THE ANATOMICAL RECORD 297:939–948 (2014)

Morphological Analysis of Lymph Nodes in Odontocetes From North and Northeast Coast of Brazil

FERNANDA MENEZES DE OLIVEIRA E SILVA,[1,2]* JULIANA PLÁCIDO GUIMARÃES,[1,2] JOCIERY EINHARDT VERGARA-PARENTE,[2] VITOR LUZ CARVALHO,[3] ANA CAROLINA OLIVEIRA DE MEIRELLES,[3] MIRIAM MARMONTEL,[4] JULIANA SHIMARA PIRES FERRÃO,[1] AND MARIA ANGELICA MIGLINO[1]

[1]Department of Surgery, School of Veterinary Medicine and Animal Science, University of Sao Paulo (FMVZ/USP), Av. Prof. Dr. Orlando Marques de Paiva, 87, Cidade Universitária, 05508-270, Sao Paulo, SP, Brazil

[2]Center for the Study of Anthropogenic Effects on Marine Resources, Aquatic Mammals Foundation (NEARM/FMA), Av. Tancredo Neves, 5655, Jabotiana, 49095-000, Aracaju, Sergipe, Brazil

[3]Marine Mammals Program, Association for Research and Preservation of Aquatic Ecosystems (PMM/AQUASIS), Av. José de Alencar, 150, Praia de Iparana, SESC Iparana, 61627-010, Caucaia, Ceará, Brazil

[4]Research Group for Amazonian Aquatic Mammals, Mamirauá Institute for Sustainable Development (GPMAA/IDSM), Estr. do Bexiga, 2584, Fonte Boa, 69550-000, Tefé, Amazonas, Brazil

ABSTRACT

The morphology and location of lymph nodes from seven species of Odontocetes, of both sexes and different age groups, were described. All animals were derived from stranding events along the North and Northeastern coasts of Brazil. After the identification of lymph nodes *in situ*, tissue samples were analyzed for light and electron microscopy. Vascular volume density (VVD) and vascular length density (VLD) were evaluated in the mesenteric lymph nodes. Lymph nodes occurred as solitary nodules or in groups, varying in shape and size. In addition to using the nomenclature recommended by *Nomina Anatomica Veterinaria*, new nomenclatures were suggested based on the lymph nodes topography. Lymph nodes were covered by a highly vascularized and innervated capsule of dense connective tissue, below which muscle fibers were observed, inconsistently, in all studied species. There was no difference in VLD among different age groups. However, VVD was higher in adults. Lymph nodes parenchyma was divided into an outer cortex, containing lymph nodules and germinal centers; a paracortical region, transition zone with dense lymphoid tissue; and an inner medulla, composed of small irregular cords of lymphatic tissue, blood vessels, and diffuse lymphoid tissue. Abundant collagen fibers were observed around arteries and arterioles. Germinal centers were more evident and developed in calves and young animals, being more discrete and sparse in adults. The morphology of lymph nodes

Grant sponsor: Sao Paulo Research Foundation (FAPESP); Grant number: 2012/01964-0.

*Correspondence to: Fernanda M. O. Silva, Department of Surgery, School of Veterinary Medicine and Animal Science, University of São Paulo, Av. Prof. Dr. Orlando Marques de Paiva, 87, Cidade Universitária, 05508-270, Sao Paulo, SP, Brazil. E-mail: fernanda_fmos@hotmail.com

Received 22 October 2013; Accepted 16 December 2013.

DOI 10.1002/ar.22871

Published online 22 January 2014 in Wiley Online Library (wileyonlinelibrary.com).

in Odontocetes was typical of that observed in other terrestrial mammals. However, new groups of lymph nodes were described for seven species occurring in the Brazilian coast. Anat Rec, 297:939–948, 2014. © 2014 Wiley Periodicals, Inc.

Key words: lymphoid system; Cetacea; anatomy; microscopy; immune system

INTRODUCTION

The lymphoid system includes all cells, tissues and organs in the body containing aggregates of lymphocytes, the main cells constituting the immune system (Eroschenko, 2008). Lymphocytes can be found as single cells, as lymphoid aggregates, as an accumulation of cells diffusely distributed in the loose connective tissue of several organ systems, or as an organized tissue, that when encapsulated originate the so-called lymphoid organs, such as lymph nodes, thymus, spleen, and tonsils (Press and Landsverk, 2006).

Lymph nodes are sentinels of the immune system due to their strategic distribution throughout the body, allowing a fast and effective immune response (Blum and Pabst, 2006; Hoorweg and Cupedo, 2008). Lymphoid organs form a highly complex network, interconnecting the immune, lymphatic and hematopoietic systems in a coordinated way, enabling an effective interaction of lymphocytes and antigen-presenting cells, which bring the antigenic substances from adjacent tissues to lymphatic centers (Van de Pavert and Mebius, 2010), thereby stimulating the innate and adaptive immunity (Janeway and Medzhitov, 2002; Parham, 2009).

Because of an increasing interest in the conservation of terrestrial and aquatic environments, researches addressing the immune system of wild species have been developed with the aim to monitor the conservation status of their habitat (Babayan et al., 2011; Pedersen and Babayan, 2011). The marine ecosystem health is a relatively new and understudied subject, largely due to its biological diversity and limiting factors that prevent a better knowledge of the ecology of habitat (Aguirre et al., 2002; Halpern et al., 2012; Bourlat et al., 2013).

Marine environments are under constant anthropogenic pressure which clearly affects aquatic animals both directly and indirectly (Epstein, 2002). There is evidence that environmental contaminants have immunotoxic and immunosuppressive effects on whales, dolphins, and seals (Beineke et al., 2005, 2006) due to bioaccumulation of persistent chemicals (Jepson et al., 1999; Siebert et al., 1999).

For most cetaceans, immunological reports are old and scarce. Simpson and Gardner (1972) obtained histological data in certain species of whales (toothed and baleen) and pinnipeds. Green (1972) and Harrison (1974) initiated anatomical and functional studies on different species of cetaceans and pinnipeds, subsequently reported by Pabst et al. (1999). Studies on the morphology of lymphoid organs (Romano et al., 1993; Cowan and Smith, 1999; Vukovic et al., 2005) and their immune system functioning (Beineke et al., 2010) were often limited to a particular species. Despite all efforts to obtain more information about the immune system of small and large cetaceans, much remains unknown (Beineke et al., 2010).

Little is known about the species occurring in Brazil regarding their immune system morphology. In order to assist in studies on the immune system of cetaceans, a prior knowledge of lymphoid organs is necessary, especially with the increasing exploitation of the Brazilian coast by oil industries. Therefore, this study aimed to describe the location and morphology of the lymph nodes in seven species of Odontocetes from both sexes and different age groups, stranded along the North and Northeastern coasts of Brazil.

