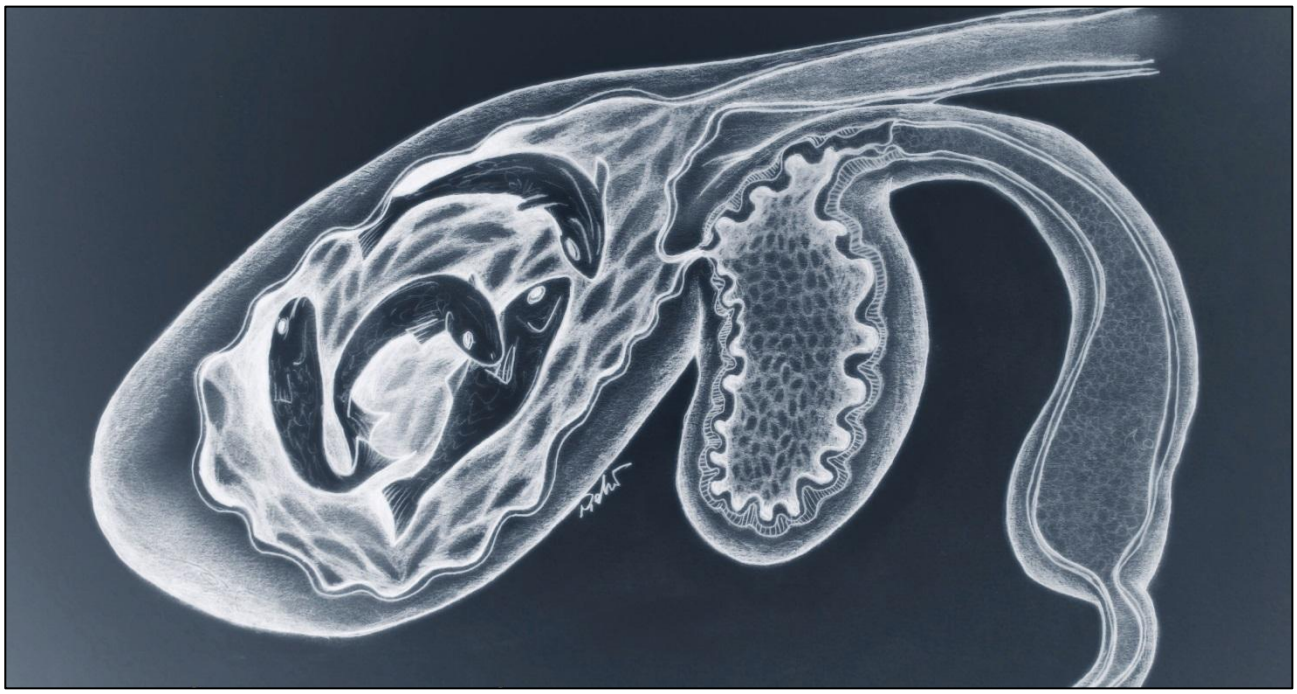
UNIVERSIDAD DE LAS PALMAS DE GRAN CANARIA

IUSA Instituto Universitario de Sanidad Animal Seguridad Alimentaria

Tesis Doctoral

Determinación de los principales datos de referencia para el examen del tracto gastrointestinal en delfines mulares (*Tursiops truncatus*) clínicamente sanos bajo el cuidado humano

Determination of the main reference data for the examination of the gastrointestinal tract in clinically healthy bottlenose dolphins (*Tursiops truncatus*) under human care



LETIZIA FIORUCCI

Las Palmas de Gran Canaria 2016

FACULTAD DE VETERINARIA

Instituto Universitario de Sanidad Animal y Seguridad Alimentaria

PROGRAMA DE DOCTORADO DE SANIDAD

"Determinación de los principales datos de referencia para el examen del tracto gastrointestinal en delfines mulares (*Tursiops truncatus*) clínicamente sanos, bajo el cuidado humano"

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Tesis Doctoral presentada por Dª: **Letizia Fiorucci**

Dirigida por los Dres. D. **Manuel Arbelo Hernández** y D. **Gabriele Brecchia**

LAS PALMAS DE GRAN CANARIA

DÑA. MARÍA SORAYA DÉNIZ SUÁREZ, SECRETARIA DEL INSTITUTO UNIVERSITARIO DE SANIDAD ANIMAL Y SEGURIDAD ALIMENTARIA DE LA UNIVERSIDAD DE LAS PALMAS DE GRAN CANARIA.

CERTIFICA

Que el Consejo de Doctores del Instituto en su sesión de fecha 13 de noviembre de 2015 tomó el acuerdo de dar el consentimiento para su tramitación, a la tesis doctoral europea titulada: **"Determinación de los principales datos de referencia para el examen del tracto gastrointestinal en delfines mulares (*Tursiops truncatus*) clínicamente sanos, bajo cuidado humano"**, presentada por la doctorando **Letizia Fiorucci** y dirigida por los Dres. D. **Manuel A. Arbelo Hernández** y D. **Gabriele Brecchia**

Y para que así conste, y a efectos de lo previsto en el Artº 73.2 del reglamento de Estudios de Doctorado de esta Universidad, firmo la presente en Las Palmas de Gran Canaria, a dieciséis de noviembre de dos mil quince.

UNIVERSIDAD DE LAS PALMAS DE GRAN CANARIA

IUSA Instituto Universitario Sanidad Animal Seguridad Alimentaria

Anexo II

UNIVERSIDAD DE LAS PALMAS DE GRAN CANARIA

Departamento/Instituto/Facultad: **Instituto Universitario de Sanidad Animal y Seguridad Alimentaria**

Programa de doctorado: **Sanidad Animal**

Título de la Tesis

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El Director,	**El Director**	**La Doctoranda,**
(firma)	**(firma)**	**(firma)**

Las Palmas de Gran Canaria, a _____ de_____ de 2015

UNIVERSIDAD DE LAS PALMAS
DE GRAN CANARIA

MANUEL A. ARBELO HERNÁNDEZ, PROFESOR CONTRATADO DOCTOR DEL DEPARTAMENTO DE MORFOLOGÍA DE LA UNIVERSIDAD DE LAS PALMAS DE GRAN CANARIA.

INFORMA:

Que la presente tesis doctoral titulada **"Determinación de los principales datos de referencia para el examen del tracto gastrointestinal en delfines mulares (*Tursiops truncatus*) clínicamente sanos, bajo cuidado humano"** realizada por Da. Letizia Fiorucci, con nº de pasaporte X9571831J, Licenciada en Veterinaria, bajo mi dirección y asesoramiento, reúne las condiciones y calidad científica para optar al grado de Doctor.

Y para que conste a los efectos oportunos, firmo la presente en Arucas, a dieciocho de noviembre de dos mil quince.

Fdo.: MANUEL A. ARBELO HERNANDEZ

UNIVERSIDAD DE LAS PALMAS
DE GRAN CANARIA

GABRIELE BRECCHIA, INVESTIGADOR DEL DEPARTAMENTO DE MEDICINA VETERINARIA DE LA UNIVERSITÀ DEGLI STUDI DI PERUGIA.

INFORMA:

Que la presente tesis doctoral titulada **"Determinación de los principales datos de referencia para el examen del tracto gastrointestinal en delfines mulares (*Tursiops truncatus*) clínicamente sanos, bajo cuidado humano"** realizada por Da. Letizia Fiorucci, con nº de pasaporte X9571831J, Licenciada en Veterinaria, bajo mi dirección y asesoramiento, reúne las condiciones y calidad científica para optar al grado de Doctor.

Y para que conste a los efectos oportunos, firmo la presente en Arucas, a dieciocho de noviembre de dos mil quince.

Fdo.: Gabriele Brecchia

UNIVERSITÁ DEGLI STUDI DI TERAMO

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Teramo, 20th. November. 2015

TO WHOM IT MAY CONCERN

This is to kindly inform you that I, Giovanni DI GUARDO, Associate Professor of *General Pathology and Veterinary Pathophysiology* at the Faculty of Veterinary Medicine of the University of Teramo (Teramo, Italy), have thoroughly read the PhD Thesis entitled "**Determinación de los principales datos de referencia para el examen del tracto gastrointestinal en delfines mulares clínicamente sanos (*Tursiops truncatus*), bajo cuidado humano"** (English translation: "Determination of the main reference data for the examination of the gastrointestinal tract in clinically healthy bottlenose dolphins (*Tursiops truncatus*) under human care"), written by Letizia FIORUCCI at the University of Las Palmas de Gran Canaria.

In this respect, I am pleased to inform you that the aforementioned PhD Thesis fulfils all the scientific requirements to be presented for being public evaluated by the Doctoral Commission at the University of Las Palmas de Gran Canaria.

Very truly yours,

Giovanni DI GUARDO

Giovanni DI GUARDO, *DVM, Dipl. ECVP*,

Associate Professor of *General Pathology and Veterinary Pathophysiology*,

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Perugia, november 13, 2015

TO WHOM IT MAY CONCERN

This is to inform that I, Elvio Lepri, professor of Veterinary Pathological Anatomy at the Department of Veterinary Medicine of the University of Perugia (Italy), have thoroughly read the PhD Thesis entitled "**Determination of the main reference data for the examination of the gastrointestinal tract in clinically healthy Bottlenose dolphins (*Tursiops truncatus*) under human care** " written by Letizia Fiorucci at the University of Las Palmas de Gran Canaria.

The above PhD thesis fulfils all the scientific requirements to be presented for being public evaluated by the doctoral commission at the University of Las Palmas de Gran Canaria.

Sincerely,
Elvio Lepri

INDEX

1. INTRODUCTION & AIMS

The sustainable balance of the marine environment is a increasing critical condition for a durable development of the planet. The protection and conservation of marine ecosystem is a global priority. For this reason, basic knowledge of the mechanisms that influence marine ecosystems are required. Throughout history have been accumulated a large quantity of biological, oceanographic, and geophysical data by which it has been possible to define risks and propose solutions for maintaining a sustainable balance between human activities and the conservation of the sea. The development of multidisciplinary research is essential, in order to preserve intact the delicate equilibrium of the marine environment. In this context, marine mammals receive a special interest, since, like human, are warm-blooded mammals, with an extended period of survival and are at the top of the food chain. They represent bio-indicators of the marine environment, providing valuable information on the degree of degradation / conservation of this habitat, from here, the importance the biomedical study of these species (Marsili, 2001).

Unfortunately, unnlike domestic terrestrial mammals, there is little information on many aspects of health of marine animals. However, in the last two decades it has made significant progress in this regard.

Cetaceans are protected species (Directive 92/43 / EEC), in particular, Bottlenose Dolphin (*Tursiops truncatus*), has been afforded special protected status under Annex II of the European Union's Habitats Directive. Several research groups in the world are working to provide baseline physiological and medical information, useful for investigations on emerging diseases and unusual mortality events in these animals. Population studies in beluga (*Delphinapterus leucas*) in Canada and several researches

concerning the mass mortality of striped dolphins (*Stenella coerulealba*) and bottlenose dolphins (*Tursiops truncatus*) in different regions of the world have provided data on the negative effect of environmental pollutants combined to infectious and non-infectious diseases on the populations and their habitat. Various projects have been developed around the world in order to define the physiological and medical parameters to define animals in a good state of health, and at the same time, give an assessment of the conditions of the marine ecosystem. In this regard, the Health and Risk Assessment (HERA) project was initiated in 2003 by the collaboration between the Harbor Branch Oceanographic Institution, the National Ocean Service, and the Center for Coastal Environmental Health and Biomolecular Research, to study by-caught the Atlantic bottlenose dolphin populations inhabiting the Indian River Lagoon, Florida, USA, and the coastal waters of Charleston, South Carolina, USA (Goldstein, 2006). Defining the health status of bottlenose dolphins is important for future management of this species and provides also information regarding the whole ecosystem where animals live (Bossart, 2003).

The study of physiology, medicine and pathology in cetaceans began to develop from the second half of the 60s, led by the work of American scientists and pathologists. At the beginning, these studies were mostly realized on animals held in captivity, although subsequently were also conducted in stranded cetaceans. Cetaceans kept under human care and cetaceans in wild life share many diseases, even if, in the first are detected some specific pathologies linked to the management and the controlled environment that have not been observed in wild animal life. At the same time, the wild cetaceans can show specific disorders related to the ocean environment and uncommon in animals kept in confined environment. Gastritis and gastric ulcers are frequently diagnosed in dolphins both under human care and in wild life. Nowadays, the ingestion of foreign bodies, such as plastic, is one of the main anthropogenic causes of strandings in free-ranging animals,

while gastric ulcers caused by problems within the social group is one of the most common findings in dolphins under human care.

Marine mammals, as several other non-domesticated species, frequently do not show early clinical signs of illness in various pathological processes. Preventive medicine in dolphins is fundamental to ensure the welfare of these animals under human care. Zoomarine mission is to work through the preventive medicine in order to ensure the welfare of housed animals. Thanks to medical behaviours, obtained by operant conditioning, the animals voluntarily submit themselves to veterinary procedures avoiding or minimizing stressful circumstances.

Diseases of the digestive system are quite common in marine mammals. Primary organs affected by these pathological processes are represented by the stomach and intestines. The main causes of these diseases are represented by pathogenic microorganisms (bacteria, viruses and fungi), tumors, foreign bodies, nutrition and stress. In this contest, it is very important to improve the knowledge about the physiology and the clinical pathology of marine mammals to prevent and treat these diseases. In addition, is also required a technological improvement of the diagnostic equipment and analytical techniques to obtain main reference data for the examination of the gastrointestinal tract. For this purpose, it could used the current knowledge, techniques and diagnostic procedures used in terrestrial mammals such as endoscopy, citology, and ultrasonography.

Therefore, the general goal of this thesis is to establish the main reference data for examining the gastrointestinal tract in clinically in healthy bottlenose dolphins (*Tursiops truncatus*), based, for the first time, on the “Guidelines for the Determination of Reference Intervals in Veterinary Species” issued by the Quality Assurance and Laboratory Standards Committee (Friedrichs et al., 2012). The use of the current knowledge available

for domestic mammals such as dogs and cats, could allow us to provide new information on cetacean species, in order to define the wild dolphin health status and, at the same time, have an assessment of the conditions of the marine ecosystem. In fact, the data collected can be useful not only to improve the management of bottlenose dolphins under human care, but, as reference values for healthy animals, may also serve to increase the knowledge on this species, and so on wild populations. Finally, they can be very useful for the clinicians who work at rehabilitation centers in diagnosing the causes of stranding in live animals, such as the foreign bodies ingestion, and thus increase the chances of survival and reintroduction into nature.

Below our specific purposes:

- to define reference baseline data for normal cytological findings of gastric juice samples in clinically healthy bottlenose dolphins;
- to assesse the existence of a correlation between healthy dolphins in the values of pH and epithelial cells, pH and WBC, and between epithelial cells and WBC;
- to combine for the first time cytology with the endoscopic, ultrasound and histological examination of stomach;
- to characterise the gastrointestinal tract of healthy bottlenose dolphins through ultrasonography;
- to define most useful acoustic windows for the examination of forestomach, fundic stomach, pyloric stomach and bowel;
- to set up a consistent and standardised method to measure the entire wall thickness in the forestomach, fundic stomach, pyloric stomach, and bowel;
- to defines the normal peristalsis of forestomach, fundic stomach, pyloric stomach, and bowel;

- to identify and to enumerate the different bacteria species such as Coliforms, E. coli, Staphylococci, Clostridium spp. and Yeasts from the gastric juice and faeces in clinically healthy bottlenose dolphins;
- to investigate potential age, sex, and facility-related variations on these parameters.

Adoption of “Guidelines for the Determination of Reference Intervals in Veterinary Species” by the entire veterinary community will improve communication and dissemination of expected clinical laboratory values in a variety of animal species and will provide a template for publications on reference intervals in order to promote quality laboratory practices in laboratories serving both clinical and research veterinarians.

2. REFERENCES REVIEW

Considering the morpho-physiological adaptations of the gastrointestinal tract of bottlenose dolphins is essential to understand some of the pathological processes that affect them. The identification of diseases in marine mammals first passes through the knowledge of normal morpho-functional aspects of these animals. To address this summary of the morphophysiological adaptations of cetaceans three basic reference books published literature on marine mammals were used:

- Handbook of ultrasonography in dolphins - Abdomen, Thorax & Eye – Saviano P. Ebook version available on Amazon Kindle Store, 2013.
- CRC Handbook of Marine Mammal Medicine (Second Edition). Section II. Edited by Leslie A. Dierauf and Frances M. D. Gulland. CRC Press, 2001.
- Marine Mammals Evolutionary Biology. Annalise Berta, James L. Sumich and Pieter Arend Folkens. Academic Press, 1999.

2.1. MAIN MORPHOLOGICAL CHARACTERISTICS OF THE GASTROINTESTINAL TRACT OF BOTTLENOSE DOLPHINS

All cetaceans are characterized by having a complex stomach with several compartments (**Figs. 1,2**). The odontocetes are fish-eating species, that is equipped with teeth all equal to each other, with whom grab the prey and then swallow it without masticate. Morphology of the gastrointestinal tract has been studied in several species of cetaceans, including bottlenose dolphins (*Tursiops truncatus*), and has been correlated

with the histological characteristics of each region (Harrison et al., 1970; Rommel & Lowenstein, 2001).

In dolphins, the esophagus is made up of a long, thick-walled tube. Its length depends on the size of the animal, constituting about a quarter of the body length in odontocetes (Rommel & Lowenstine, 2001; Berta et al., 2005).

The first chamber, called *forestomach*, is devoid of glands and is lined with a keratinized squamous epithelium, with mechanical function. It is larger in odontocetes, while it is absent in Cuvier's beaked whale (*Ziphius cavirostris*) and in La Plata Dolphin (*Pontoporia blainvillei*) (Berta et al., 2005). In delphinids, this stomach can hold several liters of water and has a muscular sphincter between the esophagus and stomach. There is not a clear delineation between the mucosa of the oesophagus and the forestomach (Dover & Van Bonn, 2001).

The ostium between the forestomach and the second chamber is located cranially in the left ventral quadrant of the first compartment (Rommel & Lowenstine, 2001; Berta et al., 2005).

The main stomach (glandular), called *fundic*, consists of a plicated mucosa, submucosa, muscularis and serosa; the mucosa is constituted by a simple columnar epithelium which invaginates to form the gastric crypts, where are located the gastric glands composed of parietal cells (acidophilic, produce HCl) and the chief cells (basophilic, produce pepsinogen) that provide the chemical part of digestion. The main stomach is equivalent to the stomach of monogastric mammals or abomasum of ruminants (Rommel & Lowenstine, 2001; Berta et al., 2005).

The connecting channel between the fundic stomach and the third chamber, called *pyloric* stomach, is intramural, small in diameter, and J-shaped (Dover & Van Bonn, 2001).

The relatively smooth pyloric stomach, (no pleats), contains typical pyloric glands and ends in a strong sphincteric muscle that regulates the flow of digesta into the duodenum of the small intestine. A strong sphincter marks the distal end of the stomach (the *pylorus*) before it connects with the duodenal ampulla. The initial part of the cetacean duodenum is expanded into a small saclike ampulla, draining the bile duct and pancreatic duct. The duodenal ampulla allows a better digestion of food, which could indicate that the enzymatic digestion has an important meaning in these animals (Rommel & Lowenstein, 2001).

In most marine mammals there is no cecum, and the different intestinal portions are not clearly defined, with a similar thickness along its entire length (Rommel & Lowenstein, 2001).

Histologically, the intestinal mucosa is formed by intestinal villi lined by cylindrical simple epithelium with micro villi and the Lieberkühn crypts. The stratigraphy from inside to outside includes: mucosa, lamina propria, submucosa, two muscular layers (transverse and longitudinal) and finally, more outside the serosa (Rommel & Lowenstine, 2001; Berta et al., 2005).

The liver of cetaceans is bilobed, there is no gallbladder (Rommel & Lowenstine, 2001; Berta et al., 2005).

The pancreas has a firm consistency, it extends transversely from side to side of the dorsal abdominal wall from the duodenum to the spleen and caudally the stomach; It

is larger in females than in males and is connected to the intestine through the main pancreatic duct (Rommel & Lowenstine, 2001; Berta et al., 2005).

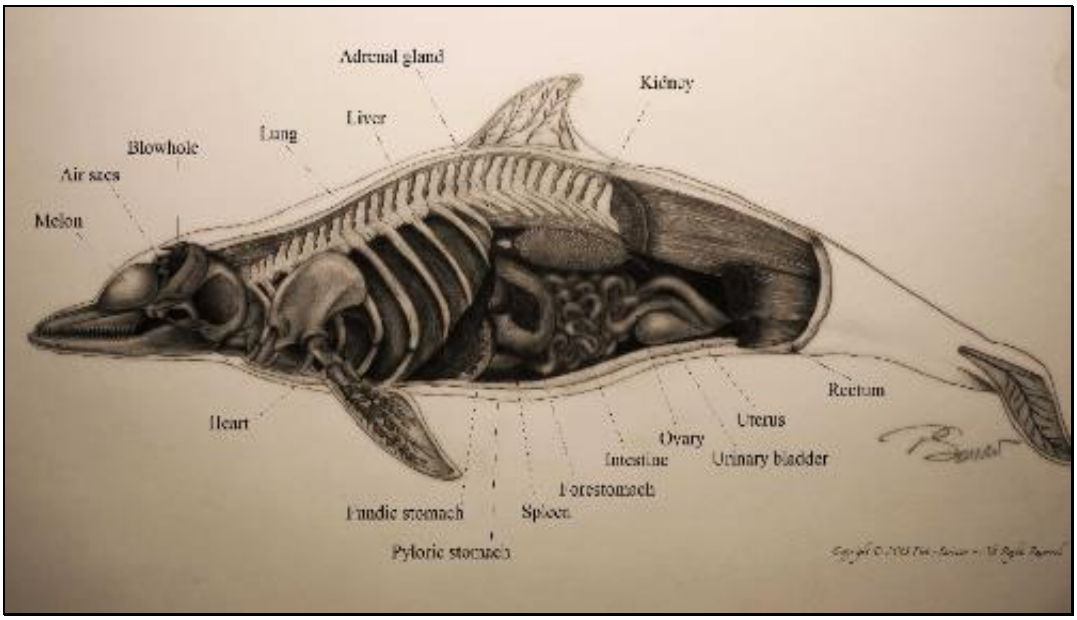


Fig. 1: Dolphin Anatomy.

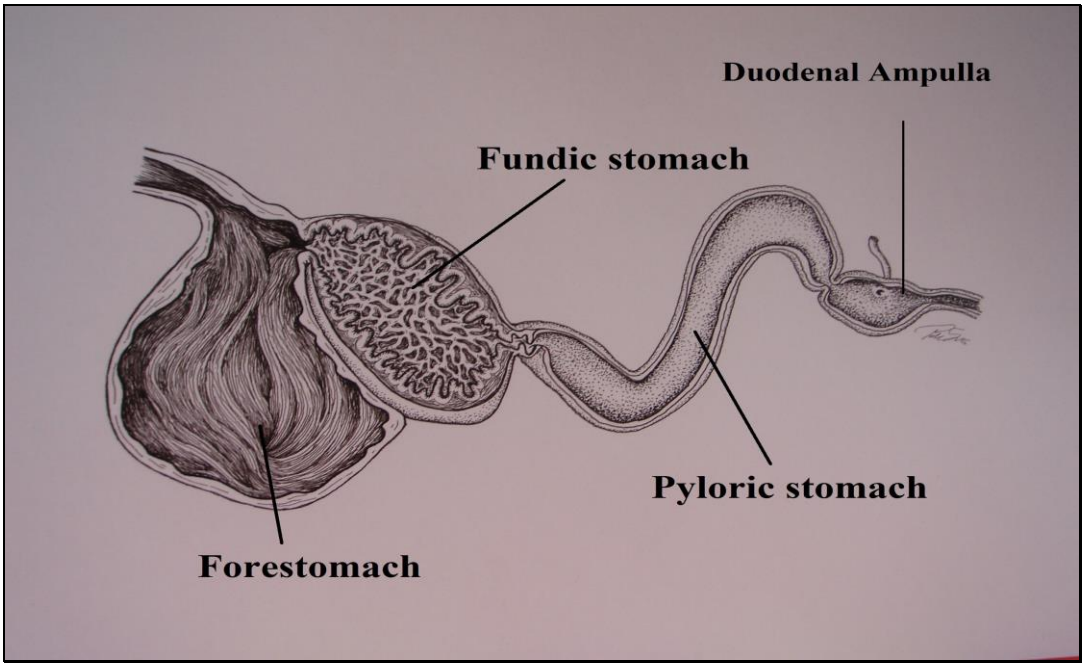


Fig. 2: Dolphin Anatomy: gastric chambers of bottlenose dolphins.

2.2 INTRODUCTION TO THE MAIN CAUSES OF DISEASE OF THE GASTROINTESTINAL TRACT OF BOTTLENOSE DOLPHINS

The study concerning the physiology, medicine and pathology in cetaceans began several decades ago, led by the work of American scientists and pathologists. First study were performed all above animals held in captivity, and subsequently were used also stranded cetaceans. Cetaceans kept under human care and cetaceans in wild life share many diseases, even if the first ones show specific diseases related to the management and the second ones related to the environment. The study of the medicine and pathology in dolphins, as in other species, requires basic knowledge of biology, anatomy and physiology of these species in addition to the basic knowledge of the scientific method.

Gastro-intestinal pathologies in marine mammals can be caused by pathogen microbic agents, parassites, tumors, foreign body and stress.

Primary gastrointestinal diseases of bacterial origin are an emerging problem in marine mammals (Saviano, 2011). Gastritis, gastric ulcers and lesions, frequently diagnosed in dolphins, may have a bacterial origin, as in human. This has recently been demonstrated by the isolation and characterization of a new species of Helicobacter spp. (*Helicobacter cetorum*) from the gastric mucosa of various species of Atlantic and Pacific toothed whales, common dolphin (*Delphinus delphis*), bottlenose dolphins (*Tursiops truncatus*) and belugas (*Delphinapterus leucas*) (Harper et al., 2000).

Rubio-Guerri et al. (2015) suggests that the adenovirus may be responsible for gastroenteritis in dolphins, porpoises and sea lions. Adenoviruses have been isolated from rectal samples in a sei whale (*Balaenoptera borealis*) in Antarctica (Smith and

Skilling, 1979), from the colon in two bowhead whales in Alaska (Smith et al., 1987) and from the intestine in belugas (*Delphinapterus leucas*) of the St. Lawrence estuary (De Guise et al., 1995).

Sweeney (1978) described hemorrhages, necrotic enteritis and peritonitis associated with *Pasteurella spp*. in cetaceans.

Sweeney (1986) suggested that *Pasteurella multocida* may cause enteritis and cause death of animals associated with intestinal bleeding and bacteremia.

Walsh et al. (1994) observed vomiting, diarrhea, gas and abdominal muscle spasms in cetaceans with gastrointestinal diseases induced by *Clostridium perfringens*. The clostridial toxin most commonly found in cetacean produced by *Clostridium perfringens* is the type A.

Furthermore, another well known cause of health problems in marine mammals are parasites.

Dailey & Brownell (1972) described as the Anisakidae family, the most common in cetaceans, can be located both in the first gastric chamber as in the third. Sometimes these nematodes can create nodules in the mucosa and submucosa. Moderate infestations rarely cause clinical signs, but severe may produce gastritis and ulcers (Dailey, 1985; Smith, 1989).

Pholeter gasterophilus is a fluke that is observed in the second and in the third gastric chamber. This parasite is deeply inserted into the submucosa, forming small blackish nodules. The mucosa over nodules usually remain intact, they contain abundant fibrous tissue around the parasite, with an eosinophilic inflammatory reaction (Woodard et al., 1969). In severe cases it may partially obstruct the intestinal transit.

Braunina cordiformis is another fluke normally found in the second and in the third gastric chamber and in the duodenal ampulla in bottlenose dolphins (*Tursiops truncatus*),

causing a slight irritation of the gastric mucosa (Delyamure, 1955; Schryver et al., 1967; Johnston and Ridgway, 1969; Zam et al, 1971).

Dailey & Stroud (1978) found a case of mixed infestation of two species of trematodes of the genus Hadwenius a harbor porpoise (*Phocoena phocoena*). Parasites were anchored in the lining of the pyloric part of the stomach and anterior duodenum, associated with hyperemia and mild bleeding. The adult tapeworms that infest the intestines of toothed cetacean (*Strobilocephalus triangularis*) can penetrate the wall of the colon and form granulomatous nodular lesions that can occlude the lumen in severe cases (Dailey, 1985).

Although not very frequent in literature, some cases of tumors of the gastrointestinal tract are listed.

Geraci et al. (1987) observed papillomas on the tongue and leiomyoma in the intestine of Atlantic white-sided dolphin (*Lagenorhynchus acutus*), De Guise et al. (1994 and 1995) observed carcinomas and adenocarcinomas in the stomach, liver and intestine of beluga (*Delphinapterus leucas*) and in the stomach of the common dolphin (*Delphinus delphis*).

Although many of the erosions, gastric and intestinal ulcers are associated with parasites or bacterial infections, also foreign bodies (Bossart et al., 1991) or stress (St. Aubin and Dierauf, 2001) have been described as possible causes of gastrointenstinal diseases.

Gastric and intestinal obstructions by ingestion of foreign bodies have been widely described in both, under human care and wild animals (Lamberts and Kohn, 1987; Kastelein and Lavaleije, 1992, Tarpley and Marwitz, 1993; Baird and Hooker, 2000) .

Intestinal volvulus associated with necrosis have been described in pantropical spotted dolphin (*Stenella attenuata*), bottlenose dolphin (*Tursiops truncatus*), bowhead whale (*Balaena mysticetus*), beluga (*Delphinapterus leucas*) and false killer whale (*Pseudorca crassidens*) (Martineau et al. 1988; Heidal and Albert, 1994; Briggs and Murname, 1995; Anderson and Rawson, 1997). Stamper et al. (2006) described a single case of successful recovery in juvenile female pygmy sperm (*Kogia breviceps*) which presented the obstruction of the stomach by plastics, mainly housed in communication between the second and third compartment stomach, producing the decreased intake and progressive weakness in the animal. These foreign bodies were extracted in several endoscopic procedures, and after the animal began to recover, eating more food and gaining weight gradually until he was released. The immediate improvement in behavior and in the post-health plastic extraction indicates a direct cause-effect relationship.

Goldstein et al. (2006) studied apparently healthy Atlantic bottlenose dolphins (*Tursiops truncatus*) inhabiting the Indian River Lagoon, Florida, USA. Sixty-two dolphins were captured, examined, and released during June 2003 and June 2004. 24% (7/29) of the dolphins examined in 2003 had evidence of neutrophilic gastric inflammation (3/29 had mild gastric inflammation, and 4/29 had severe gastric inflammation). In 2004, only 4% (1/24) of the population had mild or moderate neutrophilic gastric inflammation; no severe inflammation was present. Findings were not the result of differences in age or sex between dolphins sampled in 2003 and 2004. All animals exhibiting gastric inflammation were 8 year of age or older (average age, 14 yr). All but one animal with gastric inflammation was male. The high prevalence of severe gastric inflammation in their 2003 samples may be an indicator that pathologic stressors were affecting these individuals. The reasons responsible for the preponderance of gastric inflammation among older males were unclear.

Fair et al., (2006) studied the occurrence of gastric inflammation in dolphins from the estuarine waters around Charleston, South Carolina (CHS), USA. It was also similar

during the two years analyzed: 26% in 2003 and 29% in 2004. The prevalence of severe gastric inflammation was similar in Charleston and Indian River Lagoon dolphins (Goldstein et al., 2006). Older males comprised the majority of the animals exhibiting severe gastric inflammation; the mean age was 14 yr in both populations. Gastric inflammation may be an indicator of stress, either physiologic or pathologic.

