INVESTIGATION OF THE MORPHOLOGY AND AUTONOMIC INNERVATION OF THE LYMPHOID ORGANS IN THE PANTROPICAL SPOTTED, SPINNER, AND COMMON DOLPHINS (*STENELLA ATTENUATA*, *STENELLA LONGIROSTRIS* AND *DELPHINUS DELPHIS*) INCIDENTALLY ENTANGLED AND DROWNED IN THE TUNA PURSE-SEINE FISHERY IN THE EASTERN TROPICAL PACIFIC

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ABSTRACT

To help gain an understanding of the effects of repeated chase and encirclement on the immune system of dolphins in the Eastern Tropical Pacific (ETP), lymphoid organs including spleen, lymph nodes, thymus, and gut-associated lymphoid tissue, were collected from the pantropical spotted (*Stenella attenuata*), spinner (*S. longirostris*), and common (*Delphinus delphis*) dolphins incidentally entangled and drowned in the tuna fishery. Lymphoid organs were either snap frozen in liquid nitrogen or fixed in 10% buffered formalin and processed for general morphology using routine histological stains, or processed for autonomic innervation using immunocytochemistry.

The lymphoid organs examined displayed a characteristic morphology of a functioning and intact immune system that includes well developed white pulp areas in the spleen; lymphatic follicles with germinal centers in lymph nodes; a well-developed thymus containing lobules made up of densely packed lymphocytes; and lymphatic follicles in the submucosa extending into the lamina propria of the large intestine. There appeared to be no indication of tissue involution as has been shown after stress in other mammals. However, it is important to keep in mind that prior chase/capture history of necropsied animals was not available and the small sample size for each species makes it impossible to detect a stress-related effect in each stock. Age-dependent changes in lymphoid organ morphology may influence disease susceptibility in young vs. older animals, however, this needs further investigationAn anatomical link between the brain and immune system exists in the dolphin lymphoid organs examined, whereby stress can affect immunocompetence. Catecholamine-containing and tyrosine hydroxylase positive (the rate-limiting enzyme in norepinephrine synthesis) nerve fibers form close associations with cells of the immune system in the spleen and mesenteric lymph node. Functional implications of these nerves in regards to immunity needs further investigation.

INTRODUCTION

Dolphins, including the pantropical spotted (*Stenella attenuata*), spinner (*S. longirostris*), and common dolphins (*Delphinus delphis*) associate with yellowfin tuna (*Thunnus albacares*) in the Eastern Tropical Pacific Ocean (ETP). The tuna purse-seine fishery takes advantage of the association of the dolphins with the tuna by encircling dolphins to catch the tuna. The repeated chase and encirclement during the fishing operation is hypothesized to cause stress in dolphins. Moreover, approximately 2500-3000 dolphins are killed annually as a result of incidental entanglement (Curry and Edwards, 1998). The Necropsy Program was established to evaluate the pathophysiological condition of post-mortem specimens from tissues collected from dolphin mortalities resulting from the fishery.

In addition to the pathological examination and evaluation of major organs (described in Cowan and Curry, 2002) we investigated the general morphology and autonomic innervation of the lymphoid organs (i.e. spleen, lymph nodes, and thymus) to help gain an understanding of the effects of repeated chase and encirclement on the immune system. In mammals, stress has been reported to bring about changes in immune function, potentially leading to greater vulnerability to disease. Studies have shown that stress can cause an involution of lymphoid organs, with decreases in cellular compartmentation (Dominguez-Gerpe and Rey-Mendez, 1997 and 1998; Fukui et al., 1997).

Moreover, the anatomical link between the nervous and immune systems establishes a pathway whereby stress as "perceived" by the brain can affect immunocompetence (Felten et al., 1987; Elenkov et al., 2000). There is evidence for sympathetic postganglionic nerve fibers distributing in cellular compartments of both primary (bone marrow, thymus) and secondary (spleen, lymph nodes, gut-associated lymphoid tissue, tonsils) lymphoid organs, forming close associations with cells of the immune system (Felten et al., 1985 and 1987; Elenkov, 2000). During stress and activation of the autonomic nervous system, neurotransmitters (such as norepinephrine) released from nerve terminals synaptically or by paracrine secretion can affect the immune response. Neurochemical investigations reveal the abundant presence and release of norepinephrine from the nerves in the rodent spleen (Felten et al., 1987). Furthermore, lymphocytes (both B and T subsets) and monocytes/macrophages possess adrenergic receptors. Moreover, destroying nerves by chemical sympathectomy has resulted in measurable functional changes in the immune response (Madden et al., 1994a; Madden et al., 1994b; Felten et al., 1987).

An anatomical link between the nervous and immune systems in a cetacean, the beluga, *Delphinapterus leucas*, has previously been identified (Romano et al., 1994). Moreover, beluga lymphocytes possess beta adrenergic receptors and incubation with isoproterenol, a beta adrenergic agonist, brings about changes in the lymphocyte proliferation response *in vitro*. Given the direct innervation of lymphoid organ cellular compartments in terrestrial mammals and the beluga, as well as the evidence from a variety of disciplines for the effects of stress on the immune system, we investigated lymphoid organ morphology and autonomic innervation in lymphoid organs from dolphins incidentally drowned in the purse-seine tuna fishery in the ETP.

An independent scientific peer review of this work was administered by the Center for Independent Experts located at the University of Miami. Responses to reviewer's comments can be found in the Appendix.

METHODS

Sample Collection

Lymphoid organs including the spleen, mesenteric lymph node, thymus, gut-associated lymphoid tissue, and additional lymph nodes were collected from 57 dolphins incidentally killed in the ETP purse-seine fishery between November 1999 and September 2001. Necropsy technicians aboard the tuna vessels were trained to carry out a full dolphin necropsy during the special training courses held at the Southwest Fisheries Science Center in La Jolla (January 19-21, 1999 and June 14-15, 2001). This training included locating, collecting, and initial processing of the lymphoid organs for general light microscopy, fluorescence histochemistry, and immunohistochemistry. In addition, necropsy technicians recorded gender, morphometrics, color pattern for each dolphin, and collected teeth for aging.

Lymphoid organs were collected as soon as possible post-mortem. Post-mortem times were normally 2 hours, but ranged from 55 minutes to 11 hours. Tissues were cut into 1-2 cm^3 blocks and subsequently flash frozen in liquid nitrogen and/or fixed in 10% phosphate buffered formalin. Samples fixed in 10% buffered formalin were transferred into 0.15 M sodium phosphate buffer (pH 7.2) after 48 hrs. and stored at 4 degrees C. Tissues were stored in liquid nitrogen or phosphate buffer until further processing in the laboratory for investigation of general morphology and autonomic innervation. Routine stains were used to investigate general

morphology of lymphoid organs and catecholamine fluorescence histochemistry and immunocytochemistry were used to investigate innervation.

General Morphology of Lymphoid Organs

Lymphoid organ morphology and activity were examined using light microscopic methods. Formalin-fixed tissues were paraffin embedded, sectioned, and mounted on glass slides according to the methodology in Cowan and Curry (2002). Slides were deparaffinized and stained using routine histological stains such as Hematoxylin and Eosin (H&E), and Trichrome stain (Sigma Diagnostics) to show general morphology. Slides were examined using a Nikon microscope (Microphot-FXA) and representative sections for each lymphoid organ were photographed for documentation.

Autonomic Innervation of Lymphoid Organs

1. Sucrose-Potassium phosphate-Glyoxylic acid (SPG) fluorescence histochemistry

Fresh frozen lymphoid organs were sectioned at 16 μm using an IEC Minotome Plus cryostat between –7 to –16 degrees C. Sections were mounted on Superfrost Plus™ (Fisher) slides and processed for SPG fluorescence histochemistry (de la Torre, 1980) to examine catecholamine-containing nerve fibers in dolphin lymphoid organs. Sections were immediately examined using a Nikon microscope equipped with epi-illumination accessories. Alternate sections were stained with hematoxylin and eosin to permit visualization of lymphoid organ morphology.

2. Immunocytochemistry

In addition to the SPG fluorescence histochemistry method for identifying catecholamine-containing nerve fibers, immunocytochemistry was carried out to identify sympathetic nerves and cellular compartments. Immediately after tissues were brought back to the laboratory, those tissues stored in 0.15 M phosphate buffer were placed in 0.15 M phosphate buffer containing 30% sucrose for 24 hr. Subsequently, tissues were frozen on dry ice and stored in liquid nitrogen until further processing for immunocytochemistry.

a. Nerve Staining

Frozen 10% formalin-fixed tissues were sectioned at 40 μm using a Microm™ sliding microtome and placed in 0.15 M phosphate buffer (pH 7.2). Previously frozen bottlenose dolphin spleen and mesenteric lymph node fixed in 4% paraformaldehyde were also sectioned and used as positive controls, since staining characteristics are known. Sections were washed 6x5 min. in 0.15 M sodium phosphate buffer (pH 7.2) and subsequently incubated in 10% normal goat serum for 30 min. The sections were incubated in rabbit anti-tyrosine hydroxylase (TH) (Chemicon) diluted 1:300 in 0.15 M sodium phosphate buffer with 0.25% bovine serum albumin + 1% normal goat serum + 0.3% Triton X-100 for 24 hrs. at 4 degrees C. Additional sections were either processed with an isotype control or processed without the primary antibody.

