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Histological analysis of stranded cetaceans in the Basque Coast for the development of a cost effective sampling protocol

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1. ABSTRACT / LABURPENA

Over the past decades, there has been a growing concern regarding the presence of marine mammals in the Bay of Biscay. According to law, all endangered or vulnerable species and their habitats should be analyzed. In the Basque Coast AMBAR is the organisation that has established a stranding program and network among their volunteers in collaboration with public institutions. Due to the variability that samples of stranded cetaceans may have and the lack of a specific protocol of sampling standards for the casuistry on the Golf of Biscay, results obtained from strandings are scarce as the samples´ quality is not appropriate for advance research. Thanks to AMBAR member´s help 12 individuals that got stranded during the years 2014-2016 have been analyzed. To assess an optimal sampling and processing procedure for later histopathological assessment or other scientific purposes, during this study, different histological processing have been applied. This study has lead to the development of a cost effective protocol specific for the Basque Coast and therefore can be considered the first steps for obtaining quality samples assurance. With that purpose, a histopathological case study has been evaluated. While samples taken from inner organs are more sensitive to their processing and depend on the necropsy permission from authorities since it is not still regulated, the integument together with muscle, teeth and blubber can be always sampled independently to the casuistry. For this reason and integument being one of the most abundant tissues among the samples collected during this study, different measurements on them have been done to correlate it within the health status or nutritional condition of marine mammals. Thus, a ratio among the dermal papillae height and the epidermal thickness has been calculated. Results have shown that the ratio can be an application for the integument samples in the future. This study will help in the advance of the knowledge about cetaceans in the Bay of Biscay and would permit to establish a good protocol for processing samples as law demands.

Azken hamarkadetan, Bizkaiko Golkoko ugaztun itsastarren inguruko interesa zabaldu egin da. Legearen arabera, desagertzeko arriskuan dauden espezieak eta beraien habitatak aztertu behar dira. AMBAR da Euskal Herriko kostaldean lehorreratze programak eta boluntarioen lan-sareak antolatzen dituen erakunde bakarra, erankunde publikoengatik babestuta dagoelarik. Lehorreratzeek duten aldakortasunaren ondorioz eta laginketa protokoloa oraindik zehazteke dagoenez, orain arte lehorreratzeetatik lorturiko datuak urriak dira laginen kalitatea ez baita ikerketetarako aproposa. Elkarte honi esker, 2014-2016 urteetan zehar lehorreratuta geratu ziren 12 indibiduoen azterketa gauzatu da. Lortzen diren laginak ikerketa histopatologiko edo bestelako ikerketetan erabiliak izateko, laginketa eta prozesamendu metodologiko optimoenak bilatzeko asmoz prozesamendu histologiko ezberdinak gauzatu dira. Lan honek, euskal kostalderako laginketa protokolo kostu-efektibo posible baten garapena baimendu du eta beraz kalitatezko laginak lortzeko lehenengo pausutzat har daiteke. Gainera, laginen aplikazio posible bat testatu da. Barruko organoak, prozesazemenduarekiko eta nekropsia baimenaren menpekoak dira. Oraindik, laginketa hauek erregulatu gabe daudenez autoritateek dute nekropsiarako baimena emateko ardura. Aldiz, larrua, muskulua, hortzak eta gantza beti lagindu daitezke, ez delako nekropsiarik egin behar. Hauek, lagindu diren ehunetatik ugarienetarikoak izanda, neurketa ezberdinak egin zaizkie gantz hipodermikoari eta epidermisari, behatzeko ea neurketa hauen eta indibiduoen osasun egoeraren edota egoera nutrizionalaren artean erlaziorik dagoen. Hau frogatzeko, erratio bat kalkulatu egin da papila dermikoen altuera eta epidermisaren lodieraren artean. Emaitzek adierazten dute erratio hau lorturiko laginetan aplikazio posible bat izan dezakeela etorkizunerako. Lan honek, Bizkaiko Golkoko zetazeoen inguruko ezagumendua osatzea eta laginketa protokolo egokia zehaztea ahalbidetu du, laginketak egokiak izan daitezen, legearen esanetera.

2. INTRODUCTION

In the Marine Mammal Protection Act (MMPA, 1972) marine mammals are defined as any mammal which is morphologically adapted to the marine environment (including sea otters and members of the Sirenia, Pinnipedia and Cetacea) or primarily inhabits the marine environment (such as the polar bear). However, in this study the term marine mammals is used to refer to cetaceans, as no samples have been taken from other groups of animals. The International Whaling Convention (IWC), determined under the International Convention for the Regulation of Whaling (ICRW), was admitted in 1946 and is completely necessary for the management of the whaling industry worldwide. Another international treaty done for the protection of wild animal and plant species are the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES); and UNCLOS, United Nations Convention on the Law of the Sea that was ratified in 1982 for the conservation and preservation of the marine resources and environment. In all this document of regulations, cetaceans have an undoubted presence and relevance, indeed, in the UNCLOS the Article 194 claims that States must take necessary measures to protect and preserve rare or fragile ecosystems as well as the habitat of depleted, threatened or endangered species and other forms of marine life. Spain and the Basque Country being among the parties that have ratified this treaty, should support all the stranding networks as most of the cetaceans nowadays are endangered and their samples can provide priceless information about their health status (UNCLOS, 1982), as well as about the environment where they live.

All marine mammals are protected by law in most of the countries. These laws limit experimentation and access to marine mammals. The main sources of scientific information are obtained from aquaria or from strandings. Although the bodies may be under decompositional processes, observations made in strandings are a source of information for unaccessible wild populations. Building a reliable database with information such as populational dynamics, toxicology, histopathology, virology and other scientific research, can provide enough knowledge on cetaceans in order to enforce protective laws on them, and to obtain knowledge in ecosystem health status.

This study focuses on The Bay of Biscay (total area; 223,000 Km), which is situated between the west coast of France and the northern Spain. Scientific literature has identified the importance of this area as a critical habitat for cetacean species, where up to 30 species of cetaceans have been recorded (OSPAR, 2000). This diversity of cetaceans, makes strandings more problematic due to the wider variety of logistics needs such as, qualified personnel, specific cranes and a place to getting rid of the body. All these factors should be taken into account for the development of the stranding protocol, which is not yet established, in spite of according to law it is compulsory to take samples from the stranded cetaceans.

Animals that are about to die or enfeebled are passively guided to shore by wave and wind action. However, sometimes cetaceans may come ashore on purpose, swimming towards rocky coastlines, in order to die. Although some of them may die by natural causes, there has been an increase on the threats that can rise their mortality rate. Environmental conditions such as low sea temperature or ice entrapment, diseases, parasitic lesions, toxins, bycatch of fisheries, starvation due to a decrease in food availability, collisions with watercrafts (Laist *et al.,* 2001), contaminants, oil spills, and injuries caused by predators or humans (Geracy and Lounsbury, 2005) are some of the main causes that can increase mortality among these mammals. Therefore, the causes of

death have to be determined to maintained under control by sampling and analyzing the data collected from all the strandings.

This evaluation is extremely important since it can guide experts through the assessment of the health status of cetaceans and ecosystems. For this purpose, necropsies of the stranded bodies offer valuable information. External observations of body and organs can give information about the general health status, health diseases and presence of macroscopic parasites which can be also be correlated with the lack of appropriate nutrition. This can be supported by factors such as, body condition, serum protein levels, measurements of specific nutrients in body (Cowan, 2009) and the sloughing rate of the epidermis (Hicks *et al.,* 1985). However, in necropsies the analysis must be accompanied by histopathological assessment for a proper interpretation of the causes of death. Histopathology and toxicology accomplished using samples from the beached cetaceans offer priceless information for the assessment of the health status and to determine the cause of death. For example, microorganisms that may affect humans, such as Brucella infection and prions´ disease Creutzfeld-Jakob can be reported and therefore maintained under control by histopathological analysis (Cowan, 2009). In Spain, advance research on this field has been carried out mainly by the long-research project of the Cetacean Research Unit, University of Las Palmas de Gran Canaria (ULPGC). Collecting data under systematic necropsy and sampling, has enabled ULPGC to stored samples in the Cetacean Tissue Bank (CTB) and have been used, for example, to correlate the presence of gas bubbles with the sonar activities. (Jepson *et al.,* 2003; Bernaldo de Quirós *et al.,* 2016). Both Galicia and Gran Canaria, are two regions in Spain that have to deal with a high indice of strandings. CEMMA is the Non Governmental Organization (NGO) that acts on the strandings in Galicia, and following the law they send all the samples to an histopathological laboratory to find the possible cause of death, supported by the Galician government.

In the Golf of Biscay the NGO AMBAR (www.ambarelkartea.org) recollects data of marine mammals from two different sources. On the one hand, the whale watching programs for PhotoID research and on the other hand, stranded cetaceans have provided valuable of information during years, for example, bycatch evidences, general condition of the animal (nutritional state), as well as presence or absence of macroscopic parasites. Indeed, AMBAR has established a stranding program and network among their members, at the request of the regional government (Bizkaiko Foru Aldundia). AMBAR´s stranding program established to provide a complete management advice to public service employees, to safeguard the welfare of live stranded animals, minimize risk to public health and safety, support scientific investigation and advance public education. Since 1996, there has been a continuous collection of data on strandings done by its members as well as a continuous increase of concern in scientific research. However, the data collected regularly is not enough and samples must be collected regularly as stipulated by law. There is undoubtedly a need for grants or public financial support for NGO as well as a well defined sampling protocol that could warranty the quality of samples for future scientific purposes.

According to registered and processed data by AMBAR, in the Basque Coast 12 different species have got stranded within the last 20 years. Different pilot whales, beaked whales, fin whales, dolphins, porpoises and sperm whales are common to find stranded in our coast. The most common ones among those, are the common dolphin (*Delphinus delphis*) with the 18,8% of the strandings and the striped dolphin (*Stenella coeruleoalba*) with the 20,4% of the strandings. Three out of the five species that have been used in this study, exactly *Delphinus delphis, Tursiops truncatus* and *Phocoena phocoena,* are considered vulnerable mammal species in

Spain according to the 42/2007 law and protected by the Real Decreto 139/2011, and therefore their sampling is compulsory.

Each and every stranding have its identification paper, in which general information is registered and different body measurements are done depending on the species. Unfortunately, as the stranding program remains incomplete without the sample collection protocol and without being assumed by institutions, the necropsy permission depends on the local authorities. Therefore, only the necropsy can be done after been given pertinent permission. This procedures permit to determine possible causes of death if macroscopically any anomalies or damages are found in organs or there is presence of parasites. Apart from analyzing the overall condition of the inner organs, NGO members also get samples of tissues for deeper histopathological study.

But unfortunately, almost all the tissue samples that AMBAR sent to the histopathology laboratory for finding possible causes of deaths resulted in vain, as the sampling methodology was not appropriate. Determining a systematic and standardized protocol is indispensable before any research, nevertheless, not only the sampling methods must be determined but the acting and sample storing location should be also specified to ensure the quality and usability of the samples.

In almost all the strandings volunteers are able to collect some samples that are easier to get, such as teeth, integument, blubber and muscle, apart from the pertinent basic biometrics, as it does not depend on the necropsy which is often forbidden by authorities. However, full necropsies depend on more factor and due to the variability of the stranding, the volunteer network have encountered some complications in the sampling and storing processes in the Golf of Biscay.

1. THE STORING: Until now, samples were stored in volunteers´s private establishments. Due to this dispersal of samples, there has been a loss of samples and data. From now on, thanks to the collaboration agreement between AMBAR and Research Centre for Experimental Marine Biology and Biotechnology (PiE-UPV/EHU) samples collected from the strandings can be stored in the “Biscay Bay Environmental Biospecimen Bank” (BBEBB). This, is a magnificent progress for maintaining samples save and under some extrict guidances, as each of the individuals that get stranded can provide useful and unique information in future research.
2. SAMPLE PROCESSING: Tissues have been preserved during years in %10 ethanol. Although alcohol may be used to preserve samples for other studies, such as DNA analysis or morphological studies, it is not useful for histological analysis.
3. NECROPSY PERMISSION: As a stranding protocol is not completely accepted yet by institutions, before a necropsy is done, authorities or public services employees that are in charge of getting rid of the body, must give permission. If not, volunteers are not allowed to do the necropsy.
4. ENVIRONMENTAL FACTORS: These factors can make a necropsy impossible or worthless because it does not enable the access to the body with the full sampling equipment, or because other expensive actions should be required. a) meteorology; b) orography: beach or rocky areas, inaccessibility by road; c) tides; d) light conditions day/night.
5. COSTS: The equipment for attending a stranding is fully provided by volunteers themselves. Personal protective equipment (PPE) such as protective clothing, goggles, or every equipment needed to avoid injuries or infections are required, according to Directive 89/686/EEC. These protocols must be followed precisely

because stranded cetaceans are considered as a biohazard as they can transmit any kind of diseases. Undoubtedly, more financial aids are needed for the execution of this rutinary and compulsory (according to the UNCLOS) sampling. With financial support, fixatives and more equipment could be bought to standardize the sampling and it would be possible to employ a qualified person to attend strandings in any situation. However, due to the reduced budget, a cost effective protocol is needed.

