Involution of Lymphoid Organs in Bottlenose Dolphins (*Tursiops truncatus*) From the Western Gulf of Mexico: Implications for Life in an Aquatic Environment

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ABSTRACT

Involution of lymphoid tissues in relation to age has not been defined for bottlenose dolphins (*Tursiops truncatus*). Twenty-five bottlenose dolphins from the coast of Texas and western Louisiana were examined and complete necropsies were performed with histological samples taken of nearly all tissues. Ages ranged from several days to 27 years. The histology of four lymphoid organs—thymus, pharyngeal tonsil, mucosa-associated lymphoid tissue (MALT) of the colon, and anal tonsil—was assessed. Numerical scores were assigned to specific morphological features, thus creating an involution score. Definable and scorable features of each organ were selected for evaluation and determination of loss of lymphoid elements. Neonatal dolphins were recorded as the reference standard for no involution. The highest score for each organ represented the greatest amount of retention of tissue elements. Thus, the lower the score, the greater degree of involution. Comparing involution scores to tooth age permitted an assessment of involution over time. The greatest degree of involution was found in the MALT of the colon. The MALT of the colon declined dramatically so that after age 10 it was absent from 4 of 14 animals and minimally present in 8 others. Thymic tissue also suffered a precipitous drop in volume after about age 5, but was found in animals up to 24 years of age. Involution was moderate and variable in both pharyngeal and anal tonsils. In some animals, these tissues were reduced in volume early, and prominent in others well into adult life (over 20 years).

Key words: lymphatic system; thymus; tonsil; anal tonsil; colon mucosa-associated lymphoid tissue; lymph nodes; bottlenose dolphins; *Tursiops truncatus*

Lymphoid tissues are defined as organized collections of lymphocytes located in specific areas throughout the body (Junqueira et al., 1998). Cells of the lymphoid system, including plasma cells, are recognized as the morphological basis of the immune system. A typical mammalian immune system is constantly changing throughout growth and development. The immune system is established early on in life by dispersing immune component cells from specific organs such as the thymus and the functional equivalents of the Bursa of Fabricius (Junqueira et al., 1998). It is then maintained through the lymph nodes, bone marrow, spleen, and lymphoid cells resident in other tissues. After the development phase, the now unnecessary progenitor or formative lymphoid tissues decline in cellularity and fade or involute (Burkitt et al., 1993). Involution, in the context used here, means physiological loss of lymphoid elements from tissues or organs, progressing over time.

Several previous studies have described lymphoid organs and tissues in cetaceans (dolphins and whales). Cave (1979), Cowan and Smith (1999), Donaldson (1977), Simp-

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son and Garnder (1972), and Romano et al. (1993) have all described the location and structure of the pharyngeal tonsil, while Cowan and Smith (1995) discussed the location and morphology of the anal tonsil (a lymphoepithelial structure with close similarities to the pharyngeal tonsil but present in large numbers in the squamous lining of the anal canal in a variety of cetacean species). Cowan and Smith (1999) described the morphology of several lymphoid organs in bottlenose dolphins (*Tursiops truncatus*), including the thymus, spleen, lymph nodes (in various locations), and the gut-associated lymphoid tissues (GALTs). Simpson and Gardner (1972), Wunschmann et al. (1999), and Romano et al. (1993) also described the thymus of several other cetacean species. The GALT includes dorsal and ventral pairs of pharyngeal tonsil, anal tonsils, a sheath of well-organized lymphoid follicles in the mucosa of the distal intestinal tract (colon), and the mucosa-associated lymphoid tissues (MALTs). It was speculated that the MALTs of the colon served the functions of the vermiform appendix, which is absent from nearly all species of cetaceans (Cowan and Smith, 1999). While the lymph nodes are not recognized as involuting tissues, and the spleen of *T. truncatus* appears to enlarge with age, four tissues (pharyngeal and anal tonsils, thymus, and the distal intestinal lymphoid tissues) are said to involute with age, without specification of the relation of age to involution (Cowan and Smith, 1999).