Goldstein et al. (2012) studied 114 apparently healthy Atlantic bottlenose dolphins (*Tursiops truncatus*) inhabiting the Indian River Lagoon (IRL), Florida, and from 73 dolphins from the estuarine waters around Charleston, South Carolina (CHS) USA. Gastric, fecal, and blowhole samples were collected to assess the presence and degree of cytologic evidence of gastric inflammation from 2003 to 2007. The prevalence of moderate and severe gastric inflammation was 9.6% in the IRL and 11.0% at CHS. A case-control study of 19 dolphins with cytologic evidence of gastric inflammation and 82 with normal cytology from the combined populations was conducted by Goldstein et al. in 2012. Blood parameters evaluated included hematology, serum chemistry, serum protein electrophoresis, and stress hormones. Few differences of clinical or statistical significance were found between affected and unaffected dolphins. Serum norepinephrine and cortisol were significantly higher in cases compared to the controls, and aldosterone was marginally higher (P ¼ 0.06) based on eight cases. None of the hematologic, serum chemistry, or serum electrophoresis results were significantly different. Gastric fluid pH was not significantly different between cases and controls. There were no clinically significant aerobic-anaerobic or fungal culture results from gastric contents; bacteria cultured from both groups were considered to represent normal flora. The prevalence of inflammation did not differ by gender. Historically, cytologic evidence of gastric inflammation has constituted a marker of systemic illness in dolphins; however, there was little evidence to indicate systemic illness among affected animals in the current study (Goldstein et al., 2012). Additionally, there was an absence of hematologic and serum chemistry abnormalities indicative of systemic illness in those dolphins with evidence of gastric inflammation. The clinicopathologic parameters most frequently utilized to

determine the presence of systemic inflammatory disease in cetaceans include complete blood counts (CBCs), alkaline phosphatase, plasma fibrinogen, erythrocyte sedimentation rate, and serum iron. All of these parameters were included in the current study (Goldstein et al., 2012); however, no statistically significant differences in the mean values for these parameters were found between cases and controls. Definitive evidence of anemia was not found in the current study (Goldstein et al., 2012). However, the MCV, MCH, serum iron, and PSAT were slightly lower in cases vs. controls and were considered to be of clinical concern. In domestic animals, the most common cause of iron deficiency or low serum iron is often chronic hemorrhage. Further, a CBC will reveal a leukocytosis and anemia in cases of gastric hemorrhage, which can be associated with gastric ulceration. Thus, the lower values for MCV, MCH, and iron found in the cases may reflect chronic blood loss. In addition, iron sequestration is a common occurrence in most chronic disease states. Decreased or low serum albumin values occur with malnutrition, gastrointestinal disease, and especially parasitism. Albumin levels in the dolphins exhibiting gastric leukocytosis were similar to both those of healthy controls (4.40 vs. 4.49) and values reported for healthy dolphins from the IRL in an earlier study. It is possible that underlying conditions such as helicobacteriosis, bacterial infections, or parasitism within the gastric fundus or pylorus that could not be assessed adequately during this study could have contributed to the inflammatory process. The forestomach gastric pH did not differ significantly between dolphins with evidence of inflammation and healthy controls. This was an expected finding, as the bacteria cultured were all considered to represent normal flora. Therefore, it is unlikely that a bacterial infection or overgrowth of normal flora were the primary cause of the gastric leukocytosis. The presence of bacterial overgrowth in marine mammals typically results in increased gastric pH secondary to bacterial phagocytosis of the stomach mucosa. This is in contrast to the response in humans, which results in lowered pH. The authors considered the possibility that the cytologic findings may be an indicator of chronic stress in this population. Previous studies of IRL dolphins have reported multiple stressors, including subclinical morbillivirus infections,

orogenital papillomatosis, and lobomycosis. Results indicated that serum norepinephrine and serum cortisol were significantly elevated in cases compared to controls and that serum aldosterone was also elevated, with borderline statistical significance (Goldstein et al., 2012). Chronic physiologic stressors can result in amplified release of glucocorticoids, including cortisol, which may in turn reduce the immune system's ability to function properly (e.g., prevention of inflammatory cell exudation and decreased intracellular destruction of bacteria). Elevation in endogenous cortisol also occurs in response to pain and extreme fluctuations in body temperature. Cortisol and aldosterone, the principal glucocorticoid and mineralocorticoid measured in cetaceans, are also known to be influenced by factors such as chase, capture, and restraint. It was hypothesized that amplified catecholamine release (epinephrine, norepinephrine) occurs in dolphins with gastric leukocytosis. Elevated catecholamine values are frequently seen in mammals responding to fear, excitement, and muscle exertion. The results for norepinephrine and epinephrine were inconsistent; however, there is no clear explanation for this difference (Goldstein et al., 2012). In addition, aldosterone is greatly increased in cetaceans and pinnipeds subjected to adrenocorticol stimulation. The presence of elevated cortisol, norepinephrine, and aldosterone levels in the cases vs. controls could be an indicator that physiologic stress is a contributing factor to abnormal gastric cytology. The cases and controls in the current study (Goldstein et al., 2012) experienced the same capture and sampling process, and, thus, differences between the groups were unlikely to be related to these factors. Samples from IRL and CHS dolphins were obtained during the same month of each capture year, thus reducing the potential effects of seasonal stress. The potential roles of changes in diet, reproductive status, or environmental factors on the results could not be determined. However, cases and controls were sampled concurrently, reducing the likelihood that these factors created bias (Goldstein et al., 2012). The role of acute and chronic stress on endogenous glucocorticoids in dolphins has not been clearly established, so the current results must be interpreted with caution. The analyses of glucocorticoids and catecholamines were based on 8 cases and 48 controls. Therefore,

the power to detect statistically significant differences was limited. A larger sample size is needed to determine what role, if any, stress hormones play in the pathogenesis of gastric inflammatory disease. The data obtained by Goldstein et al.(2012), provided a basis for further investigation and evaluation of gastric cytology in wild and managed bottlenose dolphins. Cytologic examinations can provide a snapshot of potential illness prior to systemic disease. Dolphins in a managed care or rehabilitation facility exhibiting moderate to severe cytologic evidence of gastric inflammation are often treated for potential gastric ulceration. However, increased numbers of inflammatory cells in gastric fluid may not be indicative of gastric ulceration. Applying the concepts of evidence-based medicine and the results of the Goldstein's study should encourage clinicians to proceed with caution in examining the assumption that gastric leukocytosis indicates the presence of gastric ulcerations. Without the use of advanced sampling methods, such as endoscopic examination of the cetacean stomach, combined with histologic examination of stomach tissue there is no definitive way to diagnose gastric disease. Evidence-based medical techniques deemphasize such assumptions and promote a systematic clinical examination. Caution must also be exercised when examining populations at a single point in time (as opposed to serial investigation), since the long-term consequence of abnormal gastric cytology are uncertain.

2.3 BOTTLENOSE DOLPHIN UNDER HUMAN CARE: MEDICAL BEHAVIOUR

Health management of marine mammals in a controlled environment is based on three factors closely related to each other:

• Preventive medicine

• Animal welfare

• Synergy among staff

The goal of a good preventive medicine is to create and maintain optimal conditions for animal welfare. It is based on a series of routine tests, framed in a monthly medical plan. These tests make it possible to monitor the health of the animal and intervene before that occur symptoms of disease. Among the routine tests carried out are:

• Gastric juice examination

• Blowhole mucus examination

• Ultrasound examination

• Urine analysis

• Faeces analysis

• Blood analysis

Other tests that can be performed are:

1. Radiographic examination

2. Endoscopic examination

Test samples are made for voluntary behavior due to training based on operant conditioning with positive reinforcement.

This technique requires that the animal is encouraged only positively with primary (food) and secondary (games, cuddling, etc.) reinforcers. The relationship, the trust and the cooperation established between the animal and the trainer plays a major role. Trainers communicate with animals through hand signals and voice; each signal refers to a singular behaviour that the animal will be able to perform. The training involves the use of three basic tools:

• *TARGET* (the physical objective that the animal can follow, it is the instrument through which the trainer "draws" the behaviour that want to teach).

• *BRIDGE* (the connection between the start of the required behaviour and the reinforcement given at the end of it).

• *REINFORCEMENT* (it is something very positive for the animal and it is given to facilitate the behaviour).

Thanks to training, is possible to improve the quality of management of the animals and avoiding or minimizing the stressful circumstances. For this reason it is essential the synergetic work of veterinarians and trainers.

Moreover, the veterinarian staff take charge of the nutrition of the animals, taking into account their needs depending on the species, physiological/pathological status, age, sex, seasonality, etc. The best diet includes several species of fish and is based on kilocalories and kilograms. It should be also integrated with a vitamin supplementation.

2.4 BOTTLENOSE DOLPHIN UNDER HUMAN CARE: NUTRITION

Husbandry of marine mammals under human care is often more of an art than a science, primarily due to a lack of data on the nutritional and energetic requirements of most species. The most important facet of animal husbandry is meeting the daily energetics needs of the animal. The magnitude of energy required is a function of body size, age, gender, activity level, physiological and reproductive status, thermoregulatory expenses of the animal, and whether or not the animal is actively growing. These energy expenditures are collectively referred to as the daily metabolic rate. This rate can be conveniently subdivided into two components: maintenance energy and production energy. Mainteinance energy costs, which include basal (or standerd) metabolism,

thermoregulation, and the cost of locomotion, are expenses that are incurred by all animals, but do not include any investment in growth or the production of new offspring. There have been a few studies that have monitored food (or energy) intake in captive cetaceans (Kastelein and Vaughan, 1989; Cheal and Gales, 1991; Kastelein et al., 1993,1994). These studies noted a relationship between food intake and factors such as proximate composition (or energy density) of food, water temperature, growth rate of juveniles, pregnancy, and activity level. An understanding of the relative costs of each of these parameters is essential to understanding energetic constraints on marine mammals (Worthy G.A.J. 2001).

The bottlenose dolphin is the most common cetacean held in captivity. It is a cetacean odontoceta, ichthyophagous, which in the wild feeds on a wide variety of species of fish and cephalopods. A varied and balanced diet is an essential factor in maintaining the health of this species under human care. For this reason, species of fish with higher fat are supplemented with leaner fish or invertebrates, such as the squid (*Loligo vulgaris*) (Couquiaud, 2005).

A good nutritional program in human care situation must rely on a good understanding of the composition and quality of the food being offered. All food can be classified with respect to the amount of moisture, protein, and fat that it contains. Knowledge of each of three major components is of importance in assessing the true value of a diet. The water content provides information regarding the amount of preformed water that is available to the animal, the protein content influences the amount of waste heat generated as the heat increment of feeding and the urinary energy losses, and the fat content is the major determinant of the energy value of the food (Worthy G.A.J. 2001). Many prey species show age-related and seasonal changes in proximate composition, and therefore energy content. These changes are usually related to reproductive condition, with gravid females being exceptionally high in lipid content. These seasonal

changes can be extreme, as in the case of herring (*Clupea harengus*) where fat content can range from 2 to 4 % during early spring to 15 - 20 % in the winter (Stoddard, 1988). Other species, such as capelin (*Mallotus villosus*) show equally impressive changes in composition (Jangaard, 1974). Changes in energy content can lead to a great disparity in energy intake with similar amounts of different fish being consumed. As the concentration of fat increases, the protein to calorie and water to calorie ratios decrease.

Moreover, fat-soluble vitamins will rise and water-soluble vitamins will decrease with increasing fat content (Worthy G.A.J. 2001). Considering the fish supplied to the marine mammals under human care is frozen, the losses of nutrients in freezing, storing and thawing should be taken into account and compensate with a vitamin supplementation, containing hydrosoluble vitamins such as C, B1, B2, B5, B6, B8, B9, B12. The fat-soluble vitamins A, D, and E, are abundant in marine organisms (Dierenfeld et al., 1991). Therefore, as long as the fish is fresh, adequate levels of these vitamins are present, but they break down quickly in bad fish. Marine and coldwater fish store energy as polyunsaturarated fats that remain fluid at low temperatures. These fats are also unstable in the presence of oxygen, leading quickly to peroxidation and rancidity. Peroxidation consumes vitamin E in the fish, as well as affecting the marine mammal eating it by increasing its vitamin E requirents. This type of vitamin supplement should be provided at least every one hour before the first feeding session, to improve the correct absorption by the body. The dose given to each animal will depend on various factors such as the reproductive status, health, physical activity, and age (White Floyd and Francis, 1988). The diet of a young dolphin after weaning is identical to an adult (Immerzeel and Lotens, 2005).

Scombroid poisoning is a potential health azard whenever poorly preserved scombroid fish, such as mackerel, are eaten over an extended period. The clinical signs are respiratory congestion, abdominal pain, nausea, vomiting and diarrhea. These

symptoms resemble those produced by histamine or related compounds. The best prevention for scombroid poisoning is to avoid using scombroid fish species that have been stored beyond their safe shelf life (4 months) (Geraci and St. Aubin, 1980).

When basic nutritional needs are not met, animals may divert nutrients in different metabolic ways to compensate for stress, leading to decreased resistance to infectious agents and ultimately to disease. The understanding and application of specific nutritional regimes can prevent many such problems from developing.

2.5 MAIN DIAGNOSTIC FINDINGS FOR EVALUATION OF THE GASTROINTESTINAL TRACT OF BOTTLENOSE DOLPHINS

2.5.1 ENDOSCOPY

Endoscopy means “to look inside”. The term is usually reserved for examination of the interior of hollow viscera such as bronchi or the instestinal tract. Flexible endoscopes have been used in marine mammal medicine for many years, and it should be considered a required diagnostic tool in any marine mammal practice (Dover &Van Bonn, 2001). The most common use of endoscopy in marine mammals is the evaluation of the gastrointestinal tract.

An adult bottlenose dolphin requires a gastoscope with a minimum lenght of 150 cm for a thorough gastroscopy. Larger marine mammals, including killer whales (*Orcinus orca*) usually require a flexible gastroscope longer than 200 cm. Dolphin can be trained for this medical provedure, otherwise the animal should be restrained by trainers. In this case, sedation of the animal may be indicated even if gastroscopy could be performed

safety without medication. Fasting for at least 6 hours prior the examination is recommended considering normal gastric emptying time of healthy dolphins is less than 4 hours. To perform a gastroscopy, animals should be placed over a closed pore foam mattress in sternal recumbency and maintained in that position by trainers. The endoscopist should be positioned at the head of the animal. Clean terry cloth bath towels should be placed around the maxillary and mandibular rostrum. The distal scope tip should be passed over the dolphin's tongue and into the oropharynx, esophagus, and then into the forestomach. The oral cavity and esophagus of cetaceans contain a thick tenacious mucus that will readily adhere to the lens and blur the image; ensure that there is a properly functioning water irrigation valve present to prevent or correct this problem. As the gastroscope is advanced into the oropharynx, numerous punctate openings are visible in the mucosa. These are the openings of mucous glands, which lubricate food items prior the swallowing. Moving the dolphin on its right lateral recumbency, is possible to pass the ostium between the forestomach and the fundus to evaluate the second chamber. Air insufflation is required during the whole procedure in order to obtain a diagnostic image. A moderate amount of fluid is usually present in the forestomach, this fluid can be collected by aspiration for cytological examination. The connecting channel between the fundus and the pyloric chamber is intramural, small diameter and J-shaped. Therefore, it is not possible to examine the remainder of the stomach compartments or proximal small bowel of cetaceans with current technologies.

Endoscopy has the undoubted advantage of allowing not only the visual examination of the mucosal surface, but also the execution of targeted biopsy, by appropriate biopsy instruments, with the acquisition of several samples for cytological and histological evaluation,. Endoscopy, permits to accurately examine the esophagus, first stomach and second chambers, but not to reach the third compartment and the bowel with this procedure. It is always recommended collect biopsy samples, even in the absence of macroscopic mucosal alterations, since only through of the histological examination it is possible to exclude structural alterations or infiltrative diseases.

2.5.2 CYTOLOGY

Cytology, the microscopic study of cells, is a common, inexpensive, and readily available diagnostic tool that constitutes a valuable part of a medical evaluation for both terrestrial and aquatic species (Sweeney & Reddy, 2001). Jergens et al. (1998) found a high correlation between the results of cytological and histological examination of samples collected by endoscopy of the stomach, small intestine, and colon of cats and dogs. The anatomy and physiology of cetaceans render cytological sample easily obtainable. In the fasted cetaceans, the stomach almost always contains fluid, which acts as a repository for exfoliated cells that can be sampled and examined. Cytologic examinations can provide a snapshot of potential illness prior to systemic disease (Cowell et al., 1999), in fact, an elevated number of leukocytes along with increased epithelial cells, particularly basal cells and or erythrocytes in the fluid of the stomach might suggest ulceration (Mitchell et al., 2008).

Cytologic evidence of gastric inflammation is a common finding in stranded marine mammals and likely has a multifactorial etiology. Possible contributors to cetacean gastric inflammation include *Helicobacter spp*. infection, parasitic overload, ingestion of prey that irritates the stomach wall, malnutrition, and chronic physiologic stress (Goldstein et al., 2012). Currently, the most common way to identify the presence of gastric inflammation is by cytologic analysis of gastric fluid. The presence of small numbers of leukocytes (10 cells por high power field) in the forestomach of cetaceans is a normal occurrence in both captive and free-ranging dolphins. However, the finding of moderate (10–20 cells/hpf) and severe (20 cells/hpf) numbers of leukocytes, particularly neutrophils, may lead clinicians to suspect the presence of gastritis (Sweeney & Reddy, 2001).

Gastric cytology can also be altered by the swallowing of oropharyngeal or respiratory secretions, leading to a false interpretation of gastric cytology.

Marine mammals often do not show clinical signs of disease until the disease process is advanced (Sweeney & Reddy, 2001). However, cytologic abnormalities can be

indicative of illness well before the onset of clinical signs. The identification of cytological abnormalities combined with hematology and serum chemistry could serve as a useful tool by which it is possible to determine the presence of illness prior the exhibition of clinical signs (Sweeney & Reddy, 2001).

Husbandry programs at facilities that mantain marine mammals under human care, enhance their medical care by conditioning animals to allow physical examinations and collection of specimen without the need for physical restraint. Regular repetition maintans the behaviour, and allows cytological monitoring. This provides baseline data for each animal, important precursor of the effective evaluation of pathologycal cytology (Sweeney & Reddy, 2001). Following careful sampling from body fluids and aspirates for cytology, microscopic examination of the appearance and quantity of the individual cells collected can provide valuable information about the current status of the examined body systems. In the broad field of veterinary medicine, cytological evaluation is most commonly used as a post-hoc diagnostic aid, employed following the presentation of certain clinical signs from the patient. For example, an aspirate of cells may be extracted using a fine needle from a lump seen on a dog or a cat, and identifying the predominant cells can reveal whether the mass is benign or malignant (Perman, Alsaker, & Riis, 1979). Another common use of cytology occurs during urine analysis, as the identification of crystals or casts in a urine sample may be a clue toward disease associated with the kidneys and urinary tract. Cytology is rarely used as the sole means of reaching conclusive diagnoses; instead, it provides an initial context by which to direct further diagnostic procedures. Dewhurst and Skeldon (2009) found that while cytology is a very useful tool, many clinicians underuse it.

There are notable differences in the care of marine mammals compared to that of companion animals, and thus the role of cytology has evolved in order to suit the needs of this field. While the aquatic environment is the most obvious difference and provides its own unique challenges for sample collection, the most important difference in terms of animal care is the increased number of dedicated caregivers needed to train behaviours

with the animals. Historically, training innovative voluntary husbandry behaviours has not only proven to be valuable to advancing marine mammals medicine (Sweeney, 1984), it has now become an integral part of maintaining the health and welfare of these animals under human care. Animal trainers not only build meaningful relationships with the animals they care for, they also provide the animal with the opportunity to voluntarily participate in various medical and husbandry procedures, a rarity for companion animal medicine. Training provides more opportunity for physical examination with reduced stress for animal, trainer, and veterinarian (Sweeney & Reddy, 2001). This in turn provides a cornerstone from which healthcare can transition from reactive to proactive and to which true baseline values are more likely to occur. The training techniques utilised with exotic species at zoos and aquariums are unprecedented and have contributed to the development of highly sophisticated preventative healthcare programs. These programs are constantly being enhanced, and this creates an ever-increasing demand for diagnostic resources for marine mammals (Varela, Schmidt, Goldstein, & Bossart, 2007). Many facilities employ full-time teams of veterinarians and technicians, in addition to the training and husbandry staff responsible for the daily care of the animals in their charge. In this setting, cytology has taken on a new role as part of the preventative healthcare screen, and animals participate through training to offer samples of cells from their different body systems. Sweeney and Reddy (2001) noted the importance of behavioral training for cytological sampling, as maintenance and repetition of collection behaviors can provide a large bank of baseline data for each animal, thus contributing to the detection and monitoring of any known pathologies, as well as progression toward recovery. The inclusion of a cytological database for each individual animal is an invaluable resource, unique to the field of marine mammals medicine.

Varela, et. al., (2007) state that physical examination of the animal alone is not enough to assess certain problems beyond a macroscopic level, and that cytology can provide more information in the event that disease manifests itself through cellular abnormalities, rather than by outward clinical signs. It is for this reason that regular

sample collection is often a part of daily operations include in a preventative healthcare programs, as there may be cases where cytology is the first clue to an underlying pathology (Cowell, Tyler, & Meinkoth, 1999). Husbandry protocol includes monthly collection of sputum, urine, fecal, and gastric samples from each of its trained animals for cytological analysis.

The steps in cytological assessment are specimen collection, preparation, microscopic examination, and interpretation (Sweeney & Reddy, 2001). Gastric sample collection requires the use of a clean 2 cm diameter polyethylene tube similar to those used in the nasogastric intubation of equine species. The dolphin is trained to rest calmly in a 'heads-up' position directly in front of the trainer. The tube is carefully passed into the open mouth and down into the stomach (Hayward, 2003). Gastric samples are taken inserting the gastric tube into the first chamber with a mild pression and rotation. (Hayward, 2003). To ensure correct placement of the tube, the trainer may listen for sounds of gastric juice (described as 'gurgling'), or an odor may be perceived from the open end of the tube. Once in place, the trainer may apply gentle suction to the end of the tube, using a negative-pressure siphon technique, before bending the tube to prevent loss of the sample. The tube may then be slowly removed, and the sample transferred to a clean sample cup. The samples are stained in New methylene blue and evaluated for the presence of epithelial cells and wbc. Gastric samples be read as soon as possible after collection; however, samples may also be refrigerated for up to one week (Hayward, 2003). The pH of the fluid is measured by color fixed indicator strips.

Collection of fecal samples requires training the dolphin to lay calmly with its ventrum exposed from the water. A trainer's hand is used to support the peduncle at the level of the genital and anal slits. From here a narrow 0.5 diameter tube is lightly lubricated and passed into the anal slit around 13-15 cm (Hayward, 2003) and never more than 38 cm (Sweeney & Reddy, 2001). The tube is then kinked to avoid loss of sample as it is slowly removed. The sample is transferred from the tube into a sample cup. While all samples should be read as soon as possible to avoid desiccation of cellular material

(Perman, Alsaker, & Riis, 1979), faecal sample shelf life, is up to eight hours without refrigeration (Hayward, 2003).

2.5.3 ULTRASONOGRAPHY

Ultrasonography was considered a survey technique not suitable for evaluation of the gastrointestinal tract, until a short time ago. The presence of gas, food and feces, in addition to the low definition of ultrasound devices, have been an obstacle to obtaining an accurate and comprehensive evaluation of the entire gastrointestinal tract. Technological advances, combined with the ever widening experience of veterinarians, have allowed a significant improvement in the quality of the ultrasonographic findings. This diagnostic technique is currently one of the most used to assess gastrointestinal diseases in domesticated animals. Ultrasonography plays an important role in modern-day cetacean preventive medicine because it is a non-invasive technique, is safe for both patient and operator, and is also used to follow up in animals undergoing therapy. Sonographic study has also the advantages that not need of a special preparation of the animal, allows an evaluation of the entire gastrointestinal wall rather than just the mucosa as with endoscopy. Ultrasonagraphy permits to perform different measurements and not only of the wall thickness, gives an assessment of motility in real-time and finally, provides an assessment of regional disorders (peritonitis) (Tyrrell & Beck, 2006).

Ultrasonography is a diagnostic method based on the use of ultrasound and exploits the properties that allow them to move in space and to interact with the bodies and the surfaces they encounter. The ultrasound acoustic mechanical waves have frequencies range considerably higher than those perceptible by the human ear. The energy transmission is realized with the oscillation of the particles of the medium traversed by the ultrasound, which, approaching and moving away between them, creates bands of compression and rarefaction. As all the waves, even ultrasound are characterized by various parameters such as the amplitude, the wavelength and

frequency. The ultrasound machines are composed of a ultrasound source and a receiving system, both identifiable in the transducer, which transforms acoustic impulses into electrical impulses and vice versa, by software that processes the images and a screen that makes them visible. Ultrasound waves are emitted from a source consisting of piezoelectric material which, stimulated by electrical pulses, is capable of entering into vibration for a brief instant, transmitting the acoustic waves to the means surrounding and placing then in a reception phase, which allows to enter vibrating with the reflected waves (echoes) and sends it to the transducer in the form of electrical impulse. The reflected echoes are represented on the screen in an image based on a gray scale, ranging from black to white. The more intense the received echo, the clearer will be represented at the structure that generated it. There are several types of probes: linear, microconvex and sectoral or phased array (Ciaramella et al, 2013). In bottlenose dolphins the linear probe provide images with better axial and lateral resolution, require a large acoustic window and is not indicated to cover different dimensions and depth as ranging between 7.5 and 13 MHz. Not even the microconvex are suitable because they are too small.

Ultrasound meets all the requirements of an ideal diagnostic method: it is widely applicable in all subjects, it has high diagnostic accuracy independent of the function of organs and, above all, non-invasive especially in individuals trained to perform such medical behavior. An echographic image can be represented in three different ways.

• A-mode (mode amplitude): is the simplest way of representing the ultrasound signal and is kind of one-dimensional (it provides an analysis in one dimension). A mode-mode has limited applications only in ophthalmology and neurology.

• TM-mode (time motion mode): you get an image, in which each echo is represented by a light spot. It is similar to A-mode with the difference that is also recorded the movement of the echo. This method is used in eco-cardiography.

• B-mode (brightness mode): it is a classical image of a section of the body. It offers the classic ultrasound images (Ciaramella et al, 2013).

The advantages and applications of clinical diagnostic ultrasound in the field of veterinary medicine are now well established, also within the marine mammals that is undoubtedly one of the most important diagnostic techniques. It is important to recognise that ultrasound does not replace other diagnostic techniques and still remains secondary to a thorough physical examination. Ultrasound is an important addition to the information provided by physical, endoscopic and radiological examination.

Ultrasound examination of bottlenose dolphin is generally performed outdoors and in direct sunlight, so it may be difficult to see the image. This can be overcome by using a dark cover to shield the monitor or the glasses-display allowing the display without reflections. The choice of the transducer depends on the size of the patient and the area of interest. The 3.5 MHz convex probe provides a wider field size ultrasound examination of bottlenose dolphin. All transducers have the modern dynamic focusing which means the depth of the focal zone and changes along the beam axis by extending the focal zone along the depth of the image. The transducers are usually waterproof, but it can be further protected from saltwater covering them with a plastic bag containing acoustic gel and sealed with waterproof tape around the cable.

The animal training is essential for medical procedures such as ultrasound, which is now an integral part of medical behaviors that take place within the structures in a controlled environment to monitor their well-being. The ultrasound examination is performed in sternal and lateral "decubitus". The voluntary behavior of the animal in cooperation with the trainer creates a situation of no stress. However sometimes the animal may not be able to cooperate usually due to the intrinsic nature of his disease. In this scenario it is appropriate to retain the animal in shallow water rather than move it completely out of the water in such a way as to be much more comfortable, thereby reducing stress (Saviano, 2013).

Dolphins scan of the upper abdomen is relatively easy. Parasagittal and transverse planes scanning through the lateral abdominal wall provides better access to the entire abdomen. The abdomen can be divided into three sections: the cranial includes liver,

biliary system, spleen, pancreas, forestomach, second stomach (or bottom), duodenal bulb and pylorus; the middle portion contains a part of the small intestine, the kidneys and the pregnant uterus; caudal portion presents the final part of the small intestine, rectum, bladder and reproductive organs. Ultrasound can provide very useful information on most of the gastro-intestinal tract. Endoscopy results to be complementary ultrasound for clinical investigations of the different gastric compartments.

In order to examine the three gastric chasmbers with ultrasonography it is possible to use both, dorsal and lateral recumbencies. The forestomach and the second stomach are visible on the left side, while the third stomach on the right side (Saviano, 2013).

The forestomach is often difficult to be examined by ultrasound. The presence of free gas or whole food mixed with the liquid lumen may produce artifacts. However it is possible scan the forestomach changing the position of the animal to avoid so the effect of the gas. In the case where there is a suspicion of ingestion of a foreign body, the ultrasound examination should be performed while fasting: the absence of food makes the visualization of any foreign body easier. Another option is to administer water by tube. This method allows a very good scan of the lumen of the forestomach and improves visualization. The fluid in the forestomach can provide a good acoustic window to the deeper anatomy of the cranial abdomen including the spleen and pancreas (Brook et al, 2001). In order to facilitate the ultrasound examination it is important to know what is the diet of the animals. The forestomach can be observed in the sternal or left lateral decubitus, placing the probe on the right side of the animal, behind the liver and the fundus. The wall is thin and the surface of the mucosa can be evaluated only after the administration of water in fasting aniamals in order to avoid artifacts caused by gas and food residues that are usually present.

The fundus is displayed on the left side of the dolphin and it is the most similar to the stomach structure found in other mammals. The morphology and morphometry are characterized by the presence of folds whose thickening can suggest the presence of gastritis or parasitosis. The mucosa layer may appear hypoechoic in healthy animals. An

increase in size and in a dramatic hypoechogenicity, combined with an abnormal peristalsis and an abnormal pattern represents a strong indications of gastritis (Saviano, 2013). The echogenicity of the lumen during digestion is usually homogeneous and hypoechoic compared to the fundic wall and the hepatic parenchyma (Saviano, 2013).

The pyloric stomach can be seen extending laterally from the fundic stomach, then reflecting at the medial border of the right liver, to run caudomedially for a short distance within the cranial abdomen (Brook et al, 2001). The size, motility, transition of contents through the pyloric chamber may provide useful information (Brook et al, 2001). The chamber is located between the second pyloric stomach and duodenum continuing along the liver. It is linear and has an echogenic structure when it is healthy. The presence of digested food mixed homogeneously in fluid and gas translates into a more echogenic than the liver parenchyma (Saviano, 2013).

The structure of the three compartments varies widely. First the stratiographic appearance as well as the lumen should be examined and the resulting findings should then be compared with the peristalsis.

There are different patterns of the contents of the lumen which can be described in the gastrointestinal tract in dolphin:

- *Mucous pattern:* when only a hyperechoic layer can be seen on the mucosal surface due to the presence of mucus in the lumen; the mucous appears as hyperechoic without any acoustic shadows.
- *Food pattern*: when food is present inside the lumen. The forestomach usually presents a heterogeneous food pattern because in this section of the tract is possible to observe whole fish parts during the different phases of the mechanical digestion process. In addition, digestive gases may be present. In the fundic chamber the digested food is mixed with fluid and subsequently microbubbles of gas distend the lumen. The common food pattern for this section is homogeneously echoic as well as in the pyloric section. Food

pattern in the intestine instead is hypoechoic, but may have different echogenicities.

- *Fluid pattern*: it is represented by the presence of fluid in the lumen and has a typical anechoic sonographic appearance.
- *Gas pattern*: it is characterised by the presence of hyperechoic free gas as well as typical artifacts in the lumen.

Moreover, mixed models could be present: for example, a mixed model mucous-gassy pattern. The echogenicity of the light is different for various digestive processes. In dolphins is impossible to distinguish the different sections of the intestine which appears homogeneous in all its lenghts (Saviano, 2013). The advantage of ultrasound is the possibility of daily tests in animals under treatment. It is important to repeat the measurements in the same area of the stomach. The degree of dilatation of the lumen must be assessed by comparing the measurements. Three are the basic measurements necessary to evaluate the gastric apparatus with ultrasonography: the thickness of the folds, the thickness of inter-rugae and the size of the mucosal layer (Saviano, 2013). In addition, the ultrasound examination is the least invasive and most accurate way to detect the presence of ascites in dolphins. Ascites is generally a negative prognostic indicator (Dover et al, 2001).

Permeability tests are also used to evaluate integrity of the mucosal barrier along the entire length of the gastrointestinal tract in humans and in various other animals (Buddington et al., 2006). The administration of fluids in the forestomach can provide a good acoustic window to the deeper anatomy of the cranial abdomen (Brook et al, 2001). The oral administration of a solution as a contrast represents a good medium to improve the quality of the ultrasound image to mark the mucosal layer. Lesions in the forestomach and crianial portions of the fundus can be visualized by endoscopy. However, in dolphins, the fundus contains very little free gas and can be seen quite well by ultrasonography. It is easily identifiable by its position, thick wall, and distinctive rugae (Brook et al, 2001). A marked increase in rugal thickness may indicate gastritis or parasitosis (Woodard et al.,

1969) in an animal in which normal apparances are known. On the other hand, the forestomach is rarely empty and is also highly distensible, making measurements unreliable. The relatively fluid caudal bowel contents of marine mammals provide a useful “contrast medium” when examining the gastrointestinal tract (Brook et al, 2001). Gas content in caudal bowel is normally low when compared with that of other species, and changes in mural thickness are more easily seen (Brook et al, 2001).

