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MORPHOLOGY AND GROWTH OF THE VENTRICULAR MYOCARDIUM OF *SOTALIA GUIANENSIS*

(Morfologia e crescimento ventricular do miocárdio de Sotalia guianensis)

Letícia Versiani Gomes DA SILVA*[1]; Isis de Oliveira Carvalho DEMARQUE[1]; Hassan Jerdy LEANDRO[2]; Vinícius Novaes ROCHA[3]; Lupércio Araújo BARBOSA[4]; José LAILSON-BRITO[5]; Leonardo Serafim DA SILVEIRA[1]

[1]Laboratório de Morfologia e Patologia Animal, Universidade Estadual do Norte Fluminense Darcy Ribeiro (UENF), Av. Alberto Lamego, 2000, Campos dos Goytacazes/RJ. CEP: 28.013-602; [2]Lab. Microscopia (UNIFESSPA); [3]Lab. Patologia e Histologia Veterinária (UFJF); [4]Organização Consciência Ambiental (Instituto ORCA); [5]Lab. Mamíferos Aquáticos e Bioindicadores, Faculdade de Oceanografia (UERJ). *E-mail: versianileticia@gmail.com

ABSTRACT

Morphology, growth and aging of the heart, not only of Sotalia guianensis, but also of all cetaceans, are relatively unknown. The aim of the present study was to evaluate the morphology and growth of the heart and myocardium, using macro and micro-morphology, of samples collected from hearts of stranded Guiana dolphins found in the coast of Espírito Santo, Brazil. The ventricles were examined with light and electron microscopy, the hearts were measured and the volume density of cardiomyocytes, connective tissue, and blood vessels of the ventricles were determined. Statistical differences, regressions, correlations, and growth curves were applied between total length and age of animals and morphological variables. No significant difference was found between males and females. The histology and ultrastructure of the Guiana dolphin´s ventricles are similar to those found in other mammals. Type-I and -III collagen fibers were predominant in the myocardium. Total length and age correlated with select external morphometric variables such as the circumference at the coronary sulcus and the circumference at the distal third. Only the volume density of cardiomyocytes (r = 0.516) and connective tissue (r = -0.503) of the right ventricle correlated with age. The growth of height and circumference of the heart of S. guianensis occurs until approximately ten years of age, exceeding the physical maturity age reported for the species.

Keywords: *Guiana Dolphin, heart, morphometry, microscopy.*

RESUMO

A morfologia, o crescimento e o envelhecimento do coração, não só de Sotalia guianensis (boto-cinza), como também de todos os cetáceos é pouco conhecida. O objetivo do presente estudo foi avaliar a morfologia e o crescimento, utilizando macro e micromorfologia de amostras coletadas de corações de botos-cinza encontrados encalhados no litoral do Espírito Santo, Brasil. Os ventrículos foram avaliados na microscopia de luz e eletrônica, os corações foram mensurados e a densidade de volume de cardiomiócitos, tecido conjuntivo e vasos sanguíneos dos ventrículos foram determinadas. Diferenças estatísticas, regressões, correlações e curvas de crescimento foram aplicadas entre comprimento total, idade e variáveis morfológicas dos corações dos animais. A histologia e ultraestrutura dos ventrículos do boto-cinza são similares àquelas encontradas em outros mamíferos. Fibras colágenas dos tipos I e III foram predominantes no miocárdio, indicando tecido fibroso. O comprimento total e a idade correlacionaram-se com variáveis morfométricas externas, como a circunferência na região do sulco coronário e do terço distal do coração. Apenas a densidade de volume de cardiomiócitos (r=0,516) e do tecido conjuntivo (r=-0,503) do ventrículo direito apresentaram correlação com a idade. O crescimento em altura e circunferência do coração de S. guianensis ocorreu até aproximadamente dez anos de idade, excedendo a idade de maturidade física antes registrada para a espécie.

Palavras-chave: Boto-cinza, coração, morfometria, microscopia.

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INTRODUCTION

Sotalia guianensis (van Benèdèn, 1864), commonly known as Guiana dolphin, is an Odontoceti cetacean found from Honduras to the coast of Santa Catarina, Brazil (FLORES *et al*., 2018). The species has coastal habit, and populations are concentrated in estuaries and bays (LODI and BOROBIA, 2013). Such fact makes the species susceptible to anthropogenic pressures (FLORES *et al*., 2018). Stranding events occur throughout the entire area of occurrence on the Brazilian coast (SICILIANO *et al*., 2008).

