trained to voluntarily undergo medical procedures (e.g., blood sampling, ultrasound examination), thus avoiding or minimizing stress responses to restraint. It follows that the study was conducted in the absence of stress for the animals as proved also by their blood concentration of cortisol noticeably testing below the stressed condition range of 1.25 to 2.5 µg/dl (Selye, 1976; Cavigelli & Chaudhry, 2012). In dolphins, blood samples were collected from a superficial vein of tail flukes, a ridge along the dorsal or ventral surface (Pritchard et al., 1983; Geraci & Lounsbury, 2005). Regarding the Otariidae family, blood was collected from the interdigital venous sinus; while in the Phocidae family, in addition to this site, blood could be taken from the extradural vein of the epidural sinus (Geraci & Lounsbury, 2005). In

Table 2. Animal species sampled for the study. * = all dolphins were captive born with the exception of two males and three females that were wild born but hosted in facilities for many years, and ** = rescued animals. C = captive, W = wild, and sex ratio = male.female.indeterminate.

Family	Species	*n*	Birth type	Sex ratio
Delphinidae	Common bottlenose dolphin (*Tursiops truncatus*)	16*	C/W	8.8.0
Phocidae	Common seal (*Phoca vitulina*)	1	C	0.1.0
	Gray seal (*Halichoerus grypus*)	1		1.0.0
Otariidae	California sea lion (*Zalophus californianus*)	1		1.0.0
	South African fur seal (*Arctocephalus pusillus pusillus*)	1		1.0.0
Chelonidae	Loggerhead sea turtle (*Caretta caretta*)	11**	W	2.7.2
		31		13.16.2

sea turtles, blood samples were collected from the dorsal cervical sinus under manual restraint of the animals (Owens & Ruiz, 1980).

The following variables were measured: gender, age or physiological phase (juvenile, immature, adult), total length, and weight, as well as curved carapace lengths notch-to-tip (CCLn-t) in the sea turtles only (Bolten, 1999). The gender of sea turtles was determined based on sexual dimorphism of tail morphology (Casale et al., 2009); their real age was unknown.

The animals were kept under human care by observing the following guidelines: European Union (EU) Directive for Zoo Animals (Council Directive 1999/22/EU) and the Italian Zoo Regulation (Legislative Decree No. 73/2005); and following modifications of 28 May 2015 specifically related to *Tursiops truncatus* captive management, the Italian regulation for the keeping of common bottlenose dolphins in captivity (D.M. 469/2001), the European Association for Aquatic Mammals (EAAM) standards for facilities housing common bottlenose dolphins (www.eaam.org/housing_standards), the Italian guidelines for the management of stranded and rescued sea turtles (published by the Italian National Institute for Environmental Protection and Research [ISPRA] and adopted by the Ministry of Environment on a national level), and the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) regulations. The legal status of the animals was confirmed by CITES.

All protocols were approved by Stazione Zoologica "Anton Dohrn" of Naples (NA), Italy (ethical clearance by the Animal Welfare Committee, Protocol No. 3306/2020; Case 07/2020/ec AWB-SZN of 22 June 2020). All methods were carried out in accordance with relevant guidelines and regulations, and the study was carried out in compliance with the ARRIVE guidelines (https://arriveguidelines.org).

Blood Sampling

Both in humans and animals, blood sampling was carried out between 0900 and 1200 h by collecting 4 to 6 ml of venous blood into collection tubes containing the anticoagulant ethylenediaminetetraacetic acid (EDTA). Plasma was separated by centrifugation at 3,000 rpm for 15 min at room temperature and stored in cryovial at -80°C until use.

Endogenous Ouabain Quantification in Plasma

Plasma samples (2 ml) were incubated with methanol (1:1 vol, Merck KGaA; Merck, Kenilworth, NJ, USA) overnight on a rotating shaker; they were then centrifuged, and the supernatants were dried out under vacuum (Savant™ SpeedVac™ Concentration System; Thermo Fisher, Waltham, MA, USA). Dried samples were resuspended in bidistilled water–0.025% TFA (trifluoroacetic acid; Thermo Fisher), centrifuged, and the supernatants were passed by vacuum over prewashed C18 Mega Bond Elut columns (Agilent Technologies, Santa Clara, CA, USA). EO was eluted by the columns with acetonitrile 25% (Merck KGaA), and the eluate was dried under vacuum, reconstituted with RIA buffer (1% bovine serum albumin, 0.1% thimerosal, 0.5M sodium phosphate; Sigma-Aldrich/Merck; pH 7.4), and measured by a Scintillation Proximity Assay (SPA), using Yttrium Silicate (YSi) beads (Perkin Elmer, Hebron, KY, USA) conjugated with a secondary antibody. These beads contain an embedded scintillant that emits light when bounded with EO tritiated through the primary antibody. The advantages of this method are no separation steps and no use of liquid scintillant. Specifically, ouabain standard curve and plasma samples (50 µl) were incubated in a microplate with 50 µl of goat anti-rabbit YSi beads diluted in RIA buffer (the solution has to be stirred during manipulation to maintain beads in suspension), 50 µl 3H-ouabain (2nM final concentration; Perkin Elmer), and 50 µl of a rabbit polyclonal antiouabain antiserum (1:2,000 final dilution; DBA Italia, Segrate, Italy). The microplate was shaken at 1,000 rpm on a horizontal shaker at 4°C for 18 h. At the end of the incubation, the plate was counted on a Microbeta counter (Perkin Elmer) with the specific program for SPA beads, 1 min/well. EO plasma concentrations were calculated as percent displacement of the control sample, which was carried out in the absence of ouabain and EO. EO plasma concentrations were expressed in picomolar (pM) ouabain equivalent derived from the ouabain standard curve. Two samples derived from a pool of plasma were added to each assay as quality control (intra and inter-assay variability < 10%).