However, data independent to the necropsy always can be collected from a stranding. Indeed, advanced research has been done for assessment of the health status of cetaceans thanks to the information obtained through strandings. Body condition, for example, has been the main objective of several research, because is general health indicator of short term feeding success (NAMMC, 2007-2008). Blubber thickness, among other morphometrics, have been used during years as suitable indices to assess nutritional condition (Gómez-Campos *et al.,* 2011). These indices are widely use especially for mysticetis and pinnipeds (Konishi, 2006; Pitcher and Calkins, 2000). In odontocetes, these parameters have not always accurate results (Caon *et al.,* 2007). However, some research on this issue have shown that this indices are not correlated with the changes in overall body fat reserves (Aguilar *et al.,* 2007). Therefore, in this study will try to focus on the integument to determine if the samples taken are useful to assess the health status of individuals.

The integument of cetacean has been described in a number of investigations for providing information of the health status as well as for being multifunctional; among others, it protects organism from injury, works in homeostasis as a impermeable layer and is adapted for hydrodynamics. It differs from that of terrestrial mammals because it lacks hair follicles, sweat glands and because the epidermis is 20 times thicker than the one in terrestrial mammals (Geraci *et al.,* 1986). As a consequence of the constant exposure to water friction, cetacean´s epidermis have a high rate of mitosis as well as a high rate of desquamation (Hicks *et al.,* 1985). Therefore, the height of the epidermis, which is connected with the eroding, is the evidence of the health of the individual. The epidermis of cetaceans contains an outermost stratum externum or corneum, which is also known as being a parakeratotic layer. Although it is not fully cornified, it is composed of moderately flattened cells, representing a form of parakeratosis (Geraci *et al.,* 1979; Spearman, 1972). Stratum intermedium is also known as the stratum spinosun because in marine mammals stratum granulosum and stratum lucidum are absent (Parry, 1949), and stratum basale lipokeratinocytes are responsible for the producing of both keratins and lipids. Their main function is to create a barrier against disadvantageous environmental conditions, such as solar radiation, hypertonic environment and penetration by pathogens (Mounton and Botha, 2012), and are said to contribute to the mechanical strength, buoyancy, and insulation of cetacean integument (Menon *et al.,* 1986). Research on integument defences in cetaceans also has been carried out for determining their specific immune system (Mouton and Botha, 2012) and non specific response to bacteria, fungi and algae invaders (Meyer and Seegers, 2004). Also, their mechanism to reduce passive accumulation and therefore detoxification from chemicals, such as, mercury (Cowan, 2009) or persistant organic pollutants has been reported (Buckman *et al.,* 2011). It is indispensable to maintain a good tissue quality for a better comprehension of the effects of this contaminants on their vital organs (Wang *et al.,* 2016). Together with this, it has been specified that any interpretation done out of the measured levels of organic or inorganic compounds must be taken as a function of the age, and reproductive condition, of the individual (Hohn *et al.,* 1989), and therefore underlines the importance of recollecting as much information as possible from the body.

More assessments on the integument can be done. The basal layer of the epidermis is folded, producing the dermal papillae. The relationship between the height of the dermal papillae and the relative epidermal thickness is connected with the increase of the basal surface of the germinative layer, which is correlated with the proliferative capacity (Brown *et al.,* 1983). This high cell production, causes a high sloughing rate (Solokov *et al.,* 1969), which is beneficial for hydrodynamics by maintaining a smooth layer and self-cleaning surface (Hicks *et al.,* 1985). Although cetaceans are lacking of dermal glands that protect the integument from the environment, they do have a rapid exfoliation which prevents the integument to be colonized by microorganisms (Scheuplein and Blank, 1971). Therefore, it also appears to be a correlation between pox-like integument lesions and the immunosuppressive pollutants (Harzen and Brunnick, 1997) which are considered a threat for marine mammals. Although blubber thickness morphometrics themselves can give poor information, these indices together with the relative dermal papillae measurements, may provide more detail about the health status of each individual.

Samples collected by AMBAR and stored in the BBEBB of the PiE-UPV/EHU, can be used to develop high quality tools for assessment of the biological alterations in cetaceans for knowing the level of hazard that alters marine mammal´s health, perform retrospective studies and extrapolate it to the future. This has an additional importance because some of them are considered key species, which are essential for maintaining the whole marine ecosystems. Any kind of information about them can contribute to improve the assessment of the environmental health and cetaceans´ protective laws. Before trying to do any research on the tissue, first and foremost the quality of samples has to be ensured. During these years of continuous efforts from AMBAR, the results obtained from the samplings or even the tissue quality in itself are not the expected due to the lack of a sampling protocol and good practices. In order to try to solve this problem, samples of 12 individuals that got stranded during the years 2014-2016 in the Basque Coast have been analyzed under different processings.

This study not only tries to find a relation between nutritional condition or health status and epidermal characteristics but also will help on the development of a sampling protocol for the network for volunteers to respond to marine mammal strandings in a standardized and systematic way taking into account the casuistry of the Golf of Biscay. Detailed protocols for collection of data and specimens are available in stranding and dissection manuals, but the casuistry in the Basque Country cannot be comparable to the one in USA, as there are no employees in AMBAR or institutions and fundings enough to maintain a field sampling. Thus, a cost effective protocol is presented to be useful to collect samples of stranded cetaceans in the Basque Coast in collaboration between AMBAR and PiE- UPV/EHU. Moreover, first histological studies of stranded cetaceans in the Basque Coast in integument and in relation with nutritional and general health status are carried out.

3. OBJECTIVES

A) To test the histological quality of the samples obtained through the necropsies of stranded cetaceans in the period of 2014-2016, in order to develop a cost effective sampling protocol.
B) To analyze a case study for the application of histological integument samples of cetaceans to understand the relation between the papillae height ratio and the health status.

4. MATERIAL AND METHODS

4.1 Species, collection of basic data and necropsy standards

Samples were collected from 12 individuals of 5 species; *Tursiops truncatus* (Ttr), *Delphinus delphis* (Dde), *Ziphius cavirostris* (Zca), *Phocoena phocoena* (Pph) and *Stenella coeruleoalba* (Sco). All were found stranded in different places along the Golf of Biscay Coast over the period of 2014-2016 (Table 1). No invasive methods were used in this study and all samples were collected from animals that were already deceased. Regarding the biometry and necropsy protocol of stranded specimens were carried out following different protocols (Jefferson *et al.,* 1994; Pugliares *et al.,* 2007; SEC, 2000; Geraci and Lounsbury, 2005). All the strandings, in which AMBAR has collaborate, have their identification paper with all the basic measurements done according to the Sociedad Española de Cetáceos (SEC).

Table 1. Individuals that got stranded from 2014 to 2016 in the Basque Coast.

ID	Specie	Source of samples	Date	Location / Province
Ttr1	*Tursiops truncatus*	PiE	July/14	Plentzia / Biscay
Ttr2	*Tursiops truncatus*	AMBAR	29/03/15	Muskiz / Biscay
Ttr3	*Tursiops truncatus*	AMBAR	25/03/16	Bakio / Biscay
Dde1	*Delphinus delphis*	AMBAR/PiE	2014	Donostia / Gipuzkoa
Dde2	*Delphinus delphis*	AMBAR	15/03/15	Bakio / Biscay
Dde3	*Delphinus delphis*	AMBAR	13/02/16	Zurriola / Gipuzkoa
Pph1	*Phocoena phocoena*	AMBAR	19/02/15	Armintza / Biscay
Zca1	*Ziphius cavirostris*	AMBAR	21/05/15	Laga / Biscay
Sco1	*Stenella coeruleoalba*	AMBAR	14/05/15	Getaria / Gipuzkoa
Sco2	*Stenella coeruleoalba*	AMBAR	20/03/16	Sopelana / Biscay
Sco3	*Stenella coeruleoalba*	AMBAR	22/03/16	S.J. de Gaztelugatxe / Biscay
Sco4	*Stenella coeruleoalba*	AMBAR	21/04/16	Murueta / Biscay

There are two kind of strandings depending on data collected. If the necropsy cannot be done, basic information is recorded. The body measurements were taken with a measuring tape. The body length (recorded in cm), is a straight line from the tip of the maxilla (upper jaw) to the fluke notch with the animal ventral side down, parallel to the long axis of the body, without following the contours. Blubber thickness (recorded in mm) was measured after the dissection of the integument was done, behind the dorsal fin, using a measuring tape. Sexual maturity was determined with approximations of age depending on the length for every species (Geraci and Lounsbury, 2005). Other observation such as, coloration and dentition erosion are used for the determination of sexual maturity; Ad (adult), Juv (juvenile), Cri (baby) and F (fetus).

Decompositional state was catalogued with 5 states; 1 for alive animals, 2 for fresh dead animals, 3 for moderate decomposition, 4 for advanced decomposition and finally, 5 for mummified or skeletal remains. However, only samples from individuals below 4 were dissectioned for histopathology, as recommended (Geraci and Lounsbury, 2005). The nutritional condition was determined focusing on the dorsal axis muscle and blubber content along the body cataloguing on Good, Moderate or Poor. Both, nutritional condition and decomposition state were determined using the protocol of the SEC (SEC, 2000).

Further data such as, presence/absence of parasites. sex or bycatch wounds was determined by external observation. Photos of the body, mouth, urogenital zone, dorsal fin (for Photo ID), wounds and surroundings of the stranding were also taken. Finally, samples of tissues such as, integument, blubber, muscle and teeth were taken. All integument, muscle and blubber samples have been taken in the the dorso-lateral part of the body to avoid variation. Teeth, have been taken from the center of the lower mandible.

These four samples, integument, blubber, muscle and teeth have been chosen for routine banking on the BBEBB, as there is not need of doing the necropsy for their collection, avoiding the dependence of asking for the necropsy permission to the authorities. However, if AMBAR members obtain the permission to do the necropsy, tissue samples form inner organs were taken. Liver and kidney are most commonly collected ones as they are key target organs of detoxification and excretion, although organs such as lung, heart, intestine, different stomaches, gonads and spleen have also been dissectioned and processed for histology in the present study.

4.2. Histological processing

Samples were duplicated, when possible, before the processing, for the storing (freezing samples) of one of the copies frozen at -80Cº into the BBEBB for future research. Different histological processing were used depending on the sampling casuistry, mostly related to fixation. Samples were directly fixed after the necropsy or frozen in temperatures of -18Cº (in volunteers´ houses) and -80Cº (in PiE-UPV/EHU). Different fixatives such as Carnoy, Davidson, Neutral Buffered Formalin (NBF), Formaldehyde (40%) and Alcohol (10%) as a method for preservation have been used (Table 2). After fixation all samples were preserved in Alcohol (%70) until being embedded in paraffin. As Formaldehyde is a carcinogenic product, all the Personal Protective Equipment (PPE) were used strictly. Moreover, in PiE-UPV/EHU are already looking for substitutes because it has been ranged lately in category 1 of carcinogenic compounds.

Table 2. The histological procedure of each individual. After fixation samples were preserved in %70 alcohol before being embedded in paraffin. NBF: Neutral Buffered Formalin.

