METHODOLOGY AND ANALYSIS OF GAS EMBOLISM: EXPERIMENTAL MODELS AND STRANDED CETACEANS

Yara Bernaldo de Quirós Miranda. Antonio J. Fernández Rodríguez. *Histology and Veterinary Pathology, Institute of Animal Health, University of Las Palmas Gran Canaria, Spain*

"Gas Bubble Disease" or "Decompression like Sickness" was described in stranded beaked whales linked to military sonar (Fernandez et al., 2005; Jepson et al., 2003). This hypothesis raised an important public controversy and a scientific replay in *Nature 2004* (Piantadosi and Thalmann, 2004), which clearly disagreed with that interpretation, requiring investigations on analysis of the composition of the gas in the bubbles in order to approach a diagnosis of Decompression Sickness (DCS). We have developed and standardized a methodology than enables the evaluation of gas abundance, gas sampling during *in situ* necropsies, as well as their storage and transport in vacuum tubes with non-statistical significant changes in gas composition for their final analysis in the laboratory. This methodology was applied to three experimental models: putrefaction, air embolism and compression/decompression model, showing to be effective for the differentiation between putrefaction gases and "in vivo" gas embolism processes. We studied in the same way 93 marine mammals belonging to 18 different species. We found that the presence of bubbles detected within the cardiovascular system and tissues during the necropsy of stranded cetaceans is a common finding related to "*in vivo* and / or *postmortem*" process. To try to avoid these putrefactive masking gases, necropsy and gas sampling must be performed as soon as possible, before 24 hours PM as recommendation but preferably within 12 hours PM. High amounts of gas bubbles in fresh animals were very rare. At necropsy, quantity of bubbles in decomposition codes 1 and 2 stranded cetaceans was found to be more important than the merely presence versus absence of bubbles. Deep divers presented higher abundance of gas bubbles mainly composed of 70% nitrogen and 30% CO_2, suggesting a higher predisposition of these species to suffer from decompression.

1 INTRODUCTION

The impact of anthropogenic sound on the marine environment and conservation of species has increased exponentially at national and international levels resulting in a high social and scientific concern in recent years (European-Parliament, 2004).

One of the best examples of this assertion lies in whales stranding and death related to noise emissions during naval maneuvers. The beaked whales (family *Ziphiidae*) are the most often whales involved in this kind of mass stranding temporally and geographically related to the use of antisubmarine active mid-frequency sonar .

Atypical mass stranding of beaked whales (BW) had not been referenced prior to 1963 (date from which, antisubmarine acoustic technology started to be used). Since then, mass strandings have been reported associated with military maneuvers in different world geographic locations as Bonnaire 1974, Canary Islands 1985, 1988, 1989 and 2002, Greece 1996, and in Bahamas in 2000 (Cox et al., 2006).

Except in the Bahamas, where a partial pathological study was done in three whales and authors concluded a diagnosis of "acoustic trauma", none systematic pathological analysis had been previously done in other similar cases. Until 2002, the “acoustic hypothesis” (Cox et al., 2006) was the only option that had received scientific attention, especially in the United States, although this has still not been scientifically proven.

An alternative, but not exclusive, "non-acoustic" hypothesis was published by Jepson et al. (2003) and Fernández et al. (2005), after the study of the lesions in dead BWs involved in a mass stranding coincidentally with naval exercises in the Canary Islands. Our group provided a possible explanation of the relationship between anthropogenic, acoustic (sonar) activities and the stranding and death of those marine mammals.

Fourteen BWs stranded in the Canary Islands close to the site of an international naval exercise (Neo-Tapon 2002) held the 24th of September 2002. Strandings began about 4 hours after the onset of midfrequency sonar activity. Eight Cuvier's BW’s *(Ziphius cavirostris)*, one Blainville's BW *(Mesoplodon densirostris)*, and one Gervais' BW *(Mesoplodon europaeus)* were examined postmortem and studied histopathologically. No inflammatory or neoplastic processes were noted, and no pathogens were identified.