Sections were rinsed 6x5 min. in 0.15 M sodium phosphate buffer and incubated in biotin-labeled goat anti-rabbit IgG (Vector) diluted 1:6000 in 0.15 M sodium phosphate buffer containing 0.25% bovine serum albumin + 1% normal goat serum for 90 min. The sections were then incubated for 30 min. in 5% methanol and 2.5% hydrogen peroxide in 0.05 M Tris + 0.6% NaCl, pH 7.2 to inhibit endogenous peroxidase. Sections were washed 6x5 min. in 0.15 M sodium phosphate buffer and incubated in Elite ABC (Vector Labs) in 0.15 M phosphate buffer

containing 0.25% BSA for 90 min. Sections were rinsed in 175 mM Na acetate-10mM imidazole, pH 7.4, followed by incubation in a solution containing 0.03% 3,3'-diaminobenzidine + 0.1 M Nickel(II) sulfate + 7 H_20 + 0.03% H_2O_2 in 125 mM Na acetate-10 mM imidazole buffer, pH 7.4, for 5-10 min., which stains the reaction product black. To stop the reaction, sections were rinsed 6x5 min. in 0.015 M sodium phosphate buffer, and subsequently mounted on slides, dehydrated, and coverslipped for light microscopy analysis using a Nikon microscope (Microphot FXA).

b. Cellular Staining

Fresh frozen lymphoid organs were sectioned at 10 μm between –7 to –13 degrees C using an IEC Minotome Plus cryostat. Sections were mounted on Superfrost Plus™ slides and dried at room temperature for 24 hrs. Subsequently, sections were fixed in cold acetone for 5 min. and dried at room temperature for 30 min. Sections were encircled with a PAP pen (Kiyota Int., Inc.) and dried for 30 min. Slides were immersed in 0.15 M sodium phosphate buffer and incubated in 2.14% sodium periodate for 5 min. to inhibit endogenous peroxidase. Slides were rinsed in 0.15 M sodium phosphate buffer 2x3 min. and incubated in 10% normal goat serum for 30 min. Sections were incubated in 1:40 dilutions of cetacean-specific monoclonal antibodies to the cell surface markers CD2 (T cells) and CD21 (B cells) (Stott, personal communication), or with supernatant from the myeloma cell line P2X63-AG8.653 as a negative control, in 0.15 M sodium phosphate buffer for 60 min. at room temperature. Slides were rinsed in 0.15 M sodium phosphate buffer and incubated in Biotin-SP-conjugated AffiniPure F(ab')2 goat anti-mouse IgG (Immunotech) diluted 1:100 in 0.15 M sodium phosphate buffer containing 1% dolphin serum for 30 min. Subsequently, slides were then rinsed 3x3 min. in 0.15 M sodium phosphate buffer and incubated in ZyMax™ streptavidin-horseradish peroxidase (HRP) conjugate (Zymed) diluted 1:100 in 0.15 M sodium phosphate buffer for 30 min. After 3x3 min. rinses in 0.15 M sodium phosphate buffer, slides were rinsed 2x3 min. in 0.05 M Tris + 0.06% NaCl buffer (pH 7.2). Sections were incubated in 10 mM Imidazole + 0.05% 3,3'-diaminobenzidine + 0.03% H_2O_2 in 0.05 M Tris + 0.06% NaCl buffer for 5-10 min., which stains the reaction product brown. Slides were rinsed, dehydrated, and coverslipped for subsequent examination and photodocumentation with a Nikon microscope (Microphot-FXA).

c. Double Labeling

To show localization of innervation in relation to cellular components, tissue sections were labeled with antibodies for both tyrosine hydroxylase-positive nerves and cell surface proteins on lymphoid cells. Formalin-fixed tissues were sectioned at 35 μm using a Microm sliding microtome. Nerves were labeled with an antibody to tyrosine hydroxylase using the protocol described earlier. B and T lymphocytes were subsequently labeled using cetacean-specific antibodies to the cell surface markers CD21 and CD2 using a similar but slightly modified protocol for cellular staining (i.e. the cold acetone incubation was omitted). Sections were examined with a Nikon microscope (Microphot-FXA) and photographed for documentation.

Age Determinations

Ages of animals were derived from tooth dentinal growth layer groups (Chivers and Robertson, personal communication) and were confirmed using known age-length ranges for pantropical spotted (Perrin et al., 1976; Perrin and Hohn, 1994), spinner (Perrin and Gilpatrick, 1994), and common dolphins (Evans, 1994).

RESULTS

The lymphoid organs (spleen, lymph nodes, thymus, and gut-associated lymphoid tissue) from dolphins incidentally killed in the tuna fishery, were examined for general morphology, and innervation of the autonomic nervous system. Observations from trained necropsy technicians showed tissues were collected from 30 spotted dolphins (18 females and 12 males), 24 spinner dolphins (15 females and 9 males), 2 common dolphins (1 female and 1 male), and 1 dolphin with no observational data for a total of 57 dolphins examined. Age estimates (Chivers and Robertson, personal communication) ranged from <1 year (80 cm in length) to 16 years of age (188 cm in length). Table 1. lists the dolphins sampled and the lymphoid organs collected.

General Morphology

The spleens of the dolphins examined resemble a typical mammalian spleen with a stroma formed by a capsule and trabeculae, and a parenchyma consisting of white and red pulps (Figure 1). The capsule varies in thickness and is mostly comprised of connective tissue with smooth muscle in its deeper layers. Trabeculae, consisting of connective tissue and smooth muscle, conveying arteries and veins along with the reticular network form the "framework". The red pulp consists mainly of erythrocytes, sinusoids, and other cellular constituents including reticular cells, and macrophages. White pulp areas, the major immunological components of the spleen, consist of dense areas of lymphocytes surrounding a central artery or arterioles, and lymphatic follicles with or without germinal centers. Reticular cells and fibers form a network to support the white and red pulps. White pulp areas are numerous in the dolphins examined and range in size from ovoid or small spherical clusters of densely packed lymphocytes to larger masses of clustered cells. The major components of the white pulp are also present in the dolphins examined, namely periarteriolar lymphatic sheaths (PALS) and lymphoid follicles. The PALS is a sheath of lymphocytes surrounding a central artery and/or multiple arterioles, and was observed either as a solitary entity or adjacent to lymphoid follicles. The lymphoid follicles are ovoid to spherical clusters of lymphocytes and were observed with and without germinal centers. Follicles with germinal centers consist of an inner zone of pale-staining blast-like lymphocytes, bordered by smaller and darker-staining lymphocytes and an outer zone of medium to large lymphocytes. As with the PALS, follicles were observed adjacent to the PALS, but were also observed as a solitary unit. White pulp areas also contain a marginal zone separating white pulp from red pulp with reticular cells, macrophages, and lymphocytes intermixed with red blood cells.

There appeared to be no species differences in the spleens examined. Spleens from all the dolphins examined differed in the amount and size of white pulp areas vs. red pulp and stromal elements, but for the most part looked normal. There was a difference however, in the organization of the spleen from the youngest dolphin (<80 cm in length). There was no clear organization of white pulp areas into distinct follicles or PALS compared to older animals (Figure 2). In the newborn dolphin, the white pulp is diffused and not organized into well-defined spherical structures. Dense areas of lymphocytes are intermixed with red pulp.

The lymph nodes examined include the mesenteric node, the marginal nodes of the lung, nodes associated with the intestine, and lymph nodes from other unidentified locations. All nodes have the basic morphology consistent for mammalian lymph nodes (Figure 3). The nodes contain a capsule made up of connective tissue with trabeculae emanating into the parenchyma

and coursing throughout the node. Subcapsular sinsuses are present and the node has a reticular network that supports the parenchyma. The parenchyma consists of a cortex and medulla. The cortex of the nodes contains lymphocytic follicles of different sizes that are ovoid to spherical in shape embedded in diffuse lymphoid tissue. Portions of the cortical area of some nodes have an area of diffuse lymphoid tissue with no follicular organization. Germinal centers were present in different degrees in the nodes examined. The medullary region of the nodes is composed of cords of cells, primarily lymphocytes surrounded by lymphatic sinuses, blood vessels, and diffuse lymphoid tissue. Smooth muscle is frequently observed in the medullary region of the nodes. Large macrophages and eosinophils are abundant in the medullary region of some of the nodes examined. Some nodes have a definitive lobular organization partitioned by vascular connective tissue, with 4 or 5 follicles surrounding a common medullary area.