Identification code	Fixation code	Fixation
Ttr 1	NBF	NBF (24-48 h)
Ttr2	FR/NBF	Frozen (-18Cº,1 year), NBF (24-48 h)
	C´	Alcohol 10% (48h), Carnoy (4 Cº, 1h)
	NBF´	Alcohol 10% (48h), NBF (24-48 h)
Ttr3	NBF	NBF (24-48 h)
Dde 1	FR/NBF	Frozen (-18Cº, some days), NBF (24-48 h)
Dde2	NBF	NBF (24-48 h)
Dde3	FR/NBF	Frozen (-18Cº, 1 year), NBF (24-48 h)
Pph1	NBF	NBF (24-48 h)
Zca1	NBF	NBF (24-48 h)
	FR/NBF	Frozen (-80Cº), NBF for (24-48 h)
	NBF´	Alcohol 10% (48h), NBF (24-48 h)
Sco1	FR/NBF	Frozen (-18Cº, 1 year), NBF (24-48 h)

Sco2	NBF	Formaldehyde 40% (1h), NBF (24-48 h)
	FOR	Formaldehyde 40% (24-48 h)
Sco3	NBF	NBF (24-48 h)
	D	Davidson (24-48 h)
Sco4	NBF	NBF (24-48 h)

After the fixative processings, samples were processed with the Tissue Processor (LEICA ASP 300S). Following steps were used: 1. Alcohol 70% (1h), 2. Alcohol %96 (1h) 3.Alcohol %96 (1h) 4. Absolute alcohol (1h), 5. Absolute alcohol (1h), 6. IMS, 1/2 Absolute alcohol and 1/2 xilol, (1h), 7. Xilol (1h), 8. Xilol (1h), and 9. Paraffin (6h, divided in three cycles, 2 h each).

Blocks of paraffin were sectioned using the LEICA RM 2125RTS microtome. In the present study, all samples have been sectioned into 4µm (Hashimoto *et al.,* 2015). However, integument, which was difficult to process by the microtome was sectioned into 5µm sections, to facilitate the sectioning. Sections were affixed to slides, deparaffinized and stained with Haematoxylin and Acid Eosin (HE) stain following standard procedures with the aid of the autostainer of LEICA. Samples were introduced into Xilol (10 min twice), Absolute alcohol (2 min twice), Alcohol %96 (2 min), Alcohol %70 (2 min), distilled water (5 min), Haematoxilin (4 min), distilled water (4 min), Alcoholic acid (10 sec) to clean Haematoxilin excesses, distilled water (5 min), Li2CO3 (10 sec) to neutralized the samples after the Alcoholic acid, water (1 min) to stabilize samples, Alcoholic Eosine (1 min and 30 sec), cleaning (1 min and 30 sec), Alcohol %70 (5 sec), Alcohol %96 (10 sec) and Absolute alcohol first for 15 seconds and then for 20 seconds and, finally, xilol (1 min twice). Slices were mounted with DPX (Distyrene Plasticizer Xylene). All samples are stored at the PiE-UPV/EHU, The Research Centre for Experimental Marine Biology and Biotechnology.

The quality of samples has been evaluated taking into account the presence or absence of scratches and damages in tissue. Each microscope slide contain two or three copies of the main samples. In order to accept that the fixative has been the cause of tissue scratches and or artefacts overall, all the sections on the slide had to be affected. If scratches are observed in just one of the replicas of the same slide, these errors may came from the tissue processing and not from the fixative itself.

4.3 Histological observations and measurements

The overall morphology of samples were analyzed through the OLYMPUS BX61 microscope. Measurements of the thickness of the epidermis (E) and the height of the dermal papillae (P) (Fig.1) were taken by image analysis using the CELL software for Olympus. 10 measurements were made for each individual and procedure. Measurements were divided into histological processing. Following the ratio established by Jones and Pleiffer, 1994, the dermal papillae ratio was calculated doing: P/E for each measurement. First, the normality test Shapiro-Wills ($n<50$) was tested. Then frozen and NBF (FR/NBF) fixed and directly NBF fixed (F) samples were analyzed with ANOVA, as well as Duncan pos-hoc analysis to determine wether there are differences among individuals of the same species ($p>0,05$). However, individuals which samples were duplicated and exposed to different processings were separately analyzed in order to see if measurements are affected by the fixatives. Then, two tailed Student´s test was employed for comparing results between different fixatives in the same individuals. All statistical analyses were executed using IBM SSPS 21 program.

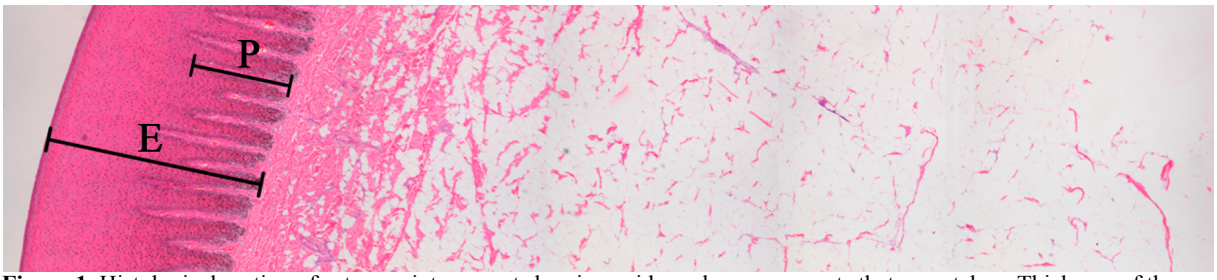

Figure 1. Histological section of cetacean integument showing epidermal measurements that were taken: Thickness of the Epidermis (E) and Height of Papillae (P).

5. RESULTS

5.1 Basic information and measurements of the each individual

All the strandings attended during this study, have their information file, from which relevant parameters have been extracted (Table 3). Due to extreme situations at strandings (time limit, meteorology…), the table is incomplete and some data, such as the parameter of blubber thickness or the body length were impossible to achieve. Samples were collected from 12 individuals by AMBAR or the PiE-UPV/EHU research group and the information indicates that the 75% of the strandings took place in Biscay. Although some individuals got stranded in an advanced state of decomposition, the 63% of the individuals had a low decompositional state (1 or 2). Some samples of individuals with a state 3 were also collected and the overall morphology of the samples was appropriate, for example Sco 2 (Table 4.). However, the integument of Dde3 with state 4 was dissectioned and several damages as gas bubbles or scratches have been observed. Although, during this project there have been more strandings than the ones shown in the table, no data or samples were collected to cetacean with really advanced decompositional state, as those samples are too degraded for their study. Another observation on the maturity indicated that a %33 of the beached animals were juvenile specimens.

Table 3. Basic data of each stranding. noD: no data; Juv: Juvenile; Ad: Adult; F: Female; M: Male.

ID	Specie	Body length (cm)	Female/ Male	Maturity	Nutritional condition	Decompositional state	Blubber thickness (mm)
Ttr1	*Tursiops truncatus*	noD	M	Adult	noD	2	noD
Ttr2	*Tursiops truncatus*	241	M	Juv	noD	2	noD
Ttr3	*Tursiops truncatus*	326	M	Ad	Good	3	23
Dde1	*Delphinus delphis*	noD	noD	noD	Good	2	noD
Dde2	*Delphinus delphis*	132	F	Ad	noD	2	noD
Dde3	*Delphinus delphis*	144	M	noD	noD	4	noD
Pph1	*Phocoena phocoena*	169	F	Juv	Good	2	15
Zca1	*Ziphius cavirostris*	640	M	Ad	Good	2	120
Sco1	*Stenella coeruleoalba*	212	M	Ad	noD	1	noD
Sco2	*Stenella coeruleoalba*	211	M	Ad	Poor	3	noD
Sco3	*Stenella coeruleoalba*	160	F	Juv	Poor	2	15
Sco4	*Stenella coeruleoalba*	177	F	Ad	Poor	3	12

5.2 Histological assessment for tissue quality

The quality of the samples obtained during this study have been analyzed to determine sampling standards for a cost effective protocol. Quality evaluation was carried out taking into account the presence or absence of scratches or damages in tissue. Among all the samples histologically assessed, only the most representative cases have been selected (Table 4). On the individuals that are not shown in the table, only basic dissections of integument muscle and blubber were done and these tissues seem to be less sensitive to the fixatives, preserving methods and dissection than inner organs. Therefore, more emphasis has been given to strandings with necropsy, as they provide more valuable information for building the protocol.

Tissue samples fixed with NBF or first frozen and then fixed with NBF (FR/NBF), Dde1, Pph1, Zca1, Sco2 and Sco3, have the highest quality observed in this study. No apparent problems have been observed in frozen samples -18Cº (for some days) and -80Cº before NBF fixation . However, little damages have been observed in the Sco1 samples which were frozen at -18Cº for a year. Alcohol %10 preserved and then NBF fixed samples (NBF´) obtained from Ttr2 have shown several fixation scratches and artefacts. These alcohol preserved samples show a slightly more appropriate quality when are fixed in Carnoy (C´). Samples treated with Formaldehyde 40% show effects of overfixation and samples fixed with Davidson have shown poorer quality in muscle (cardiac and skeletal) than the replicas fixed with NBF.

Table 4. Good (+) and bad (-) quality of samples. *: the skeletal muscle fixed with formalin was overfixed and it was impossible to cut with the microtome. Ç: impossible to cut the blubber. H.P: Histological processing.

ID	Dde1	Pph1	Ttr2			Zca1			Sco1	Sco2		Sco3	
H. P.	FR/NBF	NBF	NBF´	C´	FR/NBF	NBF	FR/NBF	NBF´	FR/NBF	FOR	NBF	D	NBF
Integument	+	+			+/-	+	+	+	+	-	+	+	+
Adipose tissue	+	+			Ç	+	+	+	+	+	+	+	+
Skeletal Muscle	+	+			+/-	+	+	-	+/-	-	+	+/-	*
Smooth Muscle	+	+	+	-						+	+	-	+
Kidney	+	+	-	+		+					+	+	+
Liver	+	+	-	-		+				-	+	+	+
Stomach	+	+	-	+		+				-	+		
Intestine	+	+	-	-						+	+	+	+
Heart	+	+	-	+								-	+
Lung	+	+	-	-		+				+	+	+	+

Muscle and integument of Ttr2 have shown that freezing samples at -18Cº for a year, before been NBF fixed, impoverishes tissue samples. Scratches (Fig.2A) have been observed between the dermal papillae and the parakeratotic layer (Fig.2A) and there is a slight displacement of the muscle fibres (Fig.2B).

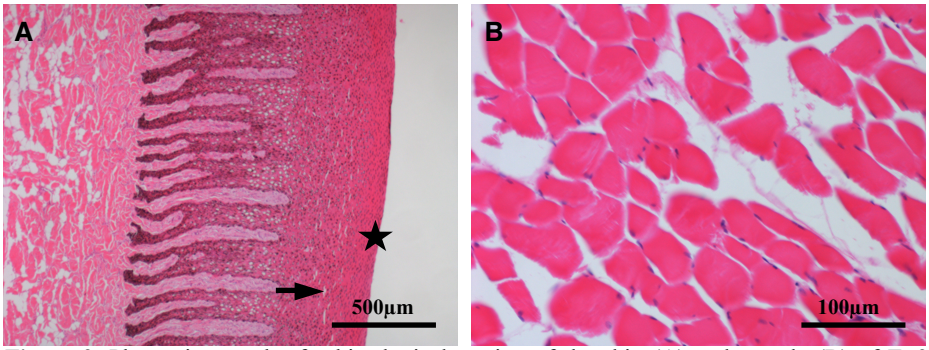

Figure 2. Photomicrograph of a histological section of the skin (A) and muscle (B) of Ttr2 fixed with NBF after been frozen. Star indicates the parakeratotic layer and Arrow indicates the scratches. H&E.

Inner organs collected from the individual Ttr2 have shown that Carnoy is more suitable for fixating samples that have been preserved in alcohol rather than NBF. However, these samples have shown a overall loss of cellular integrity comparing to samples that are not preserved in alcohol and therefore are fixated at the necropsy place. The mucosa of the stomach and hepatic lobules surrounding the central vein (Fig.3A) on the liver and in lung are rather melted or surrounded by prominent scratches (Fig.3B) and gas bubbles (Fig.3B).