MATERIAL AND METHODS

Animals and Samples

Specimens of Guiana dolphin (*Sotalia guianensis*), Tucuxi (*Sotalia fluviatilis*), Clymene dolphin (*Stenella clymene*), Spinner dolphin (*Stenella longirostris*), Boto (*Inia geoffrensis*), Melon-headed whale (*Peponocephala electra*), and Short-finned Pilot Whale (*Globicephala macrorhynchus*), of both sexes and different age groups (calf, young, and adult), stranded along the North and Northeastern coasts were sampled under Sisbio Permit n° 37369-1, Animal Bioethics Protocol 2571/2012 (Table 1). Animals' age groups were defined based on dentition and on the body measurements when teeth analyses were not possible to be performed.

Biological samples were obtained and maintained in the research institutions Aquatic Mammals Foundation – FMA (Sergipe, Brazil), Association for Research and Preservation of Aquatic Ecosystems – AQUASIS (Ceará, Brazil), and Mamirauá Institute for Sustainable Development – IDSM (Amazonas, Brazil).

TABLE 1. List of collected samples by species, according to animals' sex and age group (calf:young:adult)[a]

Species	Number of males/age group	Number of females/age group	Total number of animals evaluated
Sotalia guianensis	2:1:1	2:3:3	12
Sotalia fluviatilis	0:3:1	0:2:1	07
Stenella clymene	0:2:6	0:0:5	13
Stenella longirostris	0:1:1	1:0:2	05
Peponocephala electra	–	0:9:1	10
Inia geoffrensis	0:1:1	0:1:1	04
Globicephala macrorhynchus	1:2:0	–	03

[a]Age groups were defined based on animals' dentition and body measurements, according to Jefferson et al. (1993).

TABLE 2. Lymph nodes found in seven odontocete species, regarding their location, quantity, and size

Lymph nodes	Location	Species[a]	Quantity and average size[b]
Parotid (*Lymphonodi parotidei*)	Superficial, cranially to the parotid gland	*Sg, Sf, Sc, Sl*	1 lymph node in both antimeres; 1.5cm
Mandibular (*Lymphonodi mandibulares*)	Close to the mandible angle	*Sg, Sf, Sc, Sl, Pe, Gm, Ig*	2-4 lymph nodes in both antimeres; 1.0cm
Hyoid (*Lymphonodi hyoideus*)	Base of the tongue, near the hyoid bone	*Sg, Sf*	1 lymph node; 1.0cm
Cervical (superficial and deep) (*Lymphonoduli cervicale superficialis et profunda*)	Neck region, ventral and dorsally, between the fascia and muscles	*Sg, Sf, Sc, Sl, Pe, Gm, Ig*	1 -6 lymph nodes in both antimeres; 0.5-1.0cm Large lymphoid clusters (from the merging of several nodes), originating a single nodular mass.
Prescapular	Cranial margin of scapula. Often described as cervical (superficial)	*Sg, Pe, Gm*	1 lymph node in both antimeres; up to 1.2cm
Mediastinal (*Lymphonodi mediastinico*)	Close to the aortic arch; may be associated with thymus, thyroid, base of the heart and the esophagus entire length	*Sg, Sf, Sc, Sl, Pe, Gm, Ig*	4-8 lymph nodes; 1.0-1.5cm
Respiratory lymphocenter			
Hilar (*Lymphonodi tracheobronchiales*)	Associated to the main bronchus and its insertion into the pulmonary tissue.	*Sg, Sf, Sc, Sl, Pe, Gm, Ig*	1-4 lymph nodes; up to 2.0cm
Pulmonary (*Lymphonodi pulmonales*)	Usually located ventrally throughout the free border of the lung	*Sg, Sf, Sc, Sl, Pe, Gm, Ig*	1-3 lymph nodes in each lung; up to 6.0cm
Diaphragmatic	Attached to the diaphragm, it is located around the oesophagus and diaphragmatic hiatus. When in groups, it can extend laterally and ventrally between the pericardium and the diaphragm.	*Sg, Sf, Sc, Sl, Pe, Gm, Ig*	1-3 lymph nodes; 1.0-3.0cm
Gastrointestinal lymphocenter			
Gastric (*Lymphonodi gastrici*)	Close to the convex greater curvature of first and second stomachs	*Sg, Sf, Sc, Sl, Pe, Gm, Ig*	1-3 lymph nodes; 2.0-3.0cm
Mesenteric (*Lymphonodi mesenterici)*	A large mass of joined lymph nodes that irregularly follows the mesenteric artery	*Sg, Sf, Sc, Sl, Pe, Gm, Ig*	15-23 lymph nodes throughout small and large intestines; 1.0 -3.0cm
Mesocolic/Colic (*Lymphonodi colici*)	Usually attached to the intestinal wall, being present in the colon mesentery	*Sg, Sf, Sc, Sl, Pe, Gm, Ig*	1-3 lymph nodes; up to 1.5cm
Hepatic hilar (*Lymphonodi hepatici*)	Close to the liver hilus	*Sg, Sf, Sc, Sl, Pe, Gm, Ig*	1-2 lymph nodes; up to 2.0cm
Pancreatic	Attached to the pancreas and covered by its serous membrane	*Sg, Sf*	1 lymph node; up to 1.5cm
Renal (hilar) (*Lymphonodi renales*)	Close to the renal pelvis, attached to the capsule	*Sg, Sf, Sc, Sl, Pe, Gm, Ig*	1-2 lymph nodes in each kidney; 1.0-1.5cm
Pelvic	Attached to the abdominal wall, not covered by the peritoneum	*Sg, Sf, Sc, Sl, Pe, Gm, Ig*	2-5 lymph nodes; 1.5cm
Male and female genital (*Lymphonodi ovaricus and Lymphonodi testicularis*)	Male: close to the testicular artery and/or vein; Female: broad ligament (uterus) and proper ligament (ovary)	*Sg, Sf, Sc, Sl, Pe, Gm, Ig*	2-4 lymph nodes; 1.5cm

[a]*Sg, Sotalia guianensis; Sf, Sotalia fluviatilis; Sc, Stenella clymene; Sl, Stenella longirostris; Pe, Peponocephala electra; Gm, Globicephala macrorhynchus; Ig, Inia geoffrensis*.
[b]Average size, based on the diameter (larger diameter in elongated lymph nodes).