The nodes examined from these animals seemed to be relatively normal and active functioning lymph nodes. Mesenteric nodes and marginal nodes seem to have thin cortical areas with a much higher medullary content than other nodes which have well developed cortical areas, paracortical areas and medullary regions. Nodes do not show any consistent patterns with age. However, the node from the youngest dolphin does not have well developed follicles and germinal centers are sparse (Figure 4). Nematodes and larvae were found in the nodes of two dolphins. The mesenteric lymph node from dolphin CTC001 and a node from dolphin SBZ002 (node location unknown) showed the nematodes and larvae or the larvae alone in the middle of cortical follicles (Figure 5).

The thymus was only collected and identified from two dolphins (SJC011 and SBZ001/001A). The thymus as in other mammals is arranged in lobules separated by connective tissue septae (Figure 6). The septae convey blood vessels which are observed entering the lobule. Each lobule consists of a darker staining cortex and a paler staining medulla. The cortex consists of densely packed lymphocytes while the medulla includes these along with larger lymphocytes and epithelial components.

The large intestine from three dolphins was examined for gut-associated lymphoid tissue. Gut-associated lymphoid tissue varied from diffuse to discrete follicles along the intestine in the submucosal layer and extended into the lamina propria. The border between the muscle layer and submucosa was difficult to discern. One dolphin examined showed continuous lymphoid follicles in the submucosa/lamina propria along the length of the intestine (Figure 7).

Innervation of Lymphoid Organs

Autonomic innervation of the spleen and mesenteric lymph nodes was investigated using SPG histofluorescence to label catecholamine-containing nerves, and immunocytochemistry with an antibody to tyrosine hydroxylase (TH), the rate-limiting enzyme in norepinephrine synthesis. Since fixation with 10% formalin is not the fixative of choice for the antibodies used and the post-mortem time intervals varied, control tissues were run from a bottlenose dolphin of which staining characteristics were known.

Catecholamine fluorescence histochemistry and TH immunocytochemistry revealed innervation of stromal as well as parenchymal compartments. The capsule contains arteries and veins as well as solitary nerve profiles running parallel along the capsule, that are positively labeled. Trabeculae extending from the capsule and distributing in the red pulp, conveying arteries and veins, show abundant innervation (Figure 8a,b). The PALS contains innervation associated with the central artery (Figure 8c,d). However, nerve fibers are seen radiating out

from the central artery and coursing throughout the PALS. Moreover, nerve fibers are observed in the marginal zone and form associations with the cells in this area (Figure 8d).

The mesenteric lymph nodes showed fluorescent profiles and TH-positive staining in the capsule, septae, in the boundary between the cortex and medulla, and in the medullary region (Figure 9). Innervation in the corticomedullary zone was mostly associated with the vasculature in this region with fibers trailing into outer cortical areas or into the medulla. Nerves in the connective tissue septae also are observed entering into the outermost portion of the follicles in the cortex. The follicles themselves did not contain any staining. The medullary region contains nerves associated with arteries and veins as well as solitary nerve fibers forming close associations with the cells in the medulla.

Innervation of the thymus (from two dolphins) is mainly associated with blood vessels in the connective tissue septae and capsule (Figure 10). However, nerve fibers are observed in the outermost portions of the thymic lobule and associate with blood vessels in the medulla.

Preliminary cellular staining using immunocytochemistry to label B cells with a cetacean-specific antibody (De Guise et al., 2002) appears to label follicles in the spleen and lymph nodes (Figure 11a,b). Preliminary staining with an antibody to CD2 to label T cells did not show any positive staining in the spleen or the lymph nodes. Optimal conditions are currently being worked out on control tissues to optimize cellular staining with these antibodies. Double-labeling immunocytochemistry for nerve fibers and cells will specifically identify the cell types that sympathetic nerves associate with (Figure 11c).

DISCUSSION

The lymphoid organs examined from the spotted, spinner, and common dolphins incidentally drowned in the tuna purse-seine fishery, displayed characteristic morphology of a functioning and intact immune system. Spleen, lymph nodes, thymus, and the gut-associated lymphoid tissue examined displayed an "active" morphology including: well developed white pulp areas in spleen; lymphatic follicles with germinal centers in lymph nodes; a well-developed thymus containing lobules made up of densely packed lymphocytes; and lymphatic follicles in the submucosa extending into the lamina propria of the large intestine. There appeared to be no indication of tissue involution as has been shown after stress in other mammals (Dominguez-Gerpe and Rey-Mendez, 1997 and 1998; Fukui, et al., 1997). However, these studies report effects of stress on lymphoid organs is time dependent in relation to the stressor.

We cannot definitively determine that lymphoid organ morphology was not effected by repeated chase and encirclement since the initial morphology of the lymphoid organs (before chase and capture), and the prior history of chase and encirclement for each individual are not known. However, given reports of lymphoid organ morphology in other cetaceans (Romano et al., 1993; Cowan and Smith, 1999) there was no indication morphologically of a compromised immune system. It is important to keep in mind that changes in lymphoid organ morphology are time dependent and also may depend on the duration and intensity of the stressor as shown in other mammals (Dominguez-Gerpe and Rey-Mendez, 1997).

The spleen from the newborn dolphin was quite different from older spleens and lacked any apparent organization, suggesting age-related changes in lymphoid organ morphology as reported in other mammals (Saint-Marie and Peng, 1987; Suster and Rosai, 1990). Furthermore, age-dependent changes in lymphoid organ morphology may have implications in susceptibility to infectious agents. It is interesting to note that the few thymuses collected were from a 5 year

old and 15 year old. Even at 15 years of age there seems to be no involution of the thymus observed microscopically. This is opposite from the beluga in which the thymus was only identified in young animals (Romano et al., 1993).

The lymph nodes displayed different degrees of cortical and medullary components. The marginal and mesenteric nodes in particular seemed to have rather thin cortical areas with extensive medullary areas. Other nodes examined had rather extensive cortical areas as well as highly developed medullary areas. Architecture of the nodes in these dolphins may be location-dependent as has been described for the bottlenose dolphin (Cowan and Smith, 1999). Nematodes and larvae were found in the middle of cortical follicles in the mesenteric node and in an unidentified node in two dolphins (*S. attenuata* and *S. longirostris*) out of 57. These have not been observed in the lymph nodes of stranded bottlenose dolphins (Cowan, personal communication), and could be a characteristic infection in these species.

Autonomic nerve fibers were observed in parenchymal compartments of the spleen and mesenteric lymph node, establishing an anatomical link between the nervous and immune systems. Catecholamine-containing nerve fibers and tyrosine hydroxylase-positive nerves followed a similar distribution as in the beluga (Romano et al., 1994) forming close associations with lymphoid cells in the periarteriolar lymphatic sheath and the marginal zone in the spleen. The marginal zone is an area of antigen presentation and lymphocyte entry/exit. Therefore, neurotransmitters released during stress may affect the immune response as well as lymphocyte trafficking. Functional implications for nerves in lymphoid organs have been documented (Madden et al., 1997; Carlson et al., 1997; Felten et al., 1987). Moreover, innervation plays a role in development, aging, and certain autoimmune diseases (Bellinger et al., 1992; Madden et al., 1997). Nerve staining was not consistent from animal to animal in terms of abundance or intensity. While one can conclude this is an individual difference, staining differences could be due to post-mortem processing times and tissue handling.

While we have initiated investigations on lymphoid organ morphology and innervation of necropsied dolphins incidentally drowned in the tuna fishery, more in-depth studies of the immune system are needed from more animals. The total sample size was only 57 dolphins (30 from NE offshore spotted dolphins, 24 from eastern spinner dolphins, 3 from common dolphins), far less than the estimated 300 required to detect a stress-related effect in each stock (Gerrodette, personal communication). Future studies will focus on investigation of the lymph node morphology from different nodal groups. Necropsy technicians will be trained to collect representative nodes from various locations. In addition, the technicians will be trained to routinely collect gut- associated lymphoid tissues. Age dependent changes in lymphoid organ morphology will be investigated (e.g. thymus and gut-associated lymphoid tissue). The skin in regards to immunity will also be investigated, since it can be considered the first line of defense against invading pathogens. Cellular studies will continue to label T and B cells in the lymphoid organs as well as continuation of double labeling studies to determine the cell types that sympathetic nerves associate with. If feasible, lymphoid organ weights will be measured post-mortem, and attempts made to quantify changes in lymphoid organ morphology

The results reported from this study will be used in conjunction with other necropsy results (Cowan and Curry, 2002) and with the results from Chase Encirclement Stress Studies (CHESS) to determine the effects of repeat chase and encirclement on dolphins in the Eastern Tropical Pacific.

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Table 1. Lymphoid organs collected from necropsied dolphins in the ETP.

