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RESEARCH ARTICLE

Deciphering function of the pulmonary arterial sphincters in loggerhead sea turtles (*Caretta caretta*)

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ABSTRACT

To provide new insight into the pathophysiological mechanisms underlying gas emboli (GE) in bycaught loggerhead sea turtles (*Caretta caretta*), we investigated the vasoactive characteristics of the pulmonary and systemic arteries, and the lung parenchyma (LP). Tissues were opportunistically excised from recently dead animals for *in vitro* studies of vasoactive responses to four different neurotransmitters: acetylcholine (ACh; parasympathetic), serotonin (5HT), adrenaline (Adr; sympathetic) and histamine. The significant amount of smooth muscle in the LP contracted in response to ACh, Adr and histamine. The intrapulmonary and systemic arteries contracted under both parasympathetic and sympathetic stimulation and when exposed to 5HT. However, proximal extrapulmonary arterial (PEPA) sections contracted in response to ACh and 5HT, whereas Adr caused relaxation. In sea turtles, the relaxation in the pulmonary artery was particularly pronounced at the level of the pulmonary artery sphincter (PASp), where the vessel wall was highly muscular. For comparison, we also studied tissue response in freshwater sliders turtles (*Trachemys scripta elegans*). Both PEPA and LP from freshwater sliders contracted in response to 5HT, ACh and also Adr. We propose that in sea turtles, the dive response (parasympathetic tone) constricts the PEPA, LP and PASp, causing a pulmonary shunt and limiting gas uptake at depth, which reduces the risk of GE during long and deep dives. Elevated sympathetic tone caused by forced submersion during entanglement with fishing gear increases the pulmonary blood flow causing an increase in N_2 uptake, potentially leading to the formation of blood and tissue GE at the surface. These findings provide potential physiological and anatomical explanations on how these animals have evolved a cardiac shunt pattern that regulates gas exchange during deep and prolonged diving.

KEY WORDS: Blood flow, Decompression sickness, Diving physiology, Pulmonary blood flow, Stress

INTRODUCTION

Marine vertebrates have developed a variety of mechanisms to avoid the many potential problems of diving, such as atelectasis, transient hyperoxia, extreme hypoxia, ischemia/reperfusion, hyper- and hypotension, acid–base balance disturbances, intravascular gas bubbles and inert gas narcosis (Butler and Jones, 1997; Lutz and Bentley, 1985; Ponganis, 2015). Most research on diving vertebrates has been devoted to understanding the management of O_2 stores, but some studies have investigated the physiological traits that prevent, or at least alleviate, the pressure-related problems, such as pulmonary barotrauma, N_2 narcosis and 'the bends' (Berkson, 1967; Bert, 1870; Fahlman et al., 2017b; García-Párraga et al., 2014; Hooker et al., 2012; Moore et al., 2011; Scholander, 1940; García-Párraga et al., 2018).

Decompression sickness (DCS) is a collection of pressure related-symptoms that, by definition, reverse upon recompression treatment (Mahon and Regis, 2014). In scuba divers, continuous breathing of pressurized air results in accumulation of N_2 in the blood and tissues. As the ambient pressure is reduced upon ascent, the high levels of N_2 in the blood and tissues begin to come out of solution, leading to supersaturation in the blood. If the ascent is too rapid, the supersaturation may cause bubbles to form in the blood and tissues (Fahlman, 2017). In breath-hold diving vertebrates, N_2 uptake and removal through the lung is more complex, as increased ambient pressure causes a pulmonary shunt that correlates with dive depth as the pressure compresses the gas exchange surface (Berkson, 1967; Fahlman et al., 2017b; Kooyman and Sinnett, 1982).

A recent study revealed that bycaught turtles also experience gas emboli (GE) after entrapment at depth in fishing gear (García-Párraga et al., 2014). Diagnostic imaging showed widespread GE in the vasculature and tissues (including kidney, heart, liver and spinal cord) upon surfacing. Hyperbaric O_2 treatment alleviated the GE and observable symptoms, and these clinical findings are therefore consistent with those described in human DCS cases (Mahon and Regis, 2014). These observations are significant for sea turtle conservation as fishery bycatch is recognized as the greatest threat for most sea turtles worldwide (Lewison et al., 2013; Wallace et al., 2011). Additionally, considering that DCS probably contributes to post-release mortality, undetected cases following release from the fishing gears may lead to significant underestimates of the numbers of turtles killed by fisheries (Fahlman et al., 2017a; García-Párraga et al., 2014).

Although it is important to understand how passive lung compression alters pulmonary shunt (Fahlman et al., 2009), marine vertebrates may have developed additional mechanisms to limit N_2 uptake. In the loggerhead (*Caretta caretta*), green (*Chelonia mydas*), hawksbill (*Eretmochelys imbricata*) and leatherback (*Dermochelys coriacea*) sea turtles, a large muscular sphincter has been reported on the pulmonary artery before the insertion into the lung (García-Párraga et al., 2017; Sapsford, 1978; Wyneken, 2001). It has been speculated that this sphincter can reduce pulmonary perfusion during diving (García-Párraga et al., 2014; Sapsford, 1978). To investigate this hypothesis, we determined the vasoactive response *in vitro* (in organ baths) to

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List of symbols and abbreviations

5HT	serotonin
ACh	acetylcholine
Adr	adrenaline
DCS	decompression sickness
E_{max}	maximum plateau in the sigmoidal response
E_{min}	minimum plateau in the sigmoidal response
GE	gas emboli
LP	lung parenchyma
PASp	pulmonary artery sphincter
PEPA	proximal extrapulmonary artery
IPA	intrapulmonary arteries
SA	systemic arteries

different neurotransmitters in the autonomic innervation of isolated pulmonary and systemic arteries as well as the global response of lung parenchyma from five loggerhead sea turtles that had drowned or were euthanized by the attending veterinarian owing to severe injuries after fisheries interactions along the Valencian coast. For comparison, tissues from freshwater red-eared sliders (*Trachemys scripta elegans*) were also investigated. Our results indicate that the sphincter on the pulmonary artery contracts strongly by parasympathetic stimulation in sea turtles, and relaxes under sympathetic stimulation. The sympathetic contraction of the pulmonary artery in the red-eared slider differs from other studies showing adrenergic relaxation (A. Hougaard and T. Wang, unpublished observations; Berger, 1971; Burggren, 1977; Hicks and Comeau, 1994; Milsom et al., 1977). Thus, the dive response may restrict blood flow through the lungs and limit gas exchange, while stress/flight response could relax the sphincter resuming gas exchange. We propose that this mechanism alleviates the risk of GE during a normal dive, but may increase the risk of GE when diving sea turtles become entangled at depth.