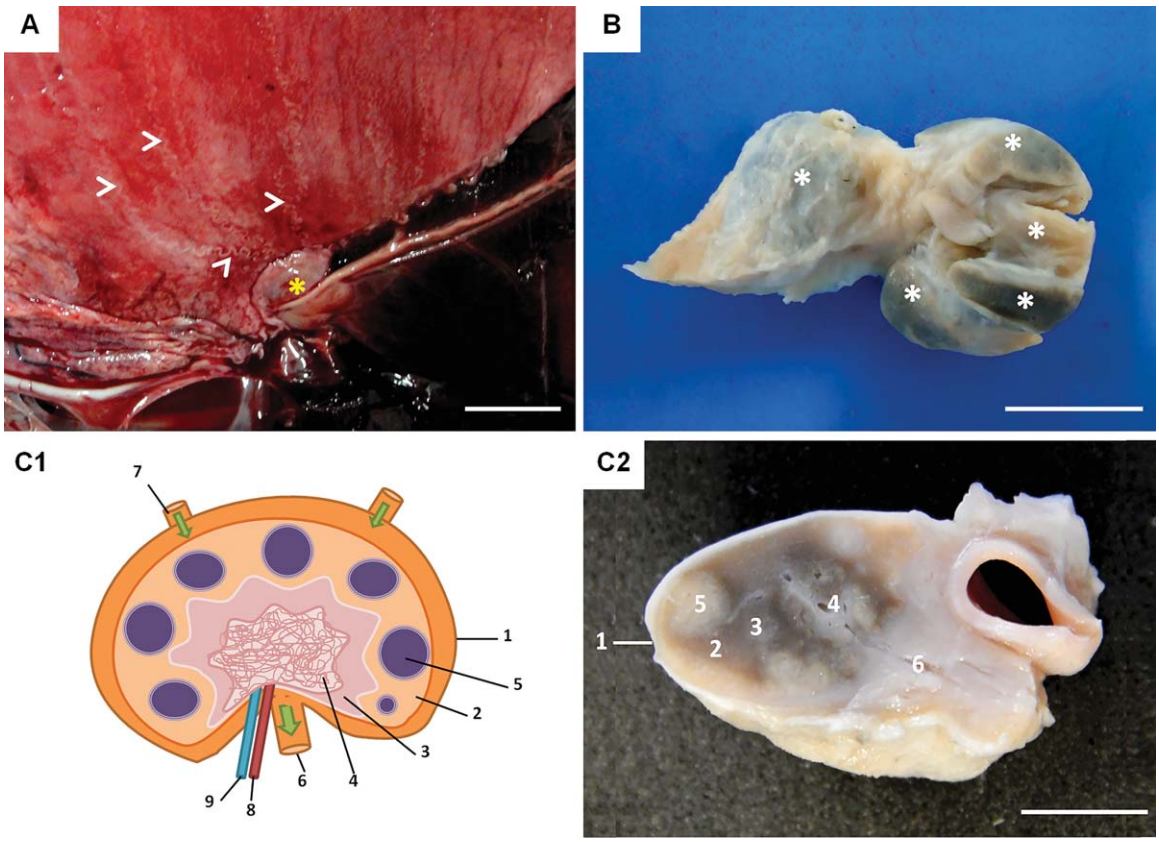

Fig. 1. **A**: Pulmonary lymph node (*) from a *Stenella clymene* calf. Lymphatic vessels indicated by arrowheads. Bar: 2 cm. **B**: Lymph nodes from an adult *Sotalia fluviatilis*. Group consists of five lymph nodes (*). Bar: 1 cm. C1. Schematic design of a lymph node. C2. Cervical lymph node from a young *Inia geoffrensis*. Bar: 1 cm. 1. Capsule; 2. Cortex; 3. Paracortex; 4. Medula; 5. Lymphoid nodule; 6. Efferent lymphatic vessel; 7. Afferent lymphatic vessel; 8. Artery; 9. Vein.

Necropsy and Macroscopic Analysis

Despite uncertainties inherent in determining the stage of decomposition, animals or carcasses were assigned to one of five basic categories (CODES 2-5; Geraci and Lounsbury, 2005). Only specimens CODES 2 and 3 were used.

Animals were positioned in the right lateral decubitus position and incision was made on the left flank for removal of skin and adipose tissue. Two additional cuts were made perpendicular to the middle of the flank on the umbilical and anal regions. A fourth cut was made parallel to the body, forming a quadrilateral (window) to gain access to the abdominal viscera and a fifth cut was made allowing access to the thoracic organs.

For macroscopic analysis, both abdominal and thoracic cavities were examined for the location of lymph nodes *in situ*. Lymph nodes were identified, nominated, photographed, and dissected. After removal, all lymph nodes were washed, cut and fixed in 10% formalin solution for light and scanning electron microscopy and in modified Karnovsky solution (2.5% of glutaraldehyde and 2% paraformaldehyde in 0.1 M sodium phosphate buffer solution) for transmission electron microscopy.

Microscopic Analysis

For light microscopy, lymph nodes were rinsed in distilled water after fixation, dehydrated in increasing ethanol solutions (70–100%), diaphanized in xylene, and embedded in Paraplast®. Sections (6 μm thick) were stained with Hematoxylin-eosin, Masson's Trichrome and Malory's stain. Slides were examined using a light microscope (Nikon Eclipse E-800).

For scanning electron microscopy, all samples were rinsed in distilled water (3 × 10 min, under rotation). Then, samples were dehydrated in ascending series of ethanol under rotation [70% (1 × 15 min); 80% (1 × 15 min); 90% (1 × 15 min) and 100% (3 × 30 min)] and critical point dried using a Blazers CPD 030 device. Thereafter, samples were mounted on metal stubs using carbon glue, coated with gold by sputting in Balzers-040 SDC and analyzed by using a scanning electron microscope (LEO 435 VP).

For transmission electron microscopy, samples were sectioned into smaller fragments and post-fixed in 1% osmium tetroxide solution with 0.1 M sodium phosphate buffer solution at 4°C for 2 hr. Then, samples were dehydrated in increasing series of ethanol (60% to absolute) and embedded in a propylene oxide solution and Spurr®

resin. Later, the solution was replaced by pure resin and included into rubber molds and placed in an oven for 36 hr for polymerization. After that, screening of all blocks was performed and 1–3 μm sections were obtained using a Reichert Ultra Cut® microtome, which were stained with Toluidine blue. Slides were examined under a light microscope in order to locate the areas of interest. Thereafter, 90 ηm ultrathin sections were obtained using a diamond knife and mounted on copper grids (200-mesh grid), which were counterstained with 4% uranyl acetate and 0.4% lead citrate solutions. Grids were examined using a transmission electron microscope (JEOL JSM-6100).