Marine mammals derive not only energy, but also metabolic water from their food. Since most fish and invertebrates consist of 60 to 80% water is a direct component of food. Metabolic water derives from the metabolism of fat, protein an carbohydrate. Supplementation of fresh water in marine mammals under human care is often necessary even if their diet is balanced and fish is of excellent quality. The reason is that frozen fish loses vitamins and water (Worthy, 2001). The preferred method for hydration in bottlenose dolphin is oral supplementation. If gastric motility is assumed to be normal, giving fresh water via stomach tube is an excellente method (Walsh and Gearhart, 2001). Moreover, another method to give water supplementation is through gelatin or ice. For this reason, the administration of an alimentary contrast medium may be a good solution to mark the mucosal layer. This procedure it is safe for the patient, can be routinely performed using medical behaviours, and over all, an homogeneus fluid provides an ideal echogenity for the examination of the digestive system.

2.5.4 MICROBIOLOGY

Microbiology (from Greek μῑκρος, mīkros, "small"; βίος, bios, "life"; and -λογία, -logia) is the study of microscopic organisms, those being unicellular (single cell), multicellular (cell colony), or acellular (lacking cells) (Madigan et al., 2006). Microbiology encompasses numerous sub-disciplines including virology, mycology, parasitology, and bacteriology. The mammalian commensal microbiota constitutes of over thousand bacterial phylotypes and the gastrointestinal tract harbors the largest amount of microbes (Suchodolski, 2014). Microbiota confers important functions including a mucosal barrier functions, a metabolic function (e.g. digestion of xenobiotics, fermentation of indigestible substrates, production of short chain fatty acids and vitamins, ions absorption) and immune regulatory functions contributing to the development and regulation of the gut immune system (Backhed et al., 2005). Microbiota also reduces the growth of pathogenic bacteria creating a physiologically restrictive environment, competing with pathogens and toxins for adherence to the intestinal epithelium and then inhibiting the bacterial translocation (Hill et al., 2010). The composition of the microbiota can be influenced by various factors, including diet, exposure to antibiotics and the well functioning of mechanisms of immune tolerance (Jernberg et al., 2007; Koenig et a al., 2011). Buck et al., (2006) collected samples from presumably health free-ranging bottlenose dolphins (*Tursiops truncatus*) of Florida, Texas, and North Carolina. Vibrios, unidentified Pseudomonads, *Escherichia coli, Staphylococcus spp*., and a large group of nonfermenting Gram-negative bacteria represented 50% of isolates. Many organisms occurred sporadically in dolphins that were sampled repeatedly, but some were consistently isolated from individual animals and may indicate the carrier state. This may indicate that, although healthy animals have a wider spectrum of associated microorganisms in low numbers, debilitated animals are characterized by a greater number of opportunists. Chan et al. (2001) examined 15 captive cetaceans monthly over a 7-yr period and found the following organisms as representing 0.2% of isolates: *Vibrio*

alginolyticus, (24.7%), *Candida albicans* (8.4%), *Proteus mirabilis* (6.5%), *Shewanella putrefaciens* (3.3%), *Morganella morganii*, (3.1%), *Staphylococcus aureus* (2.4%), and *Pseudomonas aeruginosa* (2.1%). Asper and Odell (1980) sampled 26 wild dolphins from the east coast of Florida and found *Escherichia coli*, *Staphylococcus epidermidis*, *Proteus vulgaris*, and *Pseudomonas aeruginosa* most common in blowhole samples with frequent isolation of *Saccharomyces* species.

A detailed description of the normal gastrointestinal microbiota in dolphins, is necessary to provide additional and relevant data, very useful to the clinician. The knowledge of the normal composition of the microbiota of the dolphin can be useful to understand the role of some bacteria species in the development of different infectious and inflammatory gastro-intestinal diseases, to evaluate the general health status of wild marine mammals, to favour the rehabilitation of stranded or animals under human care that show clinical signs of digestive pathologies. In addition, know the composition of the intestinal microbial flora of the dolphin might be important to prevent the spread of gastrointestinal diseases among animals and the risk of transmitting pathogens to humans. It is also important take in consideration that changes of normal microbiota probably play a crucial role in the development of different inflammatory gastro-intestinal pathologies both in human and several terrestrial animals. Cultivation based on analysis is still the most simple and inexpensive system to quantify gut bacteria. This technique does not permit detection of some species- or strain-level but is functional to evaluate physiological parameters, does not require extensive bio-informatic analysis (Sekirov et al. 2010) and can be carried out by the veterinarian as routine examination by voluntary behaviour to assess the health of the animals under human care.

3.0 MATERIALS & METHODS

Fig. 1. Bottlenose dolphin (*Tursiops truncatus*)

3.1 ANIMAL COLLECTION

In this study 48 bottlenose dolphins (*Tursiops truncatus*) hosted in three different facilities (A – B – C) were inspected. Of this group, 31 healthy bottlenose dolphins (*Tursiops truncatus*), with an age between 5 and 36 years old (mean±SE = 16±1ys), a weight between 149.9 and 266.7 kg (mean±SE = 190.1±4.3 kg) were examined and sampled. A total of 15 females and 16 males were evaluated (**Table 1**). The average water temperature in the different facilities was 23°C during all sampling period (**Tab 2**). The diet of all animals during the study consisted of capelin (*Mallotus villossus*), sprat (*Sprattus sprattus*), herring (*Clupea harengus*), mackerel (*Scomber scombrus*), horsemackerel (*Trachurus trachurus*), smelt (*Atherina boyeri*), squid (*Loligo spp*.), blue whiting (*Micromesistius poutassou*), and sandeel (*Hyperoplus lanceolatus*).

Sampling was carried out from January 2012 to December 2014.

ID	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31
Age (y)	20	15	15	11	11	22	16	19	5	7	36	35	21	9	17	17	15	11	8	34	30	25	15	15	15	10	10	7	8	14	14
Gender	M	M	M	F	F	M	M	M	M	M	F	F	F	M	M	F	F	M	M	F	F	M	F	F	M	F	M	F	F	F	M
Weight (Kg)	236,7	173,8	191,1	156,0	160,8	266,7	190,3	239,0	149,9	150,8	256,8	236,9	240,6	160,0	199,1	195,0	193,3	169,3	159,3	220,2	230,2	187,8	175,0	180,0	177,8	157,6	167,8	151,6	151,9	183,2	186,0

Table 1. Animal collection.

	FACILITY											
MONTH	A				B				C			
January	18,1	18,6	18,3	18,3	19,3	19,7	19,6	19,5	18,3	18	18,4	18,2
February	18,5	18,9	18,4	18,6	19,3	19	19,4	19,2	19	19,4	20,3	19,6
March	18,7	19,6	19,5	19,3	21,5	20,4	20,7	20,9	20,8	20,9	20,8	20,8
April	20,2	21,1	21,1	20,8	20,3	20,9	21,3	20,8	20,6	22	21,5	21,4
May	22,7	25,4	24,2	24,1	21	21,5	22,7	21,7	21,1	23,6	22,8	22,5
June	23,1	23,4	27,7	24,7	24,2	25,5	26,8	25,5	22,3	25,9	25,6	24,6
July	24,7	27	28,6	26,8	29,7	29,9	31	30,2	27,6	27,2	27,8	27,5
August	28	28	30,3	28,8	32,9	33	32,5	32,8	28,8	28	29,5	28,8
September	26,5	23,5	23,5	24,5	30,5	28,5	28	29	26,5	24,5	24	25
October	27,7	28,2	26,7	27,5	25	25	23,5	24,5	23,5	21	23,5	22,7
November	20	21	19	20,0	21	21	21	21,0	20	21	20	20,3
December	18,6	18,6	18,5	18,6	19,3	18	19,4	18,7	19,6	19,3	19,1	19,2
Annual Mean				22,7				23,7				22,6
Average water T°C	23											

Table 2. Average Water Temperature in the three different facilities (A – B – C) examined. The data listed in the table correspond to the average temperature of the days 10, 20 and 30 of each month.

Parameter	Results	Measurement units
Humidity	70.55	g/100g
Protein	15.69	g/100g
Fat	7.30	g/100g
Ash	2.18	g/100g
Sugar	1.12	g/100g
Gross Energy	187.5	Kcal/100g
TVBN (Total Volatile Basic Nitrogen)	7.2	Mg NH_3/100g
Peroxydes	39	Meq O_2/Kg
Microbiología	300	UFC/g

Table 3. The diet of all animals: mean values for the mackerel (*Scomber scombrus*) for each facility.

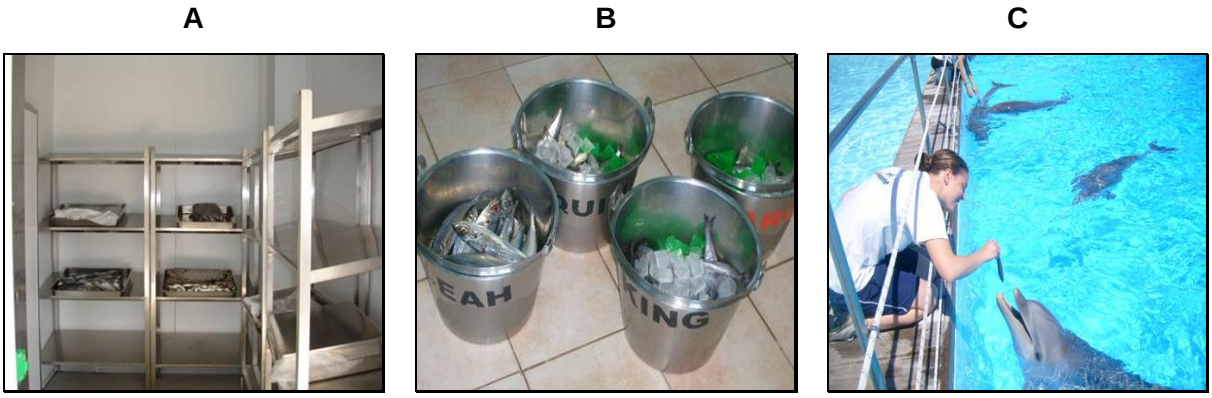


Fig. 2. The animals diet. **A:** defrosting of fish; **B:** preparation of the daily diet; **C:** feeding session.

The following criteria were selected for inclusion in the study:

• All animals were considered clinically healthy based upon a physical exam and laboratory evaluation (complete blood count and serum chemistry, urine analysis, and fecal examination) (Fair et al., 2006; Mitchell et al., 2008).

• Ultrasound evaluation of the gastroenteric tract of all the animals was performed.

• Endoscopy of the forestomach and the fundic stomach of all the animals was performed.

• Histological evaluation of the full-thickness biopsy of the forestomach and the fundic stomach of all the animals was completed.

Criteria for exclusion of individuals from the study included the following:

• Lack of good health or presence of ongoing disease

• Not complete endoscopic or ultrasonographic evaluation of the subjects

• Pregnancy

3.2 ENDOSCOPY: INSTRUMENTATION & METHODOLOGY

A portable KARL STORZ GASTRO PACK® (manufactured by KARL STORZ GmbH & Co. KG D-78532, Tuttlingen, Germany) was used for the endoscopy. The unit combines a videoendoscope with 3.25 m length (60130 PKS), a camera control unit, a monitor, a documentation unit, an insufflation pump, a keyboard, and a light source in a single system. Endoscopy was carried out as part of the annual general clinical check-up included in the veterinary preventative medicine program established for each institution. No additional endoscopic evaluations were specifically required for the purpose of this study. The bottlenose dolphins were restrained in a pool that features a lifting platform and examined individually. For gastroscopy, the standard endoscopic procedure for cetaceans (Dover & Van Bonn, 2001) was followed. To perform the procedure, all animals were examined after fasting (15 h). The animals were placed over a closed pore foam mattress in sternal recumbency and maintained in that position by trainers. The endoscopist was positioned at the head of the animal. Clean terry cloth bath towels were placed around the maxillary and mandibular rostrum. The distal scope tip was passed over the dolphin's tongue and into the oropharynx, esophagus, and then into the forestomach. Air insufflation was required during the whole procedure in order to obtain a diagnostic image. A moderate amount of fluid was present in the forestomach of every animal; this fluid was collected through aspiration and analyzed. During the endoscopy, full-thickness biopsies of the forestomach and the fundic stomach were performed to confirm normal histologic appearance. When biopsy samples are taken, the clamps should be directed perpendicular to the tissue to be grasped and pulled until it breaks off. When the stomach is deflated it is easier to take biopsy samples since reducing the tension in the lumen allows to catch the tissue firmly. Samples were fixed in neutered 10% formalin, routinely processed, embedded in paraffin wax, cut at 5 micron slices, stained with Hematoxilin and Eosin and observed under light microscope. Before

each endoscopy, the scope was cleaned and disinfected with glutaraldehyde (Glutaral, Pharmatek, Cremosano, Italy), and was flushed thoroughly before the next use.

Fig. 8: A portable KARL STORZ GASTRO PACK®.

3.3 CYTOLOGY: INSTRUMENTATION & METHODOLOGY

3.3.1 CYTOLOGY: GASTRIC SAMPLE COLLECTION AND MICROSCOPIC EVALUATION

During the endoscopic procedure, a moderate amount of fluid was present in the forestomach of each animal; this fluid was collected by aspiration into a 120 ml polypropylene container with screw cap (LP Italia, Milan, Italy) and immediately analyzed (3 to 8 mL) (Mitchell et al., 2008). The pH of the fluid was measured by color fixed indicator strips (pH-Fix 0-14; Macherey-Nagel, Duren, Germany). Standard procedure for cytologic review (**Fig. 9**) of the slides was followed (Sweeney & Reddy, 2001; Fair et al., 2006; Goldstein et al., 2006, 2012; Mitchell et al., 2008). The samples were examined after being stained with New methylene blue (NMB) and Dif-Quick (Bio-Optica, Milan, Italy).

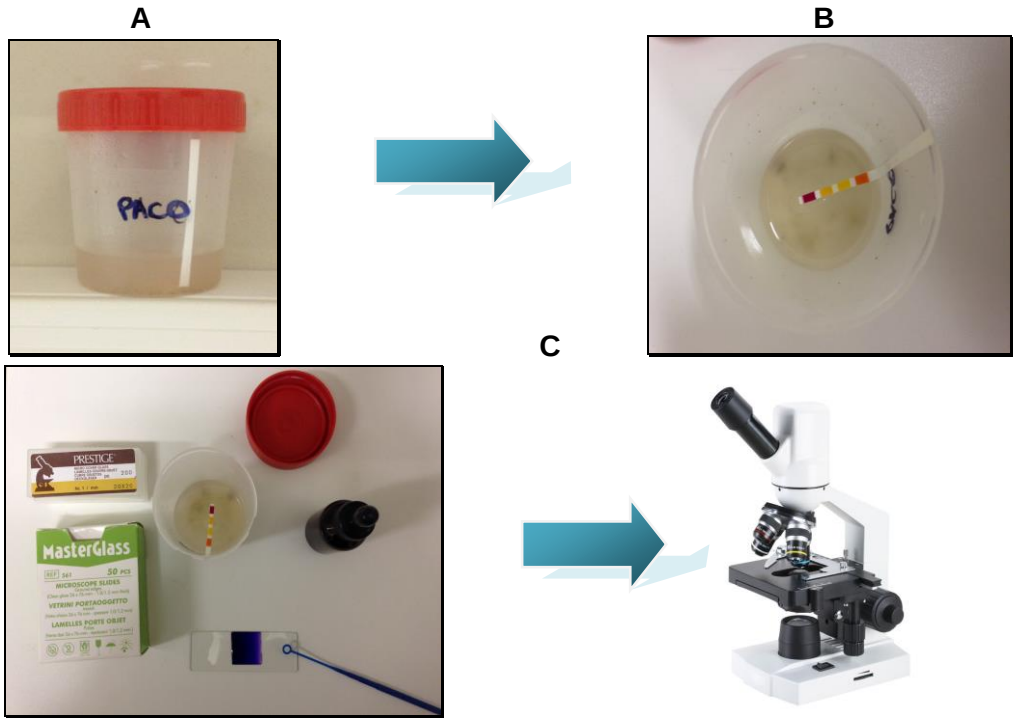


Fig. 9: Gastric juice analysis. **A:** macroscopic review; **B:** pH assessment; **C:** microscopic review.

3.4 ULTRASONOGRAPHY: INSTRUMENTATION & METHODOLOGY

A portable SonoSite 180 Plus (manufactured by SonoSite, Inc., Bothell, WA 98021, USA) with a 2 to 5 MHz convex probe (C60/5-2 Mhz transducer) was used for this ultrasonographic research project. The probe was waterproof, and the machine was covered with a transparent plastic bag to avoid accidental contact of the device with salt water. To avoid direct sunlight, a dark-colored bag was used to cover the instrument. Acoustic gel was unnecessary because water provides an excellent medium to conduct ultrasound waves. All examined animals were trained routinely for medical behaviors,

including ultrasonagraphy. Still images obtained were stored in DICOM (Digital Imaging and Communications in Medicine) format. Dorsal and both lateral recumbencies of the dolphin were used torecord videos of the gastric chambers. The acoustic windows for the three gastric chambers were identified based on knowledge of regional anatomy (Brook et al., 2001). Both the forestomach and the fundic stomach are visualized on approach from the left lateral aspect of the dolphin, while the pyloric chamber is readily visualized on the right side of the dolphin (Saviano, 2013). Cross-sectional images of the regions were made with the transducer carefully oriented perpendicular to the long axes of each chamber to avoid errors induced by oblique images (**Figures 4, 5**). Measurements were made by placing electronic internal machine calipers on the mucosal inner hyperechoic border and the outer hyperechoic layer of the serosa of the stomach wall (Pennick et al., 1989; Paoloni et al., 2002). Measurements were made between peristaltic contractions. Three fundamental measurements were taken (Saviano, 2013): (1) thickness of the plicae (the rugal folds), (2) thickness of the inter-rugal region, and (3) thickness of the wall thickness for each chamber. Measurements were always taken in the same area of the stomach to minimize errors. Since there is no clear demarcation between the small and large intestine in odontocetes, the measurements were taken in the same portion of the intestine (middle section) to standardize the methodology. The wall thickness and relative motility of the gastrointestinal tract were evaluated. In accordance to data published on dogs and cats, each chamber of the stomach was observed for 3 minutes (Pennick et al., 1989; Paoloni et al., 2002). To perform the ultrasound study, all animals were examined twice a day: the first one when fasted, and the second one after eating a whole meal (average 8 kg). Ultrasonography was performed immediately after endoscopy. The state of fullness of the stomach was classified as full (rugal folds distended) or empty (compact rugal folds).

Fig. 3: A portable SonoSite 180 Plus with a 2 to 5 MHz convex probe.

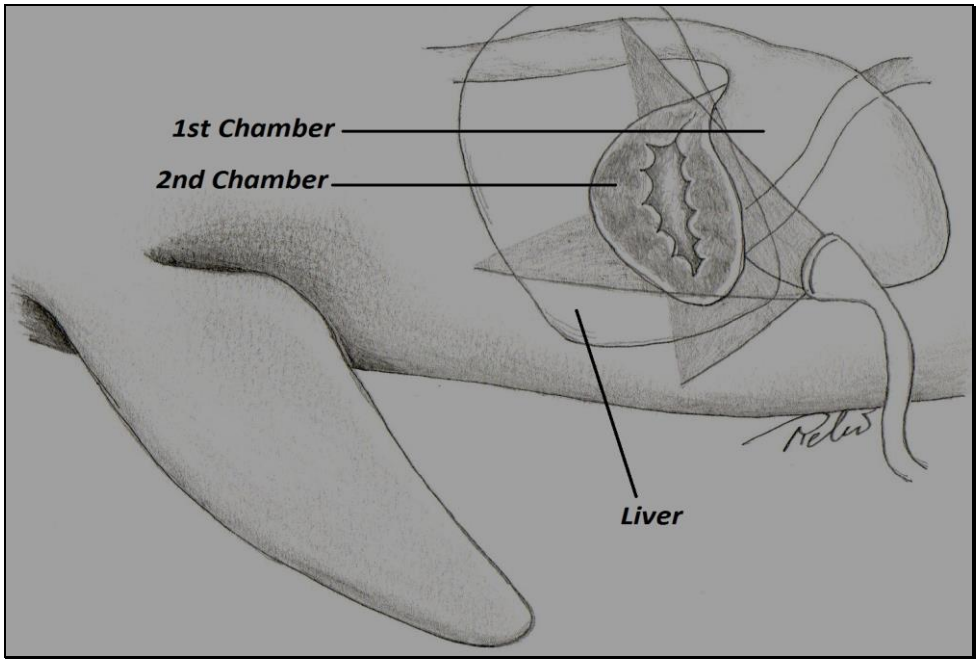


Fig. 4. Abdominal ultrasonography in bottlenose dolphins: longitudinal scan (**lateral view**) of gastric chambers (Saviano, 2013, p. 396).

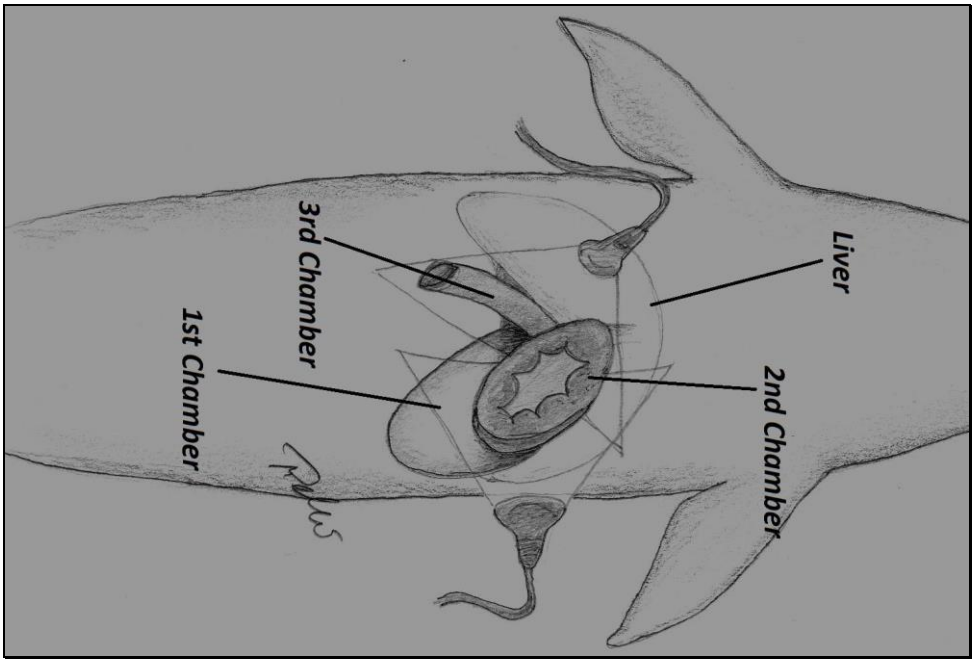


Fig. 5. Abdominal ultrasonography in bottlenose dolphins: longitudinal scan (**ventral view**) of gastric chambers (Saviano, 2013, p. 396).

Fig. 6: Abdominal ultrasonography of a bottlenose dolphins hosted at Zoomarine Italia.

3.4.1 ULTRASONOGRAPHY: ORAL ADMINISTRATION OF *GELAGAR TYPE CT 1.1* SOLUTION AS ALIMENTARY CONTRAST

The alimentary gel was prepared mixing one spoon (5 grams) of *Gelagar Type CT 1.1* with 1L of fresh water, this solution was boiled and then cooled about 4 hours in the fridge. The texture of the obtained medium was semiliquid. Oral administration of *Gelagar Type CT 1.1* solution as alimentary contrast was obtained inserting a flexible, 0.95 cm diameter tube into the first chamber with a mild pression and rotation. All the bottlenose dolphins involved in the study were trained for the procedure. The same instrument and the same procedures described in the previous chapter were used to perform the ultrasonographic examinations and all the animals were examined twice a day: the first time when fasted and the second one after the administration of the alimentary contrast medium (1 L). Ultrasonography was performed immediately after the administration of *Gelagar Type CT 1.1* solution.

A **B**

Fig. 7. A: Preparation of *Gelagar Type CT 1.1* solution; **B**: Gastric tube for hydration and gastric juice collection by behaviour.

3.5 MICROBIOLOGY: INSTRUMENTATION & METHODOLOGY

3.5.1 MICROBIOLOGY: GASTRIC SAMPLE COLLECTION

Gastric samples were taken inserting a 0,95 cm diameter sterile and flexible polyethylene tube (Pharmaplast, Milan, Italy) into the forestomach with a mild pressure and rotation. Gastric juice were collected using a negative pressure siphon technique into 120 ml polypropylene sterile container with screw cap (LP Italia, Milan, Italy) and immediately frozen at -20°C before examination.

3.5.2 MICROBIOLOGY: FAECAL SAMPLE COLLECTION

Fecal samples were collected inserting a 0,40 cm diameter sterile and flexible polyethylene tube (Pharmaplast, Milan, Italy) into the anal orifice to the intestinal tract. The region was cleaned with a sterile gauze before the procedure. In order to obtain good faecal samples, no water contamination should be achieved. Negative pressure was not necessary as the liquid fecal matter flows into the tube unassisted. Fecal samples were immediately frozen at -20°C before examination.

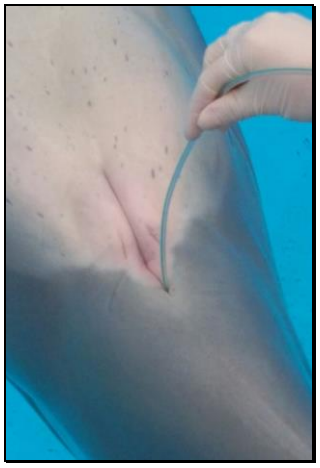


Fig. 10: Faecal collection by behaviour in bottlenose dolphin (*Tursiops truncatus*)

3.5.3 MICROBIOLOGY: CULTIVATION

1 ml of gastric juice and 1 g of feaces of each bottlenose dolphin were put into sterile tubes together with 2 mL 0.9% sterile saline solution. The stool and the gastric juice were mixed in this solution and the tubes was brought to volume (10 mL) with 0.9% sterile saline solution. Each sample (0.1 mL) was serially diluted via 10-fold dilutions (from 10-1 to 10-10). Starting from the lowest concentration, dilutions were plated and cultured on different media in triplicate using the spread plate method. Chromocult agar and Baird Parker agar (were used for the enumeration of *E. coli* and Coliforms, and Staphylococci, respectively. All the plates were incubated at 37°C, aerobically, for 24 - 48 h. Reinforced Clostridial agar enriched with 5% sheep blood and 1 mg/mL K1 vitamin, and Rose Bengal Agar were used for the enumeration of *Clostridium spp* ,and yeasts respectively. Anaerobic incubation was carried out in anaerobic jars (Oxoid) at 37°C for 48 h - 72 h. Anaerobic conditions were obtained using Anaerogen (Oxoid) and were checked using methyl blue strips as oxidation reduction indicator. The number of colonies was counted and all the data were expressed as CFUxlog/g. Log transformation of the results was carried out before statistical analysis.

3.6 STATISTICAL ANALYSIS

3.5.1 STATISTICAL ANALYSIS FOR CYTOLOGICAL REFERENCE INTERVALS

Medcalc, Version 11.6.0.0, was used to analyze the data, and p = 0.05 was used to determine statistical significance. Reference values for pH, epithelial cells (half-sum between max. and min. values of epithelial cell), and white blood cells (WBC) (half-sum between max. and min. values of WBC) were calculated from clinically healthy dolphins (n = 30). The “Guidelines for the Determination of Reference Intervals in Veterinary Species” issued by the Quality Assurance and Laboratory Standards Committee (Friedrichs et al., 2012) was used to calculate the RI. The Gaussian distribution for the values of pH, epithelial cells, and WBC was tested using D’Agostino-Pearson’s test. Outliers were examined and excluded using Horn’s algorithm (Turkey’s fences interquartile). A Spearman’s correlation coefficient (rs) was calculated to examine any correlations, biologically relevant (Sweeney & Reddy, 2001), between pH and epithelial cells pH and WBC, and epithelial cells and WBC. The t-test, assuming equal variances (more robust than Mann-Whitney in parametric statistical method), was used to compare weight, pH, epithelial cells and WBC between sexes.

3.5.2 STATISTICAL ANALYSIS FOR ULTRASOUND REFERENCE INTERVALS

For the statistical analysis, the statistical package Medcalc, Version 11.6.0.0 was used. Reference values for thickness of the forestomach mucosa, fundic stomach mucosa, rugal, inter-rugal, wall thickness of third chamber, diameter of third chamber, and thickness of the bowel’s mucosa were calculated from clinically healthy dolphins (n = 30). For the

determination of reference intervals in healthy dolphins, the “Guidelines for the Determination of Reference Intervals in Veterinary Species” issued by the American Society of Veterinary Clinical Pathologists (ASVCP) Quality Assurance and Laboratory Standards Committee were followed (Friedrichs et al., 2012). The Gaussian distribution for values of wall thickness measurements of the forestomach, fundic stomach, rugal fold, inter-rugal space, pyloric stomach, diameter of the pyloric stomach (because of its anatomical “U” shape, it was easier to evaluate compared with the others’ chambers), and wall thickness measurements of the bowel was tested using D’Agostino-Pearson’s test. Outliers were examined and excluded using Horn’s algorithm (Tukey’s fences interquartile). A Spearman’s correlation coefficient (rs) was calculated to examine any correlations that were biologically relevant (Sweeney & Reddy, 2001). Summary statistics for mucosa of the forestomach, mucosa of the fundic stomach, rugal space of the fundic stomach, inter-rugal space of the fundic stomach, wall thickness of the third chamber, diameter of the third chamber, and entire wall thickness of the bowel (n = 30) were calculated. The t-test, assuming equal variances, was used to compare the values of the forestomach mucosa thickness, the fundic stomach mucosa thickness, the thickness of the rugal space, the thickness of the inter-rugal space, the wall thickness of the third chamber, the diameter of the third chamber, and the entire wall thickness of the bowel between sexes. Also, a comparison of the *ruga* of the fundic stomach (empty/full), *inter-ruga* of the fundic stomach (empty/full), and the diameter of the third chamber (empty/full) were done (t-test paired samples). Spearman’s correlation coefficient (rs) was calculated between the thickness of the fundic mucosa and weight, thickness of the forestomach mucosa and weight, thickness of the inter-rugal space and weight, thickness of the rugal space and weight, wall thickness of the third chamber and weight, diameter of the third chamber and weight, and the thickness of the bowel mucosa and weight.