Macroscopically, whales had severe, diffuse congestion and hemorrhage, especially around the acoustic jaw fat, ears, brain, and kidneys. Gas bubble-associated lesions and fat embolism were observed in the vessels and parenchyma of vital organs. In vivo bubble formation associated with sonar exposure that may have been exacerbated by modified diving behavior caused nitrogen supersaturation above a threshold value normally tolerated by the tissues (as occurs in decompression sickness).

Alternatively, the effect of the sonar on different tissues that have been supersaturated with nitrogen gas could be such that it lowers the threshold for the expansion of in vivo bubble precursors (gas nuclei). Exclusively or in combination, these mechanisms may enhance and maintain bubble growth or initiate embolism. Severely injured whales died or became stranded and died due to cardiovascular collapse during beaching.

That study demonstrated a new pathologic entity in cetaceans. The syndrome was apparently induced by exposure to mid-frequency sonar signals and particularly affects deep, long-duration, repetitive-diving species like BWs.

The new pathological entity named "Gas Bubble Disease" or " Decompression like Sickness" in cetaceans was deeply discussed in a specific Workshop Marine Mammal Commission of the United States (2004) with the participation of international experts and one of its main conclusions was that:

"Induced Gas-bubble disease is a plausible pathologic mechanism for the morbidity and mortality seen in cetaceans associated with sonar and merits further investigations".

Simultaneously, this hypothesis raised an important public controversy and a scientific replay in *Nature 2004* (Piantadosi and Thalmann, 2004), which clearly disagreed with that interpretation, requiring investigations on analysis of the composition of the gas in the bubbles in order to approach a diagnosis of Decompression Sickness (DCS), although there is very few empirical data on composition of gas emboli produced by decompression(Armstrong, 1939; Bert, 1878; Harris et al., 1945; Ishiyama, 1983; Lillo et al., 1992; Smith-Sivertsen, 1976). There is even less information on gas composition related to post-mortem time. There are few more studies of air embolism

applied to forensic science (Bajanowski et al., 1998a; Bajanowski et al., 1998b; Dyrenfurth, 1928; Erben and Nádvornik, 1963; Keil et al., 1980; Pedal et al., 1987; Pierucci, 1985; Pierucci and Gherson, 1968; Pierucci and Gherson, 1969; Richter, 1905). However, appropriate and accurate measurements of respiratory gases while avoiding atmospheric air is difficult; with the development and sophistication of new Doppler and imaging techniques in the 1970s and 1980s, less attention was paid to gas composition.

Gas chromatography technology allows us to analyze permanent gases at the same time as hydrocarbons in a single injection; however, avoidance of atmospheric air is still a problem. Additionally, cetaceans might strand on beaches that are not easily accessible and might require execution of the necropsy *in situ.* To our knowledge it does not exist today a transportable apparatus that can measure respiratory gases as well as hydrocarbons produced by microorganism's metabolism simultaneously. For instance, analysis of hydrocarbons requires hydrogen, which is not allowed to be transport without special security measures. Although, gas extraction may occasionally be performed at the stranding site, gas analysis must take place in a laboratory. Therefore, storage and transportation of gas samples are needed.

2 STANDARDIZED METHODOLOGY

2.1 GAS SAMPLING FROM CAVITIES

The 5-mL additive-free vacutainer (BD Vacutainer® Z. ref: 367624) is directly applied to cavities with its appropriate plastic holder or adapter and a double-pointed needle. To avoid atmospheric air, the needle must first be inserted into the cavity for purging; second, the vacutainer must be pushed against the double-pointed needle; and finally, the vacutainer must be removed, always before the needle is released from the cavity. This method allows adequate sampling from sinuses, the digestive tract, and even from heart ventricles if the putrefaction of the carcass ranges from grade three to grade five. Filling the pericardial sac with distilled water is always necessary to avoid atmospheric air in cetaceans.

2.2 GAS SAMPLING FROM THE HEART CAVITIES

If the decomposition status is fresh or very fresh (decomposition code 2 or 1, respectively), then an aspirometer will be necessary to separate the gas from the blood found in the heart. The aspirometer works by differences in pressure created by the vertical displacement of the simple gas flask. If this flask is moved upwards, the water will move from