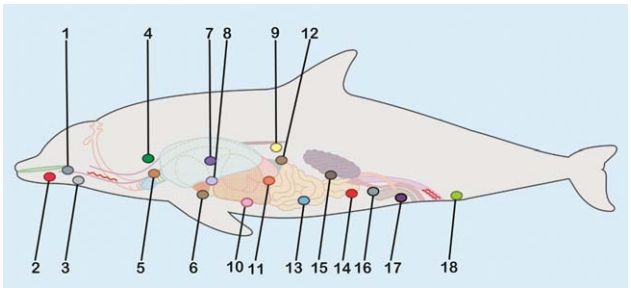

Fig. 2. Lymph nodes location in a female dolphin (Odontoceti). 1. Parotid; 2. Mandibular; 3. Hyoid; 4. Cervical; 5. Prescapular; 6. Mediastinal; 7. Pulmonary; 8. Hilar; 9. Diaphragmatic; 10. Gastric; 11. Hepatic hilar; 12. Pancreatic; 13. Mesenteric; 14. Colic; 15. Renal; 16 and 17. Genital; 18. Pelvic.

Stereological Analysis

Mesenteric lymph nodes were evaluated by stereology in order to evaluate the vascular volume density (VVD) and vascular length density (VLD). These lymph nodes were chosen due to their large amounts collected during necropsies and occurrence in all species studied, providing a better statistical analysis.

Lymph node samples, fixed in 10% formalin solution, were cut and fragments were randomly selected for light microscopic examination. Sections (6 μm thick) were not obtained in sequence, 5 cuts out of every 10 being

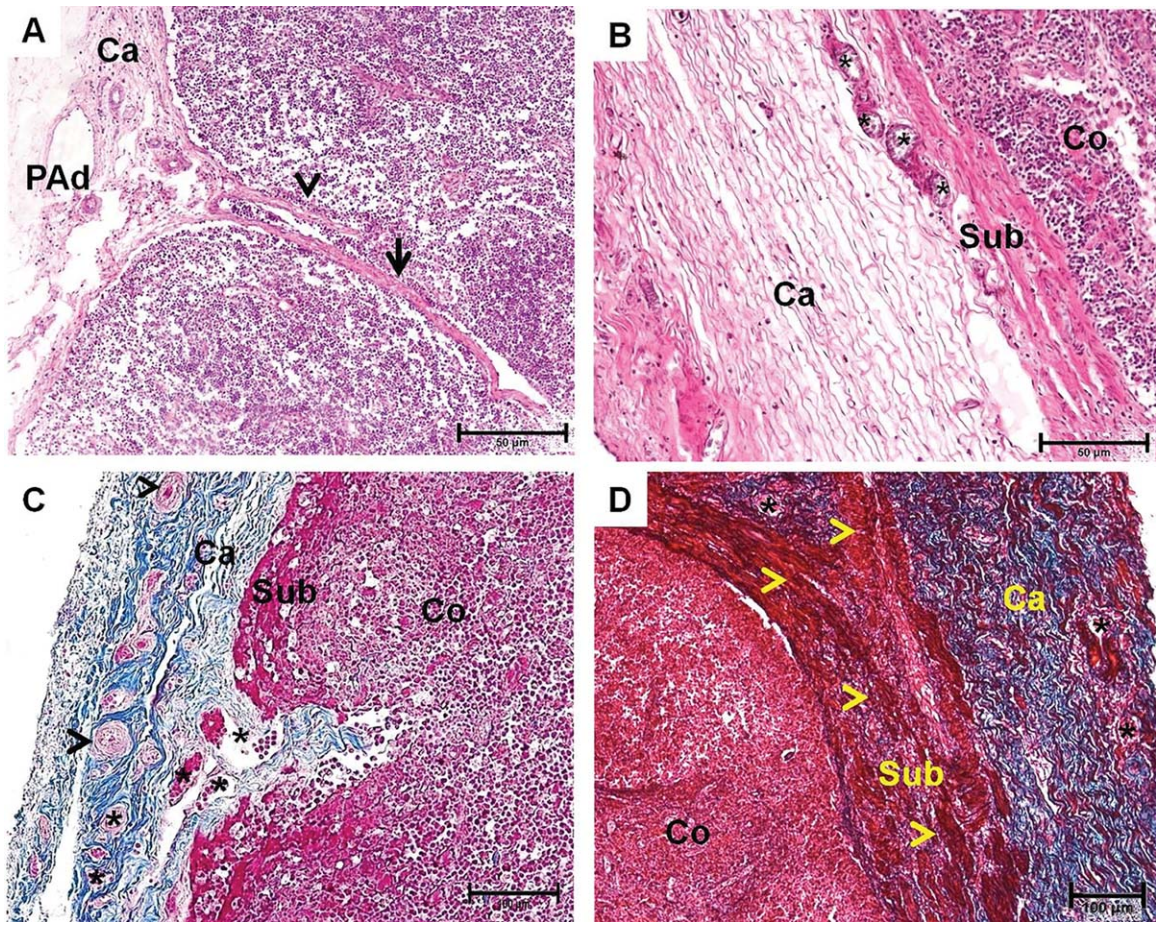

Fig. 3. Lymph nodes from *Sotalia guianensis* calves. Subcapsular zone (Sub) located between the capsule (Ca) and cortex (Co). **A**: Cervical lymph node. Thin capsule (Ca) surrounded by a pericapsular adipose tissue (PAd). Superficial and deep trabeculae (arrowhead and arrow, respectively). 40x. Hematoxylin-Eosin (HE). **B**: Mediastinal lymph node. Subcapsular sinus (Sub) composed basically by smooth muscle fibers, between a thick capsule (Cap) and the cortex (Co). 40x. HE. **C**: Mesenteric lymph node. Capsule with the presence of neuronal bundles (arrowheads) and numerous blood vessels (*). 20x. Masson's Trichrome. **D**: Renal lymph node. Smooth muscle fibers under the capsule (arrowheads). Blood vessels (*). 10x. Mallory's stain.

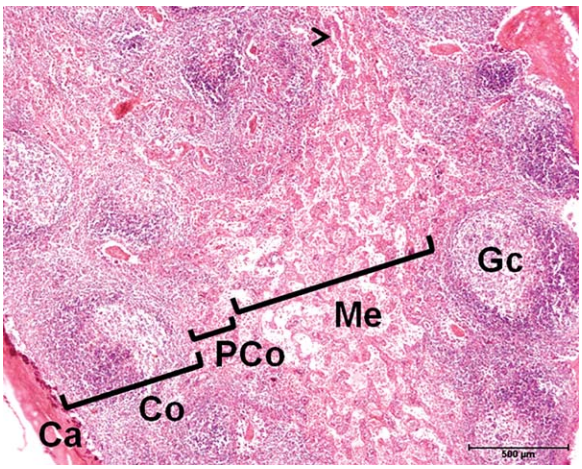

Fig. 4. Photomicrograph of a mediastinal lymph node from a young *Peponocephala electra*. Capsule (Ca), followed by a cortex (Co) with defined germinal centers (Gc), paracortical zone (PCo) and medulla (Me), formed by lymphoid cords, directed towards the hilus (arrowhead). 4x. Hematoxylin-eosin.

discarded. Slides were stained with Hematoxylin-Eosin and examined using a light microscope (Nikon Eclipse E-800). Images from lymph node capsules were analyzed by the overlapping program Stepanizer (Tschanz et al., 2011).