The cardiovascular system of cetaceans, as well as of other marine animals, is adapted to aquatic life for better oxygen transport (BRAZ *et al*., 2013). However, the knowledge about cetacean hearts and its development are scarce (MCALPINE, 1985; SEDMERA *et al*., 2003; GARRI, 2006; MACDONALD *et al*., 2007). Most research on basic knowledge of heart in this group was carried out in the past (WHITE and KERR, 1917; OCHRYMOWYCH and LAMBERTSEN, 1984; ROWLATT, 1990). Microscopically, the heart of marine mammals and cetaceans has been even less studied (VOGL and FISHER, 1976; PFEIFFER, 1990; STEWARDSON *et al*., 1999).

The three tunics that form the heart wall are the endocardium, myocardium, and pericardium. The myocardium is the thickest tunic of all and contains cardiomyocytes, which are muscle cells organized in columns (JUNQUEIRA e CARNEIRO, 2013) and supported by collagen fibers arranged in different orientations. These, in turn, compose the connective tissue that forms the stroma of the organ (SONNENBLICK *et al*., 1986; JUNQUEIRA e CARNEIRO, 2013). However, alterations in the cell proportion in the myocardium occur over the years between different species (HUDLICKA and BROWN, 1996; ÁGUILA *et al*., 1998), even in small changes (ØSTERGAARD *et al*., 2013).

Stereology allows the interpretation of a three-dimensional structure based on two-dimensional analysis (SILVA, 2007). The technique, along with morphometric results, can establish parameters for growth and the aging process, as well as reduce the level of diagnostic uncertainty (ALMEIDA and LACERDA, 2002; HOF *et al*., 2003). The aim of the present study was to examine structures of the heart of *S. guianensis* and investigate correlations between data on total length (TL), heart morphology, and age.

MATERIALS AND METHODS

Specimen acquisition and treatment

The specimens of *Sotalia guianensis* (n=28) used for this study resulted from stranding events or incidental captures in fishing nets on the coast of the state Espírito Santo, Brazil, between 2008 and 2014. The carcasses were collected and stored by the Organização Consciência Ambiental (Instituto ORCA) under the license No. 64724-2 (SisBio/ICMBio). The necropsies were performed by the team from the Animal Morphology and Pathology Laboratory of the Norte Fluminense Darcy Ribeiro State University (LMPA/UENF).

The total length was taken by measuring the distance between the notch of the tail fluke and the tip of the rostrum (NORRIS, 1961). The heart was removed from the thoracic cavity, washed in tap water, and fixed in 10% neutral buffered formalin solution. In a number of procedures, ventricle sections were fixed in a solution of 2.5% glutaraldehyde in

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0.1 M sodium cacodylate buffer (pH 7.2), and then fixed in 1% osmium tetroxide for further electron microscopy analysis. Subsequently, the samples were dehydrated in an acetone series (30%, 50%, 70%, 90%, and 100%) and embedded in Epon (812).

Epon blocks were cut in semi-thin sections (1µm thick), stained with toluidine blue and examined in a light microscope. Ultrathin sections (70nm) were cut by using a Leica Ultracut-UCT Leica Mikrosysteme GmbH, Austria ultra-microtome and counterstained with uranyl acetate and lead citrate. Afterwards, the sections were examined under a transmission electron microscope JEOL – JEM – 81011 at 80kv.

Gross measurements

The hearts were fixated in formalin (10%) for seven days, dissected, then morphometric measurements were taken: height from the aorta insertion to the apex of the heart (ABH); circumference at the coronary sulcus (CCS); circumference at the distal third (PTD); left ventricular non-septal wall thickness (LVWT), right ventricular non-septal wall thickness (RVWT); and interventricular septal thickness (IVST). Thickness was measured with a Mitutoyo´s Absolute electronic caliper, while height and circumference were measured with a measuring tape.

Microscopy and stereological analysis

Ventricles were isolated for stereological analysis by removing the sections in the same region as the ventricular free walls, but a cut was made in an isotropic random direction, ensuring that the sampling is not biased (MÜHLFELD *et al*., 2010).