Specimen	*Species*	Sex	Length (cm)	Age (years)	Lymphoid organs collected
MAG003/001	*D. delphis*	F	171	No data	Spl, Mes LN
LBZ004	*D. delphis*	M	175	6	Spl, Mar LN 1-2, LN 1-2
JCJ007	No data	No data	No data	No data	Spl, Mes LN
JCJ005	*S. attenuata*	F	80	newborn	Spl, Mar LN
MJB003	*S. attenuata*	F	119	1	Spl, Mar LN 2, Mar LN1
CEE002	*S. attenuata*	F	124	No data	Spl, Mes LN
JOG006	*S. attenuata*	F	162.6	6	Spl, Mar LN, Node 1
RGC003	*S. attenuata*	F	169	min. 5	Spl, Mes LN, Thy
RGC002	*S. attenuata*	F	172	5	Spl, Mes LN, Mar LN, Node 1, LInt
CEE001	*S. attenuata*	F	175	No data	Spl, Mes LN
MJB002	*S. attenuata*	F	175	No data	Spl, Mes LN
JCJ001	*S. attenuata*	F	179	4-5 [a]	Spl, Mes LN
JCJ008	*S. attenuata*	F	180	min. 15	Spl, Mes LN, Mar LN, Node 1
JOG001	*S. attenuata*	F	188	min. 16	Spl, Mes LN
MAG004/001B	*S. attenuata*	F	189	No data	Spl, Mes LN
LBZ002	*S. attenuata*	F	190	14	Spl, Mes LN
SJC011	*S. attenuata*	F	196	15	Spl, Mes LN, Node 1-4, Thy
JOG004	*S. attenuata*	F	197	min. 15	Spl, Nodes
MAG007	*S. attenuata*	F	198	12	Spl, Mes LN, Mar LN, Node 1-2
RGC001	*S. attenuata*	F	200.5	11	Spl, Node 1, LInt
RGV002	*S. attenuata*	F	178?	12	Spl, Node, LInt
MAG002	*S. attenuata*	M	135	1	Spl,Mes LN, Mar LN, LN 1-3, Int 2
MAG006	*S. attenuata*	M	137	1.25	Spl, Mes LN, Mar LN
JOG007	*S. attenuata*	M	158.6	4	Spl, Mar LN, Int 2, Node 1-2
SBZ002/002A	*S. attenuata*	M	163	No data	Spl, Mes LN, Mar LN
MAG005	*S. attenuata*	M	163	8	Spl, Mes LN, Mar LN, Node 1-3
JOG008	*S. attenuata*	M	167.6	7	Spl, Node 1-4
CTC004	*S. attenuata*	M	171	No data	Spl, Mes LN, Mar LN
JOG005	*S. attenuata*	M	189	9	Spl, Mes LN, Mar LN, LN 1-2
LBZ003	*S. attenuata*	M	191	12	Spl, Mes LN
JOG002	*S. attenuata*	M	196	No data	Spl, Mes LN
SBZ001/001A	*S. attenuata*	M	200	2 [a,b]	Spl, Mes LN, Mar LN, Node 1-2
ACB001	*S. attenuata*	M	205	12	Spl, Node 1-4
CEE003	*S. longirostris*	F	140	No data	Spl, Mes LN
LBZ001	*S. longirostris*	F	142	1 [b]	Spl, Node 2-3
FGO002	*S. longirostris*	F	150	1.25 [b]	Spl, Node 1
GGT002	*S. longirostris*	F	150	2 [b]	Spl, Mes LN, Mar LN, Node 1-2, LInt
MJB001	*S. longirostris*	F	154	1.5 [b]	Spl, Mes LN
MJB004	*S. longirostris*	F	155	6	Spl, Mes LN
SBZ004/002B	*S. longirostris*	F	160	No data	Spl, Mes LN
JCJ004	*S. longirostris*	F	163.5	6	Spl, Mes LN, Mar LN
CEE004	*S. longirostris*	F	164	No data	Spl, Mes LN
SJC010	*S. longirostris*	F	164.8	4	Spl, Mes LN
MAG001	*S. longirostris*	F	165	12 [a]	Spl, Mar LN 1-2, Node 1-4
RGV001	*S. longirostris*	F	171	min. 12	Spl, Mar LN, Node 1
RVC001	*S. longirostris*	F	171.5	No data	Spl, Mar LN 1-2
SBZ005/003	*S. longirostris*	F	177	No data	Spl, Mes LN
GGT001	*S. longirostris*	F	190	13	Spl, Mar LN 1-2, Mes LN 1-2, LN 1-2, Int 2
JCJ003	*S. longirostris*	M	142	No data	Spl, Mes LN
JCJ002	*S. longirostris*	M	153	13 [a,b]	Spl, Mes LN
SBZ003/001B	*S. longirostris*	M	161	No data	Spl, Mes LN, Mar LN, Node 1-2, Thy
FGO003	*S. longirostris*	M	163	2 [b]	Spl
CTC005	*S. longirostris*	M	164	7	Spl, Node 1
CTC001	*S. longirostris*	M	176	7	Spl, Mar LN, LN 1
LBZ005	*S. longirostris*	M	177	min. 8	Spl, Mar LN, Node 1, Int 2
JCJ006	*S. longirostris*	M	180	min. 14	Spl, RMar, LMar, Mes LN
JOG003	*S. longirostris*	M	193	No data	Spl, Mes LN, Mar LN, Node

Total = 57 dolphins

Legend:
Spl - spleen
Mes - mesenteric
Mar - marginal

LN - lymph node
Thy - thymus
LInt - large intestine
Int - intestine

[a] Tooth collected was inconsistent with species reported
[b] Age inconsistent with known length

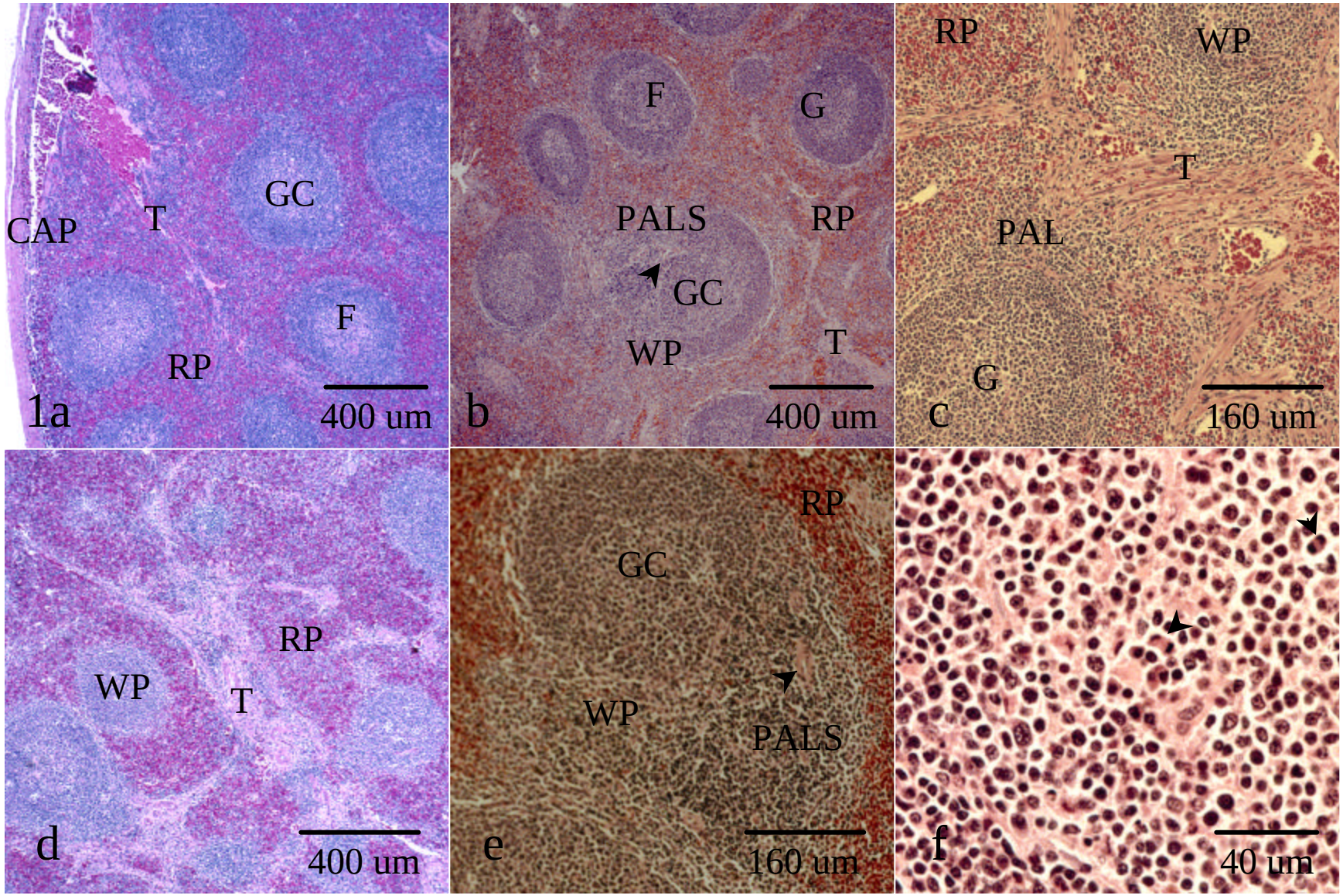

Figure 1. (a-e) Overview of *Stenella* sp. spleen morphology. The spleen is divisible into white pulp (WP) and red pulp (RP). The white pulp, the major immune component of the spleen is composed of the periarteriolar lymphatic sheath (PALS) surrounding a central artery (arrowheads). The WP contains lymphoid follicles (F) with or without germinal centers (GC). Red pulp containing trabeculae (T) forms the framework of the spleen. (f) Germinal centers are sites of B cell production and maturation and include mitotic cells (arrowheads).