3.5.3 STATISTICAL ANALYSIS FOR MICROBIOLOGY

For the determination of the Reference Intervals (RI) in healthy dolphins the "Guidelines for the determination of reference intervals in veterinary species" were followed (Friedrichs et al., 2012). RI were calculated as arithmetic mean ± 2SD for normal data (Bland 2000). Outliers were examined using Horn's algorithm (Turkey's fences interquartile). Normality of data distribution of each variable were assessed by a Shapiro-Wilk test. Where variables failed tests for normality, the median was used as the measure of central tendency and the RI given as the central 95th percentile (Friedrichs et al., 2012; Bland 2000). To evaluate reliability and biological variations, analytical (CVA) and inter-individual (CVG) variations were calculated as the mean intra-assay coefficient of variation (CV) and the CV for values between individuals, respectively (Bland, 2000; Walton, 2012). Coefficient of quartile variation (CQV) was used for non-normal data (Bonett, 2006). The CQV is a descriptive statistic that allows for model- and scale-free comparisons within and between data sets. The CQV statistic is computed as

$$\mathrm{CQV}\left(\mathrm{I(pi)}\right) = \left[\frac{(\mathrm{Q3} - \mathrm{Q1})}{(\mathrm{Q3} + \mathrm{Q1})}\right] \times 100$$

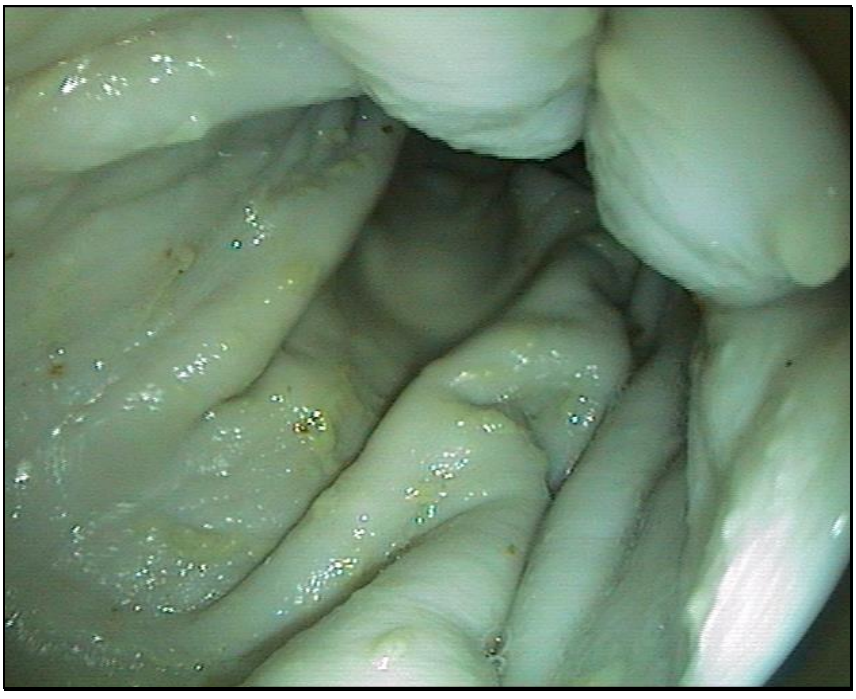
where Q1 and Q3 are the first and third quartiles of the distribution, respectively (Bonett, 2006). In order to compare means between gastric juice and fecal, paired sample t-test or Wilcoxon signed ranks test were used. To describe the relative proportion of each examined microflora component, proportions of each component are presented where the total of the examined microorganisms was set at 100%. Comparisons between gender, age, weight, and location were performed using General Linear Model procedures. In the model, intercept was excluded while age was included as covariate where appropriate. $P < 0.05$ were considered to be statistically significant. All analyses were performed with SPSS 20.0 software (SPSS, Inc., Chicago, IL) while an Excel spreadsheet was used for Turkey's fences interquartile and CV calculations.

4. RESULTS

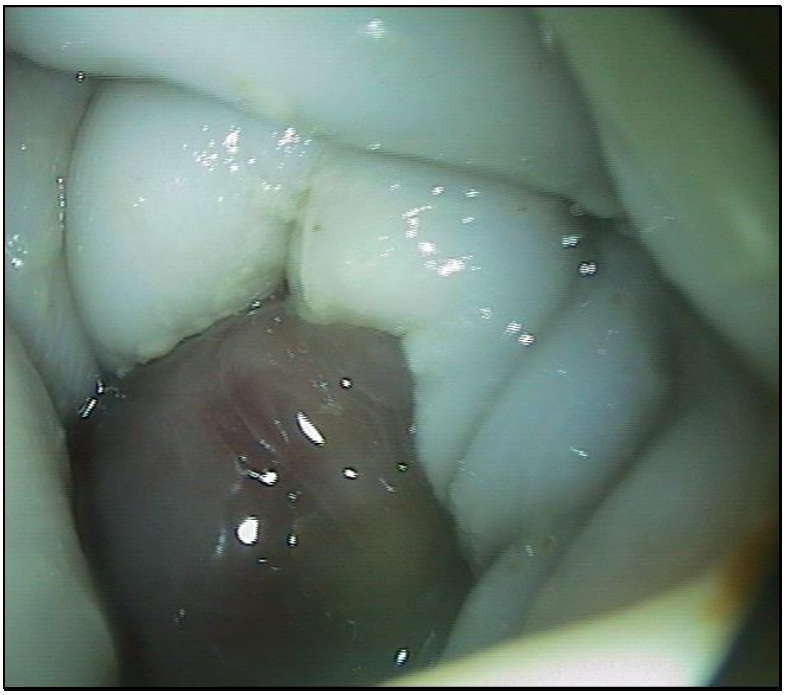
4. 1 ENDOSCOPY: REFERENCE FOR NORMAL FINDINGS

No morphological abnormalities were detected with endoscopy. The normal cetacean esophagus is seen as longitudinal folds of mucous membrane to the level of the lower esophageal sphincter. There is not a clear delineation between the mucosa of the forestomach and the esophagus. The forestomach has thick squamous epithelium-lined rugal folds that appear light pink in color during the endoscopy; the fundic portion has a mucosal surface that is deep pink to red in color. The ostium between the forestomach and the fundic stomach is located cranially in the left ventral quadrant of the first compartment (**Fig. 1**).

A



B



C

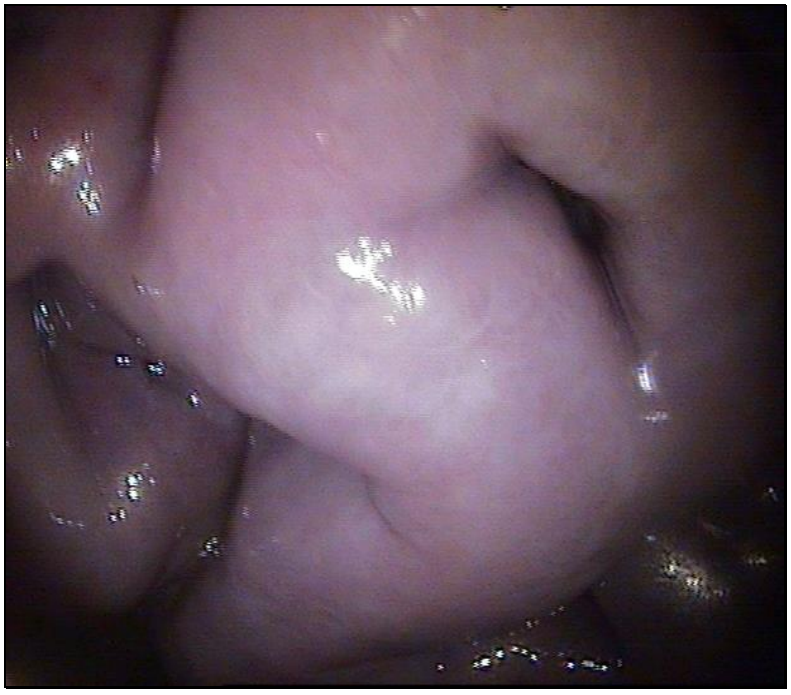


Fig. 1. A: thick squamous epithelium-lined rugal folds of the forestomach in a healthy bottlenose dolphin; **B:** the ostium between the forestomach and the fundic

chamber, located cranially in the left ventral quadrant of the first compartment; **C:** the mucosal surface of the fundic portion of the stomach in a bottlenose dolphin.

All the full-thickness biopsy samples were considered histologically normal: the forestomach showed a keratinized squamous epithelium, and the fundus consisted of mucosa, submucosa, muscular layer, subserosa, and serosa. The mucosa had a simple cylindrical epithelium forming the gastric crypts where there are glands are composed of parietal cells and chief cells.

A **B**

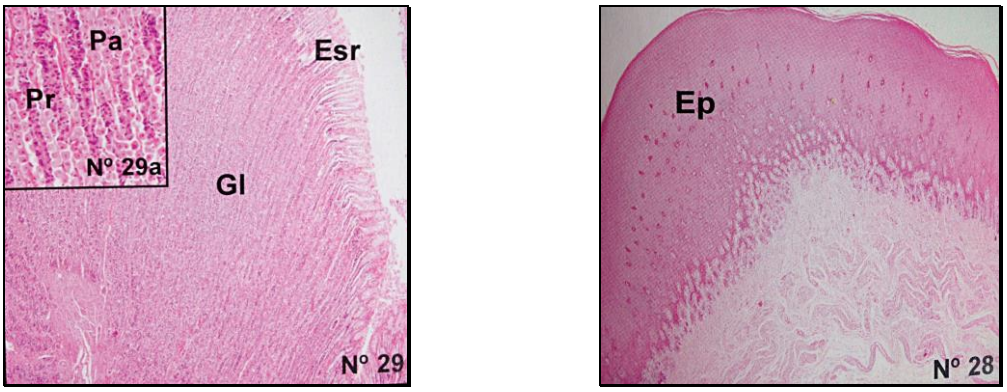


Fig. 2. A (**H/E - 4x**)**:** the forestomach is devoid of glands and is lined with a keratinised squamous epithelium (**Ep**); **B** (**H/E - 40x**)**:** the fundic stomach consists of a plicated mucosa, submucosa, muscularis and serosa; the mucosa is constituted by a simple columnar epithelium (**Esr**) which invaginates to form the gastric crypts (**Gl**), where are located the gastric glands composed of parietal cells (**Pa**) and the chief cells (**Pr**) that provide the chemical part of digestion.

4.2 CYTOLOGY: REFERENCE INTERVALS

The cytologic review of the slides showed no morphological abnormalities (**Figs. 3, 4**). Summary statistics for age, weight, pH, volume, epithelial cells, and white blood cells (WBC) for clinically healthy bottlenose dolphins (n = 30) were calculated and presented in **Table 1**. There were no outliers noted in the analysis. Reference intervals (RI) were determined using “parametric method” as recommended by the Quality Assurance and Laboratory Standards Committee (Friedrichs et al., 2012) for 20 ≤ n < 40 reference samples when the data follow Gaussian distribution (**Tab. 2**). In fact, the ASVCP protocol for establishing RI recommends parametric statistical method if Gaussian normality can be established with 90% confidence interval (CI) for reference limits (Friedrichs et al., 2012). No correlation was found between pH and the epithelial cells values (rs = 0.292, p = 0.1657), pH and WBC (rs = 0.168, p = 0.4321), epithelial cells and WBC (rs = 0.076, p = 0.7240). The t-test between the sexes (female, n = 15; male, n = 15), assuming equal variances, did not find a significant difference for weight (p = 0.6739), pH (p = 0.7707), epithelial cells (p = 0.6385), or WBC (p = 0.6968).

Statistic (n = 30)	Mean ± SD	Min.	Max.	D’Agostino-Pearson test
Age (y)	16.00 ± 8.60	6.00	36.00	p = 0.0526
Weight (kg)	187.95 ± 36.04	146.20	266.70	p = 0.1733
pH	1.83 ± 0.92	1.00	3.00	p = 0.1757
Ep. Cells/hpf	3.77 ± 1.46	2.00	7.00	p = 0.3255
WBC/hpf	2.42 ± 0.69	1.50	4.00	p = 0.4756

Table 1. Summary statistics for age, weight, pH, volume, epithelial cells, and WBC from gastric samples collected from clinically healthy bottlenose dolphins (*Tursiops truncatus*) (n = 30); SD = standard deviation, Min.-max. = minimum-maximum values.

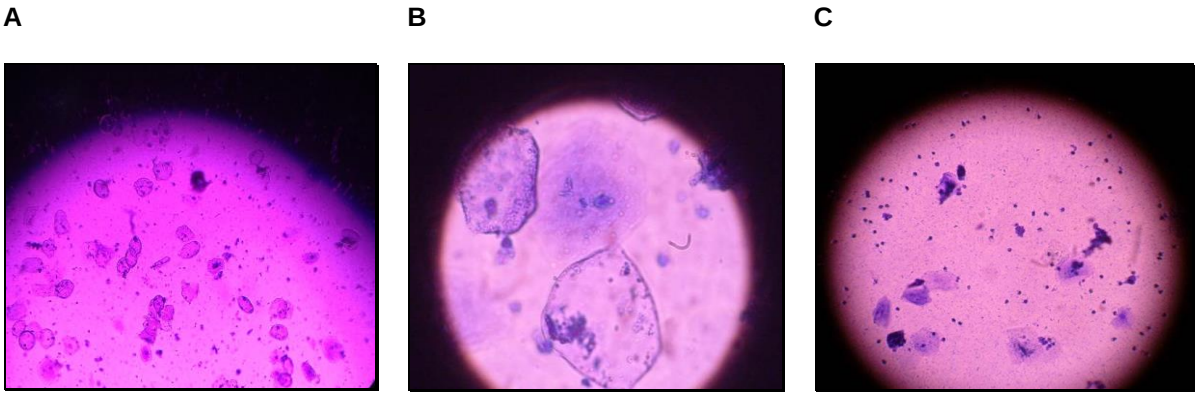


Fig. 3. A: Normal cytological findings in gastric juice of healthy bottlenose dolphins (*Tursiops truncatus*), New methylene blue (NMB), 10x; **B:** Epitelial Cells (NMB 40x); **C:** White Blood Cells (NMB 10x).

Reference intervals	Lower limit	90% CI	Upper limit	90% CI
Epithelial cells/hpf	0.91	0.05-1.77	6.63	5.77-7.49
WBC/hpf	1.07	0.67-1.48	3.76	3.56-4.17
pH	0.00	0.0-0.6	3.60	3.1-4.2

Table 2. Reference intervals (RI) determined using parametric methods for gastric samples collected from clinically healthy bottlenose dolphins (n = 30); CI = confidence interval.

4.3 ULTRASONOGRAPHY: REFERENCE INTERVALS

A consistent and standardized method to measure the entire wall thickness in forestomach, fundic stomach, pyloric stomach, and bowel was defined (**Fig. 4,5,6,7,8**).

With regard to the qualitative description of the stratigraphy of the gastrointestinal tract in all healthy bottlenose dolphins evaluated in this study, five ultrasonographic layers throughout the gastrointestinal tract were identified. The hyperechoic mucosal interface was identified in contact with the lumen, the hypoechoic mucosa, the hyperechoic submucosa, the hypoechoic muscular layer, the hyperechoic subserosa, and the serosa. The mucosal layer appears hypoechoic in all healthy animals observed.

Unfortunately, it was not possible to measure each layer with our instrumentation.

The fundic stomach is similar to the stomach of dogs and cats considering the stratigraphy and plication (Pennick et al., 1989; Paoloni et al., 2002); the mucosa appears to be slightly thicker than the other layers, as well as the submucosa, while the muscular layer appears thinner.

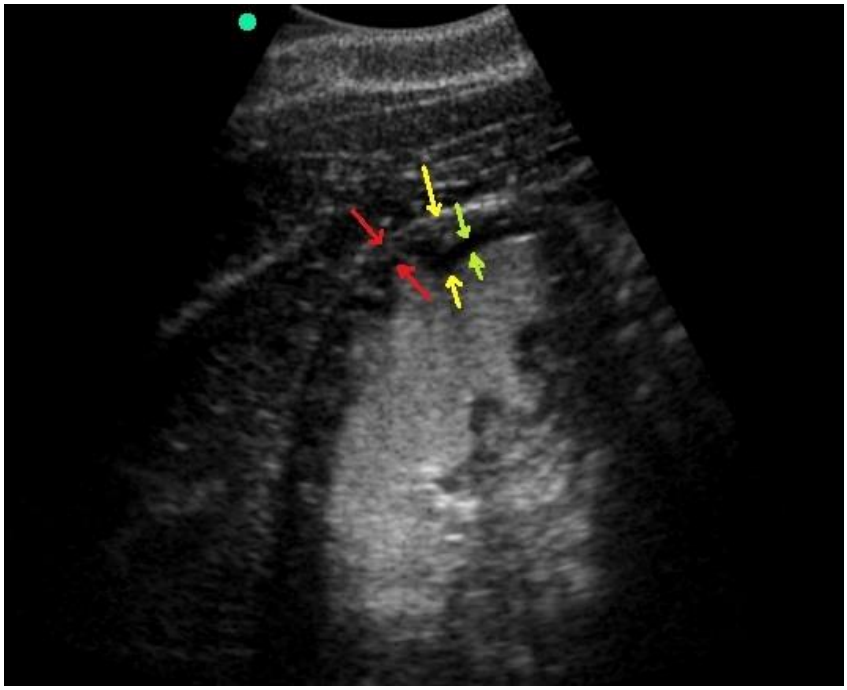


Fig. 4. Fundic ultrasound measures: Rugal folds (red), inter-rugal region (yellow), and mucosa thickness (green).

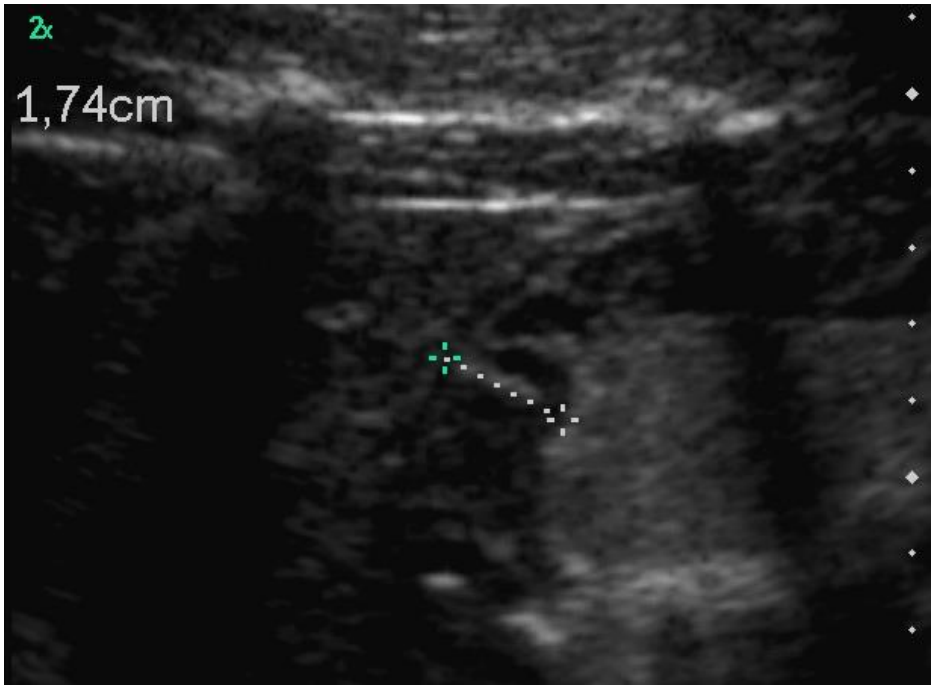


Fig. 5. Fundic ultrasound measures: Rugal folds.

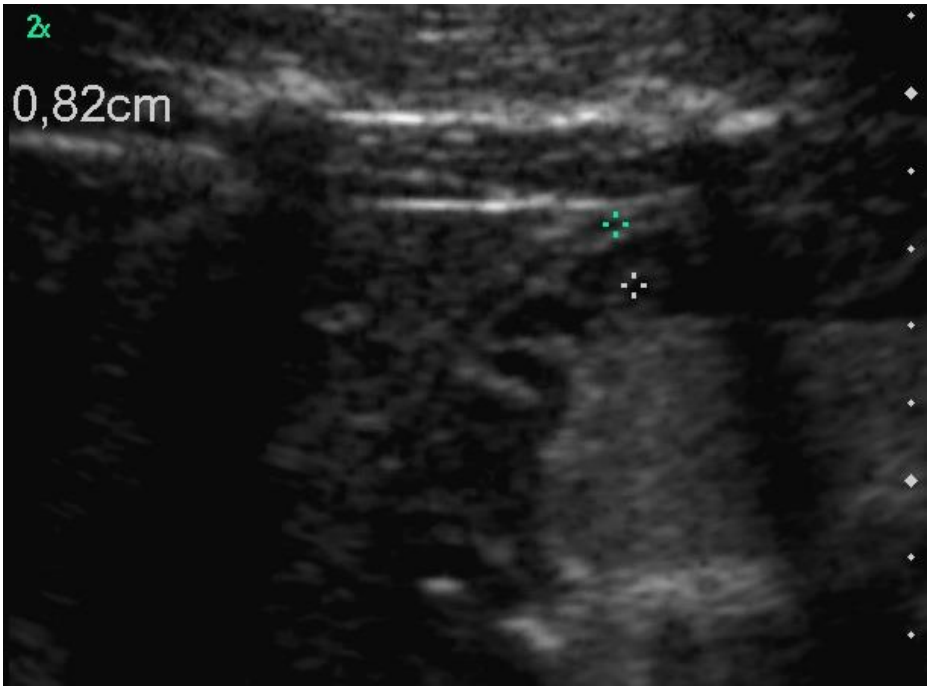


Fig. 6. Fundic ultrasound measures: Inter-rugal region.

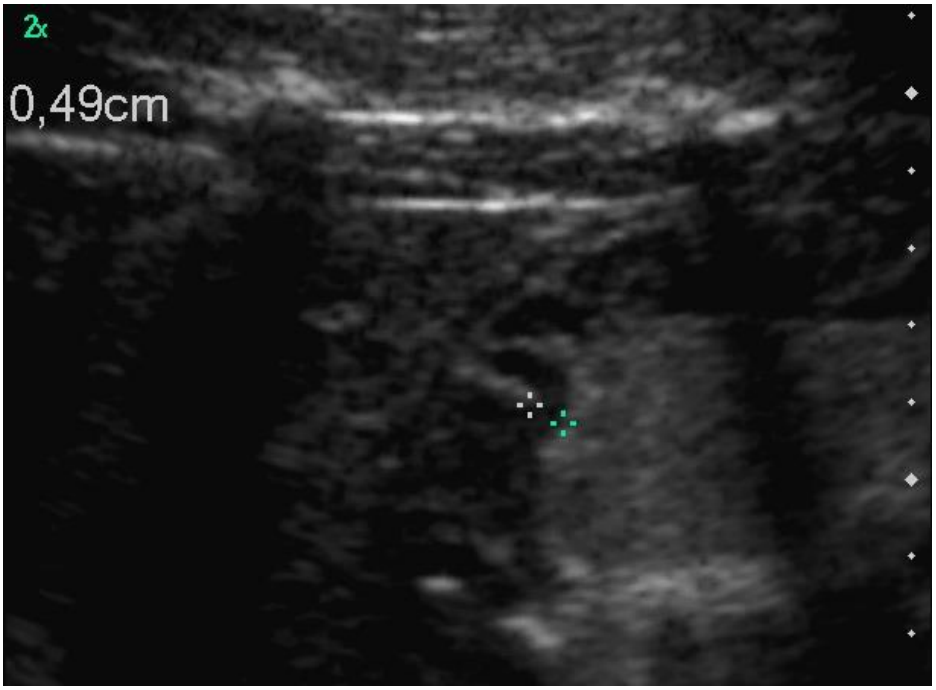


Fig. 7. Fundic ultrasound measures: mucosa thickness

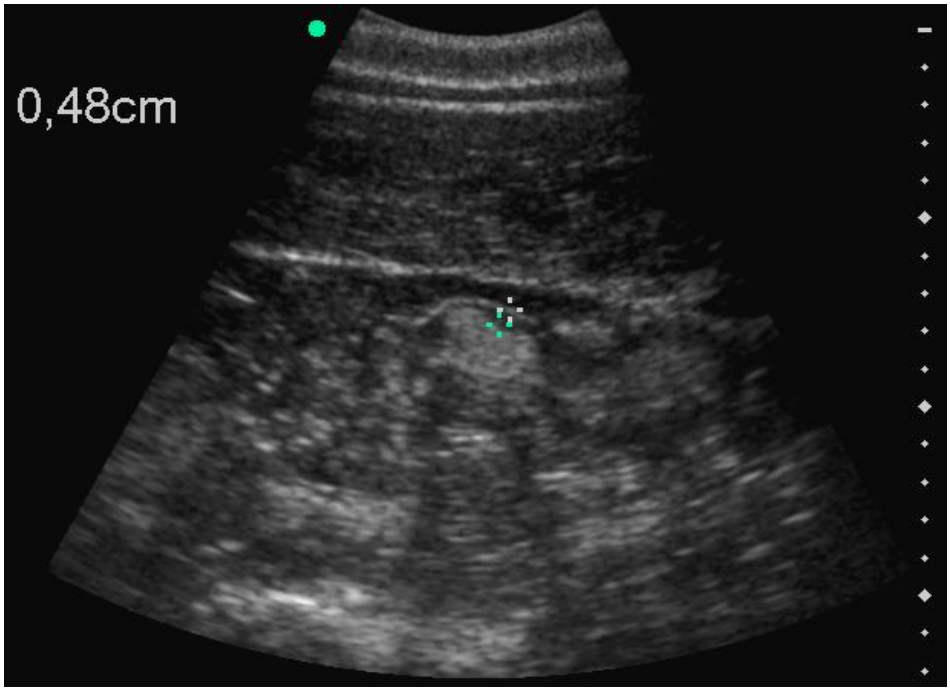


Fig. 8. Bowel ultrasound measures: entire wall thickness

Summary statistics for mucosa of the forestomach, mucosa of the fundic stomach, the rugal space, the inter-rugal space, the wall thickness of the third chamber, the diameter of the third chamber, and the entire wall thickness of the bowel (n = 30) were calculated and presented in **Tab.3**. Reference intervals for each of the ultrasonographic findings were determined (**Tab. 4**) by parametric method as recommended by the ASVCP Quality Assurance and Laboratory Standards Committee for a 20 < n < 40 reference samples (Friedrichs et al., 2012). There were no outliers. The t-test between the sexes (female: n = 15; male: n = 15) did not find a significant difference for every parameter considered in the study (thickness of the forestomach mucosa, p = 0.1521; thickness of the fundic stomach mucosa, p = 0.6616; thickness of the rugal space, p = 0.9064; thickness of the inter-rugal space, p = 0.9765; thickness of the third chamber, p = 0.3831; diameter of the third chamber, p = 0.1758; and the entire wall thickness of the bowel, p = 0.5647). The t-test for paired samples found only a significant difference in diameter of the

third chamber when it was empty/full ($p < 0.0001$). Only the thickness of the fundic stomach mucosa was moderately negative correlated with the weight ($rs = -0.589$, $p = 0.0006$). The small correlation between intestinal wall thickness and weight observed in dogs (Delaney et al., 2003) was not observed in dolphins ($rs = 0.120$, $p = 0.5284$).

Statistic (n = 30)	Mean ± SD	Min-max	Normal distribution
Forestomach wall thickness	0.52 ± 0.14	0.18-0.73	p = 0.4158
Fundic stomach wall thickness	0.52 ± 0.10	0.34-0.70	p = 0.5793
Fundic stomach ruga	1.97 ± 0.30	1.37-2.45	p = 0.4639
Fundic stomach inter-ruga	1.28 ± 0.28	0.84-1.83	p = 0.5716
Pyloric chamber wall thickness	0.36 ± 0.14	0.18-0.67	p = 0.1088
Diameter pyloric chamber	2.54 ± 0.41	2.00-3.35	p = 0.4102
Bowel wall thickness	0.51 ± 0.10	0.33-0.70	p = 0.4038

Tab. 3. Summary statistics for thickness (measured in cm) of forestomach mucosa, fundic stomach mucosa, rugal folds, inter-rugal folds, and the third chamber's mucosa; for diameter (measured in cm) of the third chamber and for thickness of the bowel's mucosa; and from ultrasonic samples collected from clinically healthy dolphins (n = 30). SD = standard deviation; Min-max = minimum-maximum values.

Reference intervals	Lower limit	90% CI	Upper limit	90% CI
Forestomach wall thickness	0.24	0.16-0.31	0.16-0.31	0.16-0.31
Fundic stomach wall thickness	0.27-0.38	0.27-0.38	0.27-0.38	0.27-0.38
Fundic stomach ruga	1.38	1.22-1.53	1.22-1.53	1.22-1.53
Fundic stomach inter-ruga	0.73	0.57-0.88	0.57-0.88	0.57-0.88
Pyloric chamber wall thickness	0.09	0.01-0.16	0.63	0.56-0.71
Diameter pyloric chamber	1.74	1.52-1.96	3.34	3.12-3.56
Bowel wall thickness	0.31	0.26-0.36	0.71	0.66-0.76

Tab. 4. Reference intervals (measured in cm) determined using parametric methods for ultrasonic samples collected from clinically healthy dolphins (n = 30); CI = confidence interval.

The forestomach and the pyloric chamber had a folding and a less noticeable peristalsis compared with the fundic stomach. An average of 3 to 4 cycles with a sequence of fundic, forestomach, and third compartment have been recorded in animals on a full stomach.

The normal luminal contents can vary and consist of food, mucus, fluid, or gas. In all healthy animals evaluated in the study, on an empty stomach, the stomach pattern could be considered variable, although there was a prevalence of a mixed pattern of gas/fluid in the forestomach, a mixed pattern between mucous/fluid in the fundic stomach, and mainly fluid in the third chamber. A food pattern is found when food is present inside the lumen: the forestomach usually presents a heterogeneous food pattern because it is the stomach in which the mechanical digestion process occurs (**Fig. 9,10,11,12**).

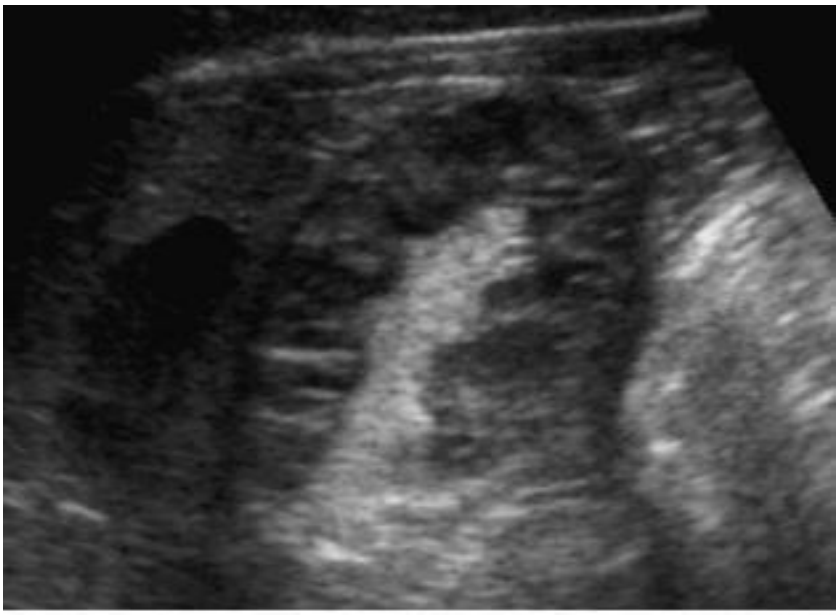


Fig. 9. The normal luminal contents: *food pattern*.

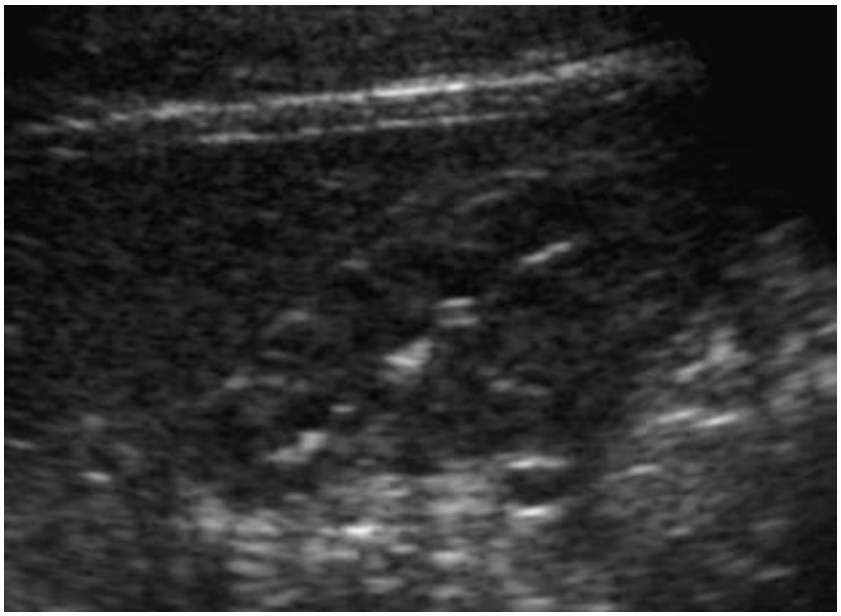


Fig. 10. The normal luminal contents: *mucous pattern*.