The process of involution has been described for many different species and it occurs as an animal ages (Lubis et al., 1982; Suster and Rosai, 1990; Romano et al., 1993). Involution of the thymus in bottlenose dolphins has been observed in beach-stranded animals along the Texas coast of the Gulf of Mexico, progressing as body size increases, which is associated with aging and frequently accompanied by the formation of epithelial cysts in the thymus (Cowan, 1994). Newman (1971) also described the formation of thymic cysts in beagles and the increasing number of cysts as age increases. Similar involution and cyst formation have been observed in the harbor porpoise (*Phocoena phocoena*) in Germany (Wunschmann et al., 1999), indicating that cyst formation is not peculiar either to species or to geographic location. The latter observers reported remnants of thymus in the harbor porpoise as late as 12 years of age. Since all animals in these reports either died of disease or carried significant parasite burdens, it is not known whether thymic involution is affected by disease.

The purpose of the study reported here is to determine involution of the thymus, pharyngeal and anal tonsils, and MALTs of the distal intestine in relation to age in bottlenose dolphins. The spleen was not included in this study as it has been determined by previous work (Cowan and Smith, 1999) that age-related involution does not occur in the spleen.

MATERIALS AND METHODS

Samples used in this study were collected by the Texas Marine Mammal Stranding Network, under the auspices of the National Marine Fisheries Service, from stranded bottlenose dolphins. These animals either beach-stranded alive and died shortly afterward; washed onto the beach dead; were accidental net-captured; or died or were euthanized during rehabilitative efforts. The collection area ranges from Cameron County at the Texas/Mexico border to Cameron Parish in Louisiana (i.e., the entire Texas Gulf Coast and part of western Louisiana).

Twenty-five bottlenose dolphins were examined for this study, including 6 sexually immature males, 3 mature males, 4 immature females, and 12 mature females ranging in age from several days to 27 years. Sexual maturity was determined by macroscopic and histological examination of the gonads for evidence of spermatozoa production in males and ovarian follicular development in females. Age was determined by counting the growth layer groups (GLGs) in the dentine of decalcified, sectioned, and stained teeth (Hohn et al., 1989). This method is widely accepted among marine mammalogists as a reliable determinant of age.

Animals were taken to a laboratory at Texas A&M University at Galveston for complete necropsy with systematic sampling of organs and tissues. All collected tissues were placed in 10% neutral buffered formalin. After fixation, samples were embedded in paraffin wax, sectioned at 5 μ, and stained with hematoxylin, phloxine, and saffron (HPS), a trichrome stain used to distinguish collagen from smooth muscle. All sections were examined using a Nikon Optiphot-2 microscope with a top-mounted 35 mm Nikon camera.

The thymus was dissected out and weighed, but these results are not considered here. While the thymus is easily defined and removed as an intact structure in young animals, in older individuals it softens and becomes nearly indistinguishable from surrounding fibroadipose tissues, and weights were not considered reliable. The structure and relationships of the other tissues under consideration did not permit weight to be used as a criterion.

A sine qua non for selection was the determination of tooth age for the animals. Animals from this group were selected with well-sampled and well-preserved tissues. Tissue sample numbers are thymus, n = 14; pharyngeal tonsil, n = 16; anal tonsil, n = 15; and colon, n = 22 (Table 2).

Involution Scoring

Histology of four lymphoid organs (thymus, pharyngeal tonsil, MALT of the colon, and anal tonsil) was assessed, and numerical scores were assigned to specific morphological features. In this scoring system, the highest number applied to an organ describes the condition in a neonate, assumed to have no involution. Numbers assigned do not indicate equal divisions along a scale, but rather represent definable features that could be used to assign a score useful in graphic representation. In the thymus, assessable features included size and conformation of lobules, differentiation between cortex and medulla, presence and degree of fibrosis, and formation of cysts. Pharyngeal tonsil scoring included bulk of lymphoid aggregates, with or without germinal centers, prominence of mucus glands, and condition of crypts. Colon MALT scoring included presence of lymphoid aggregates and presence of germinal centers. Anal tonsil scoring included lymphoid aggregates with germinal centers, prominence of mucus glands, and condition of crypts. The specific rating criteria used for each organ or tissue are listed in Table 1.

Thus, a neonate thymus with large, full, closely approximated lobules, no interlobular fibrosis, clearly differentiated cortex and medulla, and no cysts would have a score of 9, the neonate pharyngeal tonsil a score of 5, colon MALT a score of 4, and anal tonsil a score of 7. With this

TABLE 1. Numerical classification for each lymphoid tissue describing level or state of involution.