MATERIALS AND METHODS

Samples

Tissues were obtained opportunistically (under the Official Permit of Conselleria de Agricultura, Medio Ambiente, Cambio Climático y Desarrollo Rural) from five loggerhead sea turtles (*Caretta caretta* Rafinesque 1814) (Table 1) that were either recently dead or euthanized owing to injuries sustained from fisheries interaction. No animal was euthanized for the purpose of this study. Pathological examination did not reveal any apparent cardiovascular or respiratory disease besides that that could be attributed to fisheries interaction. Lungs and both pulmonary arteries were excised and placed into a refrigerated container (6–10°C) with tissue culture media (DMEM high glucose, L0102-500, Thermo Fisher Scientific) for a maximum of 48 h. Once at the laboratory, the arteries of interest were dissected, and connective tissue was removed. Sections from the following tissues were collected: (1) lung parenchyma [LP; sections of lung tissue (5×5×5 mm) from different portions of the lung that appeared homogeneous and macroscopically normal were carefully dissected and any pleural connective was tissue removed]; (2) proximal extrapulmonary artery (PEPA); (3) pulmonary artery sphincter (PASp; a thick-walled muscular section of the pulmonary artery); (4) intrapulmonary arteries (IPA; sections of the artery embedded in lung parenchyma); and (5) systemic arteries (SA; carotids).

Table 1. Morphometric data from loggerhead sea turtles used in the study

Animal ID	CCL (cm)	CCW (cm)	Fishery	M_b (kg)	Sex
CC1	53	51	Gillnet	19.2	F
CC2	36	32	Gillnet	5.9	F
CC3	35	30	Gillnet	4.8	M
CC4	27	24	Stranded	2.6	F
CC5	66	51	Trawler	19.5	F

Animal identification (Animal ID), curved carapace length (CCL), curved carapace width (CCW), type of fishery interaction (fishery), body mass (M_b) and sex (M, male; F, female) of the five turtles that were opportunistically sampled.

For comparison, in six recently euthanized red-eared slider freshwater turtles [*Trachemys scripta elegans* (Wied-Neuwied 1839)], the vasoactivity of the LP and PEPA (this species does not have an apparent PASp) was tested. These animals were euthanized as part of an eradication program of this invasive species from local natural habitats conducted by Conselleria de Agricultura, Medio Ambiente, Cambio Climático y Desarrollo Rural de la Generalitat Valenciana. These animals were captured from the wild and subsequently euthanized through an intravenous injection of sodium pentobarbital. The curved carapace length of these individuals ranged between 18 and 26 cm. The red-eared sliders in this study were neither weighed nor sexed before being used in this study.

All sections of the vessels/sphincters from either species were freed from connective tissue, and cut into 3- to 4-mm-wide rings. The tissues were returned to refrigerated Krebs–Henseleit buffer (K3753, Sigma-Aldrich) and the assays were immediately initiated.

Histology

Sections of PEPA, PASp and LP from the five loggerhead turtles were fixed in 4% paraformaldehyde for 48 h at 20°C and subsequently embedded in paraffin. The embedded tissue was cut into sections (4–6 µm) using a microtome (RM2235, Leica Biosystems). The sections were placed on slides with permanent positive charge (Dako Flex IHC Microscope Slide ref: K8020). The paraffin was removed and the tissue was dehydrated in increasing concentrations of alcohol. The tissue was stained with hematoxylin & eosin and trichrome and photographed under a light microscope (Leica DM6000).

Pharmacodynamics studies: vasoactive responses

Each ring or tissue was mounted on stirrups made out of stainless steel wire and submerged in an organ bath (10 ml, Pan-Lab, USA). One stirrup was fixed at the bottom and the other attached to a force transducer (UF1 Force Sensor; LCM Systems LM), connected to a data acquisition system (PowerLab®, ADInstruments, Castle Hill, New South Wales, Australia), which allowed the force of contraction to be measured. The tissue was mounted with an initial load of 1.2 g, and stabilized in Krebs–Henseleit buffer for 90 min at 22°C, while aerated with a mixture of 5% CO_2 and 95% O_2. Following the stabilization period, the tissue/vessels were washed with new buffer three times every 5 min to set the initial vascular tone. To determine the vasoactive response, each tissue/vessel was exposed to one of three neurotransmitters in increasing concentrations [10 nmol l^{-1} to 100 mmol l^{-1}: serotonin (5HT), acetylcholine (ACh) and adrenaline (Adr; also known as epinephrine)] (Goldie et al., 1982). In addition, in order to assess the viability of the tissues using a standard neurotransmitter, the response to histamine was tested in the LP (Ortiz et al., 1992). The contractile tension was allowed to stabilize until constant response before the next concentration was added.

The vasodilatory dose–response curves were determined by rinsing the ring as detailed above. Next, the vessel ring was contracted by

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adding 5HT in increasing concentration until a constant contraction was observed (Cortijo et al., 1997). Next, Adr was added in increasing concentration (10 nmol l^{-1} to 100 mmol l^{-1}), and the setup was allowed to stabilize before the force was recorded and the next concentration was added. Following the highest concentration of Adr, the smooth muscle relaxant papaverine (0.3 mmol l^{-1}) was added to obtain the maximum relaxation (Bardou et al., 2002). Vasodilatation was expressed as a percentage of maximum relaxation obtained with papaverine.