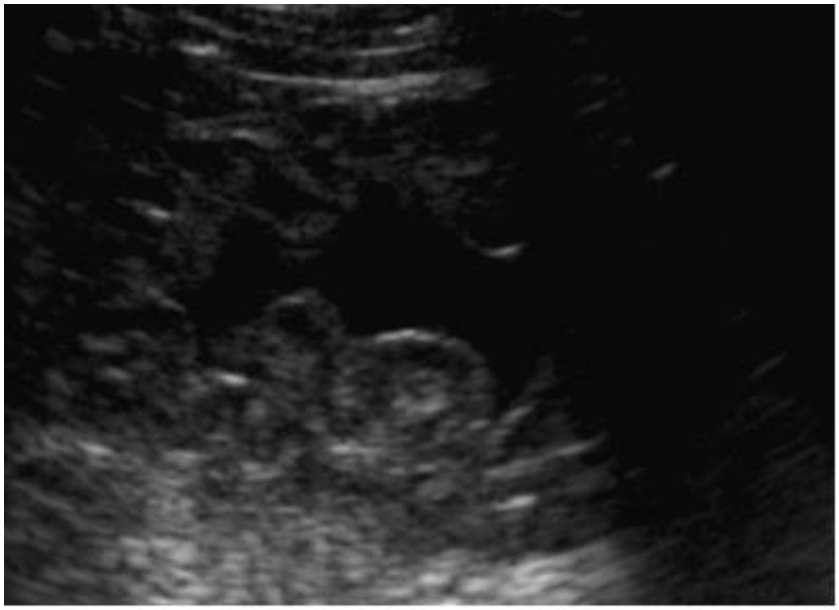


Fig. 11. The normal luminal contents: *fluid pattern*.

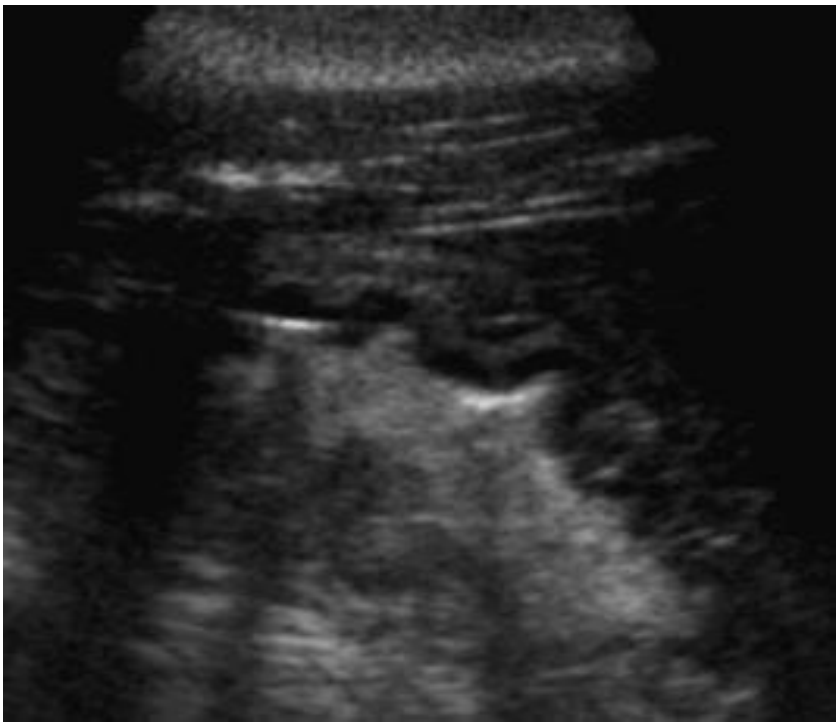


Fig. 12. The normal luminal contents: *gas pattern*.

4.3.1 ULTRASONOGRAPHY: NORMAL GASTRIC WALL THICKNESS

Thanks to *Gelagar Type CT 1.1* solution as alimentary contrast it was possible to improve the quality of the ultrasound image to mark the mucosal layer (**Fig.13**).

Five ultrasonographic layers throughout the forestomach were identified in all healthy bottlenose dolphins evaluated in this study. From the lumen to the serosal surface the hyperechoic mucosal interface in contact with the lumen, the hypoechoic mucosa, the hyperechoic submucosa, the hypoechoic muscular layer and the hyperechoic subserosa and serosa were identified. With regard to the qualitative description of the stratigraphy of the gastrointestinal tract, the mucosa layer appears hypoechoic in all healthy animals. Ultrasonographically, the forestomach wall thickness varies between 2.4 to 8 mm in

bottlenose dolphins, the results found correspond to the preovious research (Fiorucci et al., 2015).

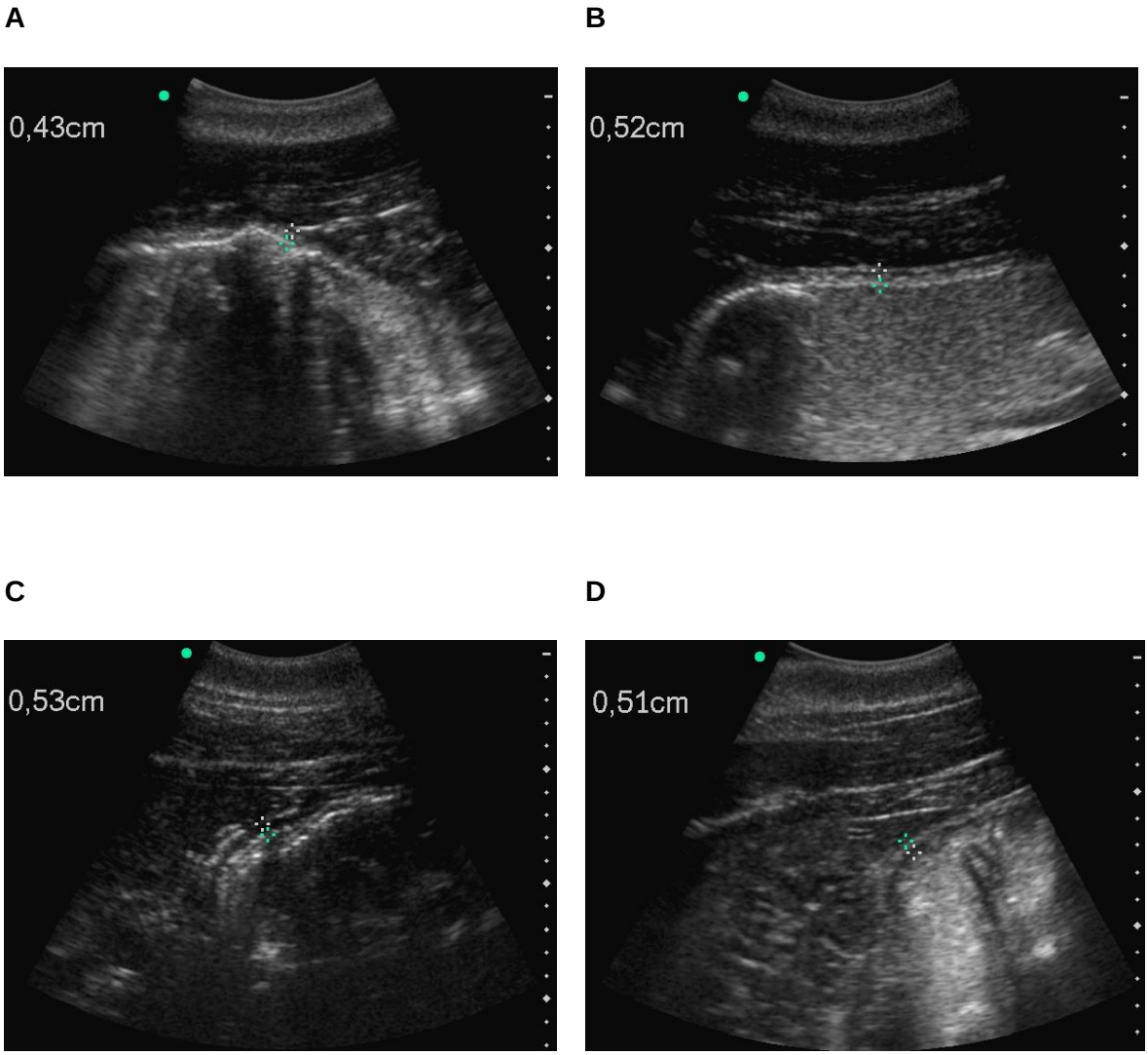


Fig. 13. A: Ultrasound image of the forestomach wall thickness in a healthy bottlenose dolphin (*Tursiops truncatus*) before administration of the alimentary contrast medium; **B:** Ultrasound image of the forestomach stomach wall thickness in a healthy bottlenose dolphin after administration of the alimentary contrast medium; **C:** Ultrasound image of the fundic stomach wall thickness before administration of the alimentary contrast medium; **D:** Ultrasound image of the fundic stomach wall thickness after administration of the alimentary contrast medium.

5.4 MICROBIOLOGY: REFERENCE INTERVALS

Body weight was significantly higher in males than in females (193±8 and 183±9 kg in male and female, respectively; P=0.004) and increased with age of the animal (b=10.3, P=0.000). A sample of gastric juice was missed; then, 31 and 30 samples of faeces and gastric juice, respectively, were analysed. One dolphin was excluded from *E. coli* evaluation on gastric juice because the value exceeded interquartile fence (1.65 - 4.83 Log CFU/gr). With regard to fecal *E. Coli*, values of seven dolphins were below or above the interquartile fence (4.14 – 5.21 Log CFU/gr) and were excluded. Due to non-normality of distribution, robust methods were used for Coliforms and yeast data on gastric juice and faeces, respectively (**Tab. 5**). Mean or median, reference intervals, and CVs for the bacteria tested in gastric juice and faecal sample are summarized in **Table 6**. The highest inter-individual biological variations (CV or CQV > 18%) were recorder for faecal Staphylococci and for yeasts both of gastric juice and feces. Microorganisms in gastric juice were not influenced by any variable considered (age, gender, facility). Faecal Staphylococci was affected by facility (F=15.21, P=0.000) being higher in dolphins reared in B than in C (5.18, and 3.51 Log CFU/gr for B, and C dolphins, respectively; P=0.000). Dolphins reared in A showed intermediate values of Staphylococci (4.41 Log CFU/gr). Faecal yeasts tended to increase as body weight of dolphins increased (b=0.011, F=6.43, P=0.017). Count of Staphylococci (t=5.31, df=29, P < 0.001) on faeces were increased compared to gastric juice samples (**Tab. 5,6; Fig. 14**).

A **B** **C**

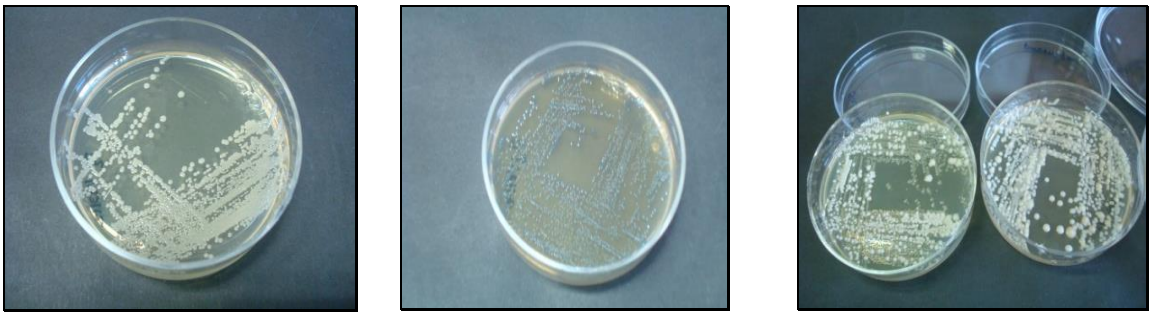


Fig. 14. A: plate with Chapman® (Merck 1.05469.0500) medium with growth of *Staphylococcus*; **B**: plate with Chromocult® medium (Merck 1.10426) with a growth of *E.coli* in blue colonies; **C**: plates with Sabouraud® medium (Liofilchem 610103) with growth of yeast.

	GASTRIC JUICE			FAECES		
	Statistic	**df**	**Sig.**	**Statistic**	**df**	**Sig.**
Coliforms	**0.846**	**30**	**0.001**	0.977	31	0.713
Escherichia coli	0.977	29	0.746	0.946	24	0.218
Staphylococci	0.940	30	0.093	0.960	31	0.289
Yeast	0.963	30	0.372	**0.862**	**31**	**0.001**
Clostridia	0.942	30	0.102	0.953	31	0.187

P-values below 0.05 are printed in bold (i.e. non-normal distribution)

Table 5. Tests of normality (Shapiro-Wilk).

		n	Mean	RI (CFU/gr)	95% confidence intervals for RI	CV_G or Cqv (%)	CV_A (%)
GASTRIC JUICE	**Coliforms**	30	5.13	3.36 – 5.91	-	11.9	1.4
	Escherichia coli	29	3.25	2.10- 4.39	[1.74, 2.46] [4.03, 4.75]	17.6	3.9
	Staphylococci	30	3.72	2.45 – 4.99	[2.05, 2.84] [4.60, 5.39]	17.1	2.6
	Yeast	30	4.49	2.75 -6.23	[2.21, 3.29] [5.70, 6.78]	19.4	2.6
	Clostridia	30	6.07	4.82 – 7.32	[4.43, 5.21] [6.94, 7.71]	10.3	2.1
FAECES	**Coliforms**	31	5.11	3.70 – 6.52	[3.27, 4.13] [6.09, 6.95]	13.8	4.5
	Escherichia coli	24	4.70	4.39 -5.01	-	1.7	3.3
	Staphylococci	31	4.62	2.62 – 6.63	[2.00, 3.23] [6.02, 7.24]	21.7	6.3
	Yeast	31	4.59	3.32 – 5.67	[2.33, 3.40] [5.85, 6.92]	19.0	5.5
	Clostridia	31	5.73	4.33 – 7.13	[3.91, 4.76] [6.71, 7.56]	12.2	4.5

Tab 6. Microflora composition (Log CFU/gr) of gastric juice and faeces of healthy bottlenose dolphins. CVA = Analytical variation. CVG = inter-individual variation. Cqv = coefficient of quartile variation.

5.0 DISCUSSION

5.1 Reference baseline data for gastric cytology in healthy bottlenose dolphins (*Tursiops truncatus*) under human care

Three types of epithelial cells are usually reported in gastric samples: (1) squamous epithelial cells from the esophagus and fundic stomach, (2) columnar epithelial cells from the second stomach, and (3) basal cells from necrosis in the first and the second chamber (Sweeney & Reddy, 2001). Only squamous epithelial cells were reported during the cytological examination of the gastric fluid of healthy animals (Sweeney & Reddy, 2001). The presence of a small number of leukocytes (< 20 cells/hpf) in the forestomach is a normal occurrence in both captive and free-ranging dolphins (Fair et al., 2006; Varela et al., 2007). However, the finding of moderate numbers of leukocytes (> 20 cells/hpf) may lead clinicians to suspect the presence of a mild gastritis (Varela et al., 2007). The current study shows that the pH value of the gastric sample from a fasted dolphin ranges from 1.0 to 3.0. The presence of bacterial overgrowth in marine mammals typically results in increased gastric pH secondary to bacterial phagocytosis; this is in contrast to the response in humans, which results in a lowered pH (Zhu et al., 2006). Furthermore, individual differences can influence the results. Thus, it seems important to set up an individual baseline, preferably by taking gastric fluid samples on a routine basis as a part of a routine medical examination. The results found in this study are in agreement with previous research (Fair et al., 2006; Goldstein et al., 2006; Varela et al., 2007; Mitchell et al., 2008). The study established a reference baseline data for normal cytological findings in gastric fluid samples of healthy bottlenose dolphins based, for the first time, on the “Guidelines for the Determination of Reference Intervals in Veterinary Species” issued by the Quality Assurance and Laboratory Standards Committee

(Friedrichs et al., 2012). Cytologic examinations can provide a snapshot of potential illness prior to systemic disease (Cowell et al., 1999); elevated leukocytes along with increased epithelial cells, particularly basal cells and or erythrocytes might suggest ulceration (Mitchell et al., 2008).

Endoscopy has traditionally been the most useful tool to examine and evaluate the upper gastrointestinal (GI) tract (Dover et al., 2001). Lesions in the forestomach and crianial portions of the fundic stomach can be visualized by endoscopy. The dolphin stomach has three divisions: (1) forestomach; (2) fundic stomach; and (3) pyloric stomach, which joins the duodenal ampulla. The forestomach has keratinised and stratified squamous epithelium, and it is the only nonglandular portion of the dolphin's stomach. The fundus consists of neck cells, chief cells, and parietal cells, whereas the pyloric portion has columnar mucous cells and argentaffin cells. The duodenal ampulla is an extension of the duodenum from which the common bile duct exits from the liver (Ridgway, 1968, 1972; Harrison et al., 1970). Disturbances of the gastrointestinal tract are encountered in both wild and under human care dolphins, and damage to the gastric mucosa, including ulcers, has been reported (Harper et al., 2000). The study showed the importance of including endoscopic evaluation of the gastric chambers and histological assessment of the mucosa of the forestomach and fundic to give more scientific value to the reference intervals found. In addition, this study underlines the importance of including endoscopy in routine testing as a procedure to complement clinical and cytological examination of the animal to monitor gastric compartments, considering that animals can be trained to perform this kind of examination.

5.2 Determination of the main reference values in ultrasound examination of the gastrointestinal tract in clinically healthy bottlenose dolphins (*Tursiops truncatus*)

The main goal of this study was to evaluate the characteristics of the gastrointestinal tract of healthy bottlenose dolphins through ultrasonography and to set up a consistent and standardized method to measure the entire wall thickness of the forestomach, fundic stomach, pyloric stomach, and bowel based, for the first time, on the "Guidelines for the Determination of Reference Intervals in Veterinary Species" issued by the Quality Assurance and Laboratory Standards Committee (Friedrichs et al., 2012).

Preventative medicine programs, and particularly examination of the digestive system in cetaceans, is one of the keys factors in health evaluation to ensure the welfare of these animals under human care. Endoscopy has traditionally been the most useful tool to examine and evaluate the upper GI tract (Dover & Van Bonn, 2001). However, ultrasonography plays an important role in modern-day cetacean preventative medicine because it is a non-invasive technique, is safe for both patient and operator, can be performed routinely using medical behaviors, and is also used to follow up on animals undergoing therapy (Saviano, 2013). Unfortunately, there is significant variability in echogenicity between individuals which may interfere with the accuracy of the measurements.

According to data published for dogs and cats, a thickened wall is the most common abnormality identified. Tumors and granulomas generally produce focal, asymmetrical thickening with disruption of normal wall layering (Kaser-Hotz et al., 1996; Paoloni et al., 2002; Monteiro & O'Brien, 2004). Other inflammatory or infiltrative diseases generally produce diffuse thickening, maintaining the wall layering. A marked increase in rugal thickness may indicate gastritis or parasitosis (Brook et al., 2001). In our experience, in the case of mild gastritis, the mucosal layer appeared slightly increased in size as well

as more hyperechoic when compared with the results of Table 1; the pattern of the lumen was abnormal (more hypoechoic), and the peristalsis was altered. Ultrasonographically, the fundic stomach wall varies between 3.3 to 7.1 mm thick in bottlenose dolphins, while it is 3 to 5 mm thick in the dog and between 2 to 6 mm in humans (Pennick et al., 1989; Paoloni et al., 2002). Therefore, some small variations exist between species.

In our opinion, the stomach of bottlenose dolphins should include images from a full stomach as well as after fasting given that the food often acts as a contrast highlighting natural structures that may otherwise be overlooked. A gas pattern is caused by the presence of hyperechoic free gas in the lumen; this condition is quite normal during the digestive process in the forestomach. A fluid pattern is represented by the presence of fluid in the lumen and has a typical anaechoic sonographic appearance. The present study showed that the fluid pattern in the forestomach could be related to a decrease in motility. Fluid pattern has been observed in animals with slow/absent peristalsis. Microbubbles of gas did not mix with fluid creating the normal homogeneous echoic aspect typical of healthy animals, causing a hypo/anaechoic pattern of the content. In companion animals, the mean number of gastric contractions is 4 to 5/min; for this reason, in order to obtain an accurate estimate of gastric contraction, the stomach should be observed for 3 min (Pennick et al., 1989; Paoloni et al., 2002).

In cetaceans, a primary wave moves from the lower esophageal sphincter to the fundus, and a secondary wave proceeds from the fundus to the lower esophageal sphincter. A rate of 3 to 4 cycles/1-min were observed in fasted animals (Dover & Van Bonn, 2001). In our study, we observed that the peristalsis wave in bottlenose dolphins with a full stomach starts from the fundic stomach, moves toward the forestomach, and then proceeds to the third chamber (Saviano, 2013). Hypomotility was associated with digestive disturbances (malabsorption syndrome), while hypermotility was observed associated with the presence of foreign materials in the forestomach (Dover & Van Bonn, 2001; Tyrrell & Beck, 2006).

Some authors reported that motility disturbance may be correlated to the perforation of the connecting channel between the second and third compartments of the multi-chambered stomach (Van Bonn, 2002). Nowadays, the detailed evaluation of the connecting channels to the pyloric chamber and to the duodenal ampulla by ultrasound is not easy to do as a consequence of their anatomical features.

Finally, ultrasonography should play a role of primary importance among routine diagnostic procedures as well as in the assessment of the gastrointestinal tract of dolphins for the reason that in this species, it may provide more information to the clinicians and is more comfortable for the subject than radiological and endoscopic examinations. However, more studies, such as in the use of contrast agents, are needed in order to increase our knowledge of the gastrointestinal physiology of this species.

5.3 Detection and enumeration of some culturable microorganisms from clinically healthy bottlenose dolphins (*Tursiops truncatus*), kept under human care

The mammalian commensal microbiota constitutes of over thousand bacterial phylotypes and the gastrointestinal tract harbors the largest amount of microbes (Suchodolski, 2014). Microbiota confers important functions including a mucosal barrier functions, a metabolic function (e.g. digestion of xenobiotics, fermentation of indigestible substrates, production of short chain fatty acids and vitamins, ions absorption) and immune regulatory functions contributing to the development and regulation of the gut immune system (Backhed et al., 2005). Microbiota also reduce the growth of pathogenic bacteria creating a physiologically restrictive environment, competing with pathogens and toxins for adherence to the intestinal epithelium and then inhibiting the bacterial translocation (Hill et al., 2010). The composition of the microbiota can be influenced by various factors, including diet, exposure to antibiotics and the well functioning of mechanisms of immune tolerance (Jernberg et al., 2007; Koenig et a al., 2011). Buck et al., (2006) collected samples from presumably health free-ranging bottlenose dolphins (Tursiops truncatus) of Florida, Texas, and North Carolina. Vibrios, unidentified Pseudomonads, Escherichia coli, Staphylococcus spp., and a large group of nonfermenting Gram-negative bacteria represented 50% of isolates. Many organisms occurred sporadically in dolphins that were sampled repeatedly, but some were consistently isolated from individual animals and may indicate the carrier state. This may indicate that, although healthy animals have a wider spectrum of associated microorganisms, debilitated animals are characterized by a greater number of opportunists. Chan et al. (2001) examined 15 captive cetaceans monthly over a 7-yr period and found the following organisms as representing 0.2% of isolates: Vibrio alginolyticus, (24.7%), Candida albicans (8.4%), Proteus mirabilis (6.5%), Shewanella putrefaciens (3.3%), Morganella morganii, (3.1%), Staphylococcus aureus (2.4%), and

Pseudomonas aeruginosa (2.1%). Asper and Odell (1980) sampled 26 wild dolphins from the east coast of Florida and found Escherichia coli, Staphylococcus epidermidis, Proteus vulgaris, and Pseudomonas aeruginosa most common in blowhole samples with frequent isolation of Saccharomyces species.

Marine mammals, as several other non-domestic species, frequently mask early clinical signs of disease. Preventive medicine program, particularly examination of the digestive system in cetaceans, is critical to ensuring the welfare of these animals under human care. Ultrasound, endoscopy and cytology are a very interesting and complementary procedure for the complete examination of the upper gastrointestinal tract (Brook et al., 2001). However, a detailed description of the normal gastrointestinal microbiota in dolphins, is necessary to provide additional and relevant data, very useful to the clinician. The data collected can be useful not only to improve the management of bottlenose dolphins under human care, but, as reference values for healthy animals, may also serve to increase the knowledge on this species, and so on wild populations, then to have more information about possible transmissible pathogens and the risks to human health. The project established, for the first time, baseline data to identify normal GI microflora in clinically healthy bottlenose dolphins (Tursiops truncatus), kept under human care. Secondly, we investigated potential age, sex, and facility-related variations on these parameters.

Bacteria found in this study have been identified previously in other species as part of their normal flora and are considered opportunistic pathogens (Kong et al., 2014; Maria et al, 2007). The exposure of dolphins to different microbes may vary from site to site depending on a variety of environmental factors, such as seasonal changes in water temperature (Buck et al., 2006). Dolphins were of different sexes, ages and habitat conditions. However, no statistically significant differences were found in any bacteria evaluated between male and female dolphins. The highest inter-individual biological variations and differences due to facility of faecal Staphylococci can be attributed to the diet, gut morphology and physiology, and other environmental factors. These distributions

may be also related to the quality of the waters in which the animals were located: enteric bacteria would be expected to be higher in chlorine artificial waters due to the higher sensibility of these bacteria to the ozone. The enteric bacteria were frequently isolated in all samples. Citrobacter freundii, Flavobacterium meningosepticum, Hafnia alvei, Klebsiella pneumoniae, Morganella morganii, Pasteurella multocida, Proteus spp., Providencia spp., Serratia spp., Vibrio spp., Yersinia enterocolitica, known pathogens in mammals as members of the large assemblage of non-fermenting gram-negative bacteria, were absent in all samples. Of particular interest was the occurrence of yeasts, especially Candida species. Candida albicans, C. tropicalis, C. parapsilosis, and C.(T.) glabrata are the most frequently encountered opportunistic fungi in humans. C. tropicalis occurred in 14.3% of animals, followed by unidentified Candida species (7.3%), C. albicans (7.0%), C. famata (3.7%), C. lusitaniae (3.0%), C.guilliermondii (1.3%), and T. glabrata (1.0%), (Buck et al., 1980). A low rate of yeasts was found in both, gastric juice and faecal samples. They tended to increase as body weight of dolphins increased but had the highest inter-individual biological variations. This study provides relevant data and description of the normal GI microbiota in bottlenose dolphins, very useful to the clinician. Cultivation based analysis is still the most simple and inexpensive system to quantify gut microbiota. This technique does not permit detection of some species or strain-level but is functional to evaluate physiological parameters, does not require extensive bio-informatic analysis (Sekirov et al. 2010) and can be carried out by the veterinarian as routine examination by voluntary behaviour to assess the health of the animals. To accomplish a normal microbiota study, it should be done with proper regard to the following key points; dolphins must not be dealing with any clinical pathology, neither infection, nor under antibiotic treatment at least during the former three weeks before the sampling scheme.

6.0 CONCLUSION

In the period between January 2012 and December 2014 we surveyed a total of 48 bottlenose dolphins (*Tursiops truncatus*) housed in three different facilities. Of this group, a total of 31 animals were considered clinically healthy and they were examined and sampled. We collected samples of gastric fluid and feces, we did abdominal ultrasound of the gastrointestinal tract, we did endoscopy of the first and second gastric chambers and biopsies of the first stomach were collected.. All animals examined were trained for this type of medical procedures.

This thesis provides important data to the entire scientific community, in details:

1. Provides reference baseline data for normal cytological findings (pH, epithelial cells and leukocytes) in gastric samples of healthy bottlenose dolphins (*Tursiops truncatus*), following, for the first time, the “Guidelines for the Determination of Reference Intervals in Veterinary Species” issued by the Quality Assurance and Laboratory Standards Committee of the American Society for Veterinary Clinical Pathology.

2. Assesses the existence of a correlation between healthy dolphins in the values of pH and epithelial cells, pH and leukocytes, and between epithelial cells and leukocytes. Our study shows that no correlation was found between pH and the epithelial cell values ($r_s = 0.292$, $p = 0.1657$), between pH and the leukocytes values ($r_s = 0.168$, $p = 0.4321$), and between epithelial cells and leukocytes values ($r_s = 0.076$, $p = 0.7240$). Also the t-test (assuming equal variances) did not find a significant difference for weight ($p = 0.6739$), pH ($p = 0.7707$), epithelial cells ($p = 0.6385$), or leukocytes ($p = 0.6968$), between sexes.

3. Evaluates the characteristics of the gastrointestinal tract of the bottlenose dolphin (*Tursiops truncatus*) through ultrasonography in clinically healthy individual defining most useful acoustic windows for the examination of forestomach, fundic stomach, pyloric stomach and bowel.

4. Defines a consistent and standardized method to measure the entire wall thickness in forestomach, fundic stomach, pyloric stomach, and bowel; describing the normal ultrasonographic appearance of these structures; and defines the normal peristalsis of forestomach, fundic stomach, pyloric stomach, and bowel. Statistical analysis of the data, collected through ultrasonography permitted the determination of a preliminary reference interval of the gastrointestinal tract for healthy animals, based for the first time, on the “Guidelines for the Determination of Reference Intervals in Veterinary Species” issued by the Quality Assurance and Laboratory Standards Committee of the American Society for Veterinary Clinical Pathology.

5. Detectes and enumerates some culturable microorganisms from clinically healthy bottlenose dolphins (*Tursiops truncatus*), kept under human care to establish for the first time, baseline data. following the “Guidelines for the Determination of Reference Intervals in Veterinary Species” issued by the Quality Assurance and Laboratory Standards Committee of the American Society for Veterinary Clinical Pathology. Coliforms, *E. coli*, Staphylococci, *Clostridium spp*. and yeasts from gastric fluid and faeces were enumerate.

7. SUMMARY

Marine mammals, as several non-domesticated species, frequently do not show early clinical signs of disease. Preventive medicine programs, and particularly examination of the digestive system in cetaceans is one of the keys facts in health evaluation and to ensure the welfare of these animals under human care. Gastroenteritis and digestive ulcers could trigger even more severe diseases in dolphins making early diagnosis critical. Cytological examination is a common, inexpensive and readily available diagnostic tool that constitutes a valuable part of a medical evaluation for both terrestrial and aquatic species. The advancement in the training of animals for medical behaviors has also made collection of many of these samples easier. The gastrointestinal system of cetaceans is complex in its anatomical structure, but similar to many terrestrial animals. Collection of a gastric sample may be performed with a variety of gastric tubes commonly used in equine medicine. Diagnostics that are commonly performed on gastric samples include cytology, pH, and microbiology. Furthermore, endoscopic examination allows the direct visualization of the gastric mucosa and the removal of biopsy specimens. For this reason, endoscopy should also be performed to confirm the medical state of the examined animal. This can provide supporting data and gives the clinicians the possibility to evaluate the animal more in an accurate way when suspected of gastrointestinal disorders. Endoscopy has been traditionally the most useful tool to examine and evaluate the upper gastrointestinal tract. Ultrasonography however, plays an important part in modern-day cetacean preventative medicine because is a non-invasive technique, is safe for both patient and operator, can be performed to check the animals routinely using the medical behaviors and also for the follow up of the animals under therapy. In spite of the abundant information on ultrasonographic appearance of stomach and bowel in healthy dogs and cats there are unfortunately not enough clinical measurements and data to facilitate optimum diagnostic possibilities in marine mammals.

The goal of this project is to describe in detail the ultrasonographic appearance of the normal gastrointestinal region including normal thickness measurements for the forestomach, fundic stomach, third chamber and bowel. Cytological and other characteristics of the component chambers have been interpreted in relation to feeding habits and digestion in dolphins. Also cytologic studies have been associated with the presence of disease (mainly gastritis) but never directly correlated with endoscopic, histopatological or ultrasonographic findings. Endoscopies are usually performed in marine mammal veterinary practice, allowing to see the macroscopic superficial appearance of the mucosa of the pharynx, esophagus, first and second stomach, highlighting the presence of ulcers, foreign bodies or any morphological alterations. However, dolphin anatomy precludes the endoscopic exploration of the connecting channel to the third chamber and any other further sections. We found ultrasound a very interesting complementary procedure along with endoscopy and cytological for the complete examination of the upper gastrointestinal tract, not only allowing to explore all chambers but also to evaluate the normal structure of the gastro-intestinal tract through the whole wall thickness. Then, ultrasonography has been described as the most effective and least invasive diagnostic modality available in small animals to detect gastrointestinal tumors.

8. RESUMEN EXTENDIDO.

INTRODUCCIÓN.