Blood and lymphatic vessels were counted in a known area called test area (TA). In order to avoid overestimation of the vessels, a rule was established: in the field to be counted, defined as a square, two lines (left and bottom edge of the square) were considered as prohibited for counting vessels touching them, and two lines (right and top edge of the square) were considered as permitted for counting vessels.

The variable VVD, expressed as percentage (%), was estimated according to the formula: VVD = Tcp/Ttp [Tcp: number of test points which touch the vessels; Ttp: total number of test points in the system test (100)]. The variable VLD, expressed in mm/mm^3, was estimated according to the formula: $VLD = 2^{(N/TA)}$ (N: number of blood vessels within the test area; TA: test area ($45{,}692.65\ \mu m^2$).

Data were normally distributed, therefore parametric, and One Way ANOVA followed by Tukey-Kramer post test for multiple comparisons (GraphPad Prism 5 Software Inc., San Diego, USA) were applied for comparisons among different age groups (calf, young and adult). Results were presented as mean ± standard deviation, and were considered significant when $P < 0.05$.

RESULTS

Macroscopic Analysis

Most lymph nodes described in this study were found consistently in all species except parotid, hyoid, prescapular, and pancreatic lymph nodes (Table 2). The lymph nodes were presented as nodules or masses of dense lymphocytic aggregates of varied shapes (elongated and rounded), located throughout an extensive drainage system (Fig. 1A,B). Lymphatic vessels were dilated and entered the nodules, blending with the lymph nodes. The parenchyma of the lymph nodes was arranged as a cortex composed of lymphatic nodules, and a medulla (Fig. 1C,D).

All lymph nodes, independent of their location, were surrounded by a capsule of connective tissue with variable thickness, which varied according to the anatomical location. The mesenteric, mediastinal, and renal lymph nodes had a thicker capsule, compared to the other lymph nodes and greater amounts of subcapsular smooth muscle. The respiratory lymphocenter, other than those specified above, had a thin layer of dense connective tissue around it.

Lymph nodes occurred in specific areas, solitary or in groups. The designation "lymphocenter" was applied to groups of lymph nodes occurring consistently in the same region. The nomenclature recommended by the *Nomina Anatomica Veterinaria* (NAV) was preconized. However, the analogy was not always possible for cetaceans. Thus, new nomenclatures were suggested, based on the topographic relationship between the lymph nodes and the anatomical structure related to them. We also take as reference the nomenclature used by Cowan and Smith (1999) for *Tursiops truncatus*. All lymph nodes mapping is illustrated in Fig. 2 and is described in Table 2. No difference was observed between males and females for gross anatomy of any lymph nodes evaluated.

Microscopy

All lymph nodes examined were covered by a collagen capsule of dense connective tissue, below which there was a subcapsular sinus, composed of loose connective tissue. Inconsistently, it was possible to observe smooth muscle fibers in the subcapsular zone in all species studied (Fig. 3B,D). From the capsule, the connective tissue trabeculae were extended into the interior of the lymph nodes (parenchyma) for a variable depth (Fig. 3A), being innervated and highly vascularized (Fig. 3C). The capsule thickness varied according to the lymph nodes anatomical location, being thicker in the renal and visceral lymph nodes (mesenteric and mediastinal, Fig. 3).

The parenchyma of the lymph node was divided into a cortical region (cortex), containing lymphoid follicles or nodules, and a medullary region (medulla), composed of small lymphocytic cords, blood vessels and diffuse lymphoid tissue (Fig. 4). Between the cortex and the medulla a transition area of dense lymphoid tissue was observed, called the paracortical zone. The medulla, a light-staining tissue due to lower cell density, was formed by the medullary cords and sinuses. These irregular and thin cords of dense lymphoid tissue branched from the paracortical area and extended into the inner area of the lymph nodes. Elongated and anastomosed cords delimited areas with lower cellularization and the presence of loose lymphoid tissue, called the medullary sinus. Dilated medullary sinuses drained the lymph nodes from the cortical zone, through the medullary cords towards the hilus, from which the efferent lymphatic vessels exit and drained the lymph nodes.

The cortex was located just below the subcapsular zone, containing lymphoid follicles (Fig. 5A,B), which generally were well differentiated into two zones: a peripheral area (mantle zone, Fig. 5C), containing a larger quantity of small and medium-sized lymphocytes