Data processing and statistical analysis

Data from pharmacodynamic studies are presented as means±s.e.m. unless otherwise specified. The total number of experiments (n) was averaged for each animal and the number of individuals included for each tissue is reported.

Vessel contractions are reported in grams (g), and relaxations are reported as a percentage change in tension as compared with the absolute change in tension during exposure to papaverine, i.e. [(measured tension–papaverine tension)/(maximum tension–papaverine tension)×100]–100.

We used common dose–response equations to fit the absolute increase (g; contraction) or relative decrease (%; relaxation) in force against $\log_{10}$-transformed neurotransmitter concentration (log mol l^{-1}). For contraction, we used the following equation:

$$F = E_{\min} + (E_{\max} - E_{\min}) \times (1 + 10^{(\log_{10}(EC_{50}) - C)})^{-1}, \quad (1)$$

where the generated force (F) is estimated by the minimum ($E_{\min}$) and maximum ($E_{\max}$) plateaus in the sigmoidal response, C is the $\log_{10}$-transformed molar concentration of neurotransmitter and $\log_{10}(EC_{50})$ is the logarithm of the EC_{50} (the neurotransmitter molar concentration at half the maximal response, i.e. half of the difference between $E_{\min}$ and $E_{\max}$). For relaxation, the dependent variable was normalized and expressed as a percentage of the minimum force following exposure to papaverine (see above).

The parameters were estimated using R version 3.2.1 (https://www.r-project.org/) including nlme package version 3.1-124 (https://cran.r-project.org/web/packages/nlme/index.html) and MuMIn package version 1.15.6 (https://cran.r-project.org/web/packages/MuMIn/index.html). Vessel and tissue responses from individual animals were treated as a random effect, which accounted for the correlation between repeated measurements on the same individual (Littell et al., 1998). We used a t-test to compare differences in estimated parameter values for different tissues. A P-value <0.05 was considered significant and a P-value <0.1 is reported as a trend.

RESULTS

Macroscopic and histological findings

The gross and histological findings observed in the PEPA were very similar in loggerheads and freshwater sliders. Histologically, these arteries show the typical morphology previously described for elastic arteries in other species, i.e. large lumen and thin wall with numerous elastic fibers and few smooth muscle cells in the tunica media (Figs 1, 2A,B). Grossly, the unique PASp previously described in the mid region of the extrapulmonary artery of loggerhead turtles was identified (Sapsford, 1978; Wyneken, 2001). The PASp occupied approximately the middle third of the total length of the extrapulmonary artery (Fig. 1). This region is characterized by a narrower lumen and thicker wall with accordion-like circumferential ridges on the luminal surface. These findings were especially evident once the whole vessel had been fixed in 4% paraformaldehyde for 48 h. Histologically, the morphology of the PASp is very different from than the rest of the PEPA. Most of the elastic fibers are replaced by thick bundles of smooth muscle arranged in circumferential folds, which extend from the tunica media to the intima. This allows the lumen to close when the muscle contracts (Fig. 2C,D). In the freshwater turtle, no gross or histological evidence of the PASp was observed. In the lung, thick bundles of smooth muscle fibers were noticeable in the submucosa of the respiratory ducts connecting the main bronchus with the faveoli in both loggerheads and freshwater sliders (Fig. 2E,F). In summary, these histological findings show that the PEPA in the freshwater slider is similar to the proximal portion of the pulmonary artery of the loggerhead sea turtle, and both species present large amount of smooth muscle in the lung. However, the strong development of the muscular component under the intimal layer at the region of the sphincter is highly specific to the sea turtle.

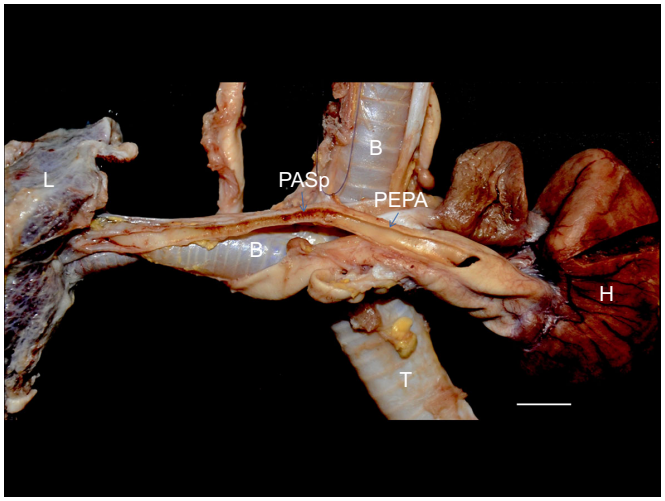

Fig. 1. Gross anatomy of the cardiopulmonary vasculature and associated structures of a loggerhead sea turtle (*Caretta caretta*). H, heart; PEPA, proximal extrapulmonary artery; PASp, pulmonary arterial sphincter; L, lung; T, trachea; B, main bronchus. Note the increased roughness of the intimal layer at the mid section of the pulmonary artery. Tissues were fixed in 4% paraformaldehyde. Scale bar: 10 mm.

Dose–response studies

In the loggerhead turtle, 5HT and ACh caused contraction in all tissues studied, but the most forceful contraction was seen at the level of the PASp (Figs 3A,B, 4, Table 2). Adr caused contraction of the SA, IPA and LP (Fig. 3C), and relaxation of the PEPA and the PASp (Fig. 3D, Table 2).

The vasoactive responses in PEPA and PASp were always in a similar direction (contraction or relaxation) for any neurotransmitter, but the contraction ($E_{\max}$) was stronger for both 5HT and ACh at the level of the PASp, where the muscular layer of the artery is thicker.