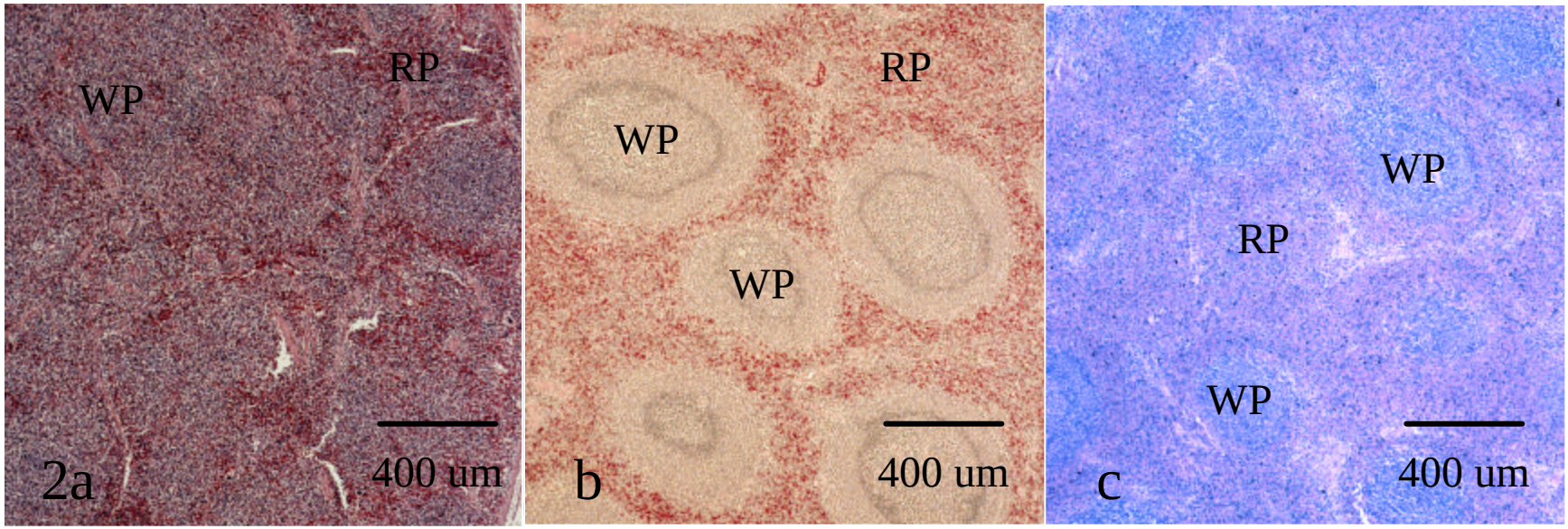

Figure 2. (a) Newborn spotted dolphin (80 cm) spleen displayed a diffused white pulp (WP) not easily distinguishable from the red pulp (RP). White pulp areas from the spleen of a one-year-old (b) and an adult estimated to be at least 16 years of age (c) are organized in distinct ovoid clusters of densely packed lymphocytes.

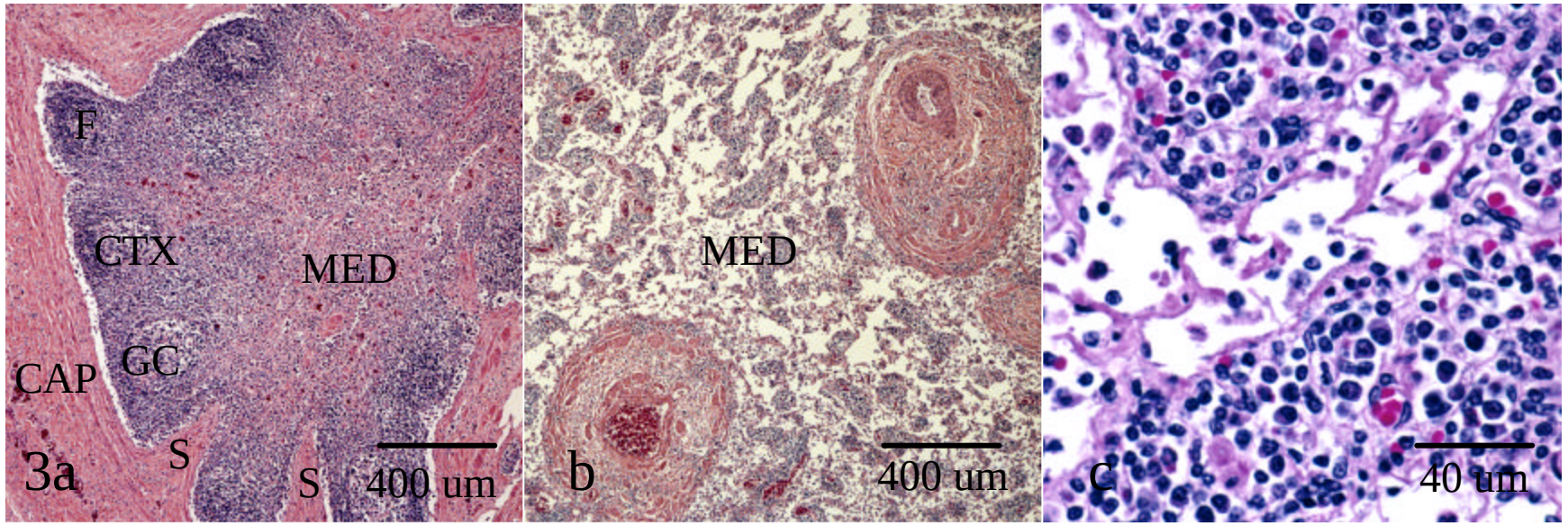

Figure 3. Overview of *Stenella* sp. lymph node morphology. (a) Connective tissue septae (S) containing smooth muscle extend from the capsule (CAP) into the cortex (CTX) of the node. The cortex contains follicles (F) with or without germinal centers (GC). (a, b) The medulla (MED) is composed of cords of cells and sinuses. (c) A variety of lymphoid cells are present in the medulla including lymphocytes, macrophages, and eosinophils.

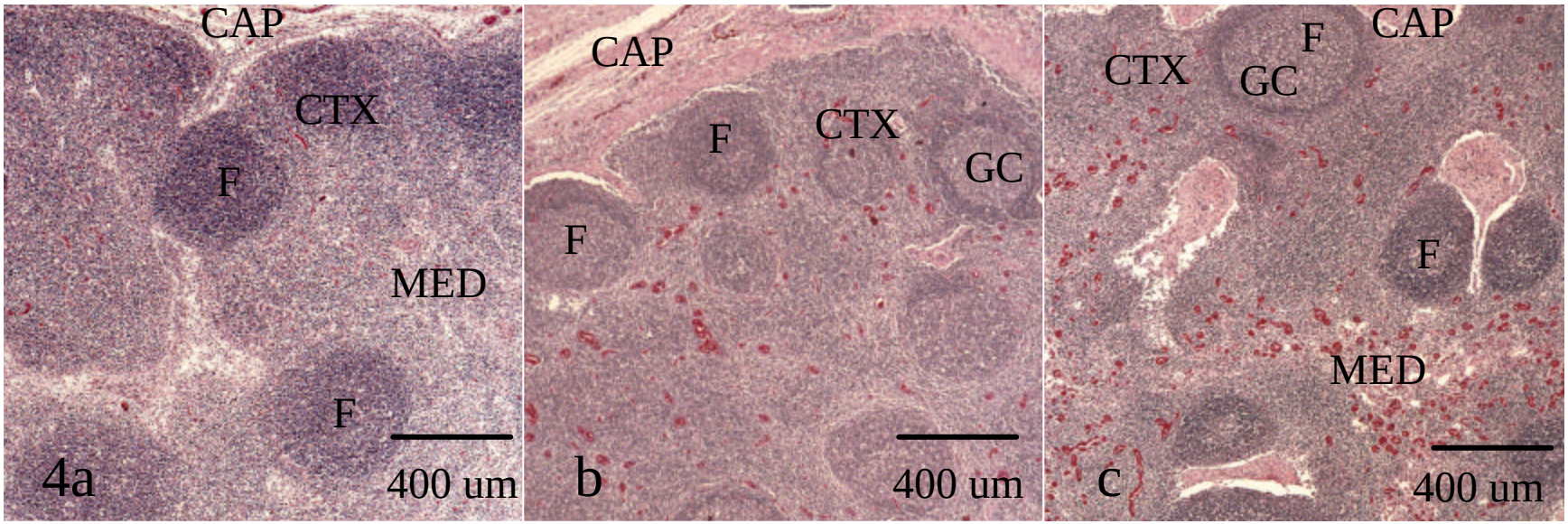

Figure 4. (a) In the newborn dolphin (80 cm), lymph node morphology was similar to that observed in the one-year-old (b) and six-year-old (c) dolphins. The lymph nodes contained a capsule (CAP), cortex (CTX) with follicles (F), and a medulla (MED). However, follicles were not as distinct in the neonate lymph node and germinal centers (GC) were rarely observed.