La estabilidad del medio marino es, cada vez más, una condición crítica para el desarrollo sostenible del planeta. La protección y conservación de los ecosistemas marinos es una prioridad mundial. Para ello se requiere un conocimiento básico de los mecanismos que influyen en los mismos. A lo largo de la historia se han ido acumulando datos biológicos, oceanográficos, geofísicos, etc. con los que se han podido definir riesgos y plantear soluciones para mantener un equilibrio duradero y un desarrollo sostenible entre las actividades humanas y la conservación de los mares. Ello nos obliga a desarrollar investigaciones multidisciplinares que confluyan en nuevas disciplinas científicas integrales que contengan al medio ambiente como núcleo central prioritario. En estas ciencias ambientales reciben un especial interés los mamíferos marinos, puesto que, como los hombres, son mamíferos homeotermos, con un amplio periodo de supervivencia y se encuentran en lo más alto de la cadena trófica. Por esta razón, constituyen unos excelentes bioindicadores del medio marino, aportando una valiosa información sobre el grado de degradación o conservación de este hábitat a través del estudio biosanitario de estas especies.

Sin embargo, al contrario que en los mamíferos terrestres domésticos, se tiene poca información sobre numerosos aspectos de la sanidad de estos animales. En las últimas décadas se ha avanzado significativamente en este sentido. Así, ejemplos como los estudios en la población de belugas (*Delphinapterus leucas*) en el Estuario de San Lorenzo en Canadá, las mortandades masivas de delfines listados (*Stenella coerulealba*) en el Mediterráneo y de delfines mulares (*Tursiops truncatus*) en la Costa Atlántica Norteamericana y Golfo de Méjico, en las que se combinan altas concentraciones de contaminantes con enfermedades infecciosas y no infecciosas, aportan datos del efecto

negativo que tanto contaminantes como agentes biológicos están produciendo sobre individuos y poblaciones a través de alteraciones metabólicas, endocrinas, reproductivas e inmunológicas, que claramente tienen como base la modificación y/o degradación de su hábitat por actividades antropogénicas.

Otro estudio que ha proporcionado datos importantes sobre la salud de los delfines mulares ha sido el proyecto de Evaluación de Salud y Riesgo (HERA) que fue iniciado en 2003 por el HBOI (Harbor Branch Oceanographic Institution) y el CCEHBM-NCCOS (Center for Coastal Environmental Health and Biomolecular Research-National Centers for Costal Ocean Science) de la NOAA (National Oceanic and Atmospheric Administration) en Estado Unidos, para estudiar las poblaciones de delfines mulares del Atlántico residentes en la Indian River Lagoon (IRL), Florida, EE.UU., y las aguas costeras de Charleston (CHS), Carolina del Sur, EE.UU. (Goldstein, 2006). Este estudio resume datos hematológicos y citológicos de los delfines de IRL y CHS. Los mamíferos marinos, como los delfines, son importantes especies clave en las zonas costeras. Pueden reflejar los efectos de los factores de estrés naturales y antropogénicos, y son a menudo vistos como centinelas para la salud del medio ambiente y de los ecosistemas. Por lo tanto, la definición de la situación sanitaria de los delfines mulares es importante tanto para el futuro de la gestión de esta especie, como para proporcionar una idea de la salud de las poblaciones de un ecosistema (Bossart, 2005).

Los cetáceos están protegidos tanto por la legislación estatal como por directivas comunitarias como la Directiva Hábitat (aprobada por la UE el 21 de mayo de 1992) relativa a la conservación de los hábitat naturales y de la fauna y flora silvestres. El delfín mular (*Tursiops truncatus*) figura, en esta directiva, en el anexo II de especies de interés comunitario para cuya conservación es necesario designar zonas geográficas especiales que deben ser objeto de medidas especiales de protección del hábitat.

El estudio de la patología en cetáceos comenzó a desarrollarse a partir de la segunda mitad de los años 60, encabezado por los trabajos de patólogos y científicos norteamericanos. Inicialmente, estos estudios se nutrieron mayoritariamente de animales mantenidos en cautividad, si bien, posteriormente se incrementaron con nuevas aportaciones procedentes de cetáceos varados. Los cetáceos mantenidos bajo cuidado humano y los de vida salvaje comparten muchas patologías, aunque en los primeros se detectan algunas diferencias específicas relacionadas con su manejo y su entorno que no han sido observadas en los animales de vida salvajes y lo mismo ocurre al contrario. La patología de los cetáceos, al igual que en otras especies, requiere conocimientos básicos de la biología, anatomía y fisiología de estas especies, además del necesario aprendizaje metodológico y científico previo de la patología animal y comparada.

OBJETIVOS.

Por tanto, el objetivo genérico de la presente tesis doctoral es la determinación, por primera vez, de los principales datos de referencia para el examen del tracto gastrointestinal en delfines mulares clínicamente sanos (*Tursiops truncatus*) bajo cuidado humano, utilizando los conocimientos actuales de la clínica y de la patología animal y/o comparada, sobre todo los conocimientos acerca la clínica de los mamíferos domésticos, lo que nos permitirá aportar nueva información sobre esta especie.

Los datos recogidos aumentarán el conocimiento sobre las caracteristicas del tracto gastrointestinal de la especie *Tursiops truncatus*, en cuanto valores de referencia para los animales sanos: la definición de la situación sanitaria de los delfines mulares no sólo es importante para el futuro de la gestión de esta especie, también proporciona una idea de la salud del ecosistema (Bossart, 2005). Esto significa que si tenemos valores de referencia basicos de hematología, citología, ecografía, etc. para determinar la salud de un animal, podemos aplicar este conocimiento a la hora de tener que evaluar la salud general de una población silvestre de un ecosistema particular (Goldstein, 2006).

Por otra parte, la citología y la ecografía, siendo técnicas menos invasivas, comparadas, por ejemplo, con la endoscopia, se pueden utilizar con éxito en los centros de rehabilitación para el diagnóstico de las causas de varamientos de animales vivos, sobre todo en caso de patologías de origen antrópico como la ingestión de plásticos y otros cuerpos extraños y así aumentar las posibilidades de supervivencia y reintroducción en la naturaleza.

En base a esto, en este estudio nos propusimos los siguientes objetivos específicos:

- Proporcionar datos básicos de referencia para los resultados citológicos normales (pH, células epiteliales y leucocitos) en muestras gástricas de delfines mulares clínicamente sanos (*Tursiops truncatus*), siguiendo, por primera vez, la “Guía para la determinación de intervalos de referencia en especies veterinaria" (“Guidelines for the Determination of Reference Intervals in Veterinary Species”), publicada por el Comité de Garantía de Calidad y de Normas de Laboratorio de la Sociedad Americana de Patología Clínica Veterinaria (Friedrichs et al., 2012).

- Evalúar la existencia de una correlación entre los valores de pH y los valores de las células epiteliales, entre los valores de pH y los valores de glóbulos blancos, y entre los valores de las células epiteliales y los valores de glóbulos blancos, en delfines clínicamente sanos.

- Combinar los estudios de citología tanto con el examen endoscópico como con el examen histológico del tejido del estómago.

- Evalúar las características del tracto gastrointestinal del delfín mular (*Tursiops truncatus*) a través de la ecografía en individuos clínicamente sanos.

    - Definir ventanas acústicas útiles para el examen del primer estómago, del estómago fúndico, del estómago pílorico y del intestino.

    - Definir el aspecto ecográfico normal del primer estómago, del estómago fúndico, del estómago pilórico, y del intestino.

    - Definir un método consistente y estandarizado para medir el grosor de la pared del primer estómago, el grosor de la pared del estómago fúndico, el

diametro del estómago pilórico, y el grosor de la pared del intestino.

- Definir el peristaltismo normal del primer estómago, del estómago fúndico, del estómago pilórico y del intestino de delfínes mulares (*Tursiops truncatus*) clínicamente sanos.

- Detectar y enumerar algunos microorganismos cultivables de delfines mulares (*Tursiops truncatus*) clínicamente sanos y mantenidos bajo cuidado humano, a través del cultivo de muestras de líquido gástrico y heces, y establecer, por primera vez, datos de referencia.

- Investigar potenciales variaciones relacionadas con los siguientes parámetros: las características de las instalaciones, la edad y el sexo de los animals.

REVISION BIBLIOGRÁFICA.

En primer lugar, trataremos de forma resumida las adaptaciones morfo-fisiológicas del tracto gastrointestinal de los cetáceos en relación a su medio natural, cuya comprensión es fundamental para la interpretación de los hallazgos diagnósticos y para entender algunos de los procesos patológicos que les afectan.

Los cetáceos que pertenecen al orden Odontoceti, como el delfín mular (*Tursiops truncatus*) son especies piscívoras, que están provistos de dientes todos iguales entre sí, con los que sujetan sus presas para luego ingerirlas sin masticar (Rommel & Lowenstine, 2001). La morfología del tracto gastrointestinal se ha estudiado en varias especies de cetáceos, incluyendo los delfines mulares (*Tursiops truncatus*), y se ha correlacionado con las características histológicas de cada región.

En los cetáceos, el esófago está formado por un tubo largo y de gruesas paredes muy distensibles. Su longitud depende del tamaño del animal, constituyendo aproximadamente un cuarto de la longitud corporal en los odontocetos (Berta y cols, 2005).

Todos los cetáceos están caracterizados por tener un estómago complejo con varios compartimentos. En la mayoría de los odontocetos y misticetos se distinguen tres porciones (**Fig. 1**).

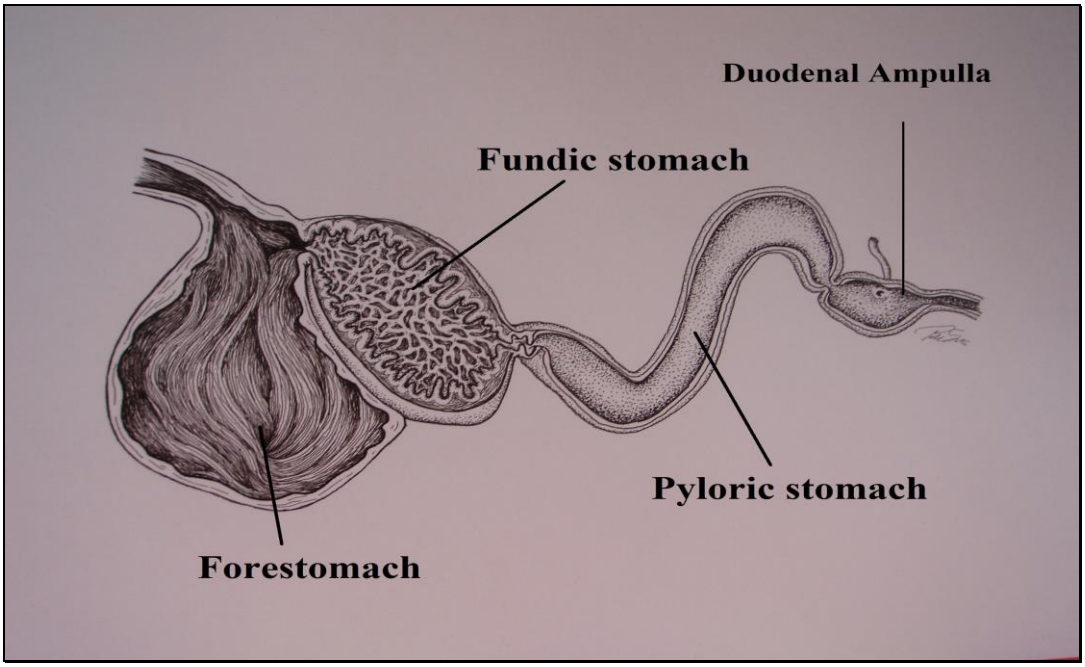


Fig. 1: los delfines mulares (*Tursiops truncatus*) están caracterizados por tener un estómago complejo con varios compartimentos.

El primer compartimento está desprovisto de glándulas y está cubierto por un epitelio escamoso queratinizado, presentando un capa muscular muy desarrollada asociada a su función mecánica. Es de mayor tamaño en odontocetos, mientras que está ausente en el zifio de Cuvier (*Ziphius cavirostris*) y en la franciscana (*Pontoporia blainvillei*). En los delfínidos, este estómago puede contener varios litros de agua y presenta un esfínter muscular entre el esófago y el estómago (Rommel & Lowenstine, 2001; Berta y cols, 2005).

El estómago principal (o glandular) presenta una gruesa mucosa plegada, compuesta por un epitelio simple cilíndrico de revestimiento superficial que se invagina para formar las criptas gástricas, donde se localizan las glándulas gástricas compuestas por las células parietales (acidófilas, producen HCl) y por las células principales (basófilas, producen pepsinógeno). En esta porción se produce la secreción de ácido clorhídrico y pepsinógenos responsables de la digestión química y enzimática del

alimento, que, a través del peristaltismo en parte se verte en el primer estómago, donde tiene lugar también la digestión química-enzimática. El estómago principal es el equivalente al cuerpo del estómago de los mamíferos monogástricos o al abomaso de los rumiantes (Rommel & Lowenstine, 2001; Berta y cols, 2005).

El estómago pilórico, presenta una mucosa relativamente lisa (sin pliegues), está caracterizado también por la presencia de criptas gástricas y numerosas glándulas pilóricas. El compartimento pilórico tiene funciones digestivas enzimáticas y desemboca en el duodeno a través de un estrecho píloro (Rommel & Lowenstine, 2001; Berta y cols, 2005).

La porción inicial del duodeno suele estar dilatada formando la ampolla duodenal, donde desemboca el colédoco y el conducto pancreático en un único conducto (conducto hepatopancreático). La ampolla duodenal permite una mejor digestión de los alimentos antes de su llegada al duodeno propiamente dicho, lo que podría indicar que la digestión enzimática tiene un importante significado en estos animales (Rommel & Lowenstine, 2001; Berta y cols, 2005).

En el delfín mular, como ocurre en los odontocetos y en la mayoría de las especies de mamíferos marinos, no existe ciego, las diferentes porciones intestinales no se encuentran claramente delimitadas, con un grosor similar a lo largo de toda su longitud (Rommel & Lowenstine, 2001; Berta y cols, 2005).

Histológicamente, la mucosa del intestino está formada por las vellosidades intestinales revestidas por un epitelio simple cilíndrico con microvellosidades y donde se encuentran las criptas de Lieberkühn. La lámina propia separa la mucosa de la submucosa, mas externamente se encuentran dos capas musculares (transversal y longitudinal) y la típica serosa (Rommel & Lowenstine, 2001; Berta y cols, 2005).

El hígado de los cetáceos es bilobulado, aunque a veces puede estar presente un tercer lóbulo. No presentan vesícula biliar (Rommel & Lowenstine, 2001; Berta y cols, 2005).

El páncreas tiene una consistencia firme, se extiende transversalmente de un lado a otro de la pared dorsal abdominal desde el duodeno al bazo y posteriormente al estómago; tiene mayores dimensiones en las hembras que en los machos y se conecta al intestino a través del conducto hepatopancreático (Rommel &Lowenstine, 2001; Berta y cols, 2005) .

Aunque mucho menos prolija que la patología de los animales domésticos y, por supuesto, que la patología humana, abordaremos a continuación la revisión bibliográfica correspondiente a las enfermedades propias del tracto gastrointestinal en cetáceos.

Las gastritis y las úlceras gástricas, lesiones diagnosticadas con frecuencia en cetáceos, pueden tener, como en los humanas, un componente bacteriano. Ésto se ha demostrado recientemente con el aislamiento y caracterización de una nueva especie de Helicobacter sp. (*Helicobacter cetorum*) a partir de la mucosa gástrica de varias especies de odontocetos (delfines de flanco blanco del Atlántico (*Lagenorhynchus acutus*) y del Pacífico (*Lagenorhynchus obliquidens*), delfines comunes (*Delphinus delphis*), delfines mulares (*Tursiops truncatus*) y belugas (Delphinapterus leucas) (Harper y cols., 2000, 2002).

Excepto las enterotoxemias por *Clostridium spp*., las enfermedades gastrointestinales primarias de origen bacteriano son un problema emergente en mamíferos marinos bajo el cuidado humano (Saviano, 2011).

Walsh y cols. (1994) determinaron que la toxina más frecuentemente encontrada en los aislamientos de *Clostridium perfringens* en cetáceos era la variante tipo A. Se han observado vómitos, diarreas, gases y espasmos musculares abdominales en cetáceos con enfermedades gastrointestinales inducidas por estas bacterias.

Sweeney (1978) describió hemorragias, enteritis y peritonitis necrótica asociadas al aislamiento de *Pasteurella spp*. en cetáceos. No obstante, en la mayoría de las ocasiones se describen muy pocas lesiones, lo que es consistente con su naturaleza sobreaguda. Las lesiones, cuando se presentan, incluyen necrosis de la grasa hipodérmica, edema cervical, petequias epicárdicas y pericárdicas y pulmones consolidados y edematosos. Los hallazgos histopatológicos incluyen esplenitis, hepatitis, bronconeumonía y neumonía intersticial, miocarditis y nefritis necróticas. Sweeney (1986) también sugirió que *Pasteurella multocida* puede causar enteritis y provocar la muerte de los animales asociada a hemorragias intestinales y una bacteriemia. El mismo autor describió una epidemia por *Pasteurella haemolytica* en un grupo de delfines, que fue controlada efectivamente con cloranfenicol, trás la muerte de uno de los animales con una traqueitis hemorrágica.

Rubio-Guerri y cols. (2015) sugiere que adenovirus puede ser responsable de gastroenteritis en delfines mulares, tal y como ocurre en leones marinos y marsopas. Además, se han aislado Adenovirus de muestras rectales de un rorcual boreal (*Balaenoptera borealis*) en la Antártida (Smith y Skilling, 1979), del colon de dos ballenas de Groenlandia (*Balaena mysticetus*) en Alaska (Smith y cols., 1987) y del intestino de belugas (*Delphinapterus leucas*) del estuario de San Lorenzo (De Guise y cols., 1995). Actualmente no se conoce la patogenicidad de estos virus aislados en cetáceos.

Se ha reconocido que los parásitos pueden causar importantes problemas de salud en los mamíferos marinos. Dailey y Brownell (1972) describieron como los

nematodos de la familia Anisakidae, los más comunes en cetáceos, se pueden localizar tanto en el primer compartimento estomacal como en el tercero. En algunas ocasiones, estos nematodos pueden dar lugar a la formación de nódulos en la mucosa y en la submucosa. Las infestaciones moderadas raramente causan signos clínicos, pero las severas pueden llegar a producir gastritis y ulceraciones (Dailey, 1985; Smith, 1989).

Pholeter gastrophilus es un trematodo que se observa en el segundo y en el tercer compartimento estomacal de los odontocetos. Este parásito se introduce profundamente en la submucosa, formando pequeños nódulos negruzcos. La mucosa que se encuentra sobre los nódulos suele permanecer intacta, éstos contienen abundante tejido conectivo fibroso alrededor del parásito, junto a una reacción inflamatoria eosinofílica y macrofágica variable (Woodard y cols., 1969). En casos graves pueden llegar a obstruir parcialmente el tránsito intestinal. *Braunina cordiformis* es otro trematodo que se encuentra normalmente en el segundo y en el tercer compartimento estomacal así como en la ampolla duodenal de los delfines mulares (*Tursiops truncatus*), produciendo una ligera irritación de la mucosa gástrica (Delyamure, 1955; Schryver y cols., 1967; Johnston y Ridgway, 1969; Zam y cols., 1971). Dailey y Stroud (1978) describieron un caso de infestación mixta por dos especies de trematodos del género Hadwenius en una marsopa común (*Phocoena phocoena*). Los parásitos se encontraban anclados en la mucosa de la porción pilórica del estómago y en el duodeno anterior, asociados a hiperemia y hemorragia moderadas.

Los cestodos adultos que infestan el intestino de los odontocetos de la especie *Strobilocephalus triangularis,* pueden penetrar en la pared del colon y formar lesiones nodulares granulomatosas, ocluyendo la luz en casos graves (Dailey, 1985).

Por último, aunque no son muy frecuentes en la literatura, enumeramos algunos casos de tumores del tracto gastrointestinal. Geraci y cols. (1987) observaron papilomas

en la lengua y un leiomioma en el intestino de un delfín de flanco blanco del Atlántico (*Lagenorhynchus acutus*), De Guise y cols. (1994 y 1995) observaron carcinomas y adenocarcinomas en el estómago, hígado e intestino de belugas (*Delphinapterus leucas*) y en el estómago de un delfín común (*Delphinus delphis*).

Aunque muchas de las erosiones y úlceras gástricas e intestinales están asociadas a parásitos o infecciones bacterianas, también se han descrito como posibles causas los cuerpos extraños (Bossart y cols., 1991) o el estrés (St. Aubin y Dierauf, 2001).

Las obstrucciones gástricas e intestinales por ingestión de cuerpos extraños han sido ampliamente descritas, tanto en animales bajo el cuidado humano como en animales salvajes (Lambertsen y Kohn, 1987; Kastelein y Lavaleije, 1992; Tarpley y Marwitz, 1993; Baird y Hooker, 2000). Los vólvulos intestinales asociados a necrosis han sido descritos en delfín moteado pantropical (*Stenella attenuata*), delfín mular (*Tursiops truncatus*), ballena de Groenlandia (Balaena mysticetus), beluga (Delphinapterus leucas) y pseudorca (*Pseudorca crassidens*) (Martineau y cols., 1988; Heidal y Albert, 1994; Briggs y Murname, 1995; Anderson y Rawson, 1997). Stamper y cols. (2006) describen un caso único de recuperación exitosa en una hembra juvenil de cachalote pigmeo (*Kogia breviceps*) en el que los medios disponibles permitieron diagnosticar la obstrucción estomacal por plásticos, alojados principalmente en la comunicación entre el segundo y el tercer compartimento estomacal, produciendo la disminución de la ingesta y una debilidad progresiva en el animal, hasta que estos cuerpos extraños pudieron ser extraídos en varios procedimientos endoscópicos, entonces el animal empezó a recuperarse, ingiriendo más alimento y recuperando peso progresivamente hasta que fue liberado. La inmediata mejora en el comportamiento y en la salud posterior a la extracción del plástico indican una relación causa-efecto directa. A pesar de las regulaciones y los avisos cautelares, cada año más de 6 millones de toneladas de basura son arrojadas al océano

(O´Hara y cols., 1994), la mayoría son plásticos y más del 90% procede de vertidos desde la costa (Faris & Hart, 1994). Al menos 23 especies de cetáceos han mostrado evidencias de haber ingerido restos de basura (Marine Mammal Commission, 2000). Los mecanismos por los cuales esta ingestión puede conllevar la enfermedad y muerte de los animales sólo puede en parte suponerse, ya que los animales enferman y mueren en el mar, sin poder observarse o aparecen varados muertos en la costa.

Factores de estrés fisiológico crónico pueden provocar una liberación amplificada de glucocorticoides, incluyendo al cortisol, que a su vez puede reducir la capacidad del sistema inmune para funcionar correctamente (por ejemplo, disminuyendo la formación y migración de células inflamatorias y la destrucción intracelular de bacterias). El aumento del cortisol endógeno también se produce en respuesta al dolor y a fluctuaciones extremas en la temperatura corporal. Los valores elevados de catecolaminas se ven con frecuencia en los mamíferos que responden al miedo, a la excitación y al sobreesfuerzo muscular. La presencia de valores elevados de cortisol, norepinefrina y aldosterona podría ser un indicador de que el estrés fisiológico es un factor que contribuye al desarrollo de una citología gástrica anormal, aunque el papel del estrés agudo y crónico de los glucocorticoides endógenos en los delfines aún no se ha establecido claramente (Goldstein y cols., 2012).

Goldstein y cols. (2006) estudiaron los delfines mulares (*Tursiops truncatus*) aparentemente sanos que residen en la Indian River Lagoon (Florida, EE.UU.). Los parámetros clínico-patológicos utilizados con mayor frecuencia para determinar la presencia de enfermedad inflamatoria sistémica en los cetáceos incluyen: recuentos sanguíneos completos, fosfatasa alcalina, fibrinógeno plasmático, velocidad de sedimentación globular y hierro sérico. Sesenta y dos delfines fueron capturados, examinados y puesto en libertad entre junio de 2003 y junio de 2004. El 24% (07/29) de los delfines examinados durante el año 2003 tenía evidencias de inflamación gástrica

neutrofílica (3/29 tenían inflamación gástrica leve y 4/29 inflamación gástrica severa). En 2004, sólo el 4% (1/24) de la población tenía inflamación gástrica neutrofílica leve o moderada y ninguno presentó inflamación severa. Los resultados mostraron que todos los animales con inflamación gástrica tenían 8 años de edad o más (edad media, 14 años). Todos menos uno de los animales con inflamación gástrica eran machos. La alta prevalencia de inflamación gástrica severa en las muestras de 2003 puede ser un indicador de que algunos factores de estrés patológicos estaban afectando a estos individuos.

Fair y cols., (2006) estudiaron la aparición de la inflamación gástrica (26% en 2003 y 29% en 2004) en los delfines de las aguas de un estuario cerca de Charleston (Carolina del Sur, EE.UU.). La prevalencia de la inflamación gástrica grave en los animales de Charleston fue similar a los de los delfines de la Indian River Lagoon (Goldstein et al., 2006). Los machos más viejos componen la mayoría de los animales que presentan inflamación gástrica grave; la edad media fue de 14 años en ambas poblaciones.

Goldstein y cols. (2012) describieron los hallazgos clínicos y patológicos del tracto gastrointestinal de 114 delfines mulares (*Tursiops truncatus*) aparentemente sanos de las mismas poblaciones residentes en la Indian River Lagoon (IRL) y de 73 delfines de las aguas del estuario cerca de Charleston (CHS). Se recogieron muestras de líquido gástrico y heces para evaluar la presencia y el grado de evidencia citológica de inflamación gástrica entre los años 2003 y 2007. La prevalencia de inflamación gástrica moderada y grave fue del 9,6% en los animales del IRL y del 11,0% en los animales del CHS. Se realizó un estudio de casos y controles de 19 delfines con evidencia citológica de inflamación gástrica y 82 con citología normal de las poblaciones combinadas. Los parámetros sanguíneos evaluados incluyeron hematología, bioquímica, electroforesis de proteínas séricas y hormonas del estrés. No se encontraron diferencias de importancia clínica o estadística entre los delfines afectados y no afectados. Ninguno de los

parámetros sanguíneos, menos las hormonas, fueron significativamente diferentes entre delfines afectados y no afectados. La noradrenalina y el cortisol sérico fueron significativamente mayores en los casos de inflamación gástrica en comparación con los controles, mientras la aldosterona era ligeramente mayor en ocho casos. El pH del líquido gástrico no fue significativamente diferente entre casos y controles. Los resultados de los cultivos microbiológicos de contenido gástrico tampoco mostraron diferencias significativas entre casos y controles. La presencia de sobrecrecimiento bacteriano en los mamíferos marinos suele producir típicamente un aumento del pH gástrico secundario a la fagocitosis bacteriana por parte de las células de la mucosa del estómago. Esto se contrapone con la respuesta en los seres humanos, en los que se observa una disminución del pH.

Los mamíferos marinos a menudo no muestran signos clínicos de la enfermedad hasta que el proceso se encuentra bastante avanzado. Sin embargo, las anormalidades citológicas pueden ser indicativas de la enfermedad mucho antes de la aparición de los signos clínicos. Históricamente, la evidencia citológica de la inflamación gástrica ha constituido un marcador de enfermedad sistémica en los delfines (Goldstein y cols., 2012).

La evidencia citológica de inflamación gástrica es un hallazgo frecuente en los mamíferos marinos varados y probablemente tiene una etiología multifactorial. Los posibles contribuyentes a la inflamación gástrica en cetáceos incluyen Helicobacter spp., otras infecciones, sobrecarga parasitaria, la ingestión de presas que irritan las paredes del estómago, la desnutrición y el estrés fisiológico crónico (Goldstein y cols., 2012). Actualmente, el método más común para identificar la presencia de inflamación gástrica es por análisis citológico de líquido gástrico. La presencia de un pequeño número de leucocitos (10 células / HPF) en el primer estómago de los cetáceos es un hallazgo normal tanto en delfines bajo el cuidado humano como en delfines salvajes. Sin embargo,

el hallazgo de un número de leucocitos, particularmente los neutrófilos, moderado (10 -20 células / HPF) y severo (>20 células / HPF), pueden llevar a sospechar la presencia de gastritis (Goldstein y cols., 2012).

Sin embargo, el aumento del número de células inflamatorias en el líquido gástrico no puede ser indicativo de ulceración gástrica. Sin el uso de métodos de diagnóstico complementarios, como el examen endoscópico, en combinación con el examen histológico de los tejidos del estómago, no es posible diagnosticar de forma definitiva la enfermedad gástrica (Goldstein y cols., 2012).

Hay que tener en cuenta que la citología gástrica puede alterarse por la deglución de las secreciones de la orofaringe o respiratorias, dando lugar a falsas interpretaciones (Goldstein y cols., 2012).

En los animales domésticos, la causa más común de deficiencia de hierro o de niveles bajos de hierro sérico es, a menudo, la hemorragia crónica. Además, un recuento sanguíneo completo revelará una leucocitosis y una anemia en casos de hemorragia gástrica, que puede estar asociada con ulceración gástrica. El secuestro del hierro es un hallazgo común en la mayoría de los casos de enfermedad crónica. Por otra parte, la disminución de los valores de albúmina sérica se asocia con estados de desnutrición, las enfermedades gastrointestinales y en especial las infestaciones parasitarias (Goldstein y cols., 2012).

Se debe tener cuidado al examinar poblaciones con un solo tiempo de muestreo, debido a que los hallazgos citológicos anormales pueden estar sujetos a cambios significativos en el tiempo (tanto las mejoras como el deterioro) que serían difíciles de evaluar sin un muestreo regular a largo plazo; este muestreo sería más fácil de realizar bajo cuidado humano (Goldstein y cols., 2012).

Crear y mantener las condiciones óptimas para el bienestar de los animales bajo el cuidado humano representa el objetivo principal de una buena medicina preventiva, ésta se basa en una serie de pruebas de rutina, enmarcadas en un plan médico mensual. Estas pruebas permiten monitorear la salud de los animales e intervenir antes de que aparezcan los síntomas de la enfermedad.

Algunas de las pruebas de rutina realizadas son:

- Exámen de líquido gástrico.
- Exámen de mucosidad nasal.
- Análisis de orina.
- Análisis de las heces.
- Análisis de sangre.
- Ecografia.
- Endoscopia.
- Rayos X.

La recogida de muestras se hace gracias al comportamiento voluntario de los animales debido al condicionamiento operante con refuerzo positivo. Esta técnica requiere que el animal sea estimulado solamente de forma positiva con refuerzos primarios (alimentos) y secundarios (juegos, caricias, etc.). La relación, la confianza y la cooperación establecida entre el animal y el entrenador juega un papel importante. Los entrenadores se comunican con los animales a través de señales visuales y auditivas; cada señal se refiere a un comportamiento singular que el animal va a ser capaz de interpretar y efectuar.

El entrenamiento implica el uso de tres herramientas básicas:

• *TARGET* (el objeto físico que el animal puede seguir, es el instrumento a través del cual el entrenador "dibuja" el comportamiento que quiere enseñar).

• *BRIDGE* (la conexión entre el inicio de la conducta requerida y el refuerzo dado al final de la misma).

• *REFUERZO* (que es algo muy positivo para el animal y se le da para facilitar el comportamiento buscado).

El entrenamiento hace que los animales bajo el cuidado humano puedan cooperar plenamente, asumir y mantener posiciones que permiten a los veterinarios llevar a cabo los procedimientos necesarios para su seguimiento sanitario. Por esta razón, es esencial el trabajo sinérgico de los veterinarios y de los entrenadores.

Por otra parte, el personal veterinario se hace cargo de la nutrición de los animales, teniendo en cuenta sus necesidades en función de la especie, del estado fisiológico o patológico, de la edad, del sexo, de la estacionalidad, etc. La mejor dieta incluye varias especies de peces, se basa en kilocalorías y kilogramos y está complementada con un suplemento vitamínico.