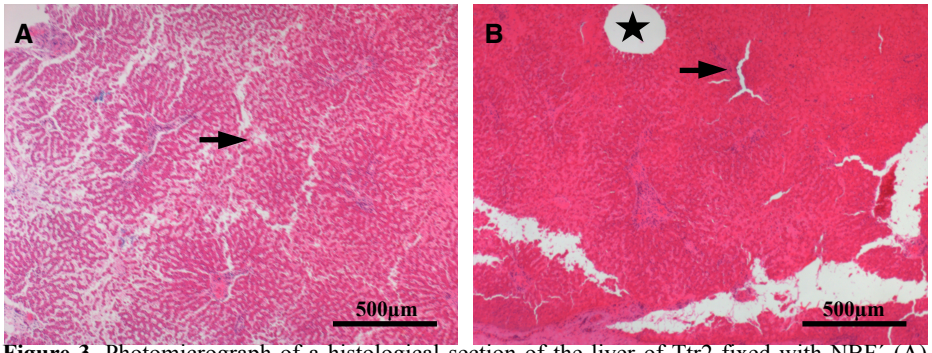

Figure 3. Photomicrograph of a histological section of the liver of Ttr2 fixed with NBF´ (A) and fixed with C´(B). Arrow in A indicates loss of cellular integrity and in B indicates fixation scratches. Star indicates gas bubbles. H&E.

Sco1 stayed stored in a particular´s fridge at -18Cº (1year) and then samples were NBF fixed. In skeletal muscle intraespecific variability was very high and different patches of tissue damages were observed (Fig.4B&Fig.4D). Few bubbles appeared under the parakeratotic layer of the epidermis (Fig.4B). Nevertheless the overall quality of the samples is acceptable. The same was observed in muscle and integument samples obtained from Dde 3 and exposed to the same procedures (FR/NBF). However, in Dde 3 the integument showed more prominent fissures and gas bubbles behind the parakeratotic layer. This is the one that have advanced decompositional state (4).

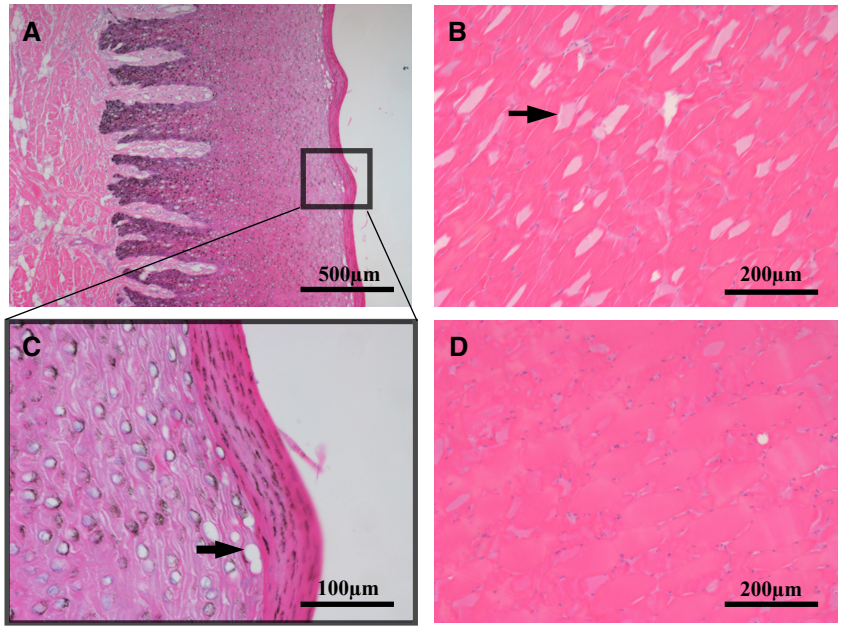

Figure 4. Photomicrograph of a histological section of the integument (A and C) and the skeletal muscle (B and D) of Sco1 frozen for a year and NBF fixed. Arrow indicates gas bubbles (A) and damages (B). H&E.

The samples of the individual Zca1 were exposed to different storing (frozen) and preservation (alcohol) methods, in order to compare and understand the effects of different procedures. In both integument (Fig.5D&F) and muscle (Fig.5A&B) samples that were NBF fixed after been frozen no change on the quality have been

identified. However, alcohol preserved samples show negative effects; skeletal muscle samples conserved in alcohol have suffered open spaces between muscle fibres (Fig.5C) and layer below the parakeratonic layer suffers from scratches (Fig.5F).

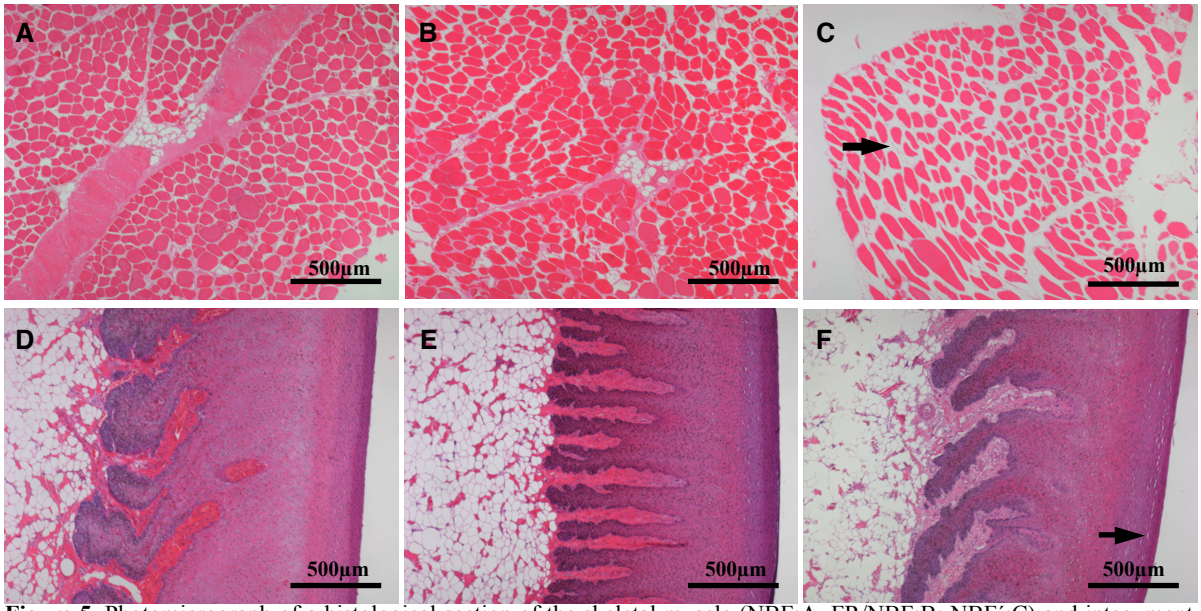

Figure 5. Photomicrograph of a histological section of the skeletal muscle (NBF:A, FR/NBF:B; NBF´:C) and integument (NBF:D, FR/NBF:E; NBF´:F) of Zca1 exposed to different processings, Arrow indicates fibre displacement (C) and small fissures under parakeratotic layer (F). H&E.

Inner organs of Zca1 were histologically processed with NBF and good quality of tissues were observed. But, only connective tissue of the capsule of each organ has been sampled. Thus, no proper tissue was achieved in the dissection of the inner organs (Fig.6).

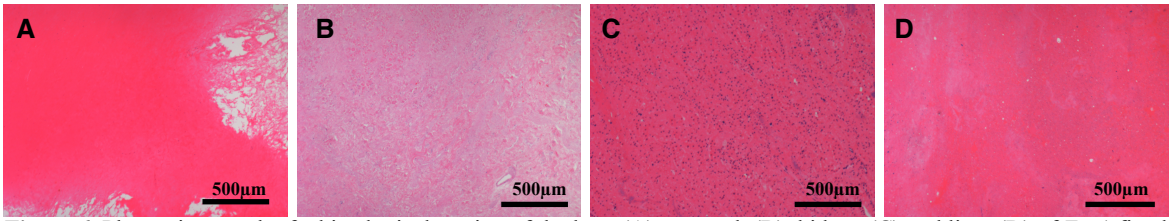

Figure 6. Photomicrograph of a histological section of the lung (A), stomach (B), kidney (C) and liver (D) of Zca1 fixed with NBF. H&E.

The liver of Sco2 was fixed in 40% Formaldehyde and presented several damages. The tissue was notably discoloured in the central area of each section (Fig.7A), as citosolic parts of the tissue were not stained. Moreover, overfixation can be observed all over the tissue (Fig.7A). The skeletal muscle fixated with 40% formaldehyde did not get stick to the slide. As a consequence, the replicas of the samples were lost during the H&E staining. Globet cells in stomach tissues, fixed with 40% Folmaldehyde, also appeared to be disintegrated. The integument samples exposed to 40% Formaldehyde showed shrinkage comparing with the replicas fixed with NBF.

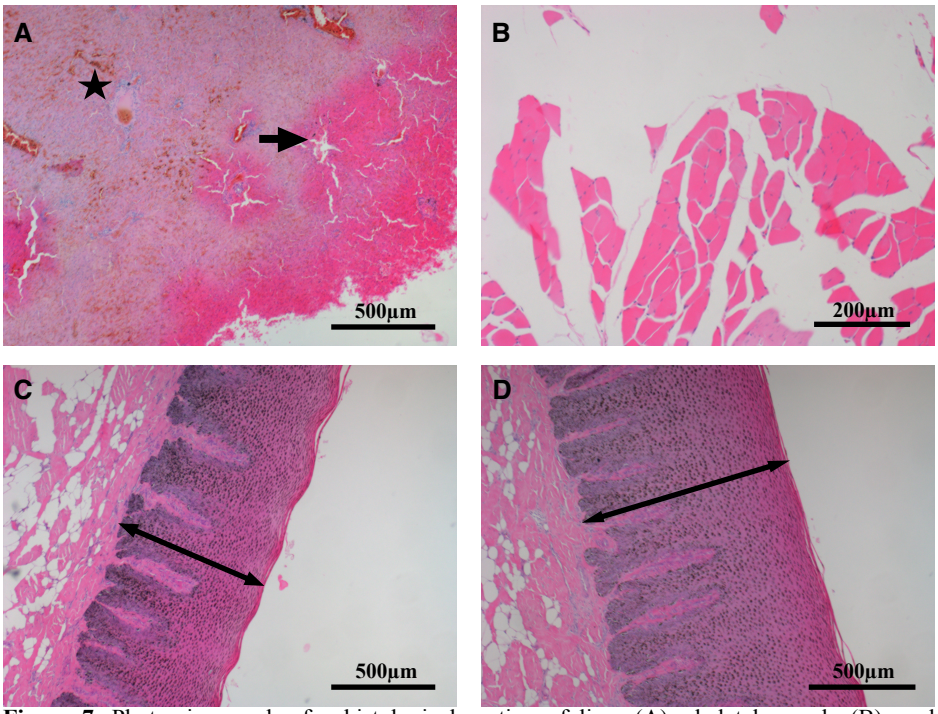

Figure 7. Photomicrograph of a histological section of liver (A), skeletal muscle (B), and integument (C) fixed in Formaldehyde 40% and integument (D) fixed in NBF of Sco 2. Arrow indicates fissures (A), Star indicates bad staining (A)and double headed arrow(C and D) indicate the epidermal thickness. H&E.

In general, samples treated with Davidson were slightly overstained. Heart samples of Sco3 fixed with Davidson that have shown great scratches or shrinkage (Fig.8A), and therefore show a lower quality comparing to the one fixed with NBF (Fig.8B). Although the cetacean tissues fixed with Davidson showed an acceptable quality of preservation, it could not be compared to the skeletal muscle treated with NBF, because it was not possible to section it with the microtome, as sections become to dust.

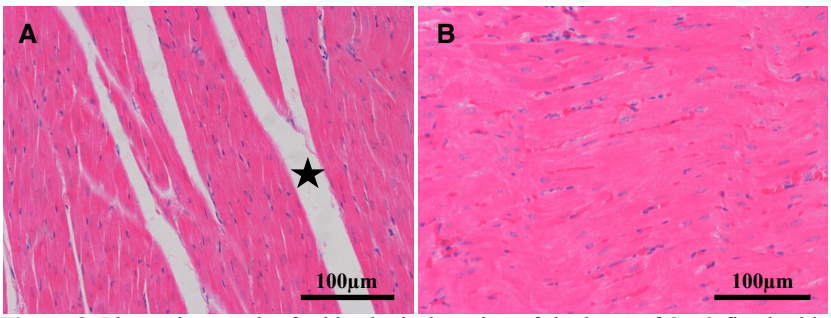

Figure 8. Photomicrograph of a histological section of the heart of Sco3 fixed with Davidson (A) and NBF (B). Star indicates shrinkage. H&E.