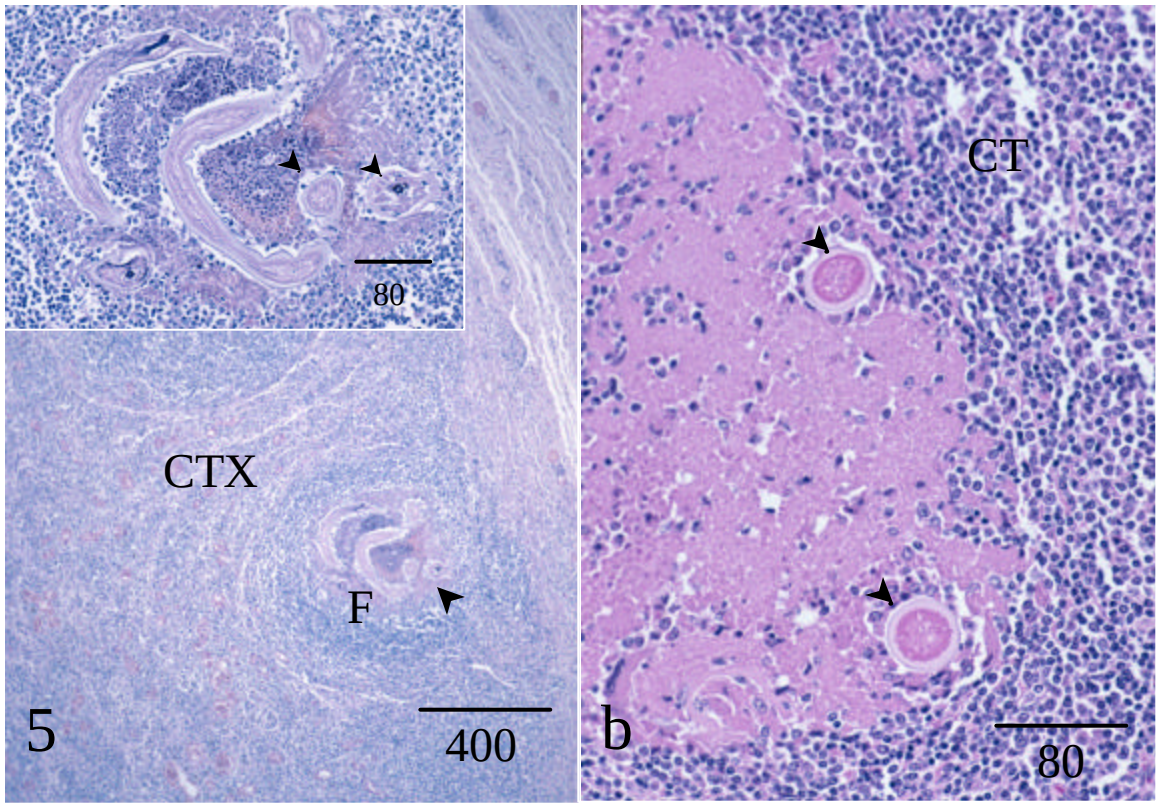

Figure 5. Nematodes (a) and larvae (arrowheads) (a, b) were found in the cortical (CTX) follicles (F) of the lymph nodes.

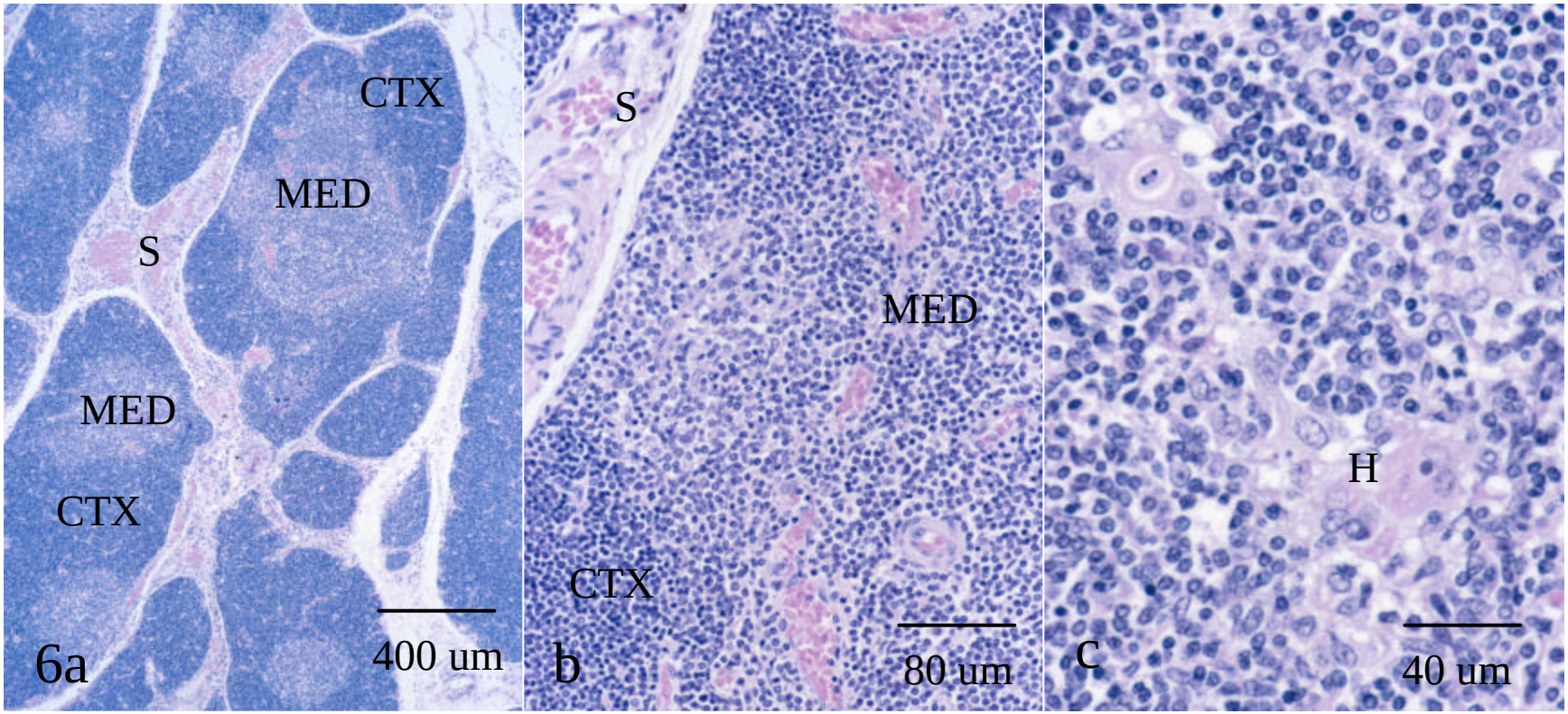

Figure 6. Overview of thymus morphology showing connective tissue septae (S), cortex (CTX), and medulla (MED) with Hassall's corpuscles (H) at low (a) and high magnification (b, c).