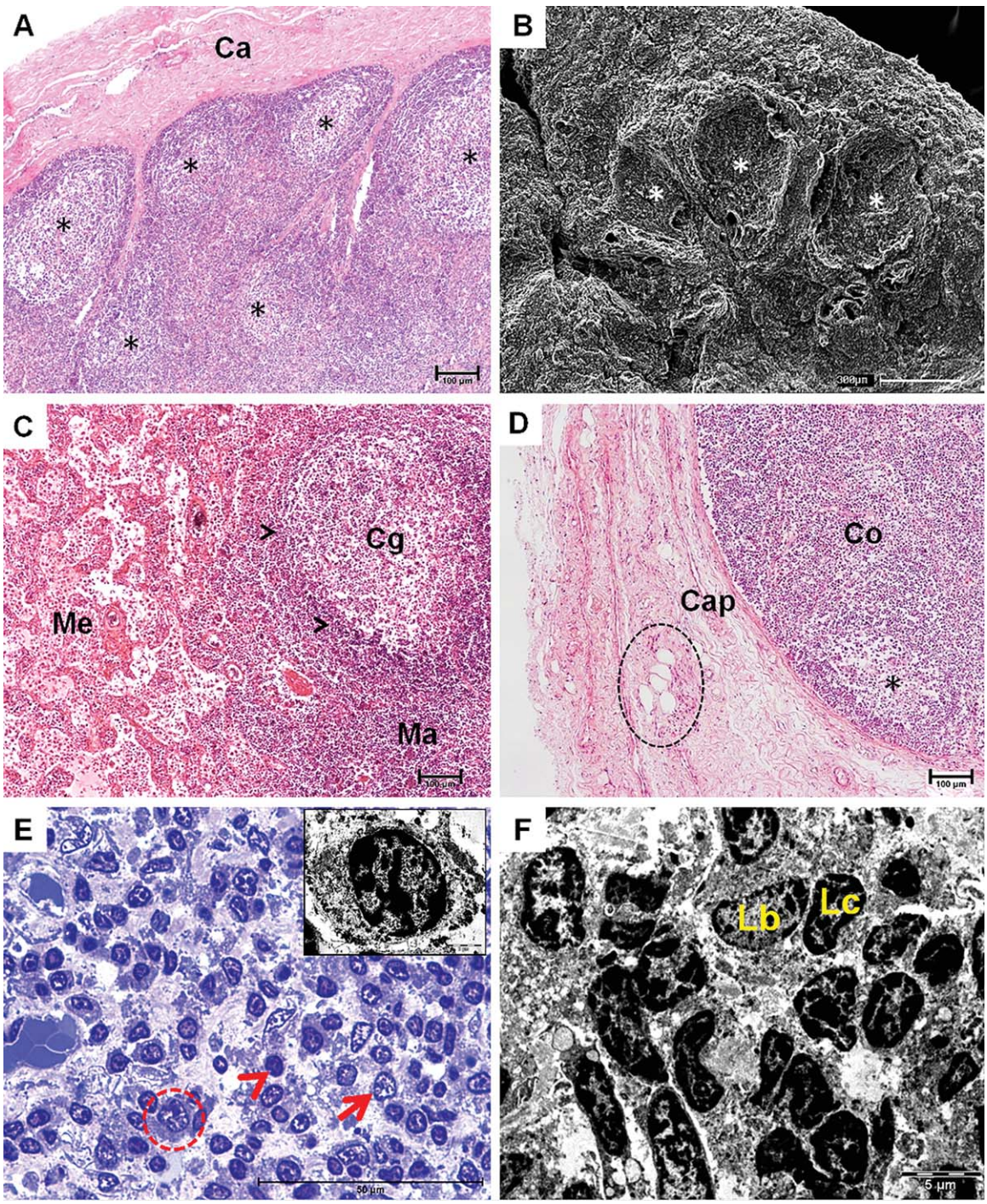

Fig. 5. **A**: Mesenteric lymph node from a young *Stenella clymene*. Thick capsule (Cap) and lymphoid follicles (*). 10x. Hematoxilin-eosin (HE). **B**: Scanning electron photomicrograph of a lymph node from a young *Sotalia fluviatilis*. Lymphoid follicles (*). **C**: Mediastinal lymph node from a young *Globicephala macrorhynchus*. Lymphoid follicles with their germinal center (Cg) delimited by the mantle zone (Ma). Medulla (Me) consisting of medullary cords and sinuses. 10x. HE. **D**: Pelvic lymph node from an adult *Sotalia fluviatilis*. Thick capsule (Cap) with the presence of pericapsular adipose tissue (highlighted). Cortex (Co) with a discrete germinal center (*). 10x. HE. **E, F**: Pulmonary lymph node from a young *Peponocephala electra*. **E**: Semi-thin section. Presence of lymphoblasts (large cell with loose chromatin; arrow) and nonactivated B-lymphocytes (arrowhead). 100x. Toluidine blue. Highlighted area: Transmission electron photomicrograph of a macrophage. 8.900x. **F**: Transmission electron photomicrograph of a lymphoblast (Lb) surrounded by numerous lymphocytes (Lc). 8.900X.

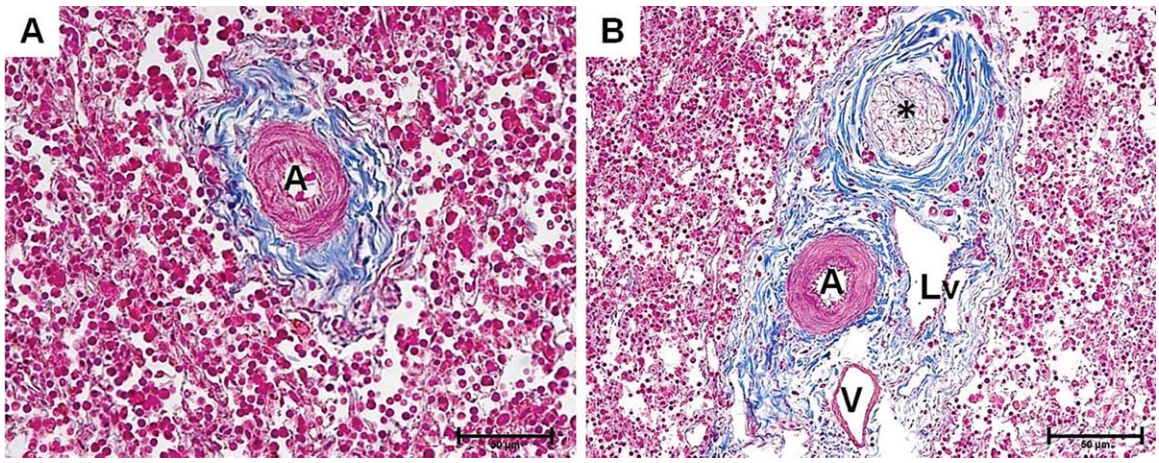

Fig. 6. Photomicrograph of the cortical region of lymph nodes from a young *Inia geoffrensis*. **A:** Pulmonary lymph node. Artery (A) surrounded by collagen fibers. **B**: Mediastinal lymph node. Cortical region. Vein (V), artery (A), lymphatic vessels (Lv), and neuronal bundle (*) surrounded by collagen fibers. A, B. 40x. Masson's Trichrome.

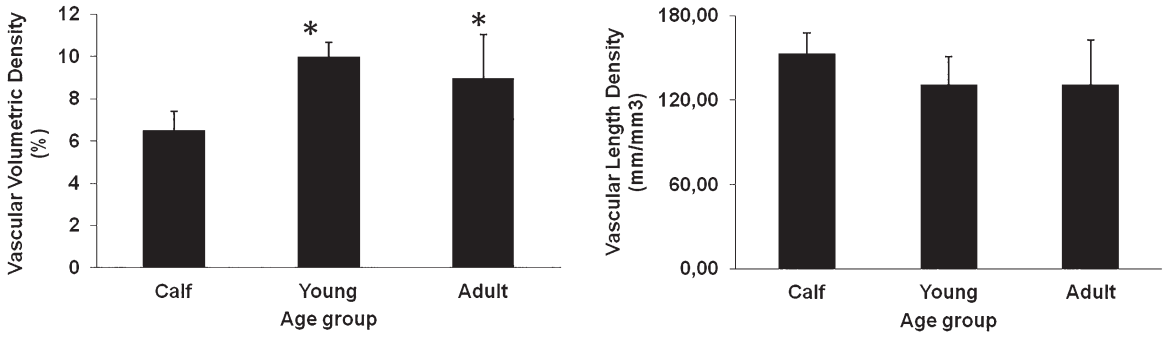

Fig. 7. Vascular volumetric density (%) and vascular length density (mm/mm3) in the capsule of mesenteric lymph nodes from calf, young, and adult Odontocetes.