In general, although fixatives, preserving methods and the dissectioning have shown to have different effects on the tissue quality, the samples that are always collected (integument, blubber, muscle and teeth) are less sensitive to these delimit factors.

Although data is not shown, during the histological observations in this study microscopic parasites were observed. Tissue samples of the stomach, blubber, and lung of the individual Sco2 have shown presence of parasites. Moreover, samples of lung obtained from Pph1 and Ttr3 individuals also showed presence of microscopic parasites. However, no correlation with the cause of death could be done because of the lack of information available of these parasites and their effects on the host bodies.

5.3 Case study with the integument of cetaceans

To assess wether samples obtained during this study are useful for research, a case study with the integument has been carried out, by calculating the ratio among epidermal thickness and dermal papillae height, in order to correlate it with individuals´ health status.

The height of the papillae with respect to the thickness of the epidermis in integument samples from Ttr2, Ttr3, Dde3, Pph1, Sco1 and Sco3, all fixed with NBF, have shown the lowest relative papillae height (values from 0,32 to 0,43). On the contrary, Ttr1, all Zca1 and Sco2 FOR have shown the highest values for this ratio (values from 0,57 to 0,48)(Fig.9). There are differences for this ratio among species, for example, comparing the porpoise (Pph1) and the beaked whale (Zca1)(Table 3).

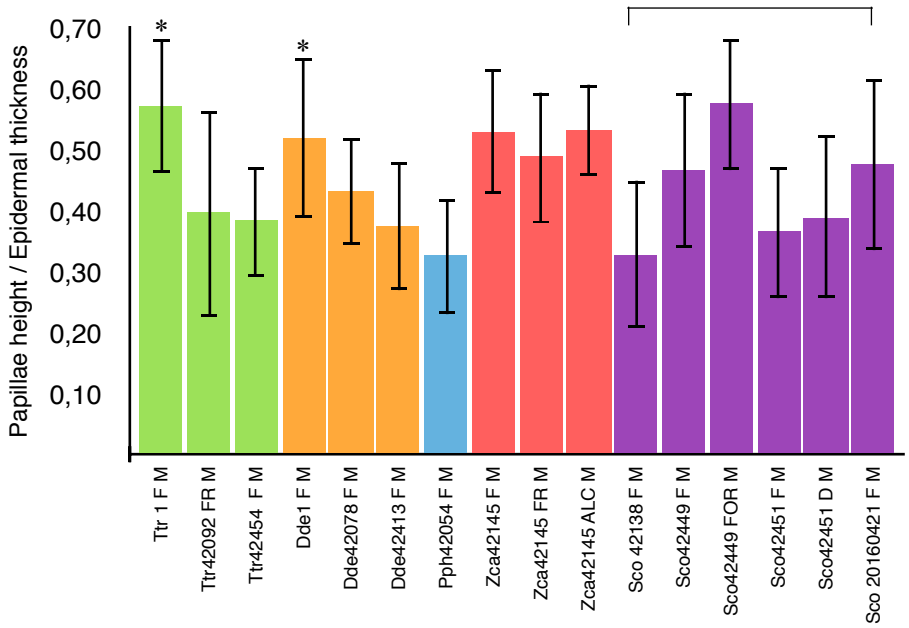

Figure 9. Bar graph showing average (M: mean) height of dermal papillae in the mid-dorsal body wall expressed as a rate of epidermal thickness (papillae height /epidermal thickness). The individual with * show differences within the individuals of the same species and square bracket indicates differences among Sco 1 and Sco4.

This ratio, have shown differences among some individuals of the same species (Fig.9). The ratio showed a normal distribution tested by Shapiro and Wills normality test. Statistics among *Tursiops truncatus* individuals have shown differences between them (ANOVA p: 0,004) and according to Duncan´s pos-hoc test Ttr1 is different to the rest (Fig.9). No information about decompositional state, nutritional condition or blubber thickness of Ttr1 was measured (Table 3). The same has been observed among *Delphinus delphis* species (ANOVA p: 0.007) where although Duncan´s tests show that Dde1 is different to the others (Fig.9). These two, Dde1 and Ttr1 are in their respective groups that have the highest relative height of papillae. Statistics on *Stenella coeruleoalba* individuals (except for Sco2 FOR and Sco3 D that were analyzed separately with Student ´s test have shown that there are differences among species of this group (ANOVA p: 0,018). Indeed, Duncan test have shown that there are differences among Sco1 and Sco4 individuals (Fig.9). This individuals have different decompositional states. Whereas Sco1 was alive when it got stranded, samples from Sco4 were taken under an advanced decompositional process (Table 3).

No significant differences were observed between samples with different storing (freezing), preservation (alcohol) or fixation methods (NBF vs Davidson and NBF vs Formaldehyde 40%). However, analyzing the epidermal thickness and dermal papillae height on their own, differences on the procedure have been found (data not shown). The epidermal thickness was significantly different comparing the three processings ; Zca1 vs Zca1 FR p:0,000, Zca1 FR vs Zca1 F´ p:0,001 and Zca1 vs Zca1 F´ p:0,006. However, papillae height differences were only significant in certain comparisons; Zca1 vs Zca1 FR p:0,046. The same was observed in Sco 3 samples fixed with NBF and Davidson and in Sco2 samples fixed with NBF and FOR.

6. DISCUSSION

6.1 Basic information of the strandings

The information obtained from this study is basic to define a good protocol for the sampling of stranded cetacean adapted to the casuistry of the Basque Coast. Analyzing the basic information recollected on the strandings, Biscay is more prone to suffer from strandings than Gipuzcoa, maybe because the stranding network in Bizkaia contains more amount of AMBAR members than Gipuzcoa. Moreover, although stranding not always provide quality samples, and therefore in this study a selection of representative data was exhaustively done, decompositional state have shown that a big percentage (%63) of stranded individuals were worth for histopathological research, as suggested by existing protocols (Geraci and Lounsbury, 2005). As a consequence, it demonstrates that good quality samples must be obtained out of the strandings with good sampling standards for a future cetaceans research.

Although the basic information to be measured in each stranding were established, NGO volunteers often result impossible to measure the blubber thickness which is representative data of the cetaceans body condition. This may happen because of the extreme conditions of the stranded (time limit, tides, permissions…) limits the proper data collecting. The determination of the nutritional condition is done by subjective observations. Although state 4 individuals in general are not advised to be sampled, the code system can easily be mistaken. As tegument muscle and blubber are less sensitive to decompositional processes and usually show a good quality, the collection of these tissues even if the animal is in a state 4 is suggested in the protocol, always noting the state as it can be a delimit factor in the study. However, a necropsy in an animal with a state of decomposition 4 won´t provide much information as the inner organs will be too degraded.

A 33% of the strandings were identified as juveniles, thus age was not a possible cause of death and more effort is required to find why apparently healthy juveniles died. With sampling protocol agreed and supported by institutions, the death causes of death could be determined as samples would be directly sent for histopathological analysis. Moeover, a part of samples could be stred in banks like BBEBB for future studies.

6.2 Histological processing and tissue quality

On this study, it was impossible to do the sampling processes under the same standards or guidelines due to the variability of strandings and the precarious situation that AMBAR volunteers have. Therefore results have shown the need of a well establish protocol, as well as the support of institutions, as this lack of help inhibits the NGO from obtaining quality samples. By the histology done on the samples obtained from strandings during this study, different advisable procedures have been determined, and there will be useful for building the cost effective protocol (ANNEX Nº 1).

Fixation as part of the tissue processing is one of the key steps behind the study of pathology and exists to prevent samples from suffering autolysis and degradation and is a essential part of the overall life of a tissue sample (Chatterjee, 2014). Many protocols give advise of using fixatives, which needs procedures that are difficult to execute on field. Although the members are experts on cetaceans, they might not be capable of manipulating toxic solutions (formaldehyde…) as they do not have a basic knowledge on fixatives and chemical compounds. The lack of accurate operating instructions of fixatives can led to more mistakes and variability in the sampling processing. However, under good standards, samples fixed on field can show good results. In fact, samples obtained from the Pph1 individual and treated with NBF in field showed really good quality, as expected (Table 4).

Use of improper fixatives can be the cause of many artefacts. According to Chatterjee (2014) fixation in alcohol results in poor staining of the epithelium, severe shrinkage and improper fixation of the connective tissue. Moreover, the study showed that the collagen bundles have an amorphous appearance that is not a result of scar formation but rather result of artefact. Therefore, alcohol is not recommended as a substitute for formalin as it also makes the tissue brittle, resulting in microtome sectioning artefacts with chattering (Chartterjee, 2014). Contrary to the advise, the NGO AMBAR has sent during years samples preserved in alcohol of low gradation (%10) to a histopathological laboratory, possibly due to the misunderstanding of protocol which advise to preserve samples on alcohol. However, the preservation should never be mistaken with fixation. Until now, no results were obtained from those samples due to their poor quality and no measures were taken to find a solution to this problem. Results of this study have shown that preserving tissue samples in alcohol at least for 48 h causes different negative damages on the tissues. On the contrary, if samples are alcohol-preserved by error, or because no resources were available, Carnoy fixative seems to be more appropriate than NBF, after observation done on the present study. In fact, Carnoy is more suitable due to its alcohol proportion as it is composed of absolute alcohol (60%), Chloroform (30%) and Acetic acid glacial (10%). The cost effective protocol would include the recommendation of moving samples as fast as possible to the PiE-UPV/EHU establishment without fixation, to continue there with the established protocol.

Histology of frozen tissue is often adequate, although it shows inferior quality NBF fixed tissue for detailed microscopic observation and analyses. SEC suggests that samples should be fixed with 10% Formaldehyde or frozen at least at -20Cº for histopathology (SEC, 2000). Indeed, samples stored in volunteers´ particular fridges at -18Cº have shown positive results, being a good option to include in the protocol. In the case of the Dde1 individual, the whole body was frozen at -18Cº until the necropsy and all tissues have shown good quality, although it must be pointed that it was really fresh individual. Moreover, results from samples frozen at -80Cº in PiE-UPV/EHU before fixation with NBF have shown good quality as well. In this study, only frozen muscle and integument have been analyzed, at ultralow temperatures. Therefore, there should be a continuity for this study in order to find wether there are effects of freezing samples on inner organs. However, there should not be any side effects, as there are tissue banks such as Pfizer, Inc.'s global tissue repository, where samples are frozen for further analysis (McDonald, 2010). Indeed, biospecimens can be stored for years to decades at ultralow temperatures from -80Cº to -150Cº. Freezing samples, apart of giving homogeneity to the catalogue, it broadens the possible target objectives that studies may have because more analysis can be done on then, such as immunohistochemistry, histopathology, genetics or chemistry. The only disadvantage of frozen samples is the need of infrastructure and the cost of storing samples. Thankfully, this equipment is available at PiE-UPV/EHU

for storing samples collected by AMBAR due to the collaboration between these entities, which has made possible to start a new catalogue of samples in the BBEBB.

Apart from the storing samples in the biobank, the results obtained leads to the conclusion that samples can be stored at particular fridge´s, for a short period of time, as plentzia may be too far from the stranding place. However, there is a strong recommendation of moving those samples to the PiE-UPV/EHU as soon as possible, to avoid the loss of data as power outage may happen. It is important to underline that biomolecules such as RNA, can easily degrade with increasing freeze-thaw cycles (Shabihkhani *et al.*, 2014). Another research done on the skeletal muscle of the buffalo, have shown that samples frozen at -18Cº and exposed to freeze-thaw cycles were affected. In fact, shrinkage and significant gaps among fibres have been observed (Sen and Sharma, 2004). If there is the need of relocating samples, moving from the particular´s fridge to the PiE-UPV/EHU, it should be rapidly done and with all the necessary prevention measures, as refrigerating samples with ice-packs to avoid the defrost of samples. Although there are other options such as using liquid nitrogen tanks on field, this is impossible without the financial support of institutions and qualified personnel. The main objective now for the NGO should be to generate a protocol for the sampling and the follow it on the PiE-UPV/EHU establishment to achieve and safe as much samples as they can.

Another factor observed during this study is that although AMBAR member are experts on cetaceans, having a basic knowledge of their anatomy is also essential for executing the necropsy. There is a huge difference between sampling a small cetacean or a whale. In the case of the Cuvier´s beaked whale (Zca1), dissections were too superficial. Indeed, all the samples of the inner organs were wrongly dissectioned, as only the connective tissue capsule of each organ was dissectioned. Therefore, all the efforts made and the time invested on this stranding were in vain and the samples of this individual were lost. However, once detected and analyzed this problem, the solution is as simple as obtaining samples from a deeper incision in big whales.