	Thymus	Pharyngeal tonsils	Anal tonsils	Colon MALT
Lobules				
3, lobules large, full, closely approximated	X			
2, full, but separated by connective tissue	X			
1, shrunken, elongated or irregular	X			
0, none	X			
Cortex/medulla				
1, clearly differentiated	X			
0, unable to clearly differentiate	X			
Collagen fibers				
3, none	X			
2, few	X			
1, moderate	X			
0, predominate	X			
Cysts				
2, none	X			
1, small, scattered	X			
0, prominent	X			
Germinal centers				
2, many, often back-to-back		X	X	X
1, few, but present		X	X	X
0, none		X	X	X
Crypts				
1, narrow, tend to be slit-like		X	X	
0, open, may be dilated		X	X	
Mucus glands				
2, none		X	X	
1, small numbers identifiable		X	X	
0, predominate over lymphoid elements		X	X	
Lymphoid aggregates				
2, continuous layer		X		X
1, interrupted layer		X		X
0, none		X		X

system, an involution score of 0 would indicate total loss of assessable features of the subject tissue (Fig. 1B, D, F, and H). Comparing involution score to tooth age permitted an assessment of involution over time.

RESULTS

Involution scores for thymus, pharyngeal tonsil, anal tonsil, and colon MALT are presented in Table 2. Since these are spectrum changes, the ends of the arrays may be compared.

Thymus

The youngest four animals (< 4 years) showed minimal involution, exhibiting easily differentiated cortex/medulla, no cysts, and no fibers with few features of tissue loss (scoring an average of 6.75 ± 2.63; Fig. 1A), while the oldest four (> 19 years) scored an average of 2.5 ± 0.58 (Fig. 1B), having an undefinable cortex/medulla, cysts, and fibrosis.

Pharyngeal Tonsil

The youngest four animals (< 4 years) scored an average of 3.0 ± 1.15 (Fig. 1C) with abundant germinal centers, no mucous glands, and crypts, while the oldest four (> 19 years) scored an average of 1.75 ± 0.5 (Fig. 1D), showing few germinal centers and abundant mucous glands.

Colon MALT

The youngest four animals (< 2.5 years) scored an average of 3.0 ± 1.15 (Fig. 1E) by having a continuous layer of lymphoid aggregates and abundant germinal centers, while the oldest four (> 19 years) scored an average of 1.00 ± 0 (Fig. 1F), showing no lymphoid aggregates or germinal centers.

Anal Tonsil

The youngest four animals (< 4.5 years) scored an average of 5.25 ± 0.96 (Fig. 1G) with a continuous layer of lymphoid aggregates, abundant germinal centers, and no mucous glands, while the oldest four (> 19 years) scored an average of 3.75 ± 0.96 (Fig. 1H), having no lymphoid aggregates or germinal centers. These scores were then tested by running two sample *t*-tests (Systat version 7.0, SPSS) to see if there were any significant differences between the oldest four and youngest four animals ($P < 0.05$ is significant). Significant differences were found in the thymus ($P = 0.045$) and colon MALT ($P = 0.041$), while there were no significant differences found in the pharyngeal tonsils ($P = 0.116$) and anal tonsils ($P = 0.069$). Although the thymus is often assumed to be completely involuted in older animals, it was present in animals as old as 24 years. No sex differences were recognized. Figure 2 depicts the involution trends of the four organs in this study. Colon MALT experienced the greatest degree of involution as it was absent from older animals (Fig. 2A).