(nonactivated B lymphocytes; Fig. 5E,F), and a central area called the germinal center (Fig. 5C,D), characterized by the presence of loose chromatin in the nuclei of large lymphocytes (lymphoblasts) (Fig. 5E,F). The outer region was dark-stained due to the accumulation of lymphocytes in this area, forming a "crown" and delimiting the germinal center (Fig. 5C).

In all studied species, the germinal centers were more evident and well developed in calves and young animals (Fig. 5A,C), being more discrete and sparse in adults (Fig. 5D). Regardless of the age group, all arteries and/or group of vessels (artery, vein, and lymphatic vessels) were surrounded by a layer of collagen fibers (Fig. 6A,B). No microscopic difference was observed between males and females for any lymph nodes in all species evaluated.

There was no difference among groups for VLD (P = 0.9717). However, a significant difference was observed for VVD between the young and adult groups (P = 0.0421) (Fig. 7).

DISCUSSION

Whales belong to the most diverse existing group of aquatic mammals and due to their particular anatomical features, such as a short neck and no pelvic bones, the location of the lymph nodes are different than described for other species (Simpson and Gardner, 1972).

A respiratory lymphocenter and the pancreatic lymph nodes were described in our study, having been poorly described in the literature (Cowan and Smith, 1999). This can be explained by the difficulty to visualize and consequently identifying and dissecting these structures during necropsies (Rommel and Lowenstine, 2001). The "lymphocenter" nomenclature used in our study was also suggested by Cowan and Smith (1999) in a study on *Tursiops truncatus*. A group of lymph nodes described in our study and not previously reported in the literature were the renal and male and female genital lymph nodes.

The capsule thickness varied according to the anatomical location of the lymph nodes and no correlation between gender and age groups was observed. Such differences have previously been described for some species of terrestrial mammals (Bacha and Bacha, 2012; Junqueira and Carneiro, 2013) in which the architecture of the lymph nodes was constant. The mesenteric, renal, and mediastinal lymph nodes had thicker capsules when compared to other nodes, with greater amount of smooth muscle fibers in their subcapsular sinuses, similar to

that described by Vukovic et al. (2005). In our study smooth muscle fibers were found inconsistently in different lymph nodes in all species evaluated. All lymph nodes from the respiratory lymphocenter, other than those specified above, were surrounded by a thin layer of dense connective tissue.

Cowan and Smith (1999) suggested that the visceral lymph nodes may be considered as contractible organs by maximizing the blood circulation in the intestinal area and hence the filtration of lymph. This description can explain the fact that we observed a greater amount of muscle fibers in the mesenteric, mediastinal, and renal lymph nodes. A significant difference in the amount of blood vessels greatly improved the blood circulation in the intestinal area in older animals, thus enhancing their immune response. Furthermore, the large amount of blood vessels running through the capsule and collagen fibers in the medullary area of the lymph nodes support the data reported by these authors.

The lymph nodes architecture was consistent with findings in other cetaceans. The cortex-medulla pattern followed the description for *Tursiops truncatus, Stenella longirostris, Delphinus delphis, Inia geoffrensis*, and *Globicephala sp*. (Simpson and Gardner, 1972; Cowan and Smith, 1999; Vukovic et al., 2005), and *Delphinapterus leucas* (Romano et al., 1993). In the literature, only *Delphinus delphis* and *Phocoena phocoena* presented a nodal morphology, which was similar to that observed in pigs and horses by Banks (1998), Bacha and Bacha (2012), and Samuelson (2006), in which the cortical and medullary areas had an inverse pattern to that observed in other terrestrial mammals.

Simpson and Gardner reported (1972) an apparent hypoactivity of secondary lymphoid tissues in aquatic mammals, shown by the low number of evident germinal centers. We proposed that this characteristic does not necessarily mean that these animals have an impaired immune response, as germinal centers in animals with infectious diseases were evident and pronounced. Thus, it is suggested that this hypoactivity may be related to the age and health status and the absence of enough antigenic stimulation.

In some studies, such hypoactivity can be extended to lymph nodes, which are considered as the main secondary lymphoid organs in mammals. In *Tursiops truncatus*, the composition of these organs is closely related to aging, since in younger animals the number of germinal centers is higher than in adults (Cavagnolo, 1979). The lack of follicular development in older animals indicates low immune function (Simpson and Gardner, 1972; Romano et al., 1993), fact that still remains uncertain for other aquatic mammals species.

In our opinion this hypo-responsiveness can be applied to lymph nodes only regarding the age of animals, since in older animals these organs had more discrete germinal centers. We observed a large amount of germinal centers in calves and young animals. This finding can be explained by the fact that, at these age groups, the immune system is developing and highly active. In older animals, although these centers were less evident, it was still possible to see some lymphatic nodules around the cortical surface of the lymph nodes, especially in the mesenteric lymph nodes. These findings were similar to that observed by Clark et al. (2005) when evaluating the involution of secondary lymphoid organs in older animals.

Our findings suggest that the morphology of the lymph nodes in Odontocetes is typical of that observed in other terrestrial mammals. However, new groups of lymph nodes have been described for the species occurring in the Brazilian coast. The lymph nodes showed similar architecture to that described in the literature for terrestrial mammals, having no inverse structure as observed in other species of Odontocetes.

ACKNOWLEDGMENTS

The authors thank the amazing staff of the Aquatic Mammals Foundation (FMA), the Association for Research and Preservation of Aquatic Ecosystems (AQUASIS), and the Mamirauá Institute for Sustainable Development (IDSM) for their dedication in sample collection and assessment. They appreciate the collaboration of J. E. Sampaio in the preparation of schematic mapping of lymph nodes used in this study.

This article employed data generated by Programa Regional de Monitoramento de Encalhes e Anomalidades na Área de Abrangência da Bacia Sergipe-Alagoas carried out by the Fundação Mamíferos Aquáticos and Petrobras, in partnership with Projeto Tamar/ICMBio, as a mitigating measure of the Federal Environmental Licensing conducted by the Brazilian Environmental Agency (IBAMA). Samples collected by IDSM's Aquavert Project were supported by Petrobras through its Petrobras Ambiental Program and Aquasis samples were collected by Projeto Manatí, sponsored by Petrobras.

LITERATURE CITED

Aguirre AA, O'Hara TM, Spraker TR, Jessup DA. 2002. Monitoring the health and conservation of marine mammals, sea turtles and their environment. In: Aguirre AA, Ostfeld RS, Tabor GM, House C, Pearl MC, editors. Conservation medicine: ecological health in practice. New York: Oxford University Press. p 79–94.

Babayan SA, Allen JE, Bradley JE, Geuking MB, Graham AL, Grencis RK, Kaufman J, McCoy KD, Paterson S, Smith KG, Turnbaugh PJ, Viney ME, Maizels RM, Pedersen AB. 2011. Wild immunology: converging on the real world. Ann NY Acad Sci 1236:17–29.