The tissue sections were stored in tissue embedding cassettes and automatically processed. The samples were dehydrated, clarified, and paraffin-embedded. The samples were transferred to a paraffin embedding station Leica EG1150H to create paraffin blocks.

Seven non-consecutive 5µm-thick sections of the sample of each ventricle were cut with a Leica RM 2145 rotary microtome. The sections were placed on histological slides and stained by using the Hematoxylin and Eosin (HE) protocol to test tissue viability, as well as Picrosirius Red to assess collagen fibers in the connective tissue of the myocardium and Masson´s trichrome stain to differentiate the conjunctive tissue from cardiomyocytes and facilitate the stereological analysis.

The types of collagen fibers in the connective tissue of the pericardium, myocardium, and endocardium of the left and right ventricles were identified with a polarized light microscope ICC50 HD coupled with a Camera Leica.

Five images of randomly selected fields were taken using a 40X objective lens on each slide and analyzed using STEPanizer software. The obtained images were analyzed with a test-system composed of 36 test-points (PT). The volume density was determined for cardiomyocytes (VvM), connective tissue (VvC), and blood vessels (VvV) according to the number of points that overlaps each examined structure divided by the total number of test-points (BEZERRA, 2008).

Electron microscopy

Fragments of heart were fixed in 2.5% glutaraldehyde (Riedel-de-Haen, Germany) in 0.1 M cacodylate buffer (pH 7.2) and post-fixed in 1% osmium tetroxide (Sigma-

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Aldrich Louis, USA). Then, the samples were dehydrated through a graded series of acetone and embedded in Epon (Embed-812). Semithin sections (1µm) were cut, stained with toluidine blue (Vetec, Rio de Janeiro, Brazil), and observed with a light microscope (Olympus BX 53F,Tokyo, Japan).

Ultrathin sections (70nm) were obtained with an ultramicrotome (Leica Ultracut-UCT, Leica Mikrosysteme Gmbh, Austria), counterstained with uranyl acetate and lead citrate, and examined with a JEOL JEM 1011 transmission electron microscope at 80 Kv.

Age estimate

The three longest and most straight teeth were collected from *S. guianensis* after carcasses were macerated at the Instituto ORCA. The teeth were processed at the the Laboratório de Mamíferos Aquáticos e Bioindicadores (MAQUA) as described by Hohn (1990).

The method used to estimate age was counting layers deposited annually on tooth dentin. Animals that presented no neonatal line were assigned the age 0, animals that presented only one neonatal line were assigned the age of 0.5 year old (DE CARVALHO *et al*., 2015), and from the neonatal line each layer was considered to represent one year of age in the estimate (HOHN, 1990). In accordance with the total length, the specimens were categorized as physically mature (TL>185cm) or immature (TL<185cm) (DE CARVALHO *et al*., 2012).

Statistical analyses

All statistical analyses were performed by using the R Studio program (CORE TEAM, 2017) and growth curves were plotted with Microsoft Excel. The distribution normality was performed by using the Shapiro-Wilk test ($p<0.05$), which indicated whether the data were parametric (bilateral t test) or non-parametric (Wilcoxon test) to assess the statistical difference between the means of females and males, matures and immatures, left and right ventricle. Pearson's correlation and simple linear regression were applied to data on heart morphology, total length, age, and stereology. Those that did not fit linear regression fitted non-linear growth models. The non-linear models of Brody, Logistic, von Bertalanffy and Gompertz were tested. The fitted models were compared based on the results of the Akaike Information Criterion.

RESULTS AND DISCUSSION

The examined specimens of *Sotalia guianensis* ranged in age from 0 to 35 years, with an average of 7 years. The total length ranged from 90 to 202cm, with an average of 162.9cm. From the 28 examined dolphins, 20 were categorized as immature and 8 as mature. Ten were females and 15 were males, while the sex of 3 animals could not be identified due to genital region decomposition. No sexual dimorphism was detected in any of the investigated variables.