Lamentablemente, el manejo de los mamíferos marinos bajo el cuidado humano es a menudo más un arte que una ciencia, principalmente debido a la falta de datos sobre las necesidades nutricionales y energéticas de la mayoría de las especies. El objetivo más importante del manejo nutricional está en el satisfacer las necesidades diarias energéticas del animal. La magnitud de energía requerida está en función del tamaño corporal, de la edad, del género, del nivel de actividad del animal, de su estado fisiológico y reproductivo, de sus gastos de termorregulación y del crecimiento activo del animal. Estos gastos de energía se denominan colectivamente como la tasa metabólica diaria. Esta tasa puede ser convenientemente dividida en dos componentes: la energía para el

mantenimiento y la producción de energía. Los gastos de energía de mantenimiento incluyen el metabolismo basal, la termorregulación y la locomoción, no incluyen ningún tipo de inversión en el crecimiento o la producción de nuevas crías. Existen pocos estudios que hayan monitoreado la absorción de los alimentos (o de la energía) en los cetáceos bajo el cuidado humano (Kastelein y Vaughan, 1989; Cheal y Gales, 1991; Kastelein et al, 1993,1994.). Estos estudios observaron una relación entre la ingesta de alimentos y factores como la composición proximal (o densidad de energía) de los alimentos, la temperatura del agua, la tasa de crecimiento de los juveniles, el embarazo y los niveles de actividad. La comprensión de los gastos relativos de cada uno de estos parámetros es esencial para la comprensión de las limitaciones energéticas en los mamíferos marinos (Worthy, GAJ 2001).

El delfín mular es el cetáceo más común en los delfinarios. Esta especie pertenece al orden de los odontocetos (*Odontoceti*) que en la naturaleza se alimentan de una gran variedad de especies de peces, crustáceos y cefalópodos. Una dieta variada y equilibrada representa un factor esencial en el mantenimiento de la salud de esta especie bajo el cuidado humano. Por esta razón, las especies de peces de mayor contenido en grasa se complementan con los pescados magros o con invertebrados, como el calamar (*Loligo vulgaris*) (Couquiaud, 2005).

Un programa nutricional adecuado debe basarse en una buena comprensión de la composición y de la calidad de la comida que se ofrece. Todos los alimentos se pueden clasificar con respecto a la cantidad de humedad, proteína y grasa que contienen. El conocimiento de cada uno de los tres componentes principales es importante para evaluar el verdadero valor de una dieta.

La humedad proporciona información con respecto a la cantidad de agua metabolica que está disponible para el animal. El contenido de grasa es el principal

determinante del valor energético de los alimentos (Worthy GAJ, 2001).

Muchas especies de presas mostraron cambios en la composición de proteínas y grasas y por lo tanto en el contenido energético, relacionados con la edad y la temporada. Estos cambios suelen estar asociados a la condición reproductiva de los peces, al presentar las hembras grávidas tasas excepcionalmente altas en contenido de lípidos. Estos cambios estacionales pueden ser extremos, como en el caso del arenque (*Clupea harengus*) donde el contenido de grasa puede variar del 2 al 4% durante la primavera temprana al 15 - 20% en el invierno (Stoddard, 1988). Otras especies, como el capelán (*Mallotus villosus*) muestran cambios igualmente destacados en composición (Jangaard, 1974). Los cambios en el contenido de energía pueden llevar a una gran disparidad en el consumo de energía consumiendo cantidades similares de diferentes peces. Según aumenta la concentración de grasa, disminuye el aporte de las proteínas y del agua. Por otra parte, el aporte de vitaminas solubles en grasa se incrementará y las vitaminas solubles en agua disminuirán con el aumento en la dieta de peces con mayor contenido de grasa (Worthy GAJ, 2001).

El pescado suministrado a los mamíferos marinos bajo cuidado humano se congela (**Figs. 2, 3, 4**), por lo tanto, a la hora de realizar la dieta diaria de los delfines, hay que tener en cuenta las pérdidas de nutrientes durante los procesos de congelación, almacenamiento y descongelación. Se compensa esta pérdida con un suplemento de vitaminas, que contiene vitaminas hidrosolubles como C, B1, B2, B5, B6, B8, B9 y B12. Las vitaminas liposolubles A, D y E, son abundantes en los organismos marinos (Dierenfeld et al., 1991). Siempre y cuando el pescado sea fresco, niveles adecuados de estas vitaminas están presentes, pero se descomponen rápidamente en peces mal conservados.

Los peces almacenan energía en forma de grasas poliinsaturadas que

permanecen fluidas a bajas temperaturas. Estas grasas poliinsaturadas también son inestables en presencia de oxígeno, lo que conduce rápidamente a la peroxidación y al enranciamiento. La peroxidación consume vitamina E en el pescado, además de afectar al metabolismo del animal aumentando sus requerimientos de vitamina E. Para mantener su eficacia y garantizar su correcta absorción, el suplemento vitamínico debe proporcionarse al menos una hora antes de la primera sesión de alimentación. La dosis administrada a cada animal dependerá de diversos factores tales como el estado reproductivo, la salud, la actividad física y la edad (Floyd y Francis, 1988).

La escombroidosis es un potencial riesgo tanto para la salud humana como para la de los delfines. Se produce cuando se administran peces escómbridos, como la caballa, mal conservados, después de un período demasiado prolongado con respecto a la fecha de pesca. Los signos clínicos son la congestión respiratoria, dolor abdominal, náuseas, vómitos y diarrea. Estos síntomas se asemejan a los producidos por la histamina o compuestos relacionados. La mejor prevención para la intoxicación por escómbridos es evitar el uso de especies de peces escombroides que se han almacenado más allá de su vida útil segura (4 meses) (Geraci y St. Aubin, 1980).

Cuando no se satisfacen las necesidades básicas nutricionales, se puede generar estrés, que lleva a una disminución de la resistencia a los agentes infecciosos y en última instancia a la enfermedad. La comprensión y la aplicación de la energética nutricional pueden prevenir muchos de estos problemas.

La microflora comensal de los mamíferos está constituida más de mil filotipos bacterianos y la mayor cantidad de microorganismos se encuentra en el tracto gastrointestinal (Suchodolski, 2014). La microflora confiere funciones importantes, incluyendo las funciones de barrera de la mucosa, una función metabólica (la digestión de xenobióticos, la fermentación de sustratos digeribles, la producción de ácidos grasos de

cadena corta, de vitaminas y los iones de absorción) y las funciones que contribuyen al desarrollo y a la regulación del sistema inmunológico (Bäckhed et al., 2005). La microflora también contribuye a reducir el crecimiento de bacterias patógenas creando un ambiente fisiológicamente restrictivo, compitiendo con los patógenos y toxinas por la adherencia al epitelio intestinal (Hill et al., 2010).

La composición de la microflora puede estar influenciada por varios factores incluyendo la dieta, la exposición a los antibióticos y el correcto funcionamiento de los mecanismos de tolerancia inmune (Jernberg et al., 2007; Koenig et una al., 2011).

Buck y cols., (2006) recogieron muestras de delfines mulares (*Tursiops truncatus*) presumiblemente sanos de Florida, Texas y Carolina del Norte (EE.UU.). *Vibrios*, *Pseudomonas* no identificadas, *Escherichia coli, Staphylococcus spp*. y un gran grupo de bacterias Gram-negativas no fermentadoras representaron el 50% de los aislamientos. Muchos organismos se observaron esporádicamente en delfines que fueron muestreados en repetidas ocasiones, pero algunos se aislaron consistentemente y esto puede indicar un estado de portador en animales individuales.

Aunque los animales sanos tienen un espectro más amplio de microorganismos asociados en números bajos, los animales debilitados se caracterizan por un mayor número de oportunistas.

Chan y cols. (2001) examinaron 15 cetáceos bajo cuidado humano durante un período de 7 años y encontraron los siguientes organismos como la representación de un 0,2% de los aislamientos: *Vibrio alginolyticus*, (24,7%), *Candida albicans* (8,4%), *Proteus mirabilis* (6,5%), *Shewanella putrefaciens* (3,3%), *Morganella morganii*, (3,1%), *Staphylococcus aureus* (2,4%) y *Pseudomonas aeruginosa* (2,1%).

Asper y Odell (1980) muestrearon 26 delfines salvajes de la costa este de Florida (EE.UU.) y aislaron *Escherichia coli, Staphylococcus epidermidis, Proteus vulgaris, Pseudomonas aeruginosa* y algunas especies de *Saccharomyces* entre las especies bacterianas más comunes.

Una amplia y mejor comprensión del papel de la microflora bacteriana fisiológicamente normal en delfines sanos es importante por varias razones, además de aportar datos científicos: apoyar al veterinario a la hora de enfrentarse con la rehabilitación de animales varados o con la gestión de animales bajo cuidado humano, contribuir a la evaluación del estado sanitario general de los mamíferos marinos de vida libre, mejorar los métodos destinados a prevenir enfermedades en los delfines y a prevenir el riesgo de transmisión humana de dichas enfermedades.

Fig. 2: descongelación del pescado**; Fig. 3:** preparación de la dieta diaria; **Fig. 4**: alimentación

MATERIAL Y MÉTODOS.

En la presente tesis doctoral se detallan estudios morfológicos y de hallazgos diagnósticos de las características del tracto gastrointestinal de 31 delfines mulares (*Tursiops truncatus*) clínicamente sanos y por primera vez se realizan valores de referencia.

En el periodo comprendido entre enero de 2012 y diciembre de 2014 inspeccionamos un total de 48 delfines mulares (*Tursiops truncatus*) alojados en tres instalaciones diferentes (A - B - C). De este grupo, un total de 31 animales que tenían de 5 a 36 años de edad (media ± SE = 16 ± 1ys) y pesaban de 149,9 - 266,7 kg (media ± SE = 190,1 ± 4,3 kg), fueron considerados clínicamente sanos, de ellos recogimos muestras de líquido gástrico y heces, hicimos ecografía abdominal, endoscopia de la primera y segunda cámara gástrica y recogimos biopsias de la mucosa del primer estómago. Se evaluaron un total de 15 hembras y 16 machos. Las hembras embarazadas no se incluyeron en el estudio (**Fig. 5**).

Fig. 5: delfín mular (*Tursiops truncatus*)

El peso corporal fue significativamente mayor en los machos que en las hembras (193 ± 8 y 183 ± 9 kg en machos y hembras, respectivamente, p = 0,004) y aumentó con la edad del animal (b = 10.3, P = 0,000).

Todos los animales se consideraron clínicamente sanos en función de los análisis clínicos de laboratorio (hemograma y bioquímica sérica, análisis de orina y de heces) y de una evaluación física completa (Fair y cols, 2006; Mitchell y cols, 2008).

La temperatura media del agua en las diferentes instalaciones fue de 23 °C durante todo el periodo de muestreo. La dieta de todos los animales durante el estudio estuvo compuesta por capelán (*Mallotus villossus*), espadín (*Sprattus sprattus*), arenque (*Clupea harengus*), caballa (*Scomber scombrus*), jurel (*Trachurus trachurus*), fundir (*Atherina boyeri*), calamar (*Loligo spp*.), bacaladilla (*Micromesistius poutassou*), y lanzón (*Hyperoplus lanceolatus*).

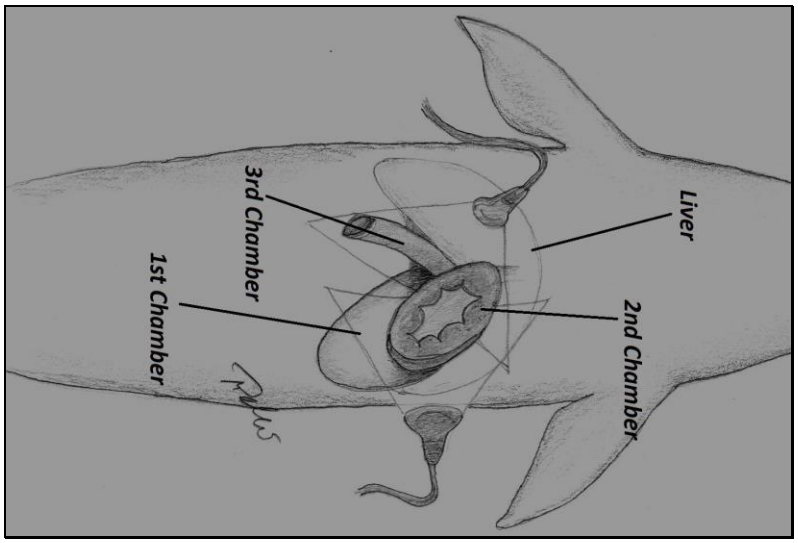
Todos los animales examinados fueron entrenados para este tipo de procedimientos médicos.

Para el **estudio ecográfico** se utilizó un portátil SonoSite 180 Plus (fabricado por SonoSite, Inc., Bothell, WA 98021, EE.UU.) con una sonda convexa 2 a 5 MHz (/ 2.5 Mhz transductor C60, **Fig. 6**). La sonda era resistente al agua y la máquina se cubrió con una bolsa de plástico transparente para evitar el contacto accidental del dispositivo con agua salada. Para evitar la luz solar directa se utilizó una bolsa de color oscuro para cubrir el instrumento. El uso de gel acústico no fue necesario porque el agua proporciona un excelente medio a través del cual se trasmiten las ondas de ultrasonido.

Fig. 6: portátil SonoSite 180 Plus con una sonda convexa 2 a 5 MHz

Las imágenes fijas obtenidas se almacenan en formato DICOM (*Digital Imaging and Communication in Medicine*), también se gravaron vídeos.

Para la realización de las ecografías se utilizaron las posiciones decubito dorsal y decúbito lateral (**Figs. 7 y 8**). Se identificaron las ventanas acústicas para las tres cámaras gástricas gracias al conocimiento de la anatomía regional (Brook et al., 2001). Tanto el primer estómago como el estómago fúndico se visualizan mejor en la cara lateral izquierda del delfín, mientras la cámara pilórica se visualiza fácilmente en el lado derecho del delfín (Saviano, 2013).



Figs. 7 y 8: ventanas acústicas para las tres cámaras gástricas.

Se realizaron imágenes de cortes transversales de las regiones con el transductor cuidadosamente orientado perpendicular a los ejes longitudinales de cada cámara para evitar errores inducidos por imágenes oblicuas. Las medidas se realizaron empezando por la capa interior de la mucosa (hiperecoica) y la capa exterior de la serosa (hiperecoica) de la pared estomacal (Pennick et al., 1989; Paoloni et al., 2002). Las medidas se hicieron entre las contracciones peristálticas. Tres medidas fundamentales fueron tomadas (Saviano, 2013): (1) espesor de los pliegues rugosos, (2) espesor de la región entre pliegues, y (3) el grosor de la pared de cada cámara. Las medidas fueron

tomadas siempre en la misma zona del estómago para minimizar los errores. Al no existir una demarcación clara entre el intestino delgado y grueso en odontocetos, las medidas se tomaron en la misma porción del intestino (sección central) para estandarizar la metodología.

Se evaluó el espesor de la pared y la motilidad relativa del tracto gastrointestinal. De acuerdo a los datos publicados en los perros y gatos, se observó cada cámara del estómago durante 3 minutos (Pennick et al., 1989; Paoloni et al., 2002).

Para realizar el estudio de ultrasonido, todos los animales fueron examinados dos veces al día: en ayuno, y después de ingerir una comida entera (una media de 8 kg). El estado de llenado del estómago fue clasificado como completo (pliegues distendidos) o vacíos (pliegues compactos).

Además se hizo un estudio ecografico utilizando un medio de contraste con la finalidad de destacar la mucosa del estómago para obtener mediciones más precisas. 5 gramos de *Gelagar Tipo CT 1,1* se mezcló con 1 litro de agua dulce, esta solución se hirvió y luego se enfrió alrededor de 4 horas en la nevera. La textura del medio obtenido era semi-liquida. La administración oral de *Gelagar Tipo CT 1,1* como solución de contraste se realizó insertando un tubo flexible de diámetro 0,95 cm en la primera cámara gástrica con una leve compresión y rotación.

Fig. 9: preparación de Gelagar Tipo CT 1,1 como medio de contraste.

La ecografía se realizó inmediatamente después de la endoscopia. Para el **estudio endoscópico** se utilizó un endoscopio portátil KARL STORZ GASTRO PACK® (fabricado por KARL STORZ GmbH & Co. KG de D-78532, Tuttlingen, Alemania, **Fig. 10**).

Fig. 10: endoscopio portátil KARL STORZ GASTRO PACK®.

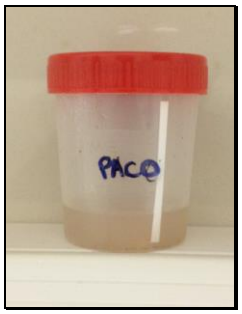
La unidad combina un videoendoscopio con 3,25 m de longitud (60.130 PKS), una unidad de control de cámara, un monitor, una unidad de documentación, una bomba de insuflación, un teclado y una fuente de luz, en un solo sistema.

La endoscopia se llevó a cabo como parte del chequeo clínico general incluido en el programa de medicina preventiva veterinaria anual establecido para cada institución. No fue necesario realizar examenes endoscópicos adicionales específicamente para el propósito de este estudio.

La endoscopia no se hizo por comportamiento voluntario debido a que se tenían que tomar muestras de la segunda cámara gástrica. Por lo tanto, los delfines mulares fueron restringidos en una piscina, que cuenta con una plataforma elevadora, para examinarse individualmente.

Antes de cada endoscopia, toda la instrumentación fue limpiada, desinfectada con glutaraldehido y se lavó a fondo antes del siguiente uso. Para la gastroscopia, se siguió el procedimiento estándar para los cetáceos (Dover & Van Bonn, 2001). Para realizar el procedimiento, todos los animales se examinaron después de un ayuno de 15 horas. Los animales fueron colocados sobre un colchón de espuma de poro cerrado en decúbito esternal y permanecían en esa posición gracias a los entrenadores. El endoscopista se colocó en la cabeza del animal. Se colocaron toallas de baño alrededor del maxilar y del mandibular para evitar daños al operador y al animal durante el procedimiento. Se pasó la punta del endoscopio sobre la lengua de los delfines, se introdujo en la orofaringe, esófago y luego en la primera cámara gástrica. Se requerió la insuflación de aire durante todo el procedimiento a fin de obtener una imagen diagnóstica.

Durante los procedimientos endoscópicos, se encontró una cantidad moderada de líquido en la primera cámara gástrica de cada animal; este líquido se recogió por aspiración en un recipiente de 120 ml con tapón de rosca de polipropileno (LP Italia, Milán, Italia) y se procesó inmediatamente (3 a 8 ml) para su **estudio citológico.**



Figs. 11 y 12: muestras de líquido de la cámara gastrica.

Figs. 13 y 14: estudio citológico del líquido gastrico: preparación de la lámina.

El pH del fluido se evaluó mediante el uso de tiras indicadoras de color fijo (pH-Fix 0-14; Macherey-Nagel, Düren, Alemania). Se siguieron los procedimientos estándar para la revisión citologica de las láminas (Sweeney y Reddy, 2001;. Bueno et al, 2006;. Goldstein et al, 2006, 2012;. Mitchell et al, 2008). Una gota de líquido gástrico de la primera cámara se recogió del recipiente con una pipeta estéril y se puso en un porta. Las muestras fueron examinadas microscópicamente después de haber sido teñidas con azul de metileno (NMB) y Dif-Quick (Bio-Optica, Milán, Italia) (**Figs. 11, 12, 13 y 14**).

Se calcularon las estadísticas correlacionando los datos de edad y peso con los parámetros de pH, volumen, células epiteliales y glóbulos blancos que se encontraron en las citologías de líquido gástrico, para cada uno de los delfines mulares clínicamente sanos (n = 30).

Durante la endoscopia también se realizaron biopsias de la primera y segunda cámara gástrica para confirmar mediante **el estudio histológico** la apariencia normal encontrada durante la observación macroscopica. Cuando se toman las muestras para la biopsia, las pinzas se deben dirigir perpendicularmente al tejido, que debe ser sujetado y traccionar hasta que se desprende. Si el órgano está desinflado, es más fácil tomar muestras de biopsia, ya que la reducción de la tensión en el lumen permite sujetar de una manera mas firme el tejido. Las muestras fueron fijadas en formalina al 10% y procesadas rutinariamente, embebidas en parafina, cortadas en secciones de 5 micras, teñidas con hematoxilina y eosina y observadas con microscopio óptico.

Para el **estudio de la microflora gastrointestinal** se tomaron muestras gástricas insertando un tubo de polietileno estéril y flexible de 0,95 cm de diámetro (Pharmaplast, Milán, Italia) en la primera cámara gástrica, con una presión leve y rotación. Gracias a una técnica a sifón de presión negativa, se recogió el líquido gástrico de la primera cámara de cada animal en contenedores de polipropileno estéril de 120 ml con tapón de rosca (LP Italia, Milán, Italia). Cada muestra fue inmediatamente congelada a -20 °C antes del examen (**Fig. 15**).

Fig.15: recogida del líquido gástrico de la primera cámara de cada animal.

Se tomaron muestras fecales insertando un tubo de polietileno estéril y flexible de 0,40 cm de diámetro (Pharmaplast, Milán, Italia) en el orificio anal. La región se limpió con una gasa estéril antes de los procedimientos. Con el fin de obtener buenas muestras fecales, debe evitarse la contaminación del agua. La presión negativa no fue necesaria ya que la materia fecal líquida fluye en el tubo sin ayuda. Las muestras de heces se congelaron inmediatamente a -20 °C antes del examen microbiológico (**Fig. 16**).

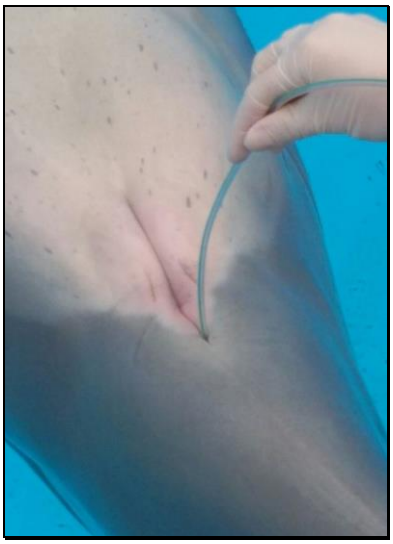


Fig. 16: toma de muestras fecales.

Se mezclaron 1 ml de líquido gástrico y 1 g de heces de cada delfín mular en tubos estériles junto con 2 ml de solución salina estéril al 0,9%. Las heces y el líquido gástrico se mezclaron en esta solución hasta que los tubos alcanzaron un volumen de 10 ml con solución salina estéril al 0,9%. Cada muestra (0,1 ml) se diluyó en series de 10 diluciones de diferencia (10^{-1} a 10^{-10}). A partir de la concentración más baja, las diluciones se sembraron y cultivaron en diferentes medios, por triplicado, utilizando el método de la propagación en placa.

Los métodos de Agar Chromocult y Agar Baird Parker se utilizaron, respectivamente, para el recuento de *E. Coli/coliformes* y *estafilococos*,. Todas las placas

se incubaron aeróbicamente a 37 °C, durante 24-48 h. Para el recuento de *Clostridium spp*. se utilizaron medios de Agar para clostridios enriquecido con un 5% de sangre de oveja y 1 mg/ml de vitamina K1 y para levaduras se utilizó el Agar Rosa de Bengala, ambos en incubación anaeróbica en frascos anaeróbicos (Oxoid) a 37ºC durante 48-72 h; se utilizaron tiras de color azul de metilo como indicador de oxidación-reducción. El número de colonias se contó y todos los datos se expresaron como CFUxlog g.

RESULTADOS Y DISCUSIÓN.

Estudio ecográfico.

Gracias a este estudio por primera vez se ha definido un método consistente y estandarizado para medir el espesor de la pared del primer estómago, la cámara fúndica, el estómago pilórico y el intestino (**Figs. 17, 18, 19 y 20**) de delfines mulares. Desafortunadamente, no fue posible medir aisladamente todas las capas de la pared (submucosa, muscular y serosa) con nuestra instrumentación.

Se determinaron valores de referencia para cada uno de los hallazgos ecográficos por el método paramétrico según lo recomendado por el Comité de Garantía de Calidad y de Normas de Laboratorio (Friedrichs et al., 2012). No hubo valores atípicos. La prueba t entre los sexos (hembras: n = 15; machos: n = 15) no mostró ninguna diferencia significativa para todos los parámetros considerados en el estudio (grosor de la mucosa de la primera cámara gástrica, p = 0,1521; grosor de la mucosa del estómago fúndico, p = 0,6616; grosor de la mucosa de los pliegues del estómago fúndico, p = 0,9064; grosor de la mucosa entre pliegues del estómago fúndico, p = 0,9765; grosor de la mucosa de la tercera cámara, p = 0,3831; diámetro de la tercera cámara, p = 0,1758; y el grosor de la mucosa intestinal, p = 0,5647). La prueba t para muestras apareadas sólo encontró una diferencia significativa en el diámetro de la tercera cámara cuando estaba vacía / llena (p <0,0001). Sólo el grosor de la mucosa fúndica del estómago fue moderadamente negativo correlacionado con el peso (rs = -0,589, p = 0,0006). La ligera correlación existente entre el grosor de la pared intestinal y el peso observado en los perros (Delaney et al., 2003) no se observó en los delfines (rs = 0,120, p = 0,5284).

El estómago fúndico es similar al estómago de perros y gatos teniendo en cuenta

la estratigrafía y desarrollo de los pliegues (Pennick et al., 1989; Paoloni et al., 2002); la mucosa parece ser ligeramente más gruesa que las otras capas, así como la submucosa, mientras que la capa muscular aparece más delgada.

Los contenidos luminales normales pueden variar y estar constituidos por alimentos, moco, líquido o gas. En todos los animales sanos evaluados en el estudio con el estómago vacío, el patrón de contenido del estómago podría ser considerado variable, aunque hubo una prevalencia de un patrón mixto de gas / líquido en la primera cámara gástrica, un patrón mixto entre moco / líquido en el estómago fúndico, y sobre todo líquido en la tercera cámara. Un patrón de alimentos se encuentra cuando el alimento está presente en el interior del lumen: la primera cámara gástrica por lo general presenta un patrón heterogéneo de alimentos debido a que es el estómago en el que se produce el proceso de digestión mecánica.

En todos los delfines mulares sanos evaluados en este estudio se identificaron cinco capas ecográficas en la primera cámara gástrica. Se identificaron, desde la interfaz de la mucosa (hiperecoica) en contacto con el lumen hasta la superficie serosa: la mucosa (hipoecoica), la submucosa (hiperecoica), la capa muscular (hipoecoica) y la subserosa y serosa (hiperecoicas). Con respecto a la descripción cualitativa de la estratigrafía del tracto gastrointestinal, la capa mucosa aparece hipoecoica en todos los animales sanos.

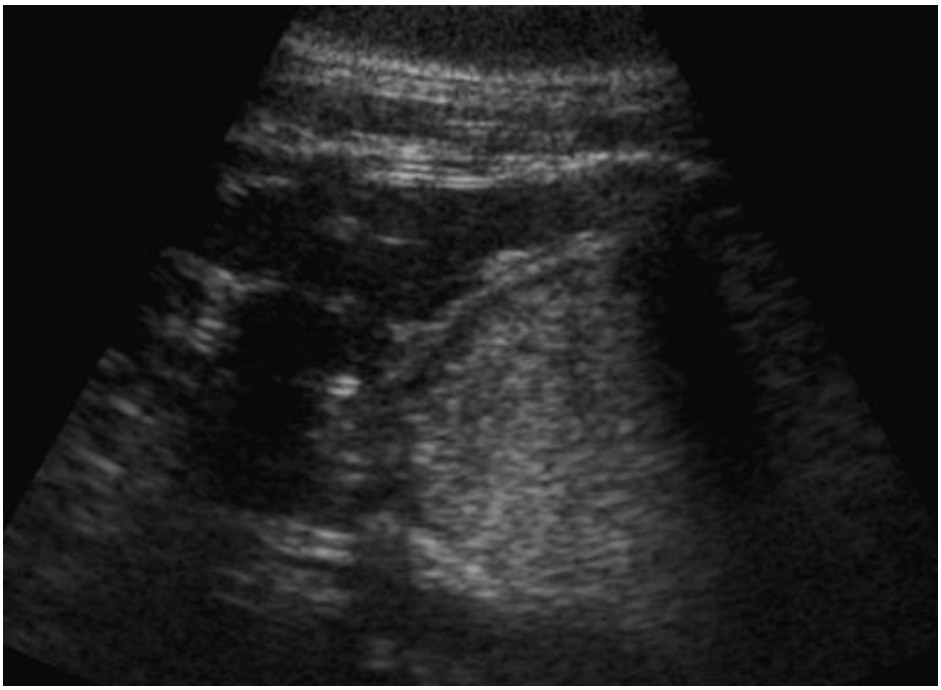


Fig. 17: Estudio ecográfico: primera cámara gástrica

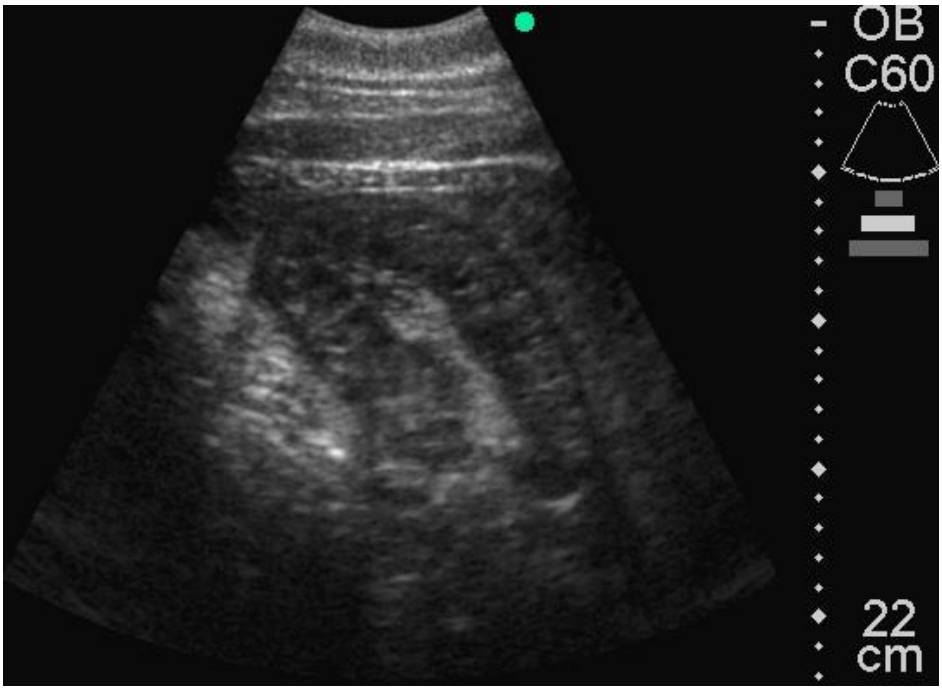


Fig. 18: Estudio ecográfico: segunda cámara gástrica

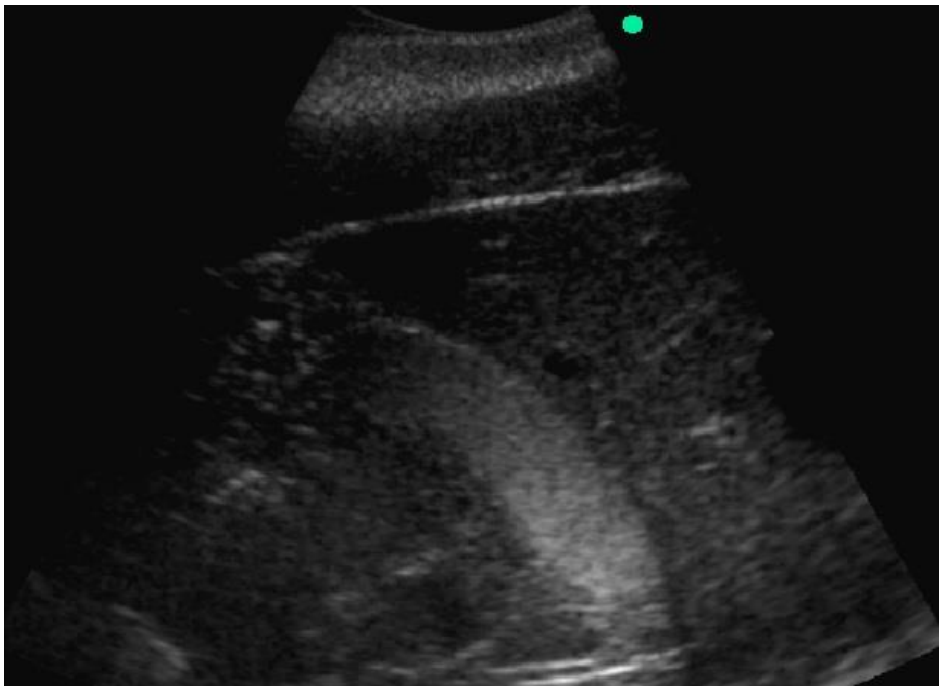


Fig. 19: Estudio ecográfico: porción pilórica.