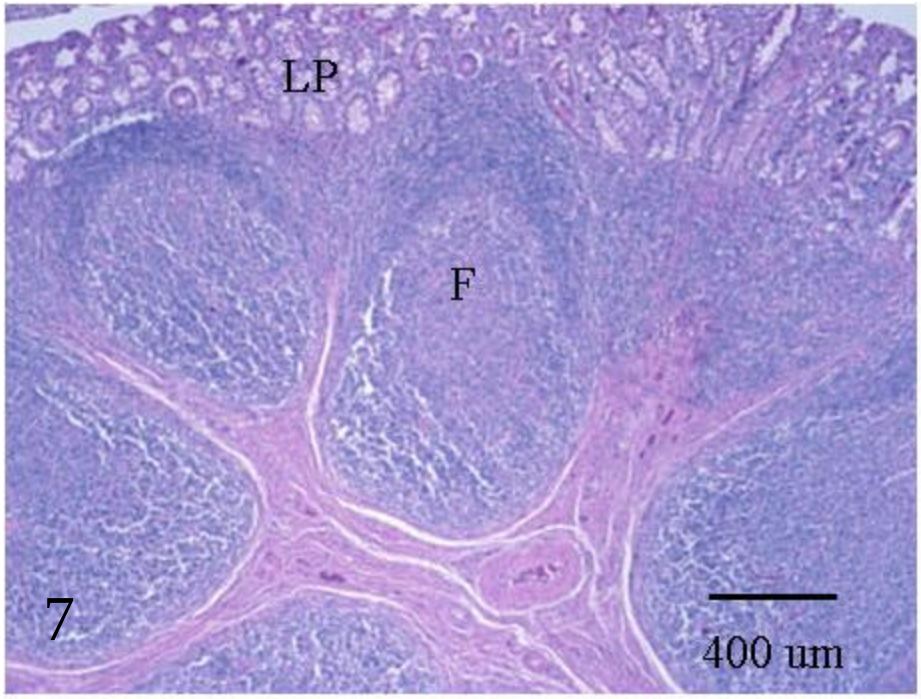

Figure 7. General morphology of gut associated lymphoid tissue (GALT) from *Stenella* sp. showing discrete lymphoid follicles (F) in the submucosa entering into the lamina propria (LP).

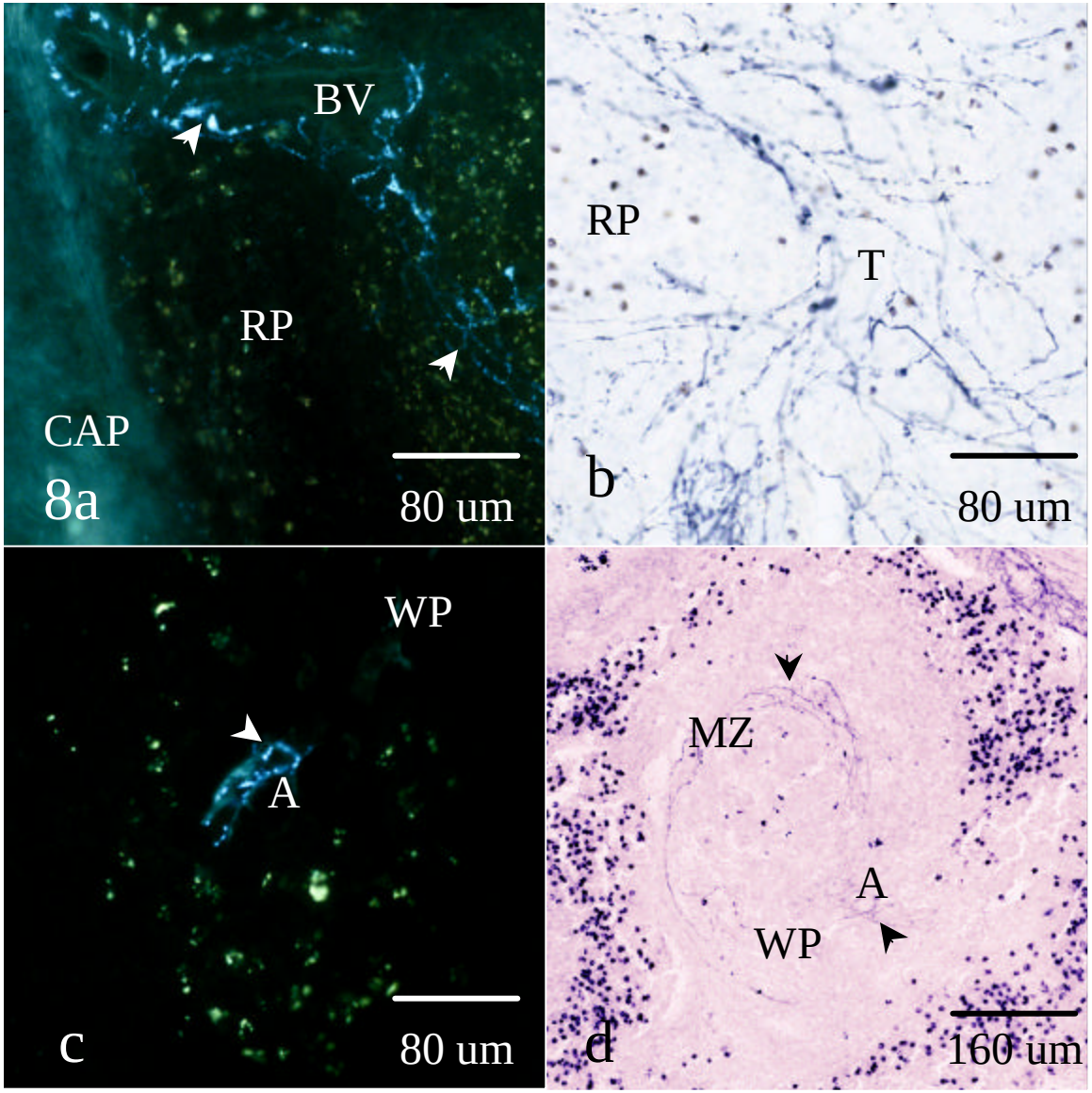

Figure 8. Innervation in the spleen. (a) Catecholamine-containing nerves (arrowheads) were observed with a blood vessel (BV) coursing from the capsule (CAP) into the red pulp (RP) (SPG method). (b) Tyrosine hydroxylase (TH)-positive nerves in the trabeculae (T) and red pulp. (c) Catecholamine-containing nerves (arrowhead) associated with the central artery (A) in the white pulp (WP) (SPG method). (d) Tyrosine hydroxylase (TH)-positive nerves (arrowheads) in the marginal zone (MZ) of the white pulp (WP). The central artery (A) of the white pulp is shown.

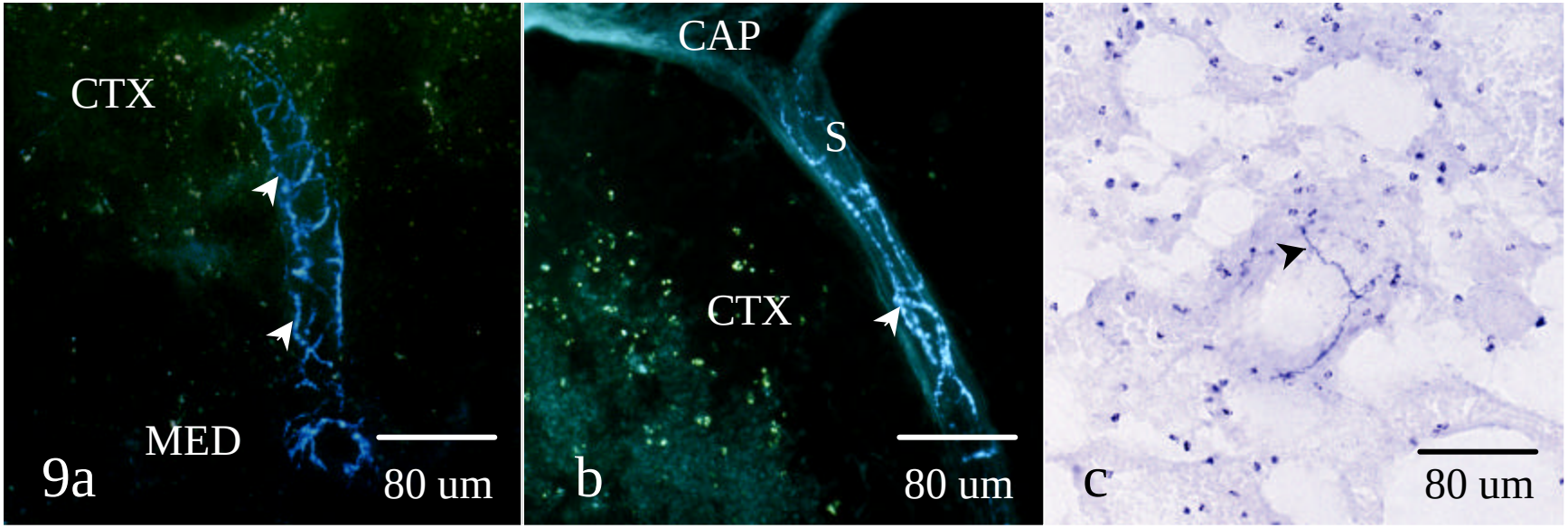

Figure 9. Innervation in the mesenteric lymph node. (a) Catecholamine-containing nerves (arrowheads) in the boundary between cortex (CTX) and medulla (MED) (SPG method). (b) Catecholamine-containing nerves (arrowhead) traveling from the capsule (CAP) into the cortical (CTX) septae (S) (SPG method). (c) Tyrosine hydroxylase (TH)-positive nerves (arrowhead) in the cellular cords of the medulla.