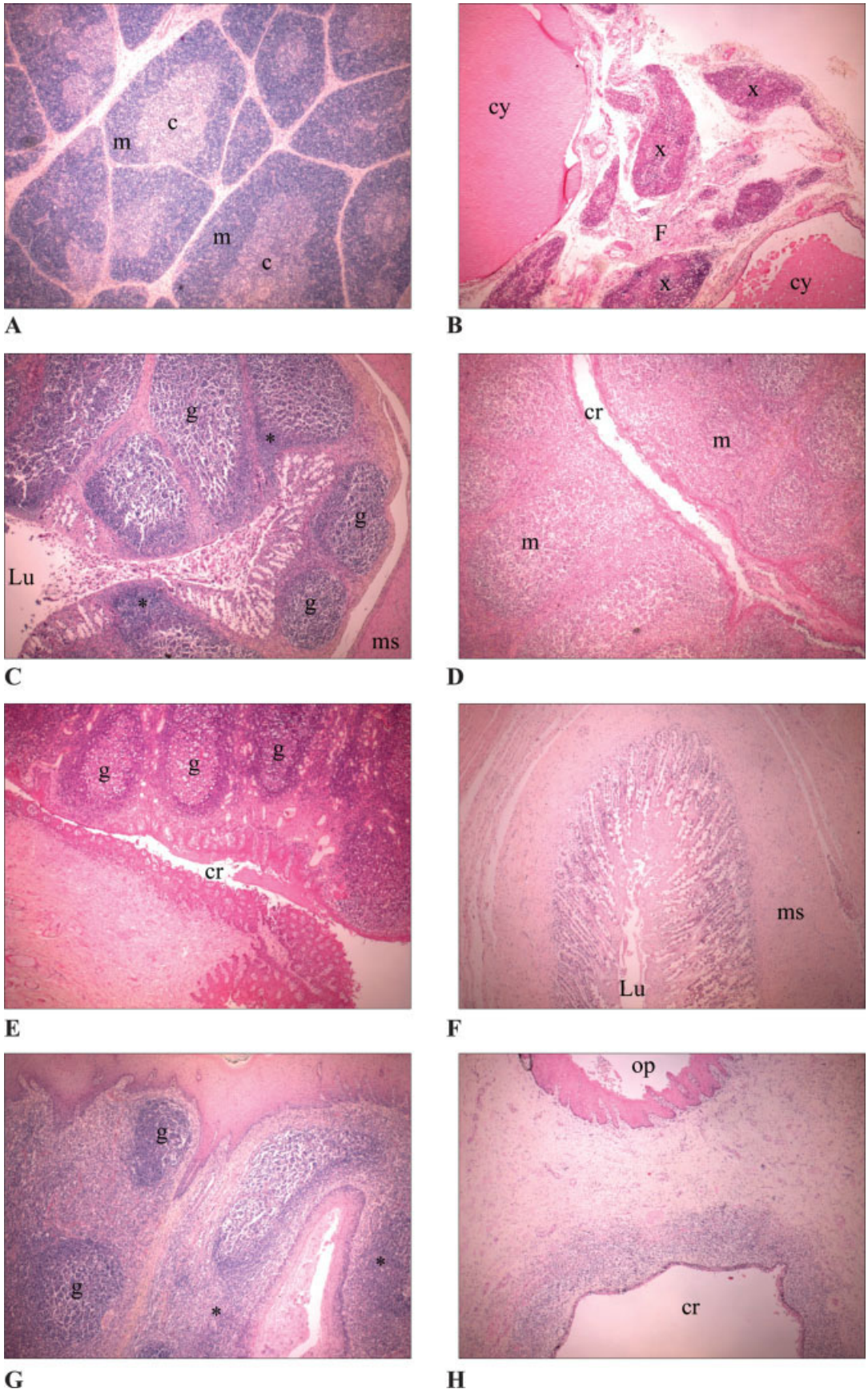

Figure 1.

TABLE 2. Involution scores for thymus, pharyngeal tonsils, anal tonsils, and colon MALT*

ID	Age (years)	Thymus	Pharyngeal tonsil	Anal tonsil	Colon MALT	Cause of death
GA947	0.01	9	NS	NS	4	Intraspecific aggression
SP190	0.33	9	4	6	4	Net entanglement
GA426	2	NS	4	NS	2	Sepsis
LA038	2.5	5	2	6	2	Net entanglement
GA705	3.5	4	NS	4	2	Heart failure, blood loss, and hydrocephalus
GA425	4	NS	2	NS	2	Inflamed liver and myocardial degeneration
PA381	4.5	NS	3	NS	NS	Hit by boat
PA409	4.5	NS	3	5	3	Net entanglement
LA040	5.5	4	2	5	1	Net entanglement and disease
PO275	7	NS	NS	NS	0	Severe arthritis and protozoan infection
SP189	8	9	5	5	2	Net entanglement
GA535	9	6	NS	6	2	Meningitis and arthritis
GA699	11	NS	NS	3	1	Septic arthritis
PA236	12	NS	NS	NS	1	Sepsis
PA292	13	4	NS	3	0	Esophageal perforation by fish spine
GA775	13	NS	NS	NS	1	Infection from rib fracture
PA397	14	NS	4	4	NS	Pleural effusion
CC110	15	3	2	4	0	Hepatitis
SP153	16	4	NS	NS	1	Disease and parasites in lung
PA368	16	NS	2	NS	NS	Hit by boat
PA387	19	3	0	3	0	Lung disease and hepatitis
GA664	19	3	1	4	1	Cardiac necrosis and hepatitis
PA361	20	2	2	5	1	Aneurysm and amyloidosis
GA710	24	2	2	3	1	Sepsis
GA881	27	NS	2	NS	1	Angiomatosis, amyloidosis and heart failure