Histamine caused contraction similar to that of 5HT and Adr at the level of the LP but much weaker than that of ACh, which clearly caused the stronger contractile response of the pulmonary tissue (Table 2).

In the freshwater turtle, ACh, 5HT and Adr caused similar vasoconstriction in PEPA, and although all three neurotransmitters induced the contraction of the LP (Fig. 3A–C, Table 1), ACh clearly provoked the strongest response.

DISCUSSION

The divergent responses in freshwater and sea turtles in the present study indicate that sea turtles have evolved a distinct mechanism to avoid pressure-related problems. By managing the temporal

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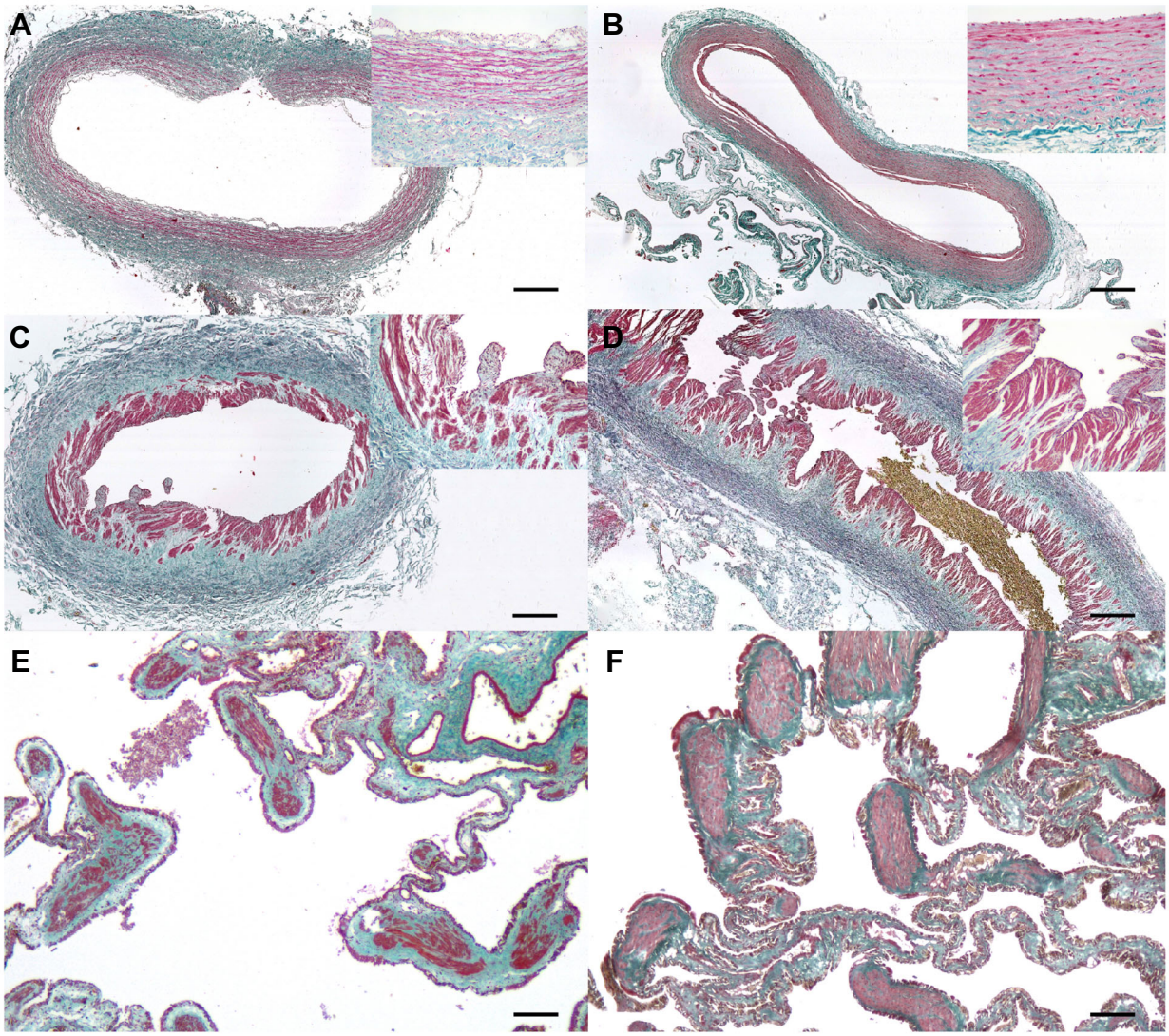

Fig. 2. Histological features of the pulmonary artery, pulmonary sphincter and lung in loggerhead sea turtles (*Caretta caretta*) and freshwater slider turtles (*Trachemys scripta elegans*); Masson's trichrome stain. PEPA in a loggerhead (A; scale bar: 550 µm) and pulmonary artery in a freshwater slider (B; scale bar: 335 µm) showing large lumen and a thin arterial wall composed of elastic fibers and collagen admixed with thin bundles of smooth muscle fibers (inserts: detail of the arterial walls). PASp of a loggerhead turtle in transverse (C; scale bar: 335 µm) and longitudinal (D; scale bar: 435 µm) sections. The tunica media shows thick bundles of smooth muscle arranged in accordion-like circumferential folds (inserts: detail of the muscular folds of the sphincter). Lung, from loggerhead (E; scale bar: 100 µm), and freshwater slider (F; scale bar: 100 µm) both revealing thick bundles of smooth muscle within the parenchyma.

ventilation–perfusion ratio in the lung, they may avoid DCS, while retaining the ability to manage pulmonary O_2 stores and CO_2 levels during dives (Fahlman et al., 2018; García-Párraga et al., 2018). Failure of this mechanism, caused by fisheries interaction, may explain the high incidence of GE during enforced submersion of turtles (Fahlman et al., 2017a; García-Párraga et al., 2014).