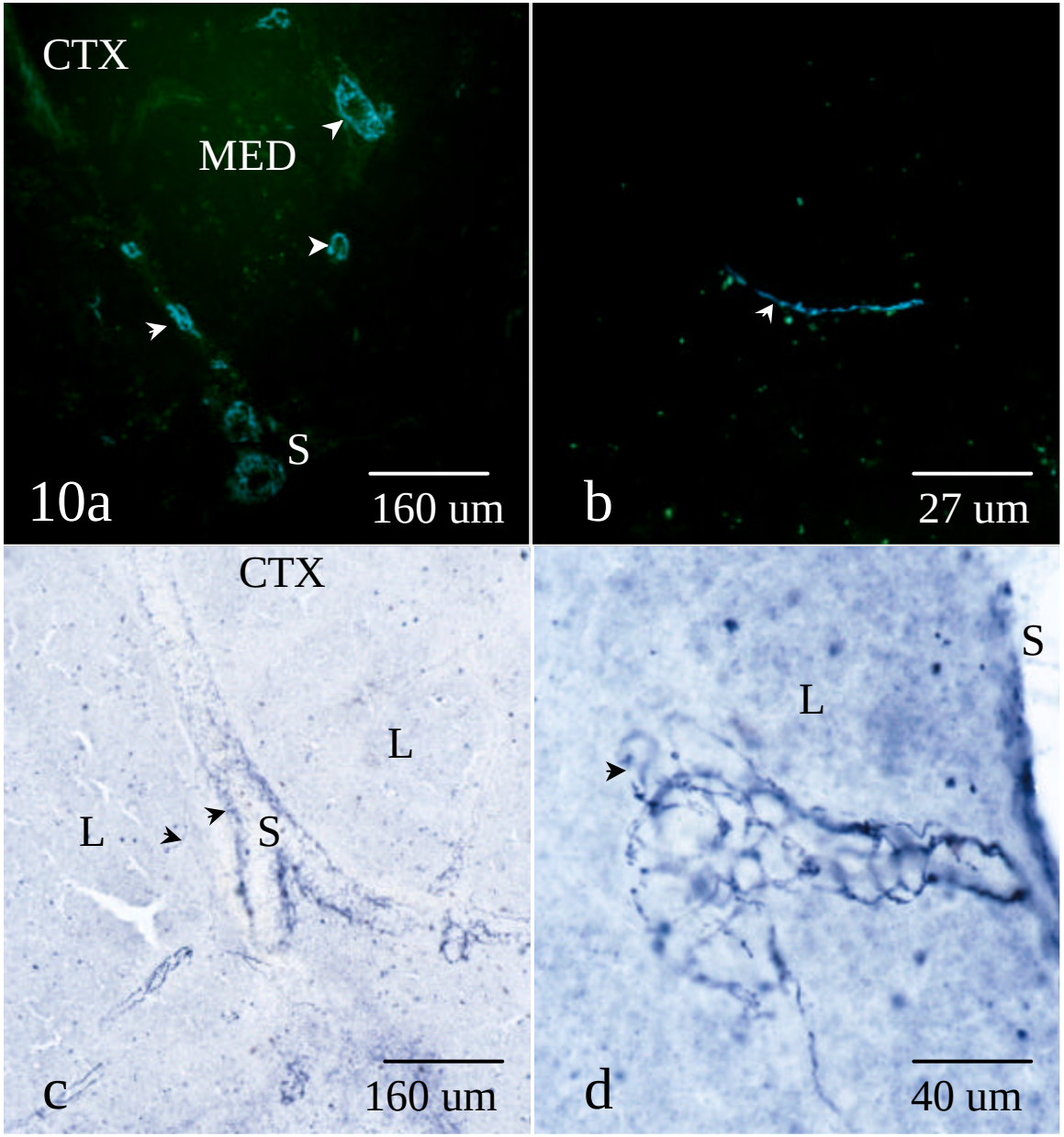

Figure 10. (a) Catecholamine-containing nerves (arrowheads) were found in the thymic connective tissue septae (S) and associated with blood vessels in the medulla (MED). The cortex (CTX) is shown adjacent to the septae. (b) Single nerve fiber (arrowhead) in the outermost portion of the thymic lobule. (c, d) Tyrosine hydroxylase (TH)-positive nerves (arrowheads) distributing with a blood vessel in the connective tissue septae (S) and entering into a lobule (L).

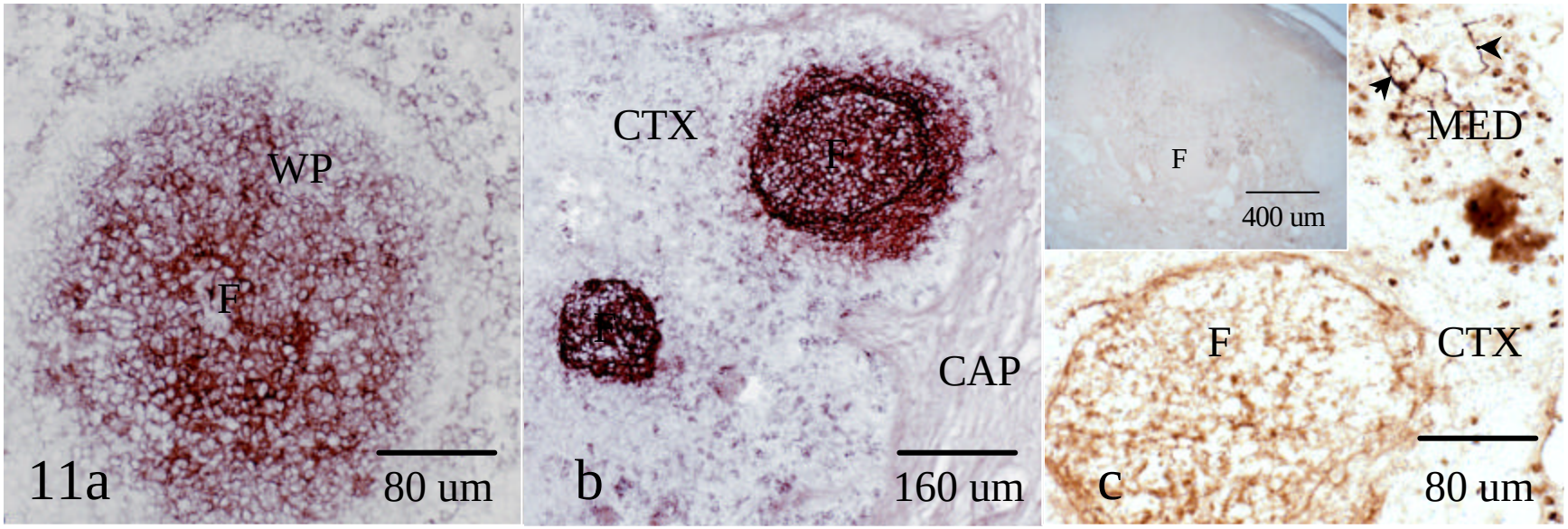

Figure 11. (a) Lymphoid follicles (F) in the splenic white pulp (WP) primarily contained B lymphocytes (seen as brown reaction product). (b) B lymphocytes were primarily in the follicles (F) of lymph node cortex (CTX). Follicles adjacent to the capsule (CAP) are shown. (c) Innervation of the mesenteric lymph node in relation to cellular components using double labeling immunocytochemistry. Tyrosine hydroxylase (TH)-positive nerves (arrowheads) associate with CD21-positive cells (seen as brown reaction product) in the medulla (MED). CD21-positive cells are also seen in the cortical (CTX) follicles (F). No cellular staining was observed in the negative control (inset).

APPENDIX – Responses to reviewer comments

Necropsy Review- CIE-S10
Sylvain De Guise

1. No Control tissues were available. We had to rely on lymphoid organ morphology from other cetacean species.
2. It is agreed that quantitative studies are needed; however, we weren't sure what we were going to get back. The processing protocol was more difficult than typical field protocols. Now that we have an idea of tissue quality we can design quantitative studies.
3. Never said that this study is to address acute changes; it's more the opposite (chronic changes). We couldn't control in study design for addressing the effects of chronic stress of repeated fishing efforts of necropsied animals.

Daniel Martineau

No Recommended Changes

Gregory Bossart

No changes. The following points raised were already mentioned in the document:
-make statement: prior history case of chase and encirclement for each necropsy case is unknown-no information provided to help determine the effects of repeated chase and encirclement-functional morphologic effects of "stress" on lymphoid organs in dolphins are likely time dependent and dependent on the duration and intensity of the stressor

Rudy Ortiz

No Recommended Changes

Janet Mann

1. Teeth *were* analyzed by Susan Chivers and Kelly Robertson for age determination.
2. We could not control for sample size of each organ received (ex. only 2 thymuses collected).