*NS, not scored. Column 1 indicates an alphanumeric field number assigned to each animal by the Texas Marine Mammal Stranding Network. The next five columns indicate the animal age in years and the overall involution scores assigned to each tissue type with a smaller number indicating a higher degree of involution. Column six describes the cause of death for each animal as determined during gross and histological examinations.

The thymus also experienced a significant degree of involution but was found in many older animals (Fig. 2B). Figure 2C and D show a much slower involution rate for pharyngeal and anal tonsils, respectively.

DISCUSSION

Involution of the thymus and certain other lymphoid tissues has been documented in harbor porpoises, humans, dogs, and cattle, but the rate at which it occurs has not been defined for *T. truncatus* (Newman, 1971; Slijper, 1979; Lubis et al., 1982; Suster and Rosai, 1990; Wunschmann et al., 1999). Because of the number of animals examined, dissecting procedures, and aging techniques, we are able to relate and describe this involution process.

It has been accepted that the thymus completely involutes as animals age. This was not found to be the case in the present study. With careful and systematic dissecting techniques, we were able to locate at least some remnant of the thymus in the majority of animals examined, even those animals as old as 24 years, a finding consistent with the observations of Wunschmann et al. (1999) in the harbor porpoise and Suster and Rosai (1990) in humans. Epithelial thymic cysts are common in older cetaceans, as reported elsewhere (Cowan, 1994; Wunschmann et al., 1999), and may contribute to the identification of thymic tissue and its discrimination from fat and soft connective tissues.

The greatest degree of involution was found in the MALT of the colon, which declined dramatically so that after age 10 it was absent from 4 of 14 animals and minimally present in 8 others. In this, it bears further resemblance to the vermiform appendix, which tends to be most amply supplied with lymphoid tissue in early life, suffering an age-related attrition, so that in later adult life lymphoid tissues that remain may be very sparse. Thymic tissue also suffered a precipitous drop in volume after about age 5, but thymic remnants were found in animals up to 24 years old. Involution was moderate and variable in both the pharyngeal and anal tonsils. In some animals, these two lymphoid tissues were reduced in volume early and prominent in others well into adult life (over 20 years). This persistence may possibly occur throughout

Fig. 1. **A:** Thymus of a neonate with large full lobes, definable cortex (c) and medulla (m), no fibers, and no cysts (60×). **B:** Thymus of a 24-year-old showing elongated/not touching lobes (x), undefinable cortex and medula, moderate fibrosis (F), and cysts (cy; 60×). **C:** Pharyngeal tonsil of a 4.5-year-old with abundant germinal centers (g), no mucus glands, and normal crypts (cr; 60×). **D:** Pharyngeal tonsil of a 20-year-old with few germinal centers, abundant mucus glands (mu), and normal crypts (cr; 60×). **E:** Large intestine (MALT) of a 1-month-old showing a continuous layer of lymphoid aggregates (asterisk) and abundant germinal centers (g; 60×). **F:** Large intestine (MALT) of a 15-year-old with no lymphoid aggregates and no germinal centers (60×). **G:** Anal tonsil of a 1-month-old showing a continuous layer of lymphoid aggregates (asterisk), abundant germinal centers (g), no mucus glands, and normal crypts (60×). **H:** Anal tonsil of a 14-year-old showing no lymphoid aggregates, no germinal centers, no mucus glands, and dilated crypts (cr; 60×). Lu, lumen; ms, muscle; op, oropharyngeal cavity.