Cardiomyocytes from *S. guianensis* ventricles had a single or two nuclei. They were arranged in multidirectional muscle bundles and supported by dense connective tissue associated with arterioles, venules, lymphatic vessels, and scarce adipocytes. The connective

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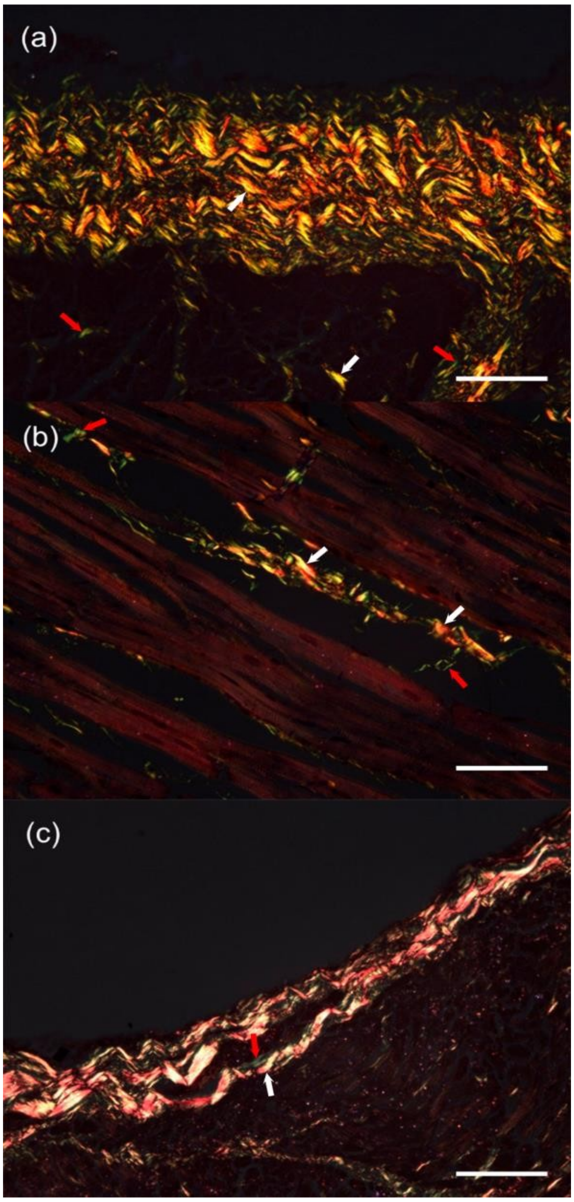
tissue spread out and formed the epicardium, with type III collagen fibers predominantly. In the endocardium, the predominant collagen fibers were type I.

The myocardium presented these two types of fibers in similar proportions (Fig. 01). In cattle, artiodactyl ungulates, there is a predominance of type III collagen fibers in more distensible tissues such as pericardium (NAIMARK *et al*., 1992), as well as of the examined *S. guianensis*. Similarly, type I and III fibers are present in the myocardium of Arctocephalus australis, the South American fur seal (GUIMARÃES *et al*., 2014). We believe that similar proportions of these fibers are necessary to maintain and balance the contractility of tissue (YANG *et al*., 1997).



(**Source:** Personal collection, 2018)

Figure 01: Photomicrograph of ventricles in longitudinal section of *Sotalia guianensis* from the Espírito Santo coast, Brazil.

Obs.: A = epicardium with predominance of type III collagen fibers; B = myocardium with type I and III collagen fibers; C = endocardium with predominance of type I fibers. Scale bar 50µm. Red arrows indicate yellow to orange fibers (type I) and white arrows indicate greenish fibers (type III).

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The electron microscopy showed that the ultrastructural organization of the cardiomyocyte is similar to other mammals (SOMMER and WAUGH, 1978). Myofibrils are organized in a banding pattern, such as striations, alternating between bands I and A, and sarcomeres are delimited by two successive Z lines. Cardiomyocytes also show an abundance of mitochondria, with varied shapes and sizes, distributed among groups of myofibrils arranged longitudinally. Predominantly, the mitochondria showed signs of degradation, with irregular and discontinuous cristae, and myelin figures (Fig. 02), which may be associated with the process of post-mortem degeneration of the tissue.