Statistical Analysis

R, Version 3.2.1 (R Core Team, 2014; https://www.r-project.org), and code programmed to specific analyses and methodological approaches were used for data analysis. Descriptive data were reported in jitter plots that show the median (thick line), the limits of the 95% confidence interval (CI; thin line), the minimum and maximum values (short horizontal lines), and each individual value (small spots). Deviation from a normal data distribution of each variable was assessed by the Shapiro-Wilk test of normality. Each one of the variables collected both in humans and animals showed a normal distribution ($p < 0.05$). Using the bivariate analysis, all data were studied looking for linear correlations between EO values and the other variables. In the case of high data dispersion, comparisons were carried out with the data divided into classes and with the analysis based

Table 3. Descriptive data concerning the variables collected in elite apnea divers (EAD), noncompetitive elite apnea divers (EADn), and control subjects (CTRL). H = height, W = weight, EO = endogenous ouabain, pM = picomolar, and SD = standard deviation.

	EAD (n = 7)				EADn (n = 11)			
	Age (y)	H (cm)	W (kg)	EO (pM)	Age (y)	H (cm)	W (kg)	EO (pM)
Min	39.00	173.00	63.00	147.00	32.00	170.00	55.00	181.00
Max	49.00	192.00	88.00	275.00	58.00	190.00	94.00	264.00
Mean	44.10	179.40	76.00	192.00	41.20	178.00	75.20	231.00
SD	4.18	6.34	7.72	42.24	7.59	5.96	11.21	23.80
	CTRL (n = 26)							
Min	26.00	168.00	69.00	73.00				
Max	58.00	193.00	102.00	299.00				
Mean	42.70	177.00	80.00	165.20				
SD	8.78	5.96	8.96	62.11				

on the aggregate corresponding to class means. The degree of correlation was assessed by both Pearson's coefficient ($\boldsymbol{\rho}$) and the coefficient per ranks of Spearman ($\boldsymbol{\rho}_s$) accordingly.

In human and animal subjects, data were studied looking for linear correlations between EO plasma concentrations and biometric data such as age, total length or height, and weight. As already mentioned, in the case of high data dispersion, comparisons were carried out with the data divided into classes: six weight classes (e.g., 124.5 to 145.5 kg, 145.6 to 166.6 kg) for dolphins, four classes of height (e.g., 170 to 174 cm, 175 to 179 cm; Istituto Nazionale di Statistica [Italian National Institute of Statistics], https://www.istat.it/en), and five (e.g., 55 to 63 kg, 64 to 72 kg) and four (e.g., 63 to 72 kg, 73 to 82 kg) classes of weight for elite apnea divers and controls, respectively.

Comparisons of EO plasma concentrations between groups were performed by the analysis of covariance (ANCOVA) to correct for confounding biometric data according to the previously performed linear correlation analyses. Moreover, concerning diving animals, as the variable gender was not equally distributed within and among marine animal categories, it was used as a covariate in the ANCOVA. *Post hoc* analyses were corrected by Bonferroni method to contain type I error.

Results

Human Study

The biometric variables and endogenous ouabain plasma concentration collected for each subject are reported in Table 1, while descriptive data (e.g., min, max, mean, standard deviation) concerning all variables are reported in Table 3.

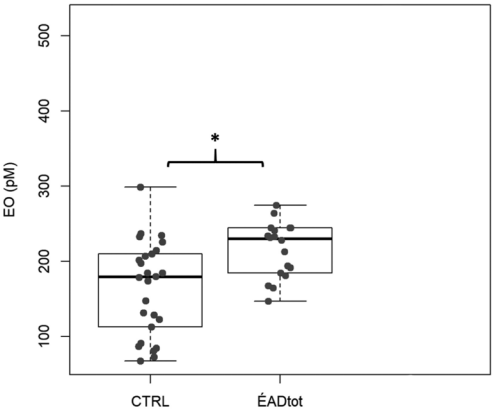

Figure 1. Jitter plots of endogenous ouabain (EO) plasma concentrations in 26 control subjects (CTRL) and 18 elite apnea divers (competitive and noncompetitive elite apnea divers grouped together [total], EADtot). EO is significantly higher in EADtot (*p = 0.034) as compared to CTRL. Bars represent means ± standard deviation (SD); pM = picomolar.

Competitive and noncompetitive elite apnea divers were grouped together as they did not differ in EO plasma concentrations and biometric variables ($p > 0.05$). In elite apnea divers, EO plasma concentrations were positively correlated to weight ($p < 0.05$) as well as in control subjects ($\boldsymbol{\rho} = 0.96, p < 0.05$). Furthermore, in controls, a trend of correlation with height ($\boldsymbol{\rho} = 0.82, p = 0.09$) was observed. Therefore, all the following analyses were corrected by these two variables.