¨Marine Mammals Ashore; Field Guide for Strandings¨ (Geraci and Lounsbury, 2005) is a protocol and a review of studies based on a good sampling. It suggests different solutions for storing and preserving samples. For gross preservation in Formaldehyde 37% (dilute on-site to a 10% formalin solution); for histopathology 10% NBF; for conserving ectoparasites Glycerin (5%) in 70% Ethanol; 70% Ethanol for storing teeth, stomach content; Alcohol-formalin-acetic fixative to preserve endoparasites and Formol-urea or saturated NaCl to preserve integument for DNA analyses. Nevertheless, more recent research on marine mammal histology and histopathology commonly use NBF for fixating the samples. NBF in marine mammals for blubber samples (Struntz *et al.,* 2004), integument-blubber (Montie *et al.,* 2008), or skeletal muscle (Sierra *et al.,* 2015; Heffer and Balos, 2007) has been used in previous research.

In this study the overall morphology of samples was acceptable using Carnoy, Davidson and NBF fixatives. However, image analysis demonstrated a significant variation among dimensions, due to shrinkage related to overfixation. These variations were observed in Sco2 and Sco3 individual´s integument samples that were duplicated and fixed with different fixatives. Some research done on the thickness of oesophageal wall using different fixatives has shown that there are differences on the thickness of this tissue. Carnoy fixed specimens had a relatively thinner oesophageal wall than the ones fixated on NBF (Howat and Beverly, 2014). Studies on a possible difference in fibre size, possibly due to the conservative methods that were employed, frozen and formalin-fixed paraffin embedded have been carried out (Sierra *et al.,* 2015). As presupposed, tissues treated

with 40% formaldehyde were overfixated and this oxerfixation has also been observed in samples of AMBAR. Indeed, the formaldehyde proportion that NBF have is %4, 10 times diluted. Although the shrinkage was evindent in the integument sample fixed with formaldehyde 40%, the ratio measured in the case study didn´t show significant differences to the integument fixed with NBF. Although samples fixed with Davidson showed good results, some tissues were more damaged than fixed with NBF. This study has revealed that NBF is the fixative that obtains the highest quality in comparison with Carnoy, Davidson, Formaldehide 40% or Alcohol %10.

According to Tiford formaldehyde fixation represents a laboratory biohazard (Titford, 2001). Indeed, UPV/EHU has recently banned this course 2015-2016 the use of formaldehyde in university teaching because it represents a danger, as it is consider to be mutagenic. Therefore, is important the use of alternatives that are available at PiE-UPV/EHU. However, taking into account the variability that stranding have, and that the sampling processes started last year, when there was not any ban towards fixatives, no alternatives were applied. In order to find out in detail the effects of the sampling procedures as well as the tissue processing methods, NBF was used but with the help of PiE-UPV/EHU staff. However, if this study has continuity, the substitute for formaldehyde should be used. Indeed, in order to determine its efficacy further research on the effects of this alternative fixative would be needed.

Histological observations have shown microparasitical presence in different organs of individuals. Even if no conclusions can be drawn out of this observations due to the lack of information available, the presence of microparasites determines that samples are useful for histopathological analysis and therefore to determine possible causes of deaths. Parasites were observed in formaldehyde fixed samples.

6.3 Case study with the integument of cetaceans

The case study with the integument of stranded cetaceans has demonstrated that histological samples obtained by AMBAR can be used in advance research. Body condition has been the main objetive of a variety of research, as any kind of information from the general health is an indicator of short term feeding success (NAMMC, 2007-2008). Blubber thickness, among other morphometrics, have been used during years as suitable indices to assess nutritional condition (Gómez-Campos *et al.,* 2011). These indices are widely in use especially for mysticetis and pinnipeds (Konishi, 2006; Lockyer *et al.,* 1985; Naess *et al.,* 1998; Pitcher, 1986; Pitcher and Calkins, 2000). In odontocetes, these parameters have not always shown accurate results (Caon *et al.,* 2007; Evans *et al.,* 2003; Lockyer, 1995; Read, 1990) and as it can be observed in the present study data, blubber thickness is a measurement done in field sometimes under bad conditions and, therefore, it is easy to loss. Indeed, some research on this issue have shown that this indices are not correlated with the changes in overall body fat reserves (Aguilar *et al.,* 2007). Although blubber thickness themselves can give poor information, these indices together with the relative dermal papillae measurements, may offer more information about the health status of each individual. Moreover, during this study problems with the loss of data, have been encountered. When only the minimum data is collected and therefore no necropsy is done, members tend to measure standard metrics of the body and the basic samples, and the measuring of the blubber thickness is easily forgotten due to the stranding conditions. In fact, is of mayor importance in the assessment of data and it should be remarked in the protocol.

Apart from the blubber thickness, the ratio among the dermal papillae height and the epidermal thickness can also be related to the health status. According to Jones and Pfeiffer (1994), the relative height of papillae remains constant, from 0,4 to 0,5, in spite of the variations of epidermal thickness and dermal papillae height may have. However, in their study three individuals out of 10 are out of that interval. Indeed, measurements done in this study have shown that the relative height of papillae in the mid-dorsal body wall varies from 0,36 to 0,57. This may be because more species have been analyzed. In the article, *Stenella coeruleoalba* had an 0.47 of average height of papillae, using only one individual, which is not enough to draw categorical conclusions. In our study, samples were obtained from 4 different individuals of this species enabling a more reliable approximation for the average height of papillae. Indeed, the results obtained from these four individuals has determined a more specific interval of the relative height of papillae. However, data of those individuals should be also added to the interval as 3 of that group were determined as being in poor nutritional condition, but as in the of Jones and Pfeiffer (1994) no data of this observation is given.

This leads to the conclusion that more samples of at least apparently healthy (good body condition, and no diseases found in the necropsy) individuals´ samples are needed to establish referential interval of this ratio for each species. Once known the common values that resident cetacean species of our coast have, it could be easily determined if a individual is in good condition or not, by comparing its ratio with the interval.

Dde1 and Ttr1 are the ones among their species that show differences in the relative height of papillae. Indeed, both individuals show the the highest rate among their groups. This can be due to the fact that the PiE-UPV/EHU research group was involved in both strandings, ensuring the good sampling. However, in order to reach reliable conclusions, more data from these individuals would be needed such as blubber thickness, state of decomposition or nutritional state. Among *Stenella coeruleoalba* individuals Sco1 and Sco4 have shown a different ratio. This differences may be due to the decompositional stage of these animals. Sco4 individual was in stage 3 and the Sco1 in stage 1. Individuals that are in an advanced decompositional processes are not worth sampling because it may seem that there are differences were there are not.

Ttr2, Ttr3, Dde3, Pph1, Sco1 and Sco3 individuals presented the lowest relative papillae height. Different factors may affect the ratio in this case. Sco3 individual, although it was in an early state of decomposition, it was described to be in poor condition (15 mm of blubber). Both measurements, blubber thickness or nutritional condition and the relative papillae height coincide on the same conclusion; the animal was too weak, both energetically, as it contained a thin blubber layer, and too exposed to the environment, as the decrease of the papillae height can be correlated to the capacity of wound healing and the colonization of microorganisms (Kabara, 1972). Moreover, it was the only juvenile among all the *Stenella coeruleoalba* and possibly different maturity stages show different values. Being a juvenile also could be the cause for the low ratio of Pph1 individual. The individual Dde3 may have a low papillae proportion due to its advanced state of decomposition (4), which may affect to the measurements. The same may have happened with Ttr3 as it was in a 3 state. This dolphin was 326cm length and had 23 mm of blubber. Although it had good nutritional condition, measurements done on the dermal papillae reached to the opposite conclusion. The low rate of Ttr2 can be because it is the only juvenile of the group. However, more data is needed for drawing more reliable conclusions.

On the contrary, Ttr1, all the samples obtained from Zca1 and Sco2 individual presented the highest values for this rate. Having a high rate for this measurement does not necessarily mean that these individuals are healthy, as

may depend on different factors. In the case of the beaked whale it is not uncommon to have bigger relative papillae height, as big whales have shown bigger rate than smaller cetaceans (Jones and Pfeiffer, 1994), and although beaked whales are not mysticetis, their body length is more similar to a whale than to a dolphin. Also, according to the basic information recorded, this individual was in a good nutritional state (120mm of blubber). Moreover, samples from Sco2 fixed with 40% formaldehyde have shown a bigger relative papillae height because the fixative has affected to the epidermal thickness but not to papillae, and therefore the rate has increased. No conclusion can be drawn for Ttr1 due to the lack of data. This need of data underlines the importance of having a good sampling protocol.

Zca1 individual´s integument samples have shown no significant differences among samples conserved in Alcohol 10%, frozen or directly fixed with NBF. Although all samples seem to be valid for this measurement, it should be underlined that the alcoholic exposure of the samples was for a short period of 48 hours and therefore this study cannot ensure that metrics do not vary over 48 h of fixation. Although in this study frozen samples have not shown any difference on the ratio, some bubbles and scratches have been found on the epidermis. Although different fixatives can affect to the measurements as during fixation tissues commonly change in volumen (Chatterjee, 2014), whatever the procedure is, the measurement of the dermal papillae ratio does not vary, which means that it can be measured in all kind of samples, as long as the decompositional stage has not affect the tissue. Although samples show histologically good quality, for these studies, more emphasis should be paid to the decompositional state.

Together with the effects of different fixatives, there are some other factors that may also affect to the height of the papillae. The epidermis height and characteristics varies all over the body, as happens in humans (Li and Urmacher, 2007). However no hedd to this variation was paid because preventive methods have been taken by sampling integument only in the dorsolateral area of each animal. Nevertheless, the maturity of the individuals may affect to the characteristics of the epidermis. Apart from that, more samples would be needed to determined wether the state of decomposition, sex or maturity are delimit factors. Another factor that may have effect on the papillae rate is the loss of surface layers of the epidermis, which is common during the tissue processing (Stromberg, 1989), and can be the cause of an increase in the relative height of papillae in this case. Although all integument samples contained the parakeratotic layer, it is true that in some cases this layer was detached. In this situations, this layer has separately measured and finally added to the epidermal thickness, in order to minimize this error.

Depending on the research, the variability encountered during this study could be reduced in the future by obtaining integument biopsies from free ranging specimens using biopsy darts launched with a crossbow (Fossi et. al., 2010). Biopsies are a good option for sampling the epidermis and hypodermic blubber, leaving factors such the state of decomposition aside. Integument samples, can provide a lot of information; the epidermis can determine the health status (Jones and Pfeiffer, 1994), the blubber is been used in research for contaminants and can give information about the nutritional state. Genetics, can determine the sex of the animal and recently, a new method was reported for determining the age, with an error of +-3,8 years using some specific fatty acids profiles in the outer blubber (NAMMC, 2007-2008).

Being able to leave aside factors that can delimit the ratio, such as maturity, nutritional condition, decompositional state or sampling procedures and processings would enable the determination of the cause for

the low or high ratio, because having a low value for the ratio would mean that the individual is weak whereas having a bigger values would mean that it is healthy.

7. CONCLUSION

From this study it can be concluded that there is a need of a specific stranding protocol to fulfill the Basque Country casuistry. AMBAR, not as other NGOs, do not have any infrastructure for the storing or for the carrying out the necropsy properly and as a consequence, the protocol must include institutions as PiE-UPV/EHU. This study has been useful for suggesting a specific cost effective stranding protocol for the Basque Country coastline in order to develop guidelines for minimizing tissue sample variability. Further information in the stranding protocol for AMBAR members is available in the ANNEX Nº1.

The case study with the integument of cetaceans has provided knowledge on the cetacean´s integument although further research in the epidermal characteristics should be carried out to establish baselines of papillae height ratio in order to correlate them with the health status of the cetaceans. Moreover, integument samples are suggested as a target tissue for research it could give complete information about the health situation in cetaceans, providing a predictive model for hazards in susceptible areas affected by anthropogenic pressure. This case study also demonstrates the possibility to perform histopathological analysis in cetaceans in the basque coast with the collaboration between AMBAR and PiE-UPV/EHU.