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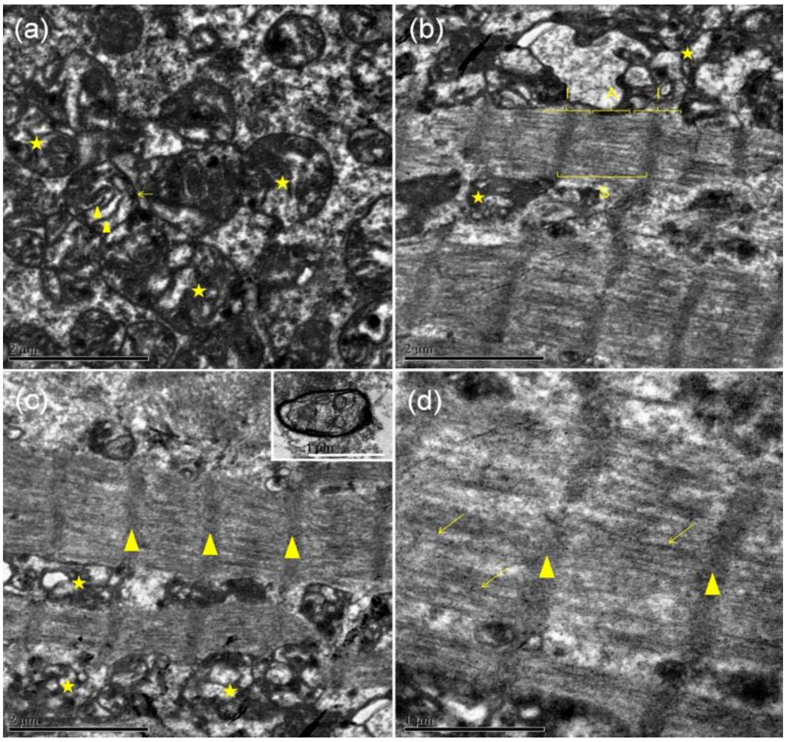

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Figure 02: Ultrastructural photomicrography of the ventricular myocardium of *Sotalia guianensis* from the Espírito Santo coast.

Obs.: a = mitochondria (stars), mitochondrial ridges (arrowheads) and myelin figures (arrow); b = sarcomeres (s) delimited by successive Z line bands (I line – "I" and A line – "A") and mitochondria (stars); c = Z lines (arrowheads) and mitochondria (stars); d = myofibrils (arrows) and Z lines (arrowheads).

Guimarães *et al*. (2014) examined the cardiac fibers of A. australis and described it as having a single or two nuclei, with elongated bundles in parallel arrangement, connected, in a three-dimensional structure, with I and A bands and Z line. The striated muscle tissue

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presents sarcoplasm with parallel myofibril bundles and great number of mitochondria between them, as was found in *S. guianensis.*

The Tab. 01 shows the heart morphometry results with mean, standard deviation, minimum and maximum values. Bisaillon *et al.* (1987) examined the hearts of Delphinapterus leucas, beluga whales, and found that the apex-base height (ABH) ranged from 18.5cm to 42.5cm and the circumference from 47.1cm to 114.0cm. The dolphins examined in this study ranged from 5.8cm to 12.8cm in heart height. However, the beluga belongs to another family and dives for a longer time and deeper than *S. guianensis*, which explains the difference. Guimarães (2009) analyzed the height between the apex and the ventral margin of the coronary sulcus of *A. australis* and found mean values of 5.7cm and 5.9cm for females and males respectively.

Table 01: Data of *Sotalia guianensis* from the Espírito Santo coast.

Variable	Mean	SD	Minimum	Maximum
Age (years)	7	8.20	0	35
TL (cm)	162.9	29.122	90	202
ABH (cm)	9.13	1.83	5.8	12.5
CCS (cm)	26.86	4.41	15.1	34.3
CDT (cm)	20.07	3.3	12.9	25.9
LVWT (cm)	1.33	0.35	0.49	2.02
RVWT (cm)	0.52	0.2	0.14	0.97
IVST (cm)	1.35	0.33	0.53	1.93

*Total length (TL); apex-base height (ABH); circumference at the coronary sulcus (CCS); circumference at the distal third region (CDT); left ventricular non-septal wall thickness (LVWT); right ventricular non-septal wall thickness (RVWT); interventricular septum thickness (IVST); standard deviation (SD). Stranded events occurred between 2008 and 2014.

A strong positive correlation was found between height of the heart (ABH) and the circumference at the coronary sulcus (r=0.8774). Two other variables were significantly correlated with height: circumference at the distal third (CDT) (r=0.5877) and left ventricular wall thickness (LVWT) (r=0.4869). Right ventricular wall thickness RVWT did not correlate with height. The correlations between the morphometric variables that this study analyzed indicate that the growth of *S. guianensis* heart is proportional in circumference and height (r=0.8774). Guimarães (2009) also found a strong correlation between height and width of A. australis heart (r=0.873 for females and r=0.950 for males).