The comparison of EO plasma concentrations between controls and elite apnea divers showed a

Table 4. Variables collected in common bottlenose dolphins (DOL), common seal (CMS), gray seal (GRS), South African fur seal (SFS), California sea lion (CSL), and loggerhead sea turtles (LST). TL = total length, W = weight, CCLn-t = curved carapace length notch-to-tip, EO = endogenous ouabain, pM = picomolar, M = male, F = female, I = indeterminate, and NA = data not available.

n	Code	Sex	Age (y)	TL (m)	W (kg)	CCLn-t (cm)	EO (pM)
1	DOL01	M	24	262.0	251.3	--	150
2	DOL02	M	18	239.0	174.4	--	103
3	DOL03	M	17	254.0	202.6	--	80
4	DOL04	M	6	229.0	152.4	--	60
5	DOL05	F	14	234.0	150.7	--	117
6	DOL06	F	14	238.0	222.1	--	52
7	DOL07	F	4	210.0	124.5	--	58
8	DOL08	M	24	247.0	200.0	--	208
9	DOL09	M	31	255.0	204.0	--	184
10	DOL10	F	38	259.0	214.0	--	140
11	DOL11	F	53	252.0	220.0	--	171
12	DOL12	F	20	283.0	198.0	--	204
13	DOL13	M	34	NA	215.0	--	269
14	DOL14	M	20	NA	225.0	--	253
15	DOL15	F	35	NA	214.0	--	155
16	DOL16	F	16	NA	158.0	--	169
17	CMS01	F	23	79.5	NA	--	174
18	GRS01	M	23	202.4	215.0	--	144
19	SFS01	M	18	96.5	165.0	--	183
20	CSL01	M	6	121.2	NA	--	211
21	LST59	M	--	78	23.9	57.5	137
22	LST81	F	--	85	34.0	67.0	66
23	LST84	F	--	93	33.8	66.0	98
24	LST85	F	--	86	29.1	61.5	34
25	LST86	F	--	99	46.6	72.0	109
26	LST87	F	--	105	54.8	75.0	71
27	LST88	M	--	83	31.7	66.0	94
28	LST89	F	--	92	42.6	71.0	87
29	LST91	I	--	81	27.4	61.5	110
30	LST01	F	--	65	16.2	52.5	178
31	LST02	I	--	50.5	8.6	41.5	164

statistically significant category effect (ANCOVA: $F(1,48) = 22.770$, $p < 10^{-6}$; Figure 1). *Post hoc* analysis revealed that EO plasma concentrations were significantly higher in elite apnea divers ($p = 0.034$) as compared to controls.

Animal Study

The biometric variables and EO plasma concentration collected for each specimen are reported in Table 4, while descriptive data concerning all variables are reported in Table 5.

EO plasma concentrations in dolphins were positively correlated to age ($\rho_s = 0.63$, $p < 0.05$), total length ($\rho_s = 0.66$, $p < 0.05$), and weight ($\rho = 0.84$, $p < 0.05$). Therefore, all the following analyses were corrected by these three variables. EO plasma concentrations in dolphins did not differ between genders ($F(1,11) = 0.597$, $p = 0.465$); and in phocids (true seals) and otariids (sea lions and fur seals), correlation analysis was not performed due to the small number of animals belonging to the four species ($n = 1$ for each).

Table 5. Descriptive data concerning the variables collected in common bottlenose dolphins (DOLs) and loggerhead sea turtles (LSTs). In pinnipeds, correlation analysis was not performed due to the small number of animals. TL = total length, W = weight, CCLn-t = curved carapace length notch-to-tip, EO = endogenous ouabain, pM = picomolar, and SD = standard deviation.

	Age (y)	TL (cm)	W (kg)	CCLn-t (cm)	EO (pM)
			DOLs (n = 16)		
Min	4.0	210.0	124.5	--	52.0
Max	53	283.0	251.3	--	269.0
Mean	23.0	246.8	195.4	--	148.3
SD	12.6	18.6	33.9	--	67.0
			LSTs (n = 11)		
Min		50.5	8.6	41.5	34.0
Max		105.0	54.8	75.0	178.0
Mean		83.4	31.7	62.9	104.3
SD		15.3	13.2	9.6	42.5

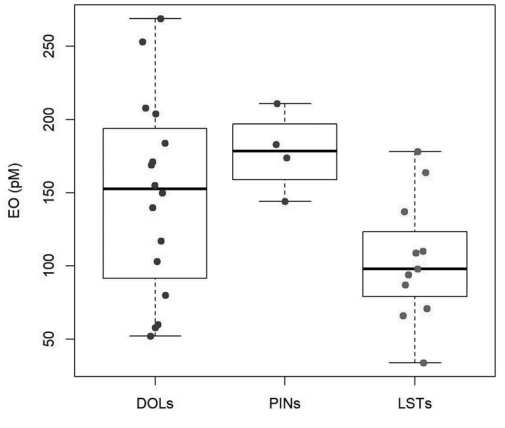

Figure 2. Jitter plots of EO plasma concentrations in 16 common bottlenose dolphins (DOLs); one common seal, one gray seal, one California sea lion, and one South African fur seal (PINs); and 11 loggerhead sea turtles (LSTs). Bars represent means ± standard deviation (SD); pM = picomolar.

For sea turtles, only total length was used for the analysis because this variable and CCLn-t were highly correlated (ρ = 0.97). In these reptiles, EO plasma concentrations were negatively correlated to total length (ρ_s = -0.65, $p < 0.05$) and weight (ρ_s = -0.66, $p < 0.05$). Therefore, all the following analyses were corrected by these two variables. EO plasma concentrations in sea turtles did not differ between genders ($F(1,8) = 0.044$, $p = 0.843$), and the comparison of EO plasma concentrations between dolphins and sea turtles did not show any significant difference ($p > 0.05$; Figure 2).

Discussion

For the first time, endogenous ouabain plasma concentrations have been measured in different models of hypoxia: one human (elite apnea divers) and six animals (five marine mammal and one marine reptile species).

Concerning elite apnea divers, we enrolled 18 divers which represented a very good size for a particular sample like this. They were healthy subjects trained in breath-holding who routinely engaged in diving practices and were able to perform remarkable high-diving/apneas. The interesting finding was that elite apnea divers showed statistically significant higher EO plasma concentrations as compared to healthy subjects without any diving experience or experience in other sports activities involving breath-holding. The increasing gradient of EO plasma concentrations from control subjects to elite apnea divers could support the protective role of this glycoside, particularly cardio-protective, in the adaptive response to hypoxia.