Also, the present study has opened a new research area as well as a new catalogue of cetaceans in the BBEBB at PiE-UPV/EHU, providing a resource that can be used for future retrospective analyses and documentation of long-term trends in ecosystem health or even for the detection of the cause of death of unusual marine mammal mortalities.

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PROTOCOLO DE RECOGIDA DE MUESTRAS

-AMBAR-

Dr. Urtzi Izagirre e Idoia Meaza
28/06/2016

Pendiente de aceptación en AMBAR por el comité de junta

Protocolo realizado en colaboración con el Estación Marina Plentzia (PiE)

1. Introducción

Mediante este documento, se establecen las normal básicas de actuación para cualquier persona que asista a un varamiento, con el propósito de asegurar una adecuada recogida de muestras. De hecho, éste es un protocolo exclusivamente de recogida de muestra para varamientos y no para animales encontrados vivos en nuestra costa. Para que fuera un protocolo mas completo, también sería imprescindible pautas de actuación, empezando desde el momento en el que se recibe la llamada de varamiento.

Sin embargo, en este caso nos vamos a centrar en que la recogida de muestras sea válida científicamente. Para alcanzar este objetivo, es totalmente necesario que la actuación en cada caso se lleve a cabo siguiendo siempre las pautas del mismo protocolo, ya que solo de esta manera se va a poder hacer uso de este material científico.

Este protocolo se ha podido realizado a partir del Trabajo de Fin de Grado en Biología de Idoia Meaza Isusi y Urtzi Izagirre Aramayona, como director del proyecto. Sin embargo, no hubiera sido posible sin la ayuda de, tanto los voluntarios de la organización sin ánimo de lucro AMBAR, como al Centro de Investigación en Biología y Biotecnología Marinas Experimentales de Plentzia (PiE). Y queda abierto a discusión, criticas con aportaciones para su mejora para facilitar la consecución del objectivo: recoger muestras para determinar causas de la muerte y promover la investigaciones futuras.

1.1. Importancia de conseguir datos de calidad

Según el tratado de UNCLOS para la protección del medio marino, todas las especies en peligro de extinción o vulnerables, al igual que su habitat, tienen que ser muestreadas y analizadas para poder por un lado, determinar factores de riesgo y por otro, poder mantenerlos bajo control (UNCLOS, 1982). Muchos de los cetáceos que varan en nuestra costa son considerados vulnerables en España. Es por esto por lo que sus varamientos tanto como varamientos de otros mamíferos marinos que pueden convivir con las especies vulnerables, deben ser analizados por ley, ya que de esta manera se pueden encontrar factores que estén afectando directamente a su salud y supervivencia. Por este motivo, las instituciones públicas deberían involucrarse en el desarrollo de un protocolo unificado y subvencionado.

Además, los animales varados, son en muchos casos una valiosa fuente de información. De hecho, conseguir muestras de cetáceos es muy complicado y muchos estudios se han hecho a partir de animales en cautividad. Sin embargo, los animales varados, aparte de no sufrir alteraciones por culpa de los estudios, pueden ofrecernos información acerca del estado de salud de poblaciones salvajes y sirven como base de muchos estudios científicos. Estudios de histopatología de las muestras de los animales varados, por ejemplo, son necesarios para descubrir las causas de muerte, que en muchos casos no se pueden determinar a simple vista. Sin embargo, para poder conseguir conclusiones fiables, es imprescindible el uso de datos o muestras de calidad.

Tenemos que ser conscientes de que cualquier animal que vare en nuestra costa es único y tiene que ser muestreado con sumo cuidado. Es más, sabiendo la compilación que supone un varamiento, las muestras tienen un gran valor científico, ya que nos pueden aportar información que de otro modo es imposible de conseguir. Todas las muestras y los datos deben ser correctamente recogidos, por que de lo contrario puede suponer una pérdida de tiempo tanto como de esfuerzo.

1.2 Objetivos de este protocolo

Durante años los voluntarios de la organización sin ánimo de lucro AMBAR, interesada en el estudio y la conservación de la fauna marina, se han esforzado en recoger muestras para posteriores análisis de histopatología. En muchos casos, las muestras no han sido recogidas de manera adecuada, suponiendo un gasto innecesario para AMBAR. Sin embargo, dado el gran trabajo que los voluntarios han hecho para conseguir una red de varamientos, estamos totalmente convencidos de que estableciendo unas simples pautas de actuación el problema se va a solucionar.

Es por esto, que el protocolo no tiene otra función que ofrecer información a los voluntarios de la asociación con el fin de intentar establecer unos estándares para todos los muestreos, de manera que las muestras sean útiles para posibles análisis de histopatología o incluso para investigación.

2. Propuestas

Determinar cuál es nuestro objetivo antes de la necropsia es imprescindible. Según el propósito se van a recoger unos datos u otros, pero todos de una manera estandarizada. Los datos que no sean recogidos según los protocolos van a ser desechados posteriormente. Es decir, la decisión de los objetivos de las muestras (según los estudios que se quieran hacer varian) va a determinar la cantidad de datos que vayamos a recoger pero no la calidad, ya que ésta se va a mantener durante el procedimiento.

Como todos los voluntarios de AMBAR sabemos, los varamientos dependen de muchas variables que en muchos casos son impredecibles. Es por esto que deberíamos asegurarnos de que todos los varamientos tengan su ficha correspondiente con los datos básicos, ya que para poder usar estos datos en investigaciones es imprescindible tener datos homogéneos.

Se agradecería, para facilitar el trabajo de recopilar la información, que la persona que haya atendido al varamiento recopile la información primero antes de pasársela a la persona encargada del almacenamiento de este material. De esta manera dividiríamos los esfuerzos y ahorraríamos material. Cuando antes se lo mandemos a la persona correspondiente, antes de quedarán los datos y muestras almacenados en lugar seguro y además, la persona que ha atendido el varamiento lo va a tener mas reciente como para poder recopilar y juntar toda la información.

En base a los problemas que nos hemos ido encontrado durante el Trabajo de Fin de Grado, hemos intentado recopilar diferentes propuestas para solucionarlo.

2.1 Etiquetado de muestras:

Todas las muestras deben ser etiquetadas con, fecha, especie, lugar, nombre muestra correspondiente y código. Este paso es totalmente necesario ya que todas las muestras mal etiquetadas son desechadas. Como consecuencia de las dificultades que hemos encontrado con el código actual, hemos propuesto una posible solución.

La propuesta es adjudicar a los individuos un código numérico. AÑOMESDIA, dos dígitos para la especie y otras dos para el número de animales varados en el mismo día. Sustituyendo así, las letras ya que suelen ser fáciles de confundir. Es decir, se podría hacer una lista con las especies han varado en nuestra costa hasta hoy en día, por ejemplo:

01 Delfín Común

02 Delfín Mular
03 Delfín Listado
….

De esta manera, podríamos catalogar hasta 99 especies diferentes. Suficientes, ya que en estos 20 años de AMBAR han varado un total de 12 especies.

A continuación pondríamos otro dos dígitos para especificar el número de animales varados en el mismo día, por si tenemos que atender a un varamiento en masa. Si en el mismo día varasen dos delfines listados, por ejemplo, seria así;
1. Individuo sería: 201606220301
2. Individuo sería: 201606220302

Si hay otros factores que sean importantes, se pueden añadir con otros dos dígitos más detrás. De esta manera, si todos los miembros tenemos el listado de las especies, todo voluntario podría poner el nombre a las muestras. Y lo mas importante, ese nombre se conservaría dentro de AMBAR y del laboratorio, sin tener que cambiarlo, facilitando la búsqueda de muestras en el catálogo.

2.2 Conservación y traslado de las muestras al PiE

Lo ideal, sería fijar las muestras en el sitio del varamiento; una replica fijándola en formalina y la otra congelándola en nitrógeno liquido. Sin embargo, como ese procedimiento requiere de personal cualificado y material que AMBAR no dispone. En este apartado se va a proponer otra alternativa a estos procedimientos, aunque en un futuro no se descarta el uso de éstos si se consiguen subvenciones o colaboraciones con entidades como el PiE.

NO vamos a introducir las muestras en ningún tipo de fijador, ni alcohol, ni agua marina. Directamente las muestras deberíamos llevarlas al PiE para que allí la persona responsable las introduzca en el biobanco (congelador -80Cº). Así, congelando las muestras mantenemos un gran abanico de análisis que se pueden hacer en las muestras, como histología, inmunohistoquímicas, análisis químicos, técnicas moleculares, genética… En cambio, si solo fijamos las muestras, solo vamos a conseguir resultados en histología.

Durante el trayecto del varamiento a plentzia, es recomendable mantener las muestras frías. Podemos introducirlas en un recipiente con hielos para poder mantenerlas frías. Hay que tener en cuenta que las muestras pueden ser portadoras de patógenos y como consecuencia, hay que mantener todas las EPIs necesarias. Además, hay que recalcar, que no puede pasar mucho tiempo desde que se hace la disección hasta que llegan al centro, ya que el proceso de descomposición de los tejidos se acelera una vez fuera del cuerpo. El PiE está abierto las 24 h del día, así que la hora no debería ser un problema.

En caso de que el varamiento haya sido en Guipúzcoa o lejos de Plentzia, o no tenemos suficiente tiempo como para trasladarlas a Plentzia, recomendamos congelarlas. Hay que recordar, que las muestras son Sin embargo, no es conveniente que estén congeladas durante un largo periodo (en casa de particulares) ya que por un lado cualquier fallo eléctrico desecharía la muestra que tanto esfuerzo a supuesto y por otro lado es fácil que la persona que ha recogido la muestra se olvide de su existencia.

Además analizando mediante histología unas muestras congeladas durante un año, hemos visto que los tejidos sufren de fisuras, aunque si el periodo de congelación es inferior, las muestras presentan mejor calidad.

Para mover las muestras del congelador de casa al PiE, hay que mantener las muestras congeladas. En el futuro, se podría hacer otra colaboración con el equipo científico del PiE para poder disponer de tanques de nitrógeno liquido y hacer la congelación de las muestras en el mismo sitio del varamiento. Sin embargo, para esto necesitaríamos subvenciones además de personal cualificado, ya que el mal uso de nitrógeno liquido puede suponer un peligro. A pesar de esto, otra posible alternativa para el traslado de muestras congeladas es el uso de ice-packs que pueden ser proporcionadas por el PiE.

2.3 TRES niveles de actuación en los varamientos

Vamos a establecer TRES tipos de niveles en los varamientos para facilitar la recogida de muestra y conseguir datos homogéneos. Completar el primer nivel va a ser imprescindible para poder acceder al segundo y la decisión de qué nivel realizar va a estar determinado por el tiempo meteorológico, tiempo disponible para la necropsia, permisos para realizar la necropsia, la experiencia de los voluntarios y el material con el que dispongan.

1° NIVEL: INFORMACIÓN BÁSICA

Éste nivel es imprescindible y básico para todos los varamientos. No lleva mucho tiempo para realizarlo ya que no hay que realizar la necropsia completa para recolectar los datos. Ademas, no requiere de permisos para necropsia ya que solo se van a tomar muestras de disecciones superficiales. Es muy importante tener en cuenta que el no tomarnos el tiempo necesario para rellenarla puede hacer que el varamiento haya sido en vano.

ANTES DE EMPEZAR.

a) Estado de descomposición:

Antes de coger ninguna muestra, tenemos que observar en qué estado de descomposición se encuentra el cuerpo. De 1 al 5; 1 vivo; 2 extremadamente fresco, animal recién muerto; 3 autólisis moderada con perdida de piel o pelo, pene evaginado en machos; 4, autólisis avanzada, pérdida casi total de piel o pelo, cuerpo hinchado; 5, momificado, restos de animal.

En estados 1, 2 y 3 siempre cogeremos muestra de tegumento, grasa, músculo y dientes. Como es difícil delimitar el estado de manera precisa, ya que es bastante subjetivo, en todo los caso que tengamos duda cogeremos muestra, y si el animal está entre el estado 3 y 4 también, ya que hemos visto que estos tejidos se conservan bastante mejor que los órganos internos. Por lo que es mejor, recoger la muestra y después de analizarla decidir si desecharla o no, dependiendo de la degradación y autólisis que haya sufrido. Aun y así, es imprescindible, y más si nos encontramos antes un cuerpo en estado 4, anotar el estado en el bote de las muestras al igual que el la ficha del varamiento, para que quede constancia de esta variable.

b) Material:

Aunque no lleguemos a hacer la necropsia, tener los correspondientes EPIs es imprescindible. Si no tenemos material suficiente no podremos empezar con las mediciones. Es por esto, que cerciorarse de llevar siempre en nuestro vehículo el material básico es importante.