One of the correlations the study found between the total length and the morphometric variables (Tab. 02) is that the circumference at the coronary sulcus (CCS) (r=0.7923), the height (ABH) (r=0.7006), and the interventricular septal thickness (IVST) (r=0.7006) were most correlated with TL.

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Table 02: Correlation and regression between total length (TL) and morphometric variables of the heart of *Sotalia guianensis* from the Espírito Santo coast.

	p	r (Pearson)	R²
ABH	<0.0001	0.7006	0.4713
CCS	<0.0001	0.7923	0.6866
CDT	0.002	0.5542	0.2805
LVWT	0.037	0.3948	0.1234
RVWT	0.552	-0.1172	-0.0165
IVST	<0.0001	0.7006	-0.0257

Obs.: Stranded events occurred between 2008 and 2014.

Results like this were also reported for A. australis (GUIMARÃES, 2009) and indicate that the heart growth follows body growth. Garri (2006) investigated possible cardiac adaptations of *S. guianensis* to diving but did not perform correlation tests. However, he reported greater interventricular septal thickness in adult dolphins than in calves, therefore, the correlation data of the present study corroborates this finding.

Age showed a significant correlation (Tab. 03) between the circumference at the coronary sulcus (CCS) (r=0.6215), height (ABH) (r=0.596) and the circumference at the distal third (CDT) (r=0.5543), allowing the conclusion that the increase in heart size is proportionally adjusted through time to a certain point (between 10 and 15 years), then there is a stabilization of growth. This time of stabilization of heart growth exceeds the age at which the species is considered physically mature, at seven years old (RAMOS *et al*., 2000).

Table 03: Correlation between age and morphometric variables of the heart of *Sotalia guianensis* from the Espírito Santo coast.

	p	r (Pearson)
ABH	0.0008	0.596
CCS	0.0004	0.6215
CDT	0.002	0.5543
LVWT	0.1018	0.3156
RVWT	0.4605	-0.1453
IVST	0.3958	0.1669

Obs.: Stranded events occurred between 2008 and 2014.

The growth curves of these variables were explained by the Brody’s non-linear model (Fig. 03). The left ventricular wall thickness (LVWT) and right ventricular wall thickness (RVWT) had no linear correlation with age, but non-linear growth models (Fig. 04) explained their growth.

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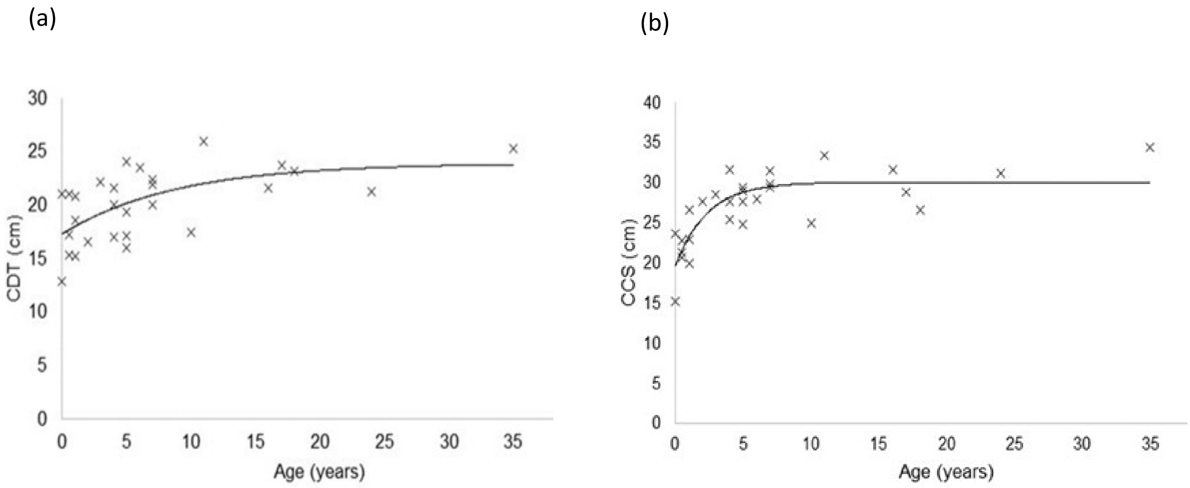

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Figure 03: Brody´s non-linear growth curve of *Sotalia guianensis* from the Espírito Santo coast.