In elite apnea divers and control subjects, EO was positively correlated to weight. Also, a trend of correlation with height was observed in the controls. Ukkola et al. (2001) observed correlations between cortisol, a steroid hormone as EO, and other similar variables (e.g., body mass index).

Regarding diving animals, this study demonstrated for the first time that EO is expressed by different taxa, phylogenetically distant from each other, which normally perform long apneas in their natural habitats without reporting neurological damages. In dolphins and sea turtles, EO was correlated to total length and weight even if

in opposite directions: positive correlation in dolphins and negative correlation in sea turtles. Also, EO plasma concentrations were not significantly different between marine mammals, although higher concentrations were observed in pinnipeds as compared to dolphins (Figure 2). Although living in a controlled environment does not require long apneas to forage or escape from a predator, in the wild, pinnipeds show better diving/apnea performances than dolphins. Dolphins usually dive for about 1 min in shallow depths with a maximal depth of 390 m for up to 8 min (Ponganis, 2011), while pinnipeds typically dive for about 4 min in shallow depths with the deepest dive to 268 to 500 m for up to 8 to 30 min depending on the species (Thompson & Fedak, 1993; Gjertz et al., 2000; Schreer et al., 2001; Eguchi & Harvey, 2005; Moore et al., 2009). However, the low number of pinnipeds and only one blood sample for each animal (dolphins and pinnipeds) did not allow the development of hypotheses on this difference.

In the case of wild sea turtles, it was not possible to predict when they would be rescued, so 11 specimens represent a significant number for the sampling/data collection period (July 2016 to June 2017). These animals had lower EO plasma concentrations as compared to dolphins and pinnipeds (Figure 2), even though they are known to spend most of their lives submerged except for brief lapses of time (Pritchard, 1971; Lutcavage & Lutz, 1997). However, the difference in EO plasma concentrations between sea turtles and marine mammals was not significant. In sea turtles, the role of EO might not be central for the adaptation to hypoxia, while other peculiar physiological adjustments not observed in the marine mammals might occur. For instance, sea turtles can maintain inalterable brain ATP levels for at least 2 h of total anoxia (Lutz et al., 1984), thanks to the suppression of the Na^+/K^+-ATPase pumps. Contrary to this, in mammals, depletion of ATP occurs within a few minutes (Lutz & Bentley, 1985; Hochachka, 1986). As the ability to save ATP consumption plays a central role in the brain's adaptation to hypoxia, in dolphins and pinnipeds, higher EO plasma concentrations might be explained by the fact that mitochondrial ATP consumption is reduced by EO via Na^+/K^+-ATPase pump inhibition.

The low number of pinnipeds, the absence of a control group, and, in both human and animal models, the lack of long-term monitoring (at least 24 to 48 h) of the EO plasma concentrations collected at different time points (T_0, T_1, . . . T_n) do not allow conclusions to be reached. Indeed, variations of EO plasma concentrations might be examined using at-sea analyses following dives by trained dolphins as studies of oxygen and other gas utilization dynamics were performed by the U.S. Navy in the past (Elsner et al., 1966). Such an experimental setting could be done in those facilities where the animals could be trained to stay on the pool bottom for a few minutes.

Another option could be collecting blood samples from live wild cetaceans and pinnipeds stranded along the Italian coast, but these events are extremely rare, especially for pinnipeds. The Mediterranean basin hosts only one pinniped species, the Mediterranean monk seal (*Monachus monachus*; Hermann, 1779), which is the most endangered seal species (Karamanlidis & Dendrinos, 2015). During the sampling/data collection period, only one live stranded cetacean has been recorded in Italy (Istituto Zooprofilattico Sperimentale del Piemonte Liguria e Valle d'Aosta–Centro di Referenza Nazionale per le Indagini Diagnostiche sui Mammiferi Marini Spiaggiati [IZSTO–C.Re.Di. Ma.], 2016, 2017). Blood samples collected from stranded specimens may not be reliable as these animals are, in most cases, not healthy, stressed, or remain alive for only a few hours (Petrella et al., 2021). However, new experimental techniques for investigating the unique complexity of biologic systems that results from the diversity of interactions and regulatory networks are now available. Indeed, it is possible nowadays to monitor thousands of molecules simultaneously and generate real-time pictures of any biological system in any condition thanks to the development of the "omics" technologies (Mancia, 2018).

For all mentioned reasons, our findings have been reached by analyzing peculiar samples, both human and animal, which are not easy to collect and represent a useful hint for future investigations on (1) other human models of hypoxia (e.g., free-diving fishermen, people living at very high altitudes or living with pulmonary pathologies); (2) the same species involved in this study but with a larger sample (captive cetaceans and pinnipeds, rescued sea turtles); and (3) other animal models of hypoxia—for example, freshwater tortoises or hippopotamus (*Hippopotamus amphibius*)—to try to identify novel molecules useful to treat very disabling pathological conditions such as idiopathic pulmonary arterial hypertension or obstructive sleep apnea. Finally, these results might represent a valid preliminary indication for approaching the biological mechanisms involved in cell protection from the damage by hypoxia.