We show that the PEPA, IPA and PASp contract when exposed to 5HT and ACh, which is qualitatively similar to the pulmonary artery in humans and other mammals (Cortijo et al., 1997; Furchgott and Bhadrakom, 1953; Van Nueten et al., 1985), and in line with previous studies with turtles and other reptiles (Berger, 1971; Burggren, 1977; Milsom et al., 1977; Taylor et al., 2009; Wang, 2011). The contraction of the PASp was more intense than in other regions of the pulmonary artery or compared with the red-eared slider also studied (Tables 2, 3). We propose that this is due to the large amount of muscular bundles in the thicker wall of the PASp (Fig. 2A–D). We speculate that this high level of contraction at this particularly thick portion of the pulmonary artery is likely to cause complete closure of the lumen in the living sea turtle. Exposure to Adr caused vasoconstriction of all tissues from the freshwater red-eared slider (Fig. 3D). In the sea turtle, IPA, SA and LP constricted when exposed to Adr, but both portions of the extrapulmonary artery dilated (PEPA and PASp; Fig. 3D). During the initial stages of this work, we exposed the LP to histamine and 5HT, which are standard neurotransmitters used to evaluate vascular response in human tissues and other animal models, including reptiles (Kiniwa and Tasaka, 1989; Parsons and Ganellin, 2006; Reite, 1970; Skovgaard et al., 2018). This allowed us to assess the response in comparison with other species previously studied and ensure that the tissues were still viable.

The dive response is a conserved reflex observed in all vertebrates and is a set of cardiovascular changes caused by increased parasympathetic tone causing bradycardia, and peripheral vasoconstriction resulting in reduced cardiac output (Berkson, 1966; Bert, 1870; Lutz and Bentley, 1985). It is believed that these cardiovascular responses are used to conserve available O_2 for vital

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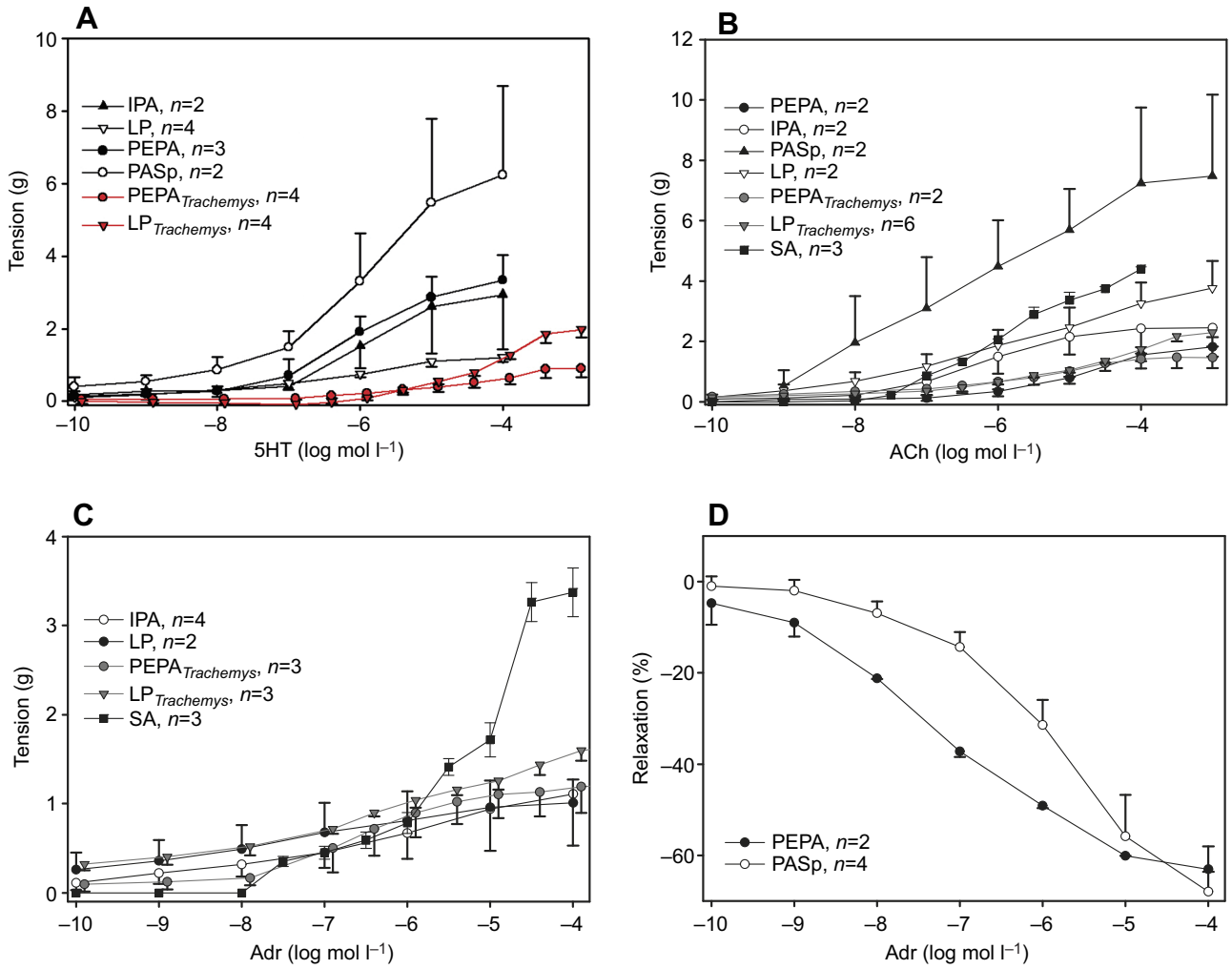