Obs.: (a) circumference at the distal third (CDT); (b) circumference at the coronary sulcus (CCS). Stranded events occurred between 2008 and 2014.

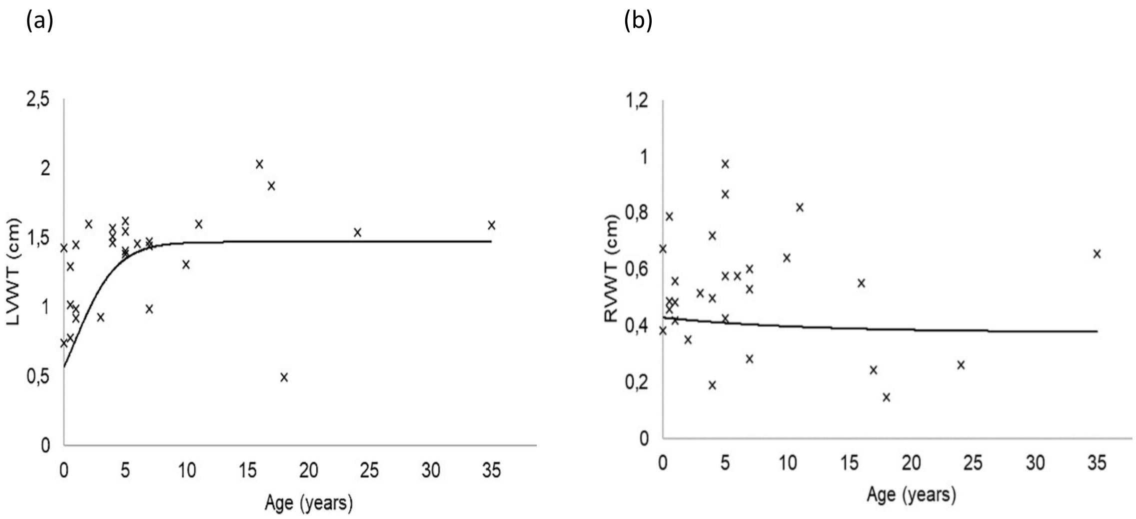

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Figure 04: Growth curves of ventricular wall thickness of *Sotalia guianensis* from the Espírito Santo coast.

Obs.: (a) Logistic non-linear curve of left ventricular wall thickness (LVWT); (b) von Bertalanffy non-linear ofright ventricular wall thickness (RVWT). Stranded events occurred between 2008 and 2014.

The growth RVWT followed a linear trend, thus it is not safe to establish a relationship between this variable and the age of *S. guianensis*. The diving behavior and the duration of the breath-holding change in the species (GARRI, 2006) over the years, hence the increase in the left ventricle thickness until a certain age may be associated with a greater effort by the left ventricular chamber to pump blood to the rest of the dolphin body during a longer diving apnea.

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No significant difference was found between the means of the stereological variables of the left and right ventricles, but the data are shown separately to facilitate the discussion of the results. Tab. 04 shows in detail all the stereological data the present study evaluated. To this date, no other studies were found in the literature on the use of stereology to investigate cetacean hearts. Therefore, similar to the present study, Xavier-Vidal and Madi (1999) found no significant difference between stereological data from male and female human fetuses.

Table 04: Data of volume density, connective tissue volume density and blood vessel volume density (VvV) of the left and right ventricles of *Sotalia guianensis*.

	Left ventricle				**Right ventricle**			
	Mean	**SD**	**Min**	**Max**	**Mean**	**SD**	**Min**	**Max**
VvM	0.5429	0.048	0.44	0.62	0.5575	0.0675	0.43	0.7288
VvC	0.4446	0.046	0.36	0.55	0.4254	0.0595	0.2655	0.56
VvV	0.0128	0.007	0	0.03	0.0174	0.0176	0	0.064

*Volume density (VvM), connective tissue volume density (VvC), blood vessel volume density (VvV), standard deviation (SD). Data of *Sotalia guianensis* from the Espírito Santo coast. Stranded events occurred between 2008 and 2015.