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Literature Cited

Antolovic, R., Bauer, N., Mohadjerani, M., Kost, H., Neu, H., Kirch, U., Grünbaum, E. G., & Schoner, W. (2000). Endogenous ouabain and its binding globulin: Effects of physical exercise and study on the globulin's tissue distribution. *Hypertension Research*, *23*, S93-S98. https://doi.org/10.1291/hypres.23.Supplement_S93

Asher, R. J., & Helgen, K. M. (2010). Nomenclature and placental mammal phylogeny. *BMC Evolutionary Biology*, *10*, 102. https://doi.org/10.1186/1471-2148-10-102

Auer, R. N., & Sutherland, G. R. (2002). Hypoxia and related conditions. In D. I. Graham & P. L. Lantos (Eds.), *Greenfield's neuropathology* (pp. 233-280). Arnold.

Bignami, E., Casamassima, N., Frati, E., Lanzani, C., Corno, L., Alfieri, O., Gottlieb, S., Simonini, M., Shah, K. B., Mizzi, A., Messaggio, E., Zangrillo, A., Ferrandi, M., Ferrari, P., Bianchi, G., Hamlyn, J. M., & Manunta, P. (2013). Preoperative endogenous ouabain predicts acute kidney injury in cardiac surgery patients. *Critical Care Medicine*, *41*, 744-755. https://doi.org/10.1097/CCM.0b013e3182741599

Boero, J. A., Ascher, J., Arregui, A., Rovainen, C., & Woolsey, T. A. (1999). Increased brain capillaries in chronic hypoxia. *Journal of Applied Physiology*, *86*, 1211-1219. https://doi.org/10.1152/jappl.1999.86.4.1211

Bolten, A. B. (1999). Techniques for measuring sea turtles. In K. L. Eckert, K. A. Bjorndal, F. A. Abreu-Grobois, & M. Donnelly (Eds.), *Research and management techniques for the conservation of sea turtles* (Publication No. 4, pp. 110-114). IUCN/SSC Marine Turtle Specialist Group.

Butler, P. J. (2004). Metabolic regulation in diving birds and mammals. *Respiratory Physiology & Neurobiology*, *141*, 297-315. https://doi.org/10.1016/j.resp.2004.01.010

Butler, P. J., & Jones, D. R. (1997). Physiology of diving of birds and mammals. *Physiological Reviews*, *77*, 837-899. https://doi.org/10.1152/physrev.1997.77.3.837

Caine, D., & Watson, J. D. (2000). Neuropsychological and neuropathological sequelae of cerebral anoxia: A critical review. *Journal of the International Neuropsychological Society*, *6*, 86-99. https://doi.org/10.1017/S1355617700611116

Casale, P., d'Astore, P. P., & Argano, R. (2009). Age at size and growth rates of early juvenile loggerhead sea turtles (*Caretta caretta*) in the Mediterranean based on length frequency analysis. *Journal of Herpetology*, *19*, 29-33.

Cavigelli, S. A., & Chaudhry, H. S. (2012). Social status, glucocorticoids, immune function, and health: Can animal studies help us understand human socioeconomic-status-related health disparities? *Hormones and Behavior*, *62*, 295-313. https://doi.org/10.1016/j.yhbeh.2012.07.006

Costa, D. P., Kuhn, C. E., Weise, M. J., Shaffer, S. A., & Arnould, J. P. Y. (2004). When does physiology limit the foraging behavior of freely diving mammals? *International Congress Series*, *1275*, 359-366. https://doi.org/10.1016/j.ics.2004.08.058

Davenport, J., Fraher, J., Fitzgerald, E., McLaughlin, P., Doyle, T., Harman, L., Cuffe, T., & Dockery, P. (2009). Ontogenetic changes in tracheal structure facilitate deep dives and cold water foraging in adult leatherback sea turtles. *The Journal of Experimental Biology*, *212*, 3440-3447. https://doi.org/10.1242/jeb.034991

Di Bartolo, V., Balzan, S., Pieraccini, L., Ghione, S., Pegoraro, S., Biver, P., Revoltella, R., & Montali, U. (1995). Evidence for an endogenous ouabain-like immunoreactive factor in human newborn plasma coeluted with ouabain on HPLC. *Life Sciences*, *57*, 1417-1425. https://doi.org/10.1016/0024-3205(95)02104-Q

Dvela-Levitt, M., Ami, H. C., Rosen, H., Shohami, E., & Lichtstein, D. (2014). Ouabain improves functional recovery following traumatic brain injury. *Journal of*

Neurotrauma, *31*, 1942-1947. https://doi.org/10.1089/neu.2014.3544

Eguchi, T., & Harvey, J. T. (2005). Diving behavior of the Pacific harbor seal (*Phoca vitulina richardii*) in Monterey Bay, California. *Marine Mammal Science*, *21*, 283-295. https://doi.org/10.1111/j.1748-7692.2005.tb01228.x

Eisner, D. A., & Smith, T. W. (1992). The Na-K pump and its effectors in cardiac muscle. In H. A. Fozzard, E. Haber, R. B. Jennings, A. M. Katz, & H. E. Morgan (Eds.), *The heart and cardiovascular system* (pp. 863-902). Raven Press.