Fig. 3. Mean (±s.e.m.) vessel and tissue response (contraction or relaxation) of the extra-pulmonary artery (PEPA), pulmonary arterial sphincter (PASp), intrapulmonary artery (IPA), lung parenchyma (LP) and systemic artery (SA) in response to different neurotransmitters in loggerhead sea turtles (*Caretta caretta*). (A) Serotonin (5HT), (B) acetylcholine (ACh) and (C,D) adrenaline (Adr). Tissues from freshwater sliders are identified in the legend as '*Trachemys*'; all others are loggerhead sea turtle tissues. *n* indicates the number individual turtles in each group used for each regression. Contraction is expressed as mean±s.e.m. contraction (g) against $\log_{10}$-transformed neurotransmitter concentration (log mol l^{-1}). For relaxation, data are expressed as the relative decrease (%) as compared with relaxation with papaverine (0.3 mmol l^{-1}).

organs, such as the heart and the brain, and that reduced blood flow and a reduction in the function of certain organs, e.g. the kidney, help to reduce the overall rate of O_2 consumption (Lutz and Bentley, 1985; Scholander, 1940). Voluntary aerobic dives in loggerhead sea turtles are known to range from approximately 7 min for smaller animals (2–15 kg) up to 27 min for larger individuals (20 kg) (Lutz and Bentley, 1985). Loggerhead sea turtles dive exclusively on inhalation, and it has been suggested that the lung is a major source of available O_2 (Lutz and Bentley, 1985). However, access to O_2 requires that the faveoli remain open to allow gas exchange whenever the lung is perfused. As the pressure increases with depth the faveoli compress, causing a passive pulmonary shunt that reduces gas exchange (Berkson, 1967), similar to alveolar collapse in marine mammals (Scholander, 1940). Consequently, to use the pulmonary O_2 stores, dives should be shallower than the depth where the faveoli collapse and gas exchange ceases. However, a recent hypothesis suggests that management of the ventilation–perfusion ratio provides a mechanism to selectively exchange gases of different solubility (Fahlman et al., 2018; García-Párraga et al., 2018).

In a previous study, a pulmonary shunt was shown in turtles diving in a hyperbaric chamber that increased with depth, and the author estimated that faveolar collapse occurred at depths >80 m (Berkson, 1967). In loggerhead sea turtles, 90% of their time is spent either at the surface or at depths shallower than 40 m (Polovina et al., 2004). Thus, the majority of dives in loggerhead sea turtles are shallower than the depth at which passive compression faveoli collapse prevents gas exchange. Consequently, N_2 would continuously be taken up in this species, increasing the risk for GE even during natural dives. However, in turtles, central vascular shunting is possible (Hicks et al., 1996), which would cause an air/blood mismatch that would serve to alter gas uptake or removal even in the inflated lung (Farhi, 1967; Farhi and Yokoyama, 1967; West, 1962). Although pulmonary blood flow can be significantly reduced, and even abolished in freshwater turtles or rattlesnakes during diving and vagal stimulation (Milsom et al., 1977; Taylor et al., 2009; Wang, 2011; Wang et al., 2001), the well-developed sphincter appears unique to sea turtles and allows for complete pulmonary bypass solely by regulation of the tone on the extrinsic portion of the pulmonary artery. The PASp in particular, and also

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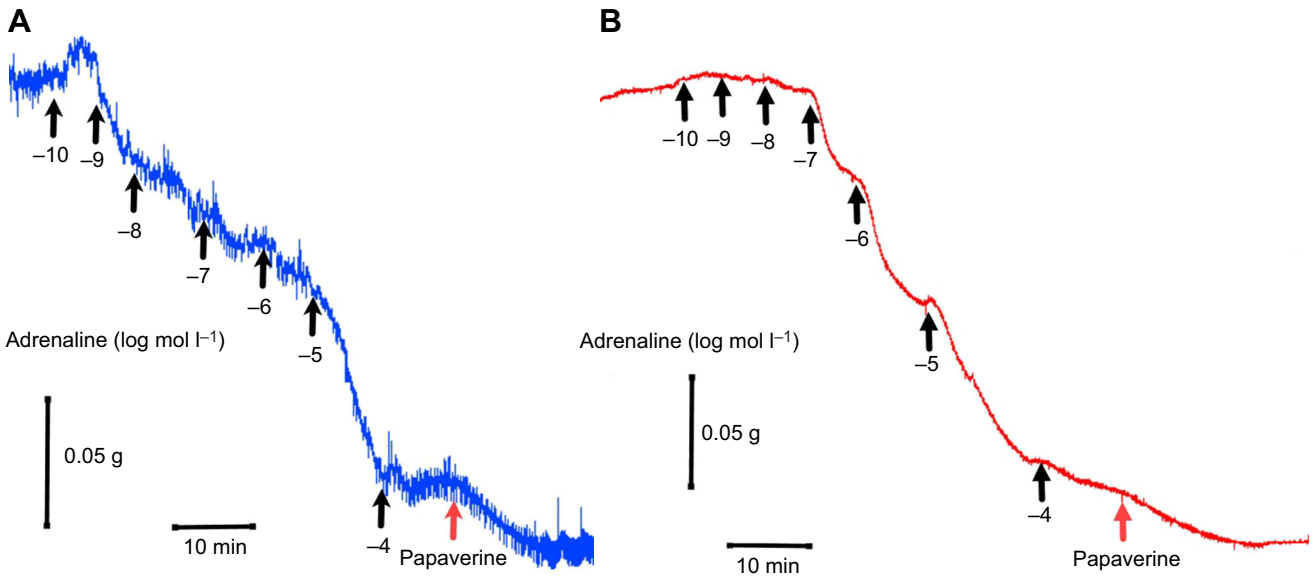

Fig. 4. Representative data trace showing the effects of adrenaline on tension development in the loggerhead sea turtle (*Caretta caretta*). Data are shown for the (A) extrapulmonary artery and the (B) pulmonary artery sphincter. Insets shown the scale for time and tension.

the LP, contracts under parasympathetic tone. Complete closure of the muscular PASp would avoid pulmonary perfusion, inducing a right to left (R–L) intracardiac shunt, redirecting all cardiac output to the systemic circulation. Contraction of the LP would be facilitated by the large number of smooth muscular bundles interspersed throughout the parenchyma (Fig. 2E). The contraction would help to manage the ventilation/perfusion mismatch to minimize uptake of gases with low solubility (N_2), while permitting exchange of gases with higher solubility (O_2 and CO_2) (Farhi and Yokoyama, 1967; West, 1962). A similar mechanism has recently been suggested for marine mammals (Fahlman et al., 2018; García-Párraga et al., 2018).