The present study shows 55% of cardiomyocyte, 42% of connective tissue, and 1% of blood vessels in the composition of the myocardium. Østergaard *et al*. (2013) used thedissector method to study the left ventricle of giraffes and found VvM to be approximately 90% in the myocardium and VvV approximately 5%, which indicates a high capacity and need for tissue oxygenation to perform contraction. However, the study did not analyze the right ventricle of these animals. Xavier-Vidal *et al*. (1993) examined the left ventricle of human fetuses and found volume densities corresponding to 67.67% of cardiomyocytes, 25.94% of connective tissue, and 6.39% of blood vessels.

Both ventricles showed the volume densities of cardiomyocytes and connective tissue having a strong and negative correlation. Tab. 05 shows the correlations between the stereological variables of the left and right ventricles.

Table 05: Correlations between cardiomyocyte volume density, connective tissue volume density, and blood vessel volume density of ventricles of *Sotalia guianensis*.

		Left ventricle			**Right ventricle**		
		VvM	**VvC**	**VvV**	**VvM**	**VvC**	**VvV**
VvM	p	-	<0.0001	0.1134	-	<0.0001	0.0007
	r	-	-0.9797	-0.3095	-	-0.9686	-0.6019
VvC	p	-	-	0.5114	-	-	0.0315
	r	-	-	0.1294	-	-	0.4072

*Cardiomyocyte volume density (VvM), connective tissue volume density (VvC), blood vessel volume density (VvV). Data of *Sotalia guianensis* from the Espírito Santo coast. Stranded events occurred between 2008 and 2015.

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The stereological variables in the left ventricle showed no significant correlation with the total length of the examined *S. guianensis* specimens: volume density of cardiomyocytes presented r = 0.1846, volume density of connective tissue presented r = -0.1970, and volume density of blood vessels presented r = -0.0475. In the left ventricle, no significant correlation was found between age and stereological variables, but there was a non-linear relationship between growth and cardiomyocyte volume density (Fig. 05). Changes in the myocardium over time are not very well documented (GRAHAM e BERGMANN, 2016), but it is known that these cells grow mostly by hypertrophy (ÁGUILA, 1998).

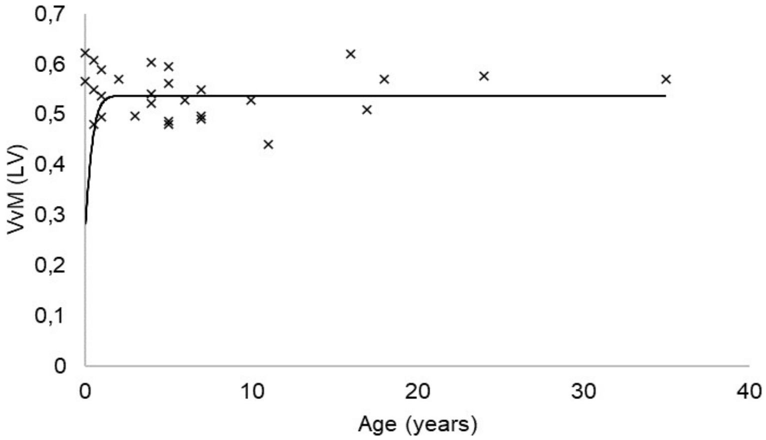

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Figure 05: Logistic non-linear growth curve of cardiomyocyte volume density (VvM) of the left ventricle of *Sotalia guianensis* from the Espírito Santo coast.

Obs.: Stranded events occurred between 2008 and 2014.

In the right ventricle, a correlation between age and cardiomyocyte volume density (VvM) was observed (r = 0.5160), while the relationship between age and connective tissue volume density showed a moderate and negative correlation, r = -0.5036. Bisaillon et al. (1987) suggested that research carried out in cetaceans should pay attention to the right ventricle. Therefore, it is known that the older the specimen, the slightly greater volume of the right ventricular wall will be occupied by cardiomyocytes.

The comparison with the aforementioned studies shows that *S. guianensis* has similar proportions between the volume density of cardiomyocytes and the volume density of the connective tissue. However, further examinations of other cetacean species are necessary to draw better conclusions about composition, growth, and aging of the myocardium in the group.

CONCLUSIONS

The external morphology, histomorphology, and ultrastructure of the heart of *Sotalia guianensis* are similar to those of other mammals, despite being a diver one. The volume density of cardiomyocytes and connective tissue of the right ventricle were related to the aging of the species, which may be associated with changes in the myocardium over the years.

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