El-Masri, M. A., Clark, B. J., Qazzaz, H. M., & Valdes, R., Jr. (2002). Human adrenal cells in culture produce both ouabain-like and dihydroouabain-like factors. *Clinical Chemistry*, *48*(10), 1720-1730. https://doi.org/10.1093/clinchem/48.10.1720

Elsner, R., Kenney, D. W., & Burgess, K. (1966). Diving bradycardia in the trained dolphin. *Nature*, *212*(5060), 407-408. https://doi.org/10.1038/212407a0

Falkowska, A., Gutowska, I., Goschorska, M., Nowacki, P., Chlubek, D., & Baranowska-Bosiacka, I. (2015). Energy metabolism of the brain, including the cooperation between astrocytes and neurons, especially in the context of glycogen metabolism. *International Journal of Molecular Sciences*, *16*, 25959-25981. https://doi.org/10.3390/ijms161125939

Ferrandi, M., Minotti, E., Salardi, S., Florio, M., Bianchi, G., & Ferrari, P. (1993). Characteristics of an ouabain-like factor from Milan hypertensive rats. *Journal of Cardiovascular Pharmacology*, *22*, S75-S78. https://doi.org/10.1097/00005344-199322002-00024

Ferretti, G., & Costa, M. (2003). Diversity in an adaptation to breath-hold diving in humans. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology*, *136*, 205-213. https://doi.org/10.1016/S1095-6433(03)00134-X

Geraci, J. R., & Lounsbury, V. J. (2005). *Marine mammals ashore: A field guide for strandings* (2nd ed.). E. John Schmitz and Sons Inc.

Gjertz, I., Kovacs, K. M., Lydersen, C., & Wiig, Ø. (2000). Movements and diving of adult ringed seals (*Phoca hispida*) in Svalbard. *Polar Biology*, *23*, 651-656. https://doi.org/10.1007/s003000000143

Graur, D., & Higgins, D. G. (1994). Molecular evidence for the inclusion of cetaceans within the order Artiodactyla. *Molecular Biology and Evolution*, *11*, 357-364.

Hindell, M. A., Slip, D. J., Burton, H. R., & Bryden, M. M. (1992). Physiological implications of continuous, prolonged, and deep dives of southern elephant seals. *Canadian Journal of Zoology*, *70*, 370-379. https://doi.org/10.1139/z92-055

Hochachka, P. W. (1986). Defense strategies against hypoxia and hypothermia. *Science*, *231*, 234-241. https://doi.org/10.1126/science.2417316

Hochachka, P. W. (2000). Pinniped diving response mechanism and evolution: A window on the paradigm of comparative biochemistry and physiology. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology*, *126*, 435-458. https://doi.org/10.1016/S1095-6433(00)00231-2

Horner, P. J., & Gage, F. H. (2000). Regenerating the damaged central nervous system. *Nature*, *407*, 963-970. https://doi.org/10.1038/35039559

Iatrino, R., Lanzani, C., Bignami, E., Casamassima, N., Citterio, L., Meroni, R., Zagato, L., Zangrillo, A., Alfieri, O., Fontana, S., Macrina, L., Delli Carpini, S., Messaggio, E., Brioni, E., Dell'Antonio, G., Manunta, P., Hamlyn, J. M., & Simonini, M. (2018). Lanosterol synthase genetic variants, endogenous ouabain, and both acute and chronic kidney injury. *American Journal of Kidney Diseases*, *73*, 504-512. https://doi.org/10.1053/j.ajkd.2018.10.012

Irwin, D. M., Kocher, T. D., & Wilson, A. C. (1991). Evolution of the cytochrome b gene of mammals. *Journal of Molecular Evolution*, *32*, 128-144. https://doi.org/10.1007/BF02515385

Istituto Zooprofilattico Sperimentale del Piemonte Liguria e Valle d'Aosta (IZSTO)–Centro di Referenza Nazionale per le Indagini Diagnostiche sui Mammiferi marini spiaggiati (C.Re.Di.Ma.). (2016). *Rilievi diagnostici post mortem nei cetacei spiaggiati in Italia – Report* [Post mortem diagnostic findings in stranded cetaceans in Italy – Report]. IZSTO–C.Re.Di.Ma.

IZSTO–C.Re.Di.Ma. (2017). *Rilievi diagnostici post mortem nei cetacei spiaggiati in Italia – Report* [Post mortem diagnostic findings in stranded cetaceans in Italy – Report]. IZSTO–C.Re.Di.Ma.

Karamanlidis, A., & Dendrinos, P. (2015). *Monachus monachus* (errata version published in 2017). In International Union for Conservation of Nature (Ed.), *The IUCN red list of threatened species 2015: e.T13653A117647375*. IUCN.

Kawamura, A., Guo, J., Itagaki, Y., Bell, C., Wang, Y., Haupert, G. T., Jr., Magil, S., Gallagher, R. T., Berova, N., & Nakanishi, K. (1999). On the structure of endogenous ouabain. *Proceedings of the National Academy of Sciences of the United States of America*, *96*, 6654-6659. https://doi.org/10.1073/pnas.96.12.6654

Komiyama, Y., Nishimura, N., Munakata, M., Mori, T., Okuda, K., Nishino, N., Hirose, S., Kosaka, C., Masuda, M., & Takahashi, H. (2001). Identification of endogenous ouabain in the culture supernatant of PC12 cells. *Journal of Hypertension*, *19*, 229-236. https://doi.org/10.1097/00004872-200102000-00009

LaManna, C., Vendel, L. M., & Farrell, R. M. (1992). Brain adaptation to chronic hypobaric hypoxia in rats. *Journal of Applied Physiology*, *72*, 2238-2243. https://doi.org/10.1152/jappl.1992.72.6.2238

Laredo, J., Hamilton, B. P., & Hamlyn, J. M. (1994). Ouabain is secreted by bovine adrenocortical cells. *Endocrinology*, *135*, 794-797. https://doi.org/10.1210/endo.135.2.8033829

Li, J., Khodus, G. R., Kruusmägi, M., Kamali-Zare, P., Liu, X. L., Eklöf, A. C., Zelenin, S., Brismar, H., & Aperia, A. (2010). Ouabain protects against adverse developmental programming of the kidney. *Nature Communications*, *27*, 1-42. https://doi.org/10.1038/ncomms1043