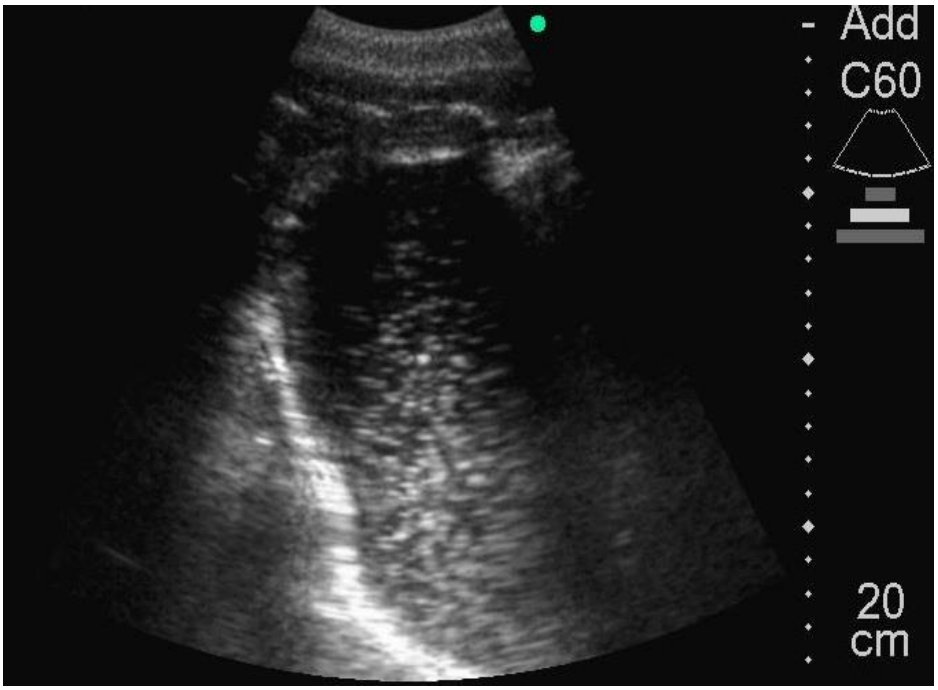


Fig. 20: Estudio ecográfico: intestino

Con respecto a los resultados del grosor total de las páredes, ecográficamente, el grosor de la pared de la primera cámara gástrica varió entre 2,4 a 8 mm en delfines mulares clínicamente sanos, el grosor de la pared de la segunda cámara gástrica varió

entre 3,3 a 7,1, mientras que es de 3 a 5 mm de espesor en el perro y entre 2 a 6 mm en los seres humanos (Pennick et al., 1989; Paoloni et al., 2002). Por lo tanto, existen algunas pequeñas variaciones entre las especies.

Según los datos publicados para perros y gatos, la anomalía más comúnmente identificada es el engrosamiento de la pared. Tumores y granulomas generalmente producen un engrosamiento focal asimétrico con una interrupción de la distribución normal de capas (Kaser-Hotz et al, 1996; Paoloni et al, 2002; Monteiro y O'Brien, 2004). Otras enfermedades inflamatorias o infiltrativas generalmente producen engrosamiento difuso, con mantenimiento de la estratificación de la pared. Un marcado aumento en el grosor de los pliegues puede indicar gastritis o parasitosis (Brook et al., 2001). En nuestra experiencia, en el caso de gastritis leve, la capa mucosa apareció ligeramente aumentada de grosor, así como más hiperecoica cuando se compara con los valores de referencia establecidos; el patrón de la luz era anormal (más hipoecoica) y la peristalsis estaba alterada.

En nuestra opinión, la ecografía del estómago de los delfines mulares debe incluir imágenes del estómago lleno, así como después de un ayuno, ya que el alimento a menudo actúa como un contraste, destacando estructuras naturales que de otra manera pueden pasarse por alto (**Fig. 21**).

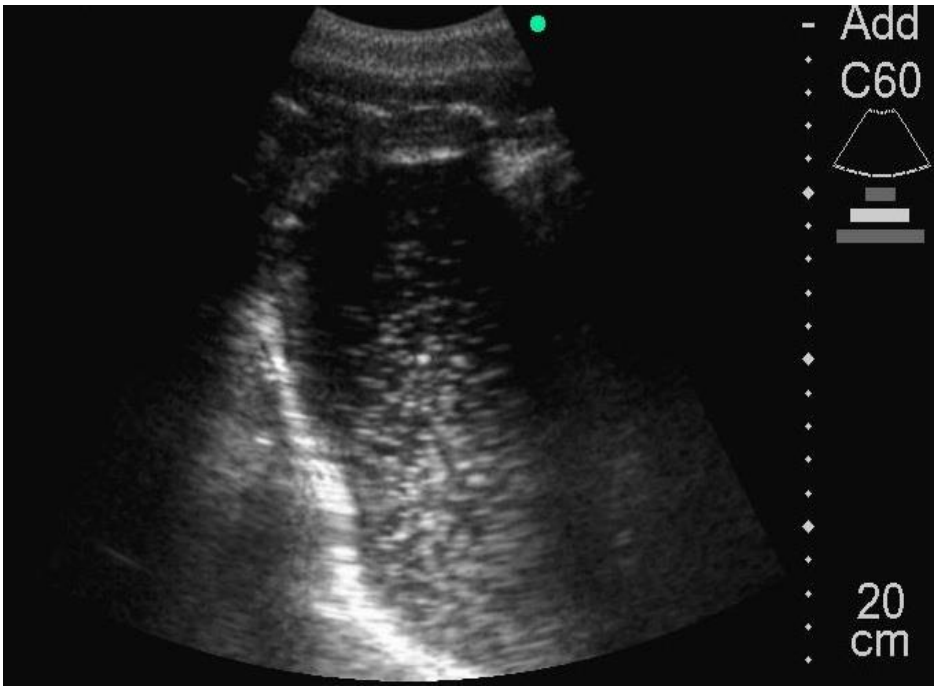


Fig. 21: Estudio ecográfico: primera cámara gástrica después de la administración de alimento que actúa como un medio de contraste.

Un patrón gaseoso está causado por la presencia de gas libre hiperecoico en el lumen, esta condición es bastante normal durante el proceso digestivo en la primera cámara gástrica. Un patrón líquido está representado por la presencia de fluido en el lumen y tiene un aspecto ecográfico anecoico típico. El presente estudio demostró que el patrón líquido en la primera cámara gástrica podría estar relacionado con una disminución en la motilidad. Este patrón se ha observado en animales con peristalsis lenta / ausente (Saviano, 2013).

La primera cámara gástrica y la cámara pilórica tenían un plegamiento y un peristaltismo menos notable en comparación con el estómago fúndico. Se ha registrado una media de 3 a 4 ciclos con una secuencia de segunda, primera cámara gástrica y tercer compartimento en los animales con el estómago lleno.

En animales en ayunas se han observado secuencias de 3 a 4 ciclos / min (Dover

& Van Bonn, 2001). En nuestro estudio, se observó que la ola de peristaltismo en los delfines mulares con el estómago lleno se inicia desde el estómago fúndico, se mueve hacia la primera cámara gástrica y luego continúa a la tercera cámara (Saviano, 2013).

En los animales de compañía, el número medio de contracciones gástricas es de 4 a 5 / min; por esta razón, con el fin de obtener una estimación precisa de la contracción gástrica, el estómago debe ser observado durante 3 min (Pennick et al., 1989; Paoloni et al., 2002). En los cetáceos, una onda peristáltica primaria se mueve desde el esfínter esofágico inferior hacia el fundus, y una onda secundaria procede desde fundus hacia el esfínter esofágico inferior.

La hipomotilidad se asocia con trastornos digestivos (síndrome de mala absorción), mientras que se observa hipermotilidad asociada con la presencia de materiales extraños en la primera cámara (Dover & Van Bonn, 2001; Tyrrell & Beck, 2006). Algunos autores describen que las alteraciones de la motilidad pueden estar correlacionadas con la perforación del canal de comunicación entre el segundo y el tercer compartimento en estómagos de múltiples cámaras (Van Bonn, 2002). Hoy en día, la evaluación detallada por ultrasonido de la comunicación de la porción fúndica a la cámara pilórica y de ésta a la ampolla duodenal es complicada de llevar a cabo debido a sus características anatómicas.

En conclusión, la ecografía debe desempeñar un papel de primera importancia entre los procedimientos del diagnóstico de rutina, así como la evaluación del tracto gastrointestinal de los delfines, ya que en esta especie, puede proporcionar mucha información al veterinario clínico y es más cómoda de realizar desde el punto de vista del manejo, comparada, por ejemplo, con los exámenes radiológicos.

El estudio ecográfico tiene la ventaja de que no necesita preparación especial, no

es invasivo, es seguro para ambos, paciente y operador, se puede realizar de forma rutinaria mediante conductas médicas y también se puede utilizar para el seguimiento de los animales sometidos a terapia (Saviano, 2013).

Ademas, permite la evaluación de la totalidad de la pared gastrointestinal en lugar de sólo la mucosa, con la instrumentación adecuada permite medir el grosor de las distintas capas, evalúa en tiempo real la motilidad, permite la evaluación de los trastornos regionales (peritonitis) y permite la visualización de cuerpos extraños gástricos, que aparecen como una interfaz hiperecoica claramente definida con sombreado distal (Tyrrell & Beck, 2006).

Sin embargo, se necesitan más estudios con el fin de aumentar nuestro conocimiento de la fisiología gastrointestinal en esta especie.

Estudio endoscópico e histológico.

La endoscopia no mostró alteraciones morfológicas. El esófago normal está formado por un tubo largo y de gruesas paredes donde se observan pliegues longitudinales de mucosa blanquecina. No hay una distinción clara entre la mucosa de la primera cámara gástrica y el esófago. La primera cámara gástrica tiene gruesos pliegues rugosos que aparecen de color rosa pálido durante la endoscopia; la porción fúndica tiene una superficie mucosa que es de color rosa oscuro a roja. La comunicación entre la primera y la segunda cámara gástrica se encuentra cranealmente, en el cuadrante ventral izquierdo del primer compartimiento (**Figs. 21, 22 y 23**).

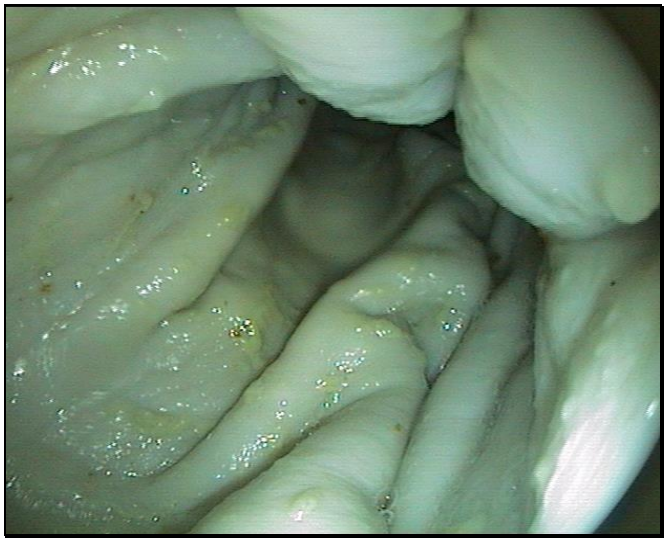


Fig. 21: Estudio endoscópico: primera cámara gástrica.

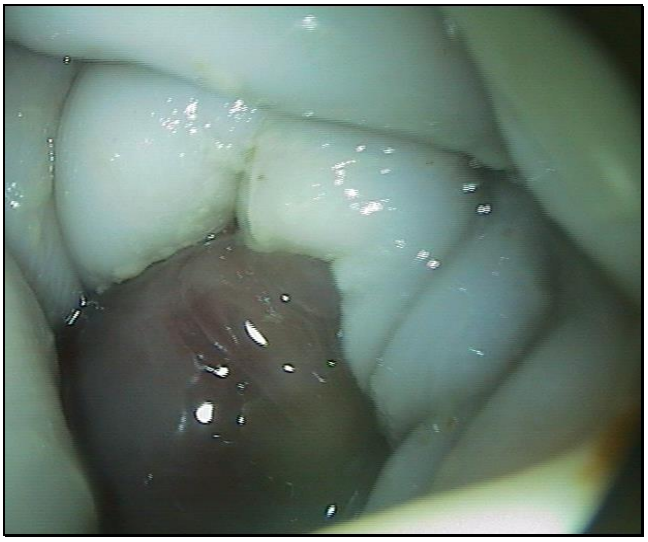


Fig. 22: Estudio endoscópico: comunicación entre la primera y la segunda cámara gástrica.

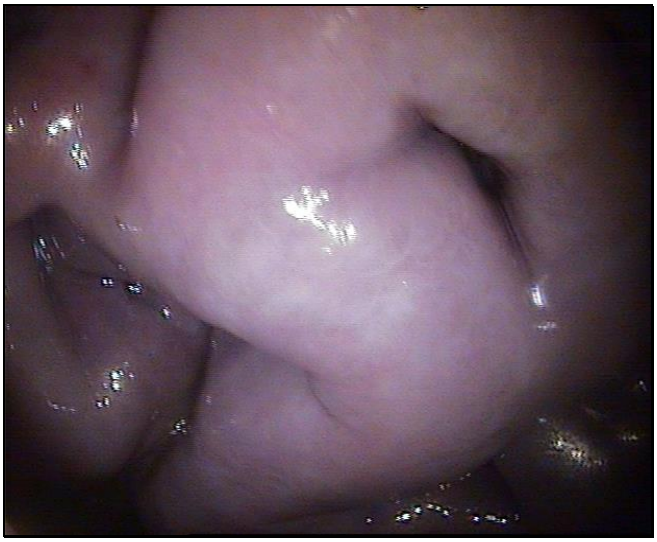


Fig. 23: Estudio endoscópico: segunda cámara.

Todas las muestras de biopsia se consideraron histológicamente normales. La mucosa de la primera cámara gástrica mostró un epitelio escamoso estratificado queratinizado normal. La pared de la segunda cámara gástrica o glandular mostró diferentes capas. Comenzando desde el interior se encuentró la mucosa, que consta de una membrana basal, de una sola capa de epitelio simple cilíndrico, de una lámina propia y de una capa muscular (*la muscularis mucosae*); la submucosa, donde se alojan las glándulas (compuestas por la células principales y parietales); la capa muscular y serosa (**Figs. 24 y 25**).

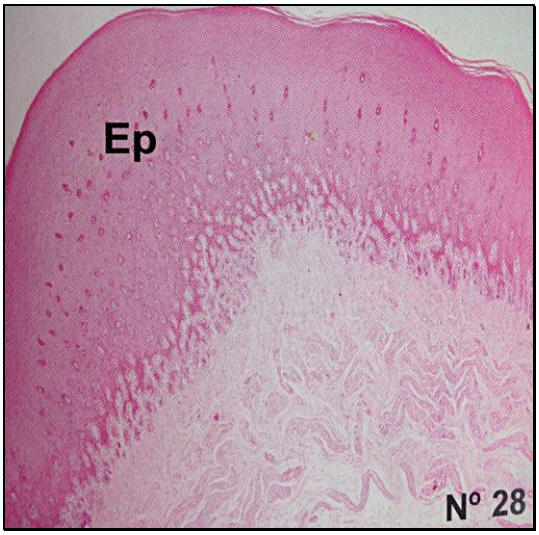


Fig. 24: Estudio histológico: la primera cámara gástrica mostró un epitelio escamoso estratificado queratinizado normal (H/E 4x).

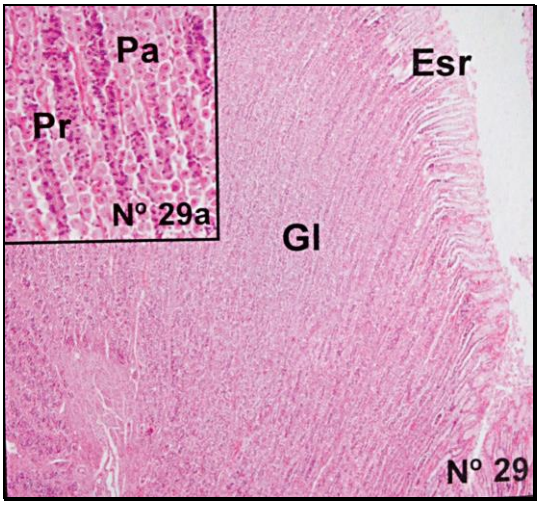


Fig. 25: Estudio histológico: la segunda cámara gástrica mostró un epitelio simple de revestimiento (Esr) y glándulas gástricas (Gl). Células parietales (Pa) y células principales (Pr) (H/E 40x).

Estudio citológico del líquido gástrico.

No se observaron valores atípicos en el análisis citológico del líquido gástrico. Los valores de referencia se determinaron utilizando el método paramétrico según lo recomendado por el Comité de Garantía de Calidad y de Normas de Laboratorio (Friedrichs et al., 2012). No se encontró correlación entre el pH y los valores de las células epiteliales (rs = 0,292, p = 0,1657), entre el pH y los valores de leucocitos (rs = 0,168, p = 0,4321), ni entre los valores de células epiteliales y leucocitos (rs = 0,076, p = 0,7240). La prueba t entre los sexos (hembras n = 15; machos n = 15), suponiendo varianzas iguales, no mostró una diferencia significativa para el peso (p = 0,6739), pH (p = 0,7707), células epiteliales (p = 0,6385) o leucocitos (p = 0,6968).

En las muestras gástricas generalmente se reportan tres tipos de células epiteliales: (1) las células epiteliales escamosas de esófago y del primer estómago, (2) las células epiteliales columnares del segundo estómago, y (3) las células basales en la primera y en la segunda cámara (Sweeney y Reddy, 2001). Durante el examen citológico del líquido gástrico de los animales sanos sólo se encontraron células epiteliales escamosas (**Fig. 26**).

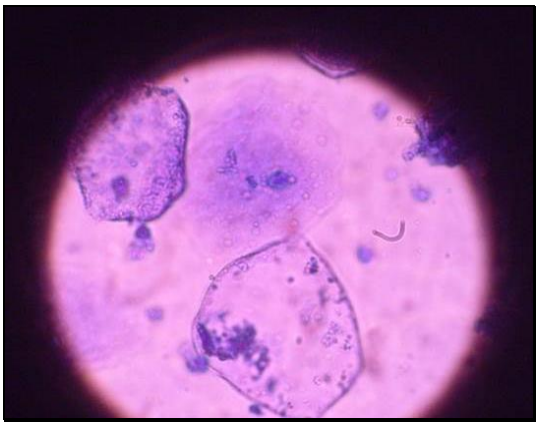


Fig. 26: Estudio citológico del líquido gástrico: células epiteliales escamosas del esófago y del primer estómago.

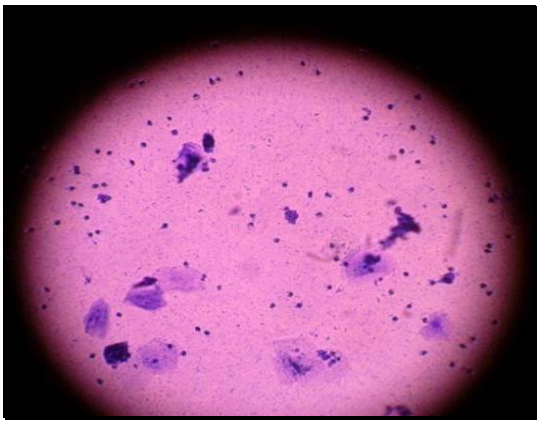


Fig. 27: Estudio citológico del líquido gástrico: leucocitos.

El presente estudio muestra que los números de leucocitos (**Fig. 27**) presentes en

el líquido gástrico de delfines mulares clínicamente sanos varía entre 0.84 y 4.11 células / HPF. La presencia de un pequeño número de leucocitos (<20 células / HPF) en la primera cámara gástrica es una ocurrencia normal tanto en delfines bajo el cuidado humano como en delfines de vida libre (Fair y cols, 2006;. Varela et al., 2007). Sin embargo, el hallazgo de un número moderado de leucocitos (> 20 células / HPF) puede llevar a sospechar la presencia de una gastritis leve (Varela et al., 2007).

El presente estudio muestra que el valor del pH del líquido gástrico de un delfín en ayunas varía de 1,0 a 3,0. Los resultados encontrados en este estudio coinciden con los de investigaciones previas (Feria et al, 2006; Goldstein et al, 2006; Varela et al, 2007; Mitchell et al, 2008).

Por otro lado, las diferencias individuales pueden influir en los resultados (Varela, 2007). Por lo tanto parece importante, para establecer una línea basal individual, tomar muestras de líquido de las cámaras gástricas de forma rutinaria como parte de un examen médico preventivo.

El objetivo principal de este estudio fue establecer, por primera vez, datos de referencia para los resultados citológicos normales en muestras de líquido de las cámaras gástricas de delfines mulares sanos, segùn el Comité de Garantía de Calidad y de Normas de Laboratorio (Friedrichs et al., 2012). La adopción de estas directrices por toda la comunidad veterinaria mejorará la comunicación y difusión de los valores laboratoriales clínico esperados para una gran variedad de especies animales, y proporcionará un formato estandarizado para las publicaciones de los valores de referencia, a fin de promover prácticas de calidad en los laboratorios que sirven tanto a los veterinarios clínicos como a los estudios de investigación (Friedrichs et al., 2012).

Los exámenes citológicos pueden proporcionar una instantánea de una

enfermedad gástrica potencial antes de la enfermedad sistémica (Cowell et al., 1999; Goldstein 2012); leucocitos elevados, junto con el aumento de las células epiteliales, células basales o eritrocitos podrían sugerir ulceración (Mitchell et al., 2008). Las alteraciones del tracto gastrointestinal se encuentran tanto en los delfines bajo el cuidado humano como en los de vida libre, y el daño a la mucosa gástrica, incluyendo úlceras, se ha descrito ampliamente (Harper et al., 2000).

Sin embargo, el aumento del número de células inflamatorias en el líquido gástrico no puede ser por si solo indicativo de ulceración gástrica. Sin el uso de métodos de diagnóstico complementarios, como el examen endoscópico, en combinación con el examen histológico de los tejidos del estómago, no es posible diagnosticar una enfermedad gástrica de forma definitiva (Goldstein y cols., 2012).

Nuestro estudio demuestra la importancia de incluir la evaluación endoscópica de las cámaras gástricas y la evaluación histológica de la mucosa de la primera y segunda cámara gástrica para dar más valor científico a los valores de referencia realizados. Además, este estudio pone de relieve la importancia de incluir la evaluación endoscopica, por lo menos de la primera cámara gástrica, en las pruebas de rutina para controlar los compartimentos gástricos de los delfines, siendo un exámen que complementa a los exámenes clínico y citológico, teniendo también en cuenta que los animales pueden ser entrenados para tal procedimiento.

Estudio de la microflora gastrointestinal.

Ecografía, endoscopia y citología son procedimientos muy interesantes y complementarios para el exámen completo del tracto gastrointestinal superior (Brook et al., 2001). Sin embargo, para obtener un amplio conocimiento del tracto gastrointestinal,

es necesaria una descripción detallada de la microbiota normal de los delfines, lo cual proporciona datos adicionales y relevantes muy útiles para el clínico.

El objetivo de este estudio fue establecer, por primera vez, los datos de referencia para identificar la microflora normal del tracto gastrointestinal en delfines mulares clínicamente sanos (*Tursiops truncatus*), mantenidos bajo cuidado humano. En segundo lugar, se investigó las potenciales variaciones entre los resultados del estudio de la microflora gastrointestinal asociados a distintos parámetros relacionados con las instalaciones, la edad y el sexo.

Una muestra de líquido gástrico se perdió, por lo que 31 y 30 muestras de heces y líquido gástrico, respectivamente, fueron analizadas. Un delfín fue excluido de la evaluación de *E. coli* en el líquido gástrico ya que el valor superaba la barrera intercuartil (1,65-4,83 Log UFC / gr). Con respecto a *E. Coli*, los valores de siete delfines estaban por debajo o por encima de la barrera intercuartil (4,14 a 5,21 log ufc / gr) y fueron excluidos. Debido a la falta de normalidad de la distribución, se utilizaron métodos robustos para *Coliformes* y levaduras en el líquido gástrico y heces, respectivamente. Los microorganismos en el líquido gástrico no estuvieron influenciados por ninguna variable considerada (edad, sexo, instalaciones).

El porcentaje de estafilococos fecales se vio afectado por las instalaciones ($F = 15.21$, $P = 0,000$) siendo mayor en los delfines criados en B que en C (5,18 y 3,51 log ufc / gr para B y C, respectivamente; $p = 0,000$). Los delfines criados en A mostraron valores intermedios de estafilococos (4,41 log ufc / gr). Los valores de levaduras fecales tendían a aumentar a medida que el peso corporal de los delfines aumentaba ($b = 0,011$, $F = 6.43$, $P = 0,017$). La cantidad de estafilococos en las heces ($t = 5,31$, $df = 29$, $p < 0,001$) estaba aumentaba en comparación con la de las muestras de líquido gástrico.

Las bacterias que se encontraron en este estudio se han identificado previamente en otras especies como parte de su flora normal y se consideran patógenos oportunistas (Kong y cols, 2014;. María y cols, 2007). La exposición de los delfines a diferentes microorganismos puede variar de un sitio a otro en función de diferentes factores ambientales, tales como los cambios estacionales en la temperatura del agua (Buck y cols., 2006). Los delfines muestreados eran de diferentes sexos, edades y condiciones del hábitat. Sin embargo, no se encontraron diferencias estadísticamente significativas en las bacterias evaluadas entre delfines machos y hembras.

Las mayores variaciones de estafilococos fecales fueron debidas a las distintas instalaciones y pueden atribuirse también a las diferencias biológicas entre individuos, a la dieta, a la morfología y fisiología intestinal y a otros factores ambientales. Estas variaciones pueden estar también relacionadas con la calidad del agua en las que se encuentran los animales; se esperaría que las bacterias entéricas sean más numerosas en instalaciones que utilizan cloro como método de desinfección del agua, debido a la mayor sensibilidad de estas bacterias al ozono.

Las bacterias entéricas se aislaron con frecuencia en todas las muestras. Sin embargo *Citrobacter freundii, Flavobacterium meningosepticum, Hafnia alvei, Klebsiella pneumoniae, Morganella morganii, Pasteurella multocida, Proteus spp., Providencia spp., Serratia spp., Vibrio spp. y Yersinia enterocolitica,* patógenos conocidos en mamíferos miembros de la gran familia de las bacterias gram-negativas non fermentativas, estaban ausentes en todas las muestras.

De particular interés fue la aparición de levaduras, especialmente las especies de Candida. *Candida albicans, C. tropicalis, C. parapsilosis y C. (T.) Glabrata* son los hongos oportunistas encontrados con mayor frecuencia en los seres humanos. Buck y cols., (1980) aislaron *C. tropicalis* en el 14,3% de los animales examinados, seguido por

especies de Candida no identificadas (7,3%), *C. albicans* (7,0%), *C. famata* (3,7%), *C. lusitaniae* (3,0%), *C.guilliermondii* (1,3%) y *C. glabrata* (1,0%). Se encontró una baja tasa de levaduras, tanto en el líquido gástrico como en las muestras fecales, en nuestro estudio. Las levaduras tienden a aumentar a medida que el peso corporal de los delfines aumenta, pero presentó las variaciones biológicas entre individuos más altas.

La metodología de análisis de cultivos es todavía el sistema más simple y barato para cuantificar la microflora intestinal. Esta técnica no permite la detección de algunas especies, o llegar a identificar al nivel de cepa, pero es funcional para evaluar los parámetros fisiológicos, no requiere de un extenso análisis bio-informático (Sekirov et al. 2010) y puede ser llevado a cabo por el veterinario, como exámen de rutina, para evaluar la salud de los animales, ya que las muestras fecales pueden obtenerse fácilmente por comportamiento voluntario de los animales entrenados.

Al fin de obtener un estudio válido de la microflora normal deben tenerse en cuenta los siguientes puntos clave: 1) los delfines no deben estar afectados por ninguna patología o infección sub-clínica; 2) los delfines no deben estar bajo tratamiento con antibióticos, por lo menos durante las tres semanas anteriores al plan de muestreo.

Los datos recogidos en esta tesis doctoral aumentan el conocimiento sobre las características del tracto gastrointestinal de la especie *Tursiops truncatus,* en cuanto se han establecido, por primera vez, valores de referencia para los animales sanos, por lo tanto pueden ser útiles para mejorar tanto la gestión de los delfines mulares bajo cuidado humano, y así como para aplicar este conocimiento a las poblaciones de vida libre. De hecho, pueden ser útiles a los veterinarios que trabajan en centros de rehabilitación y en el diagnóstico de las causas de los varamientos de animales vivos y así aumentar las posibilidades de supervivencia y reintroducción en la naturaleza.

CONCLUSIONES.

En el período comprendido entre enero de 2012 y diciembre 2014, 48 delfines mulares (*Tursiops truncatus*) alojados en tres instalaciones diferentes. De este grupo, 31 animales fueron considerados clínicamente sanos y fueron examinados. Los delfines se sometieron a la recogida de muestras de líquido gástrico y heces, ecografía abdominal, endoscopia y se obtuvieron biopsias gástricas del primer y segundo estómago.

Todos los animales examinados fueron entrenados para este tipo de procedimientos médicos para minimizar el estres, excepto para la toma de biospias, ,.

Esta tesis aumenta el conocimiento clínico sobre los delfines mulares en cuanto a que:

1. Proporciona datos básicos de referencia para los resultados citológicos normales (pH, células epiteliales y leucocitos) en muestras gástricas de delfines mulares sanos (*Tursiops truncatus*), siguiendo, por primera vez, la “Guía para la determinación de intervalos de referencia para especies veterinarias" publicada por el Comité de Garantía de Calidad y de Normas de Laboratorio de la Sociedad Americana de Patología Clínica Veterinaria.

2. Evalúa la existencia de una correlación entre los valores de pH y de células epiteliales, los valores de pH y de leucocitos, y los valores de células epiteliales y de leucocitos. Los resultados mostraron que no se encontró correlación entre los valores de pH y de células epiteliales ($r_s = 0,292$, $p = 0,1657$), los valores de pH y de leucocitos ($r_s = 0,168$, $p = 0,4321$), y

entre los valores de células epiteliales y de leucocitos (rs = 0,076, p = 0,7240). También la prueba t (suponiendo varianzas iguales) no encontró una diferencia significativa para los valores de peso (p = 0,6739), de pH (p = 0,7707), de las células epiteliales (p = 0,6385), y de los leucocitos (p = 0,6968), entre sexos.

3. Evalua las características normales del tracto gastrointestinal del delfín mular (*Tursiops truncatus*) a través de la ecografía en animales clínicamente sanos, definiendo las ventanas acústicas más útiles para el exámen del primer estómago, del estómago fúndico, del estómago pílorico y del intestino.

4. Define un método estandarizado para medir el grosor de la pared del primer estómago, del estómago fúndico, del estómago pílorico y del intestino; y define el peristaltismo normal del primer estómago, del estómago fúndico, del estómago pílorico y del intestino. El análisis estadístico de los datos obtenidos en el estudio ecográfico ha permitido la determinación de intervalos de referencia preliminares para el tracto gastrointestinal en delfines mulares (*Tursiops truncatus*) clínicamente sanos, siguiendo, por primera vez, la “Guía para la determinación de intervalos de referencia para especies veterinarias" publicada por el Comité de Garantía de Calidad y de Normas de Laboratorio de la Sociedad Americana de Patología Clínica Veterinaria.

5. Identifica, enumera y establece, por primera vez, los datos de referencia. para algunos microorganismos cultivables de delfines mulares clínicamente sanos (*Tursiops truncatus*), siguiendo, por primera vez, la

“Guía para la determinación de intervalos de referencia para especies veterinarias" publicada por el Comité de Garantía de Calidad y de Normas de Laboratorio de la Sociedad Americana de Patología Clínica Veterinaria. Se detectó y enumeró la presencia de coliformes, E Coli, estafilocos y clostridios en el líquido gástrico y las heces.

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"La Medicina è un'Arte che Esercitiamo in Attesa di Scoprirla"

(ÉMILE DESCHAMPS)

LETIZIA FIORUCCI

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