Material: bisturi, metro, botes para las muestras, guantes, bolsas de plástico, mascarilla, alcohol para desinfectar, un recipiente de plástico y fichas de varamientos (SEC, 2000)

DATOS IMPRESCINDIBLES.

1. FOTOS. Estas son las fotos que más nos interesan y que más información pueden aportar.
   A) Entorno del varamiento
   B) Foto del animal extendido de manera que se vea la longitud del animal (es recomendable poner algún objeto al lado del animal para tenerlo como referencia)
   C) Todas las marcas que pensemos que son por pesquería, de peleas o parasíticas.
   D) Zona urogenital y boca
   E) Aleta dorsal para Photo ID

2. FICHA. Todos los varamientos tienen que tener su correspondiente ficha proporcionada por la SEC, eligiéndola según el animal varado. Intentar rellenar en orden todas las casillas que aparecen. Importante medir la capa de grasa hipodérmica. Normalmente como las mediciones hacemos antes de coger muestras y para esta medición es necesario hacer una incisión debajo de la aleta dorsal nos olvidamos de realizarla.

3. MUESTRAS. Antes de coger muestras, nos tenemos que cerciorar de que tenemos todo el material a nuestra disposición y, hielos preparados para enfriar las muestras (que estarán en sus respectivos botes) para el camino al PiE. Una vez que extraigamos la muestra del cuerpo, el proceso de descomposición se acelerará.

   En todos los varamientos, en el que el animal esté en un código de descomposición inferior o igual al 3 vamos a proceder a coger muestras (si dudamos entre 3 o 4 también cogeremos muestra de estos cuatro tejidos pero anotaremos tanto en la ficha como en el bote de cada muestra el estado de descomposición, ya que puede ser una variable en los posteriores estudios). Las muestras obligatorias a recoger son **dientes, tegumento, grasa hipodérmica y músculo.** Estas muestras van a ir directamente al Biobanco de Especímenes del Golfo de Vizcaya en el PiE, BBEBB. Esto va a asegurar el correcto almacenaje de las muestras para futura investigaciones retrospectivas, manteniendo las muestras en un lugar idóneo. Es imprescindible que todos cojamos las muestras en el mismo sitio para minimizar la variabilidad de las muestras.

   Los dientes: vamos a cogerlos en la zona media, aproximadamente, de la mandíbula inferior y en el caso de que el animal no presente dientes o solamente aparezcan en otra zona, los cogeremos pero anotaremos bien en la ficha y bien en el bote correspondiente la zona de donde han sido cogidos.
   Piel, grasa y músculo: vamos a coger estas muestras del lomo debajo de la aleta dorsal. En este caso, nos da igual que las muestras se introduzcan en el mismo bote, pero en el caso de los órganos es importante separarlos entre sí.

   Piel y grasa juntas: 4 unidades de 2 cm cuadrados.
   Músculo: 4 unidades de 1 cm cuadrado
   Piel, grasa, músculo: A parte, cogeremos un buen filete (15cmx8cm aprox) de músculo. Para facilitar esta disección se puede coger el músculo pegado al tegumento y grasa, cogiendo los tres tejidos de interés.

4. TODA LA INFORMACIÓN QUE PAREZCA IMPORTANTE SE ADJUNTARÁ COMO ANEXO.

2º NIVEL: NECROPSIA Y RECOGIDA AVANZADA DE MUESTRAS

Éste nivel es complementario al primer nivel. Y sólo se podrá realizar una vez acabado el primero. En cuanto tengamos el permiso de la necropsia, es recomendable llamar al PiE (946018448) para poder informar a la persona responsable en el centro de que las muestras van a ser trasladadas al PiE. Es recomendable llamar antes de comenzar para dejar margen de tiempo a la persona encargada ya que va a ser el/la responsable de hacer las replicas para cada órgano (ya que esto es muy complicado hacerlo en el mismo sitio de la necropsia).

ANTES DE EMPEZAR:

Antes de comenzar con la necropsia, debemos observar si el estado de descomposición es el adecuado para la recogida de muestra. Los estados 1 y 2 son idóneos para asegurar una muestra de calidad. Sin embargo, vamos a tomar estado de descomposición 3 como limite para que la recogida de muestra. Si se da el caso, y recogemos muestras de un cuerpo con estado 3, deberemos anotar bien en el bote como en la ficha del varamientos el estado de descomposición, ya que puede ser una variable importante en los estudios.

Es necesario tener:

A) Permiso pertinente para la necropsia.
B) Material necesario para abrir el cuerpo y dejarlo todo recogido después.
C) Miembros cualificados con conocimiento y experiencia suficiente como para diseccionar correctamente el cuerpo de una manera ordenada, es decir, que voluntarios que hayan cursado los cursos anuales que AMBAR proporciona a los miembros.

MUESTRAS A RECOGER:

Antes de coger las muestras, nos tenemos que cerciorar de que tenemos todo el material a nuestra disposición y hielos preparados para enfriar las muestras (que estarán en sus respectivos botes) para el camino al PiE. Una vez que extraigamos la muestra del cuerpo, el proceso de descomposición se acelerará.

Una vez que hagamos la necropsia, vamos a intentar coger muestra de todos los órganos posibles, pero sobre todo riñón, hígado, corazón, estómago, intestino, pulmón y gónadas. Cada órgano irá en su correspondiente bote y con su código. Las muestras, igual que las recogidas en el Nivel Nº1 van a ser rápidamente trasladadas a las instalaciones del PiE, manteniéndolas frías, en un recipiente con hielos.

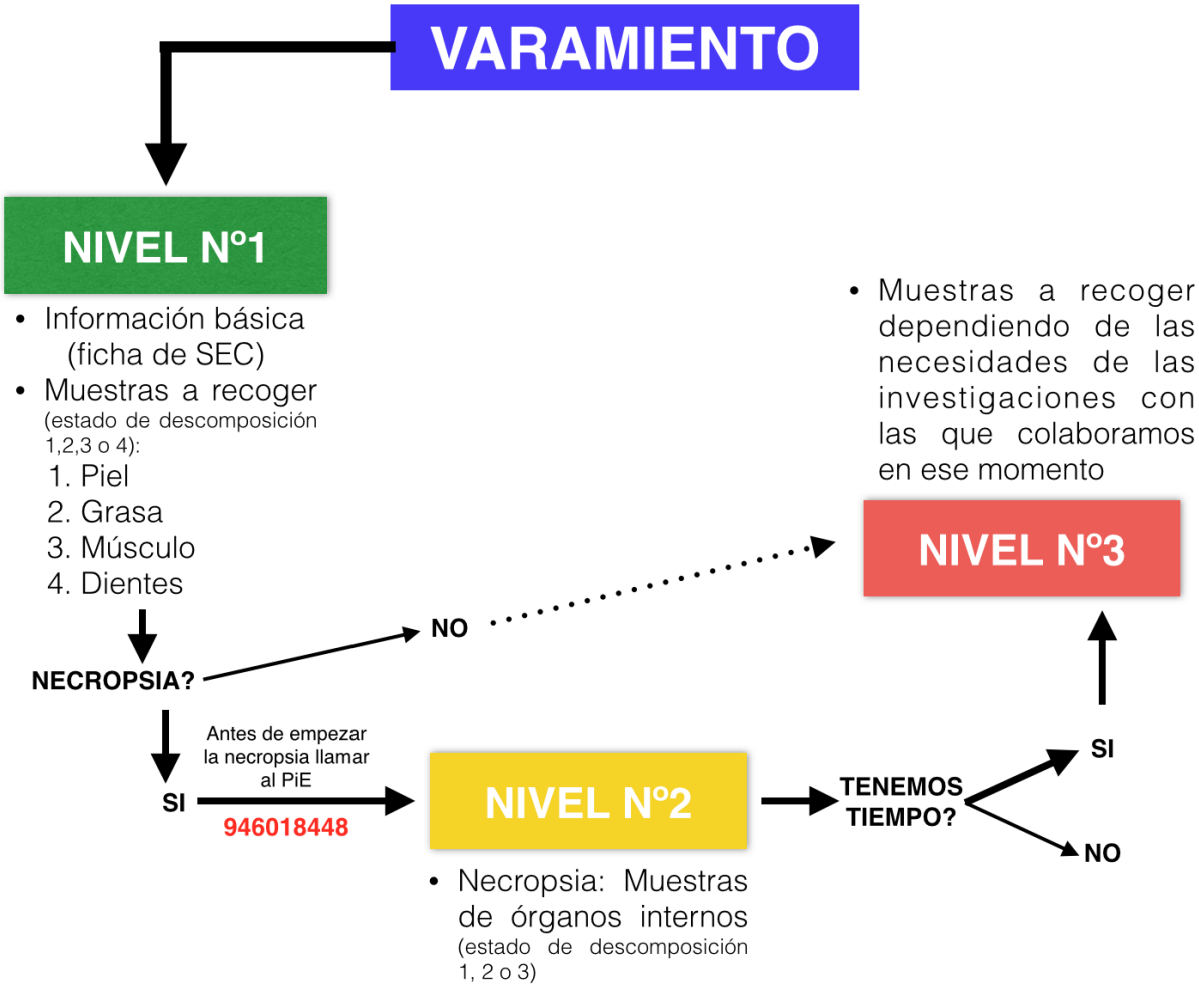
En el caso de que encontremos alguna patología en los órganos, intentaremos coger más muestras, o muestra mas dañada por esa patología y las anotaremos en la ficha del Nivel Nª1. En un futuro, se podría acordar con los miembros del PiE para tener siempre formalina preparada y en el caso de que animal varado tenga una patología se podría coger una muestra del órgano, fijarla y mandarla al laboratorio de

histopatología. Sin embargo, sería necesaria algún tipo de subvención para poder conseguirlo.

Necropsia en varamientos de cetáceos grandes: Hay que tener sumo cuidado con la recogida de muestras de órganos de animales grandes. Las disecciones, deben ser más profundas de lo normal ya que si no es muy probable que estemos cogiendo muestra del tejido conectivo que envuelven a los órganos

3º NIVEL: MUESTRAS PARA ESTUDIOS AJENOS A AMBAR

AMBAR está actualmente involucrado y colabora en muchos estudios científicos, cediendo muestras a los grupos de investigación correspondientes. Para poder seguir manteniendo ésta fuente de investigación, es importante que los voluntarios de AMBAR tengan constancia de qué muestras son de interés para esos estudios. Sin embargo, la recolecta de estas muestras se hará siempre después de completar el rutinario nivel 1 y la recogida de los órganos internos en el nivel 2. Y es que, primero deberíamos dar preferencia a recolectar las muestras básicas para poder tener suficientes datos como para hacer investigación y después coger muestras para otras investigaciones. Si no, estas colaboraciones podrían dar lugar a que la base de datos sea caótica y muy heterogénea.



2.4 Recogida de muestras en varamientos no comunes; focas, tortugas marinas y tiburones

Aunque no estemos acostumbrados a hacer la necropsia o simplemente a coger muestra de estos animales, se ha tomado la determinación de recoger muestra para congelar y posteriormente, con mas detenimiento pensaremos qué hacer con ella. Ya que si no cogemos muestra, la información de ese animal se va a perder.

3. Previsión para el futuro

Si el presente protocolo funciona, más adelante se podrían aplicar diferentes métodos de fijación. De ésta manera, podríamos obtener dos copias de la misma muestra, por un lado la congelada y por otro lado la fijada, que perdura más. Además, una vez habiendo aprendido a usar los fijadores, las muestras podrían ser enviadas al laboratorio de histopatología para poder analizar las posibles causas de la muerte. Sin embargo éste es un proceso que tenemos que introducirlo poco a poco, ya que el uso de fijadores puede llegar a causar errores irremediables y en consecuencia, la pérdida de las muestras.

Por mi parte, recomendaría introducir en el curso de varamientos anual información sobre los modos de fijación, sus limitaciones y factores a tener en cuenta. Por otro lado, si conseguimos hacer una catálogo con muestras de calidad en el biobanco BBEBB, más adelante se podría investigar con las muestras recogidas.

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