Lindholm, P., Sundblad, P., & Linnarsson, D. (1999). Oxygen-conserving effects of apnea in exercising men. *Journal of Applied Physiology*, *87*, 2122-2127. https://doi.org/10.1152/jappl.1999.87.6.2122

Loenarz, C., Coleman, M. L., Boleininger, A., Schierwater, B., Holland, P. W., Ratcliffe, P. J., & Schofield, C. J. (2011). The hypoxia-inducible transcription factor pathway regulates oxygen sensing in the simplest animal, *Trichoplax adhaerens*. *EMBO Reports*, *12*, 63-70. https://doi.org/10.1038/embor.2010.170

López-Mendilaharsu, M., Rocha, C. F. D., Domingo, A., Wallace, B. P., & Miller, P. (2009). Prolonged deep dives by the leatherback turtle *Dermochelys coriacea*: Pushing their aerobic dive limits. *Marine Biodiversity Records*, *35*, 1-3. https://doi.org/10.1017/S1755267208000390

Lutcavage, M. E., & Lutz, P. L. (1997). Diving physiology. In P. L. Lutz & J. A. Musick (Eds.), *The biology of sea turtles* (pp. 276-296). CRC Press.

Lutz, P. L., & Bentley, T. B. (1985). Respiratory physiology of diving in the sea turtle. *American Society of Ichthyologists and Herpetologists*, *3*, 671-679. https://doi.org/10.2307/1444761

Lutz, P. L., McMahon, P., Rosenthal, M., & Sick, T. J. (1984). Relationships between aerobic and anaerobic energy production in turtle brain in situ. *American Journal of Physiology*, *247*, 740-744. https://doi.org/10.1152/ajpregu.1984.247.4.R740

Mancia, A. (2018). New technologies for monitoring marine mammal health. In M. C. Fossi & C. Panti (Eds.), *Marine mammal ecotoxicology: Impacts of multiple stressors on population health* (pp. 291-320). Academic Press. https://doi.org/10.1016/B978-0-12-812144-3.00011-5

Meir, J. U., & Ponganis, P. J. (2009). High-affinity hemoglobin and blood oxygen saturation in diving emperor penguins. *The Journal of Experimental Biology*, *212*, 3330-3338. https://doi.org/10.1242/jeb.033761

Mitz, S. A., Reuss, S., Folkow, L. P., Blix, A. S., Ramirez, J-M., Hankeln, T., & Burmester, T. (2009). When the brain goes diving: Glial oxidative metabolism may confer hypoxia tolerance to the seal brain. *Neuroscience*, *163*, 552-560. https://doi.org/10.1016/j.neuroscience.2009.06.058

Moore, M. J., Bogomolni, A. L., Dennison, S. E., Early, G., Garner, M. M., Hayward, B. A., Lentell, J., & Rotstein, D. S. (2009). Gas bubbles in seals, dolphins, and porpoises entangled and drowned at depth in gillnets. *Veterinary Pathology*, *46*, 536-547. https://doi.org/10.1354/vp.08-VP-0065-M-FL

Owens, D. W., & Ruiz, G. J. (1980). New methods of obtaining blood and cerebrospinal fluid from marine turtles. *Herpetologica*, *36*, 17.

Paci, A., Marrone, O., Lenzi, S., Prontera, C., Nicolini, G., Ciabatti, G., Ghione, S., & Bonsignore, G. (2000). Endogenous gigitalislike factors in obstructive sleep apnea. *Hypertension Research*, *23*, S87-S91. https://doi.org/10.1291/hypres.23.Supplement_S87

Petrella, A., Mazzariol, S., Padalino, I., Di Francesco, G., Casalone, C., Grattarola, C., Di Guardo, G., Smoglica, C., Centelleghe, C., & Gili, C. (2021). Cetacean morbillivirus and *Toxoplasma gondii* co-infection in Mediterranean monk seal pup, Italy. *Emerging Infectious Diseases*, *27*(4), 1237-1239. https://doi.org/10.3201/eid2704.204131

Pierre, S. V., Yang, C., Yuan, Z., Seminerio, J., Mouas, C., Garlid, K. D., Dos-Santos, P., & Xie, Z. (2007). Ouabain triggers preconditioning through activation of the Na^+, K^+-ATPase signaling cascade in rat hearts. *Cardiovascular Research*, *73*, 488-496. https://doi.org/10.1016/j.cardiores.2006.11.003

Ponganis, P. J. (2011). Diving mammals. *Comprehensive Physiology*, *1*, 447-465. https://doi.org/10.1002/cphy.c091003

Ponganis, P. J. (2015). *Diving physiology of marine mammals and seabirds*. Cambridge University Press. https://doi.org/10.1017/CBO9781139045490

Pritchard, P. C. H. (1971). The leatherback or leathery turtle. *IUCN Monograph*, *1*, 1-39.

Pritchard, P., Bacon, P., Berry, F., Carr, A., Fletemeyer, J., Gallagher, R., Hopkins, S., Lankford, R., Marquez, R., Ogren, L., Pringle, W., Jr., Reichart, H., & Witham, R. (1983). *Manual of sea turtle research and conservation techniques* (2nd ed.; K. A. Bjorndal & G. H. Balazs [Eds.]). Center for Environmental Education.

Qvist, J., Hill, R. D., Schneider, R. C., Falke, K. J., Liggins, G. C., Guppy, M., Elliot, R. L., Hochachka, P. W., & Zapol, W. M. (1986). Hemoglobin concentrations and blood gas tensions of free-diving Weddell seals. *Journal of Applied Physiology*, *61*, 1560-1569. https://doi.org/10.1152/jappl.1986.61.4.1560

R Core Team. (2014). *A language and environment for statistical computing*. R Foundation for Statistical Computing.