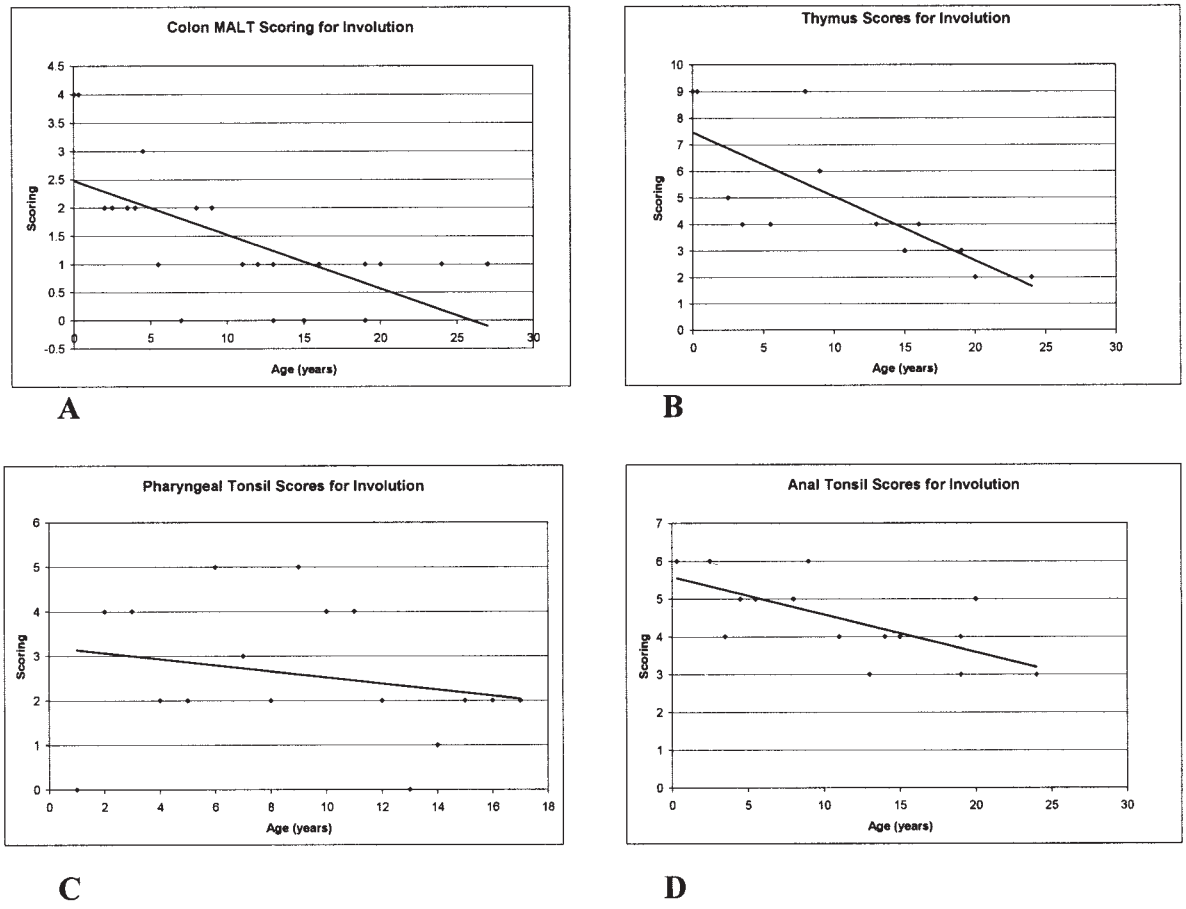

Fig. 2. **A–D:** Each graph shows the distribution of involution scores compared to the age of the animal. A lower score indicates a higher degree of tissue involution.

life because the tonsils are the first point of contact with environmental antigens.

The appearance of mucus glands in the involuting pharyngeal and anal tonsils is a special problem. It is not clear whether they have always been present, merely masked by the abundant lymphoid elements to become apparent on depletion of the lymphoid elements, or whether they develop as the lymphoid tissues fade out. We tend toward the latter interpretation, since some part of the mucus glands should be identifiable in very young animals, yet we have not found them. This implies that the primary function of these tonsillar tissues actively change over time, transforming from primarily immune to primarily lubricating and mechanically protective structures. This question requires further study.

The findings presented here may be of some value in providing an initial baseline for interpretation of findings in other bottlenose dolphins, in which immunosuppression from toxic exposure or chronic viral infection may be suspected. Even though the causes of stranding and/or death in some animals in this study were associated with bacterial infections (e.g., septic arthritis) or diseases (e.g., hepatitis), there were no noticeable differences in involution scores when compared to those animals that were killed by net entanglements or hit by boats. Our histological examinations did not reveal significant infections or tissue reactions typical of toxic exposures, so we cannot definitely rule out low-grade exposure. Also, organic toxicological studies were not performed.

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