Bacha WJ, Bacha LM. 2012. Color atlas of veterinary histology. 3rd ed. New Jersey: Wiley.

Banks WJ. 1998. Histologia Veterinária Aplicada. 3rd ed. São Paulo: Editora Manole.

Beineke A, Siebert U, McLachlan M, Bruhn R, Thron K, Failing K, Müller G, Baumgärtner W. 2005. Investigations of the potential influence of environmental contaminants on the thymus and spleen of harbor porpoises (*Phocoena phocoena*). Environ Sci Technol 39:3933–3938.

Beineke A, Siebert U, Baumgartner W. 2006. The immune system of marine mammals. Part 2: Morphology, immunophenotyping and pathology of lymphoid organs. Tierarztl Prax Ausg K Klientiere Heimtiere 34:201–207.

Beineke A, Siebert U, Wohlsein P, Baumgärtner W. 2010. Immunology of whales and dolphins. Vet Immunol Immunopathol 133:81–94.

Blum KS, Pabst R. 2006. Keystones in lymph node development. J Anat 209:585–595.

Bourlat SJ, Borja A, Gilbert J, Taylor MI, Davies N, Weisberg SB, Griffith JF, Lettieri T, Field D, Benzie J, Glöckner FO, Rodríguez-Ezpeleta N, Faith DP, Bean TP, Obst M. 2013. Genomics in marine monitoring: new opportunities for assessing marine health status. Mar Pollut Bull 74:19–31.

Cavagnolo RZ. 1979. The immunology of marine mammals. Dev Comp Immunol 3:245–257.

Clark LS, Turner JP, Cowan DF. 2005. Involution of lymphoid organs in bottlenose dolphins (*Tursiops truncatus*) from the western Gulf of Mexico: implications for life in an aquatic environment. Anat Rec A Discov Mol Cell Evol Biol 282:67–73.

Cowan DF, Smith TL. 1999. Morphology of the lymphoid organs of the bottlenose dolphin, *Tursiops truncatus*. J Anat 194:505–517.

Epstein PR. 2002. Biodiversity, Climate change and emerging infectious diseases. In: Aguirre AA, Ostfeld RS, Tabor GM, House C, Pearl MC, editors. Conservation medicine: ecological health in practice. New York: Oxford University Press. p 27–38.

Eroschenko VP. 2008. Di Fiore's atlas of histology with functional correlations. Philadelphia: Lippincott Williams & Wilkins.

Geraci JR, Lounsbury VJ. 2005. Marine mammals ashore: a Field Guide for Strandings. Baltimore: National Aquarium in Baltimore.

Green RF. 1972. Observations on the anatomy of some cetaceans and pinnipeds. In: Ridgway SH, editor. Mammals of the Sea: Biology and Medicine. Springfield: Charles C. Thomas, p 247–295.

Halpern BS, Longo C, Hardy D, McLeod KL, Samhouri JF, Katona SK, Kleisner K, Lester SE, O'Leary J, Ranelletti M, Rosenberg AA, Scarborough C, Selig ER, Best BD, Brumbaugh DR, Chapin FS, Crowder LB, Daly KL, Doney SC, Elfes C, Fogarty MJ, Gaines SD, Jacobsen KI, Karrer LB, Leslie HM, Neeley E, Pauly D, Polasky S, Ris B, St Martin K, Stone GS, Sumaila UR, Zeller D. 2012. An index to assess the health and benefits of the global ocean. Nature 488:615–620.

Harrison RJ. 1974. Functional Anatomy of Marine Mammals, 1st ed. New York: Academic Press.

Hoorweg K, Cupedo T. 2008. Development of human lymph nodes and Peyer's patches. Semin Immunol 20:164–170.

Janeway CA Jr, Medzhitov R. 2002. Innate immune recognition. Annu Rev Immunol 20:197–216.

Jefferson TA, Leatherwood S, Webber MA. 1993. FAO Species Identification Guide. Marine Mammals of the World. Rome: FAO.

Jepson PD, Bennett PM, Allchin CR, Law RJ, Kuiken T, Baker JR, Rogan E, Kirkwood JK. 1999. Investigating potential associations between chronic exposure to polychlorinated biphenyls and infectious disease mortality in harbor porpoises from England and Wales. Sci Total Environ 243-244:339–348.

Junqueira LC, Carneiro J. 2013. Junqueira's Basic Histology: Text and Atlas. 13th ed. New York: The McGraw-Hill Companies, Inc.

Parham P. 2009. The Immune System. 3rd ed. New York: Garland Science.

Pabst DA, Rommel SA, McClelland WA. 1999. Functional anatomy of marine mammals. In: Reynolds JE, Rommel SA, editors. Biology of Marine Mammals. Washington: Smithsonian Institution Press. p 15–72.

Pedersen AB, Babayan SA. 2011. Wild immunology. Mol Ecol 20: 872–880.

Press C McL, Landsverk T. 2006. Immune System. In: Eurell JA, Frappier BL, editors. Dellmann's Textbook of Veterinary Histology. 6th ed. Iowa: Blackwell Publishing. p 134–154.

Romano TA, Felten SY, Olschowka JA, Felten DL. 1993. A microscopic investigation of the lymphoid organs of the beluga, *Delphinapterus leucas*. J Morphol 215:261–287.

Rommel AA, Lowenstine LJ. 2001. Gross and Microscopic Anatomy. In: Dierauf L, Gulland FMD, editors. CRC Handbook of Marine Mammal Medicine. Florida: CRC Press. p 149–150.

Samuelson DA. 2006. Textbook of veterinary histology. Philadelphia: Saunders Elsevier.

Siebert U, Joiris C, Holsbeek L, Benke H, Failing K, Frese K, Petzinger E. 1999. Potential relation between mercury concentrations and necropsy findings in cetaceans from German waters of the North and Baltic Seas. Mar Pollut Bull 38:285–295.

Simpson JG, Gardner MB. 1972. Comparative microscopic anatomy of selected marine mammals. In: Ridgway SH, editor. Mammals of the sea. Biology and Medicine. Springfield: Charles C. Thomas Publisher. p 298–418.

Tschanz SA, Burri PH, Weibel ER. 2011. A simple tool for stereological assessment of digital images: the STEPanizer. J Microsc 243: 47–59.

Van de Pavert SA, Mebius RE. 2010. New insights into the development of lymphoid tissues. Nat Rev Immunol 10:664–674.

Vukovic S, Lucić H, Gomercić H, Duras Gomercić M, Gomercić T, Skrtić D, Curković S. 2005. Morphology of the lymph nodes in bottlenose dolphin (*Tursiops truncatus*) and striped dolphin (*Stenella coeruleoalba*) from the Adriatic Sea. Acta Vet Hung 53:1–11.