Ramirez, J. M., Folkow, L. P., & Blix, A. S. (2007). Hypoxia tolerance in mammals and birds: From the wilderness to the clinic. *Annual Review of Physiology*, *69*, 113-143. https://doi.org/10.1146/annurev.physiol.69.031905.163111

Rebel, T. P. (1974). *Sea turtles and the turtle industry of the West Indies, Florida and the Gulf of Mexico*. University of Miami Press. https://doi.org/10.2307/1442710

Ridgway, L., & McFarland, K. (2006). Apnea diving: Long-term neurocognitive sequelae of repeated hypoxemia. *The Clinical Neuropsychologist*, *20*, 160-176. https://doi.org/10.1080/13854040590947407

Rourke, S. B., & Adams, K. M. (1996). The neuropsychological correlates of acute and chronic hypoxemia. In I. Grant & K. M. Adams (Eds.), *Neuropsychological assessment of neuropsychiatric disorders* (pp. 379-402). Oxford University Press.

Schneider, R., Wray, V., Nimtz, M., Lehmann, W. D., Kirch, U., Antolovic, R., & Schoner, W. (1998). Bovine adrenals contain, in addition to ouabain, a second inhibitor of the sodium pump. *Journal of Biological Chemistry*, *273*, 784-792. https://doi.org/10.1074/jbc.273.2.784

Schoner, W., & Scheiner-Bobis, G. (2007). Endogenous and exogenous cardiac glycosides: Their roles in hypertension, salt metabolism, and cell growth. *American Journal of Physiology–Cell Physiology*, *293*, 2. https://doi.org/10.1152/ajpcell.00098.2007

Schorr, G. S., Falcone, E. A., Moretti, D. J., & Andrews, R. D. (2014). First long-term behavioral records from Cuvier's beaked whales (*Ziphius cavirostris*) reveal record-breaking dives. *PLOS ONE*, *9*(3), e92633. https://doi.org/10.1371/journal.pone.0092633

Schreer, J. S., Kovacs, K. M., & O'Hara Hines, R. J. (2001). Comparative diving patterns of pinnipeds and seabirds. *Ecological Monographs*, *71*, 137-162. https://doi.org/10.1890/0012-9615(2001)071[0137:CDPOPA]2.0.CO;2

Selye, H. (1976). *The stress of life*. McGraw-Hill Book Company.

Simonini, M., Pozzoli, S., Bignami, E., Casamassima, N., Messaggio, E., Lanzani, C., Frati, E., Botticelli, I. M., Rotatori, F., Alfieri, O., Zangrillo, A., & Manunta, P. (2015). Endogenous ouabain: An old cardiotonic steroid as a new biomarker of heart failure and a predictor of mortality after cardiac surgery. *BioMed Research International*, *2015*, 714793. https://doi.org/10.1155/2015/714793

Simonini, M., Lanzani, C., Bignami, E., Casamassima, N., Frati, E., Meroni, R., Messaggio, E., Alfieri, O., Hamlyn, J., Body, S. C., Collard, C. D., Zangrillo, A., & Manunta, P. (2014). A new clinical multivariable model that predicts postoperative acute kidney injury: Impact of endogenous ouabain. *Nephrology Dialysis Transplantation*, *29*, 1696-1701. https://doi.org/10.1093/ndt/gfu200

Sokoloff, L. (1976). Circulation and energy metabolism of the brain. In G. J. Siegel, R. W. Albers, R. Katzman, & B. W. Agranoff (Eds.), *Basic neurochemistry* (2nd ed., pp. 388-413). Little Brown & Co.

Thompson, D., & Fedak, M. A. (1993). Cardiac responses of grey seals during diving at sea. *The Journal of Experimental Biology*, *174*, 139-164. https://doi.org/10.1242/jeb.174.1.139

Tymiak, A. A., Norman, J. A., Bolgar, M., Di Donato, G. C., Lee, H., Parker, W. L., Lo, L. C., Berova, N., Nakanishi, K., Haber, E., & Haupert, G. T., Jr. (1993). Physicochemical characterization of an ouabain isomer isolated from bovine hypothalamus. *Proceedings of the National Academy of Sciences of the United States of America*, *90*, 8189-8193. https://doi.org/10.1073/pnas.90.17.8189

Ukkola, O., Gagnon, J., Rankinen, T., Thompson, P. A., Hong, Y., Leon, A. S., Rao, D. C., Skinner, J. S., Wilmore, J. H., & Bouchard, C. (2001). Age, body mass index, race and other determinants of steroid hormone variability: The HERITAGE family study. *European Journal of Endocrinology*, *145*, 1-9. https://doi.org/10.1530/eje.0.1450001

Williams, T. M., Zavanelli, M., Miller, M. A., Goldbeck, R. A., Morledge, M., Casper, D., Pabst, D. A., McLellan, W., Cantin, L. P., & Kliger, D. S. (2007). Running, swimming and diving modifies neuroprotecting globins in the mammalian brain. *Proceedings of the Royal Society B: Biological Sciences*, *275*, 751-758. https://doi.org/10.1098/rspb.2007.1484

Wyneken, J. (2007). The structure of cardiopulmonary systems of turtles: Implications for behavior and function. In J. Wyneken, V. L. Bels, & M. H. Godfrey (Eds.), *Biology of turtles* (pp. 213-223). CRC Press.

Yalaz, M., Levent, E., Olukman, M., Calkavur, S., Akisu, M., & Kultursay, N. (2013). Role of digoxin-like immunoreactive substance in the pathogenesis of transient tachypnea of the newborn. *BioMed Research International*, *2013*, 704763. https://doi.org/10.1155/2013/704763