Table 2. Results from pharmacokinetic studies in selected loggerhead sea turtle tissues under different neurotransmitters

Neurotransmitter	Tissue	E_{max} (g)	$-\log_{10}(EC_{50})$	*N* (*n*)
5HT	PEPA	2.91±0.26	6.06±0.23	3 (6)
	PASp	8.15±1.45	5.91±0.48	2 (6)
	IPA	3.12±0.56	5.73±0.41	2 (6)
	SA	NA	NA	NA
	LP	1.45±0.21*	5.73±0.52	4 (6)
ACh	PEPA	2.02±0.12*	4.76±0.14	2 (4)
	PASp	9.56±1.46	5.39±0.98	2 (8)
	IPA	2.51±0.16*	6.28±0.20	2 (6)
	SA	4.45±0.12¶	5.84±0.06	3 (5)
	LP	4.53±0.55¶	5.38±0.48	2 (6)
Adr	PEPA	–59±3%‡	7.14±0.21*	2 (3)
	PASp	–64±2%‡	5.89±0.09*	4 (10)
	IPA	1.64±1.50	5.48±4.03	2 (4)
	SA	6.17±0.93	4.35±0.29	3 (4)
	LP	1.34±0.40	7.79±1.47	2 (13)
Hist	PEPA	NA	NA	NA
	PASp	NA	NA	NA
	IPA	NA	NA	NA
	SA	NA	NA	NA
	LP	1.38±0.15	6.31±0.32	4 (5)

Non-linear least-squares regression showing the fitted values (means±s.e.m.) for the upper plateau (E_{max}) and the logarithm of the EC_{50} [$\log_{10}(EC_{50})$] contractile response of different tissues from loggerhead sea turtles during exposure to serotonin (5HT), acetylcholine (ACh), adrenaline (Adr) and histamine (His): proximal segment of extrapulmonary artery (PEPA), pulmonary arterial sphincter (PASp), internal segment of pulmonary artery (IPA), lung parenchyma (LA) and systemic arteries (SA). E_{max} indicates the sensitivity, or the maximal contractile force (‡ indicates the relative degree of relaxation as compared with papaverine), and $-\log_{10}(EC_{50})$ is the concentration for half of the maximal response. NA indicates no data available. All regressions were significant at $P<0.01$. *N* is the number of animals and *n* is the number of rings from the animals included. A *t*-test was used to compare the parameter response between PASp and the other vessels and tissues (*$P<0.05$, ¶$P<0.1$).

The undivided chelonian heart provides for intracardiac shunts, and it is well established that pulmonary blood flow is reduced during diving, causing R–L shunts, while pulmonary flow increases during intermittent ventilation, where a L–R shunt may dominate (Burggren, 1977; Shelton and Burggren, 1976; Wang et al., 1997, 2001). The changes in pulmonary blood flow are mediated by the parasympathetic innervation, where increased tone elevates pulmonary vascular resistance through vagal innervation of the smooth muscle within the wall of the pulmonary artery (Burggren, 1977; Hicks, 1998; Shelton and Burggren, 1976; Wang et al., 1997, 2001). Our observation that the pulmonary vessels from sea turtles exhibit intense vasoconstriction when exposed to 5HT and ACh suggests that the PASp is an important anatomical vasomotor structure driving the cardiac shunt in sea turtles. The vasoconstriction would significantly reduce, or possibly prevent, pulmonary blood flow. In the sea turtle, autonomic regulation of pulmonary blood flow seems to have evolved extensively and led to the development of the PASp in comparison with the pond turtles that normally perform rather shallow dives. In deeper diving species, such as the leatherback sea turtle, the PASp has a much thicker muscular wall with longitudinal folds as compared with the shallower diving loggerhead sea turtle (García-Párraga et al., 2017; Wyneken, 2001). The PASp is virtually absent in the freshwater turtle, and we hypothesize that this anatomical feature provides a crucial physiological adaptation for deep and long breath-hold dives in sea turtles. However, complete pulmonary bypass is still possible despite less innervation and smooth muscle in pulmonary vessels in freshwater turtles (Burggren and Shelton, 1979; White et al., 1989).

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Table 3. Results from pharmacokinetic studies in red-eared slider selected tissues under different neurotransmitters

	5HT			ACh			Adr		
	E_{max} (g)	$\log_{10}(EC_{50})$	N (n)	E_{max} (g)	$\log_{10}(EC_{50})$	N (n)	E_{max} (g)	$-\log_{10}(EC_{50})$	N (n)
PEPA	1.53±0.32	3.99±0.29	4(6)	1.70±0.52	5.30±1.05	2(3)	1.19±0.07	6.78±0.19	3(5)
LP	2.48±0.17	4.15±0.12	4(8)	4.32±0.76	3.44±0.52	6(11)	2.19±0.17	5.73±0.37	3(3)

Results from non-linear least squares regression showing the upper plateau (E_{max}) and the logarithm of the EC_{50} [$\log_{10}(EC_{50})$] contractile response of different tissues from red-eared slider turtles during exposure to serotonin (5HT), acetylcholine (ACh) and adrenaline (Adr): proximal segment of extrapulmonary artery (PEPA) and lung parenchyma (LP). The E_{max} indicates the sensitivity, or the maximal contractile force, and $-\log_{10}(EC_{50})$ is the concentration for half of the maximal response. N is the number of animals and n is the number of rings from the animals included.

Although the pulmonary circulation is under strong parasympathetic modulation, Adr released during sympathetic stimulation opens the sphincter in the sea turtles, causing gas exchange to resume. In fact, the relaxation of the PEPA in response to Adr occurred *in vitro* even in the presence of ACh in the tissue bath. This could be a physiological mechanism to occasionally access pulmonary O_2 stores and/or eliminate excess CO_2 during prolonged dives or during increased demands (García-Párraga et al., 2018; Malte et al., 2016a,b; Wang et al., 1997, 2001; Wang and Hicks, 1996a). Tightly regulated matching of ventilation and perfusion could allow short periods of controlled lung perfusion to preferentially exchange O_2 and CO_2 with minimal N_2 exchange (Fahlman et al., 2018; Farhi, 1967; Farhi and Yokoyama, 1967; West, 1962). However, if perfusion of the lung is maintained during certain times at depths shallower than the faveolar collapse depth, the decreasing ventilation–perfusion ratio would increase N_2 uptake, which in turn would increase the risk of GE after surfacing. Once venous bubbles form, a R–L shunt would pass bubbles directly to the systemic circulation without the ability for the lung to act as a filter (Bove, 1998; Cross and Jennings, 1994), increasing risk for GE to enter the systemic circulation (Fahlman et al., 2017a; García-Párraga et al., 2014; Vann et al., 2011).

In fact, different studies correlate exercise with breathing frequency, pulmonary blood flow and heart rate in green sea turtles (Butler et al., 1984; Southwood Williard, 2013; West et al., 1992). An increase in heart rate, catecholamine release and lung perfusion has been observed in a number of reptiles following exercise/capture, also supporting our observations (Shelton and Burggren, 1976; Wang and Hicks, 1996b; Wang et al., 2001; West et al., 1992; White and Ross, 1966).

We propose that when a sea turtle is caught in a net, the confinement stress and the increased muscular activity as the turtle attempts to escape elevate the sympathetic tone and may elevate circulating catecholamines (Comeau and Hicks, 1994; Hicks and Comeau, 1994; Overgaard et al., 2002; Shelton and Burggren, 1976). However, it is important to note that we do not know the physiological concentrations of adrenaline and noradrenaline in stressed or entangled sea turtles, which would be important variables to assess. In stressed freshwater turtles, the peak Adr concentration is around 10^{-8} mol l^{-1} (Wasser and Jackson, 1991), resulting in approximately 20% and 5% relaxation of PEPA and PASp, respectively, which may be sufficient to increase pulmonary flow and gas exchange and elevate the risk of GE. In addition, the vasoactive effects of noradrenaline should be also considered in future studies, as its vascular concentration can be significantly higher than that of adrenaline in stressed freshwater turtles (Wasser and Jackson, 1991). Prolonged sympathetic tone and circulating catecholamines would reverse the diving bradycardia, increase cardiac output and reduce the R–L shunt at depth. These responses would elevate transfer of N_2 from the lungs and increase absorption in the tissues. Thus, with prolonged entrapment in the fishing nets at depth, enough N_2 may be stored in the tissues for GE to be formed during rapid ascent to the surface (Fahlman, 2017; Fahlman et al., 2017a; García-Párraga et al., 2014).

Failure of the physiological mechanism that normally regulates ventilation and blood flow through the lungs, conserves O_2 and minimizes N_2 uptake can result in GE during ascent. Increased activity, resulting in lactic acid buildup, has been shown in bycaught turtles even during short submersion episodes (Stabenau et al., 1991; Stabenau and Vietti, 2003). We propose that entanglement and bycatch may result in increased activity, heart rate, sympathetic tone and lung perfusion, which result in failure of the ventilation–perfusion mismatch that regulates gas exchange during diving in sea turtles (Comeau and Hicks, 1994; Hicks and Comeau, 1994; Hopkins et al., 1996; Overgaard et al., 2002; Shelton and Burggren, 1976). Pulmonary sphincters have been identified in several sea turtle species (García-Párraga et al., 2017; Sapsford, 1978; Wyneken, 2001). This anatomical feature may be a conserved trait related to deep diving that provides a strategy to reduce diving-related problems while, at the same time, providing selective access when needed to the pulmonary O_2 store.

Numerous studies reveal significant physiological disturbances of sea turtles entangled in fishing gear (Harms et al., 2003; Lutz and Dunbar-Cooper, 1987; Snoddy et al., 2009; Snoddy and Southwood Williard, 2010; Stabenau and Vietti, 2003; Wyneken et al., 2013). Only recently have there been reports indicating that fisheries interaction may result in GE. In previous work, we described the presence of GE following fisheries interaction (García-Párraga et al., 2014), and the prevalence and potential risk factors in the fisheries along the Valencian coast (Fahlman et al., 2017a). The present work provides a potential mechanism for how the normal physiological function of the PEPA and PASp responds during entrapment in fishing gear, which can lead to disruption and failure of normal physiological regulation, increase pulmonary blood flow and cause GE to form. To test our hypothesis, we would need to measure the circulating concentration of Adr or the pulmonary blood flow in entangled sea turtles. In fact, there are a number of potential risk factors that may vary the severity of this trauma; fishing depth, the duration that the fishing gear is deployed, water temperature and ascent rate may be a few (Fahlman et al., 2017a). Future work is aimed at further defining these risk variables and their importance in order to establish effective mitigation measures for fisheries.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: D.G.-P.; Methodology: D.G-P., J-L.O., J.O.; Formal analysis: A.F.; Investigation: T.L., J.-L.O., J.O.; Resources: D.G., J.-L.O., J.-L.C.-P., J.C., A.F.; Data curation: A.F.; Writing - original draft: A.F.; Writing - review & editing: D.G.-P., T.L., T.W., J.-L.O., J.O., J.-L.C.-P., J.C.; Visualization: A.F.; Supervision: D.G.-P., J.-L.O., J.C., A.F.; Project administration: A.F.; Funding acquisition: A.F.

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Data availability

All data are freely available on the Open Science framework (OSF) at: https://osf.io/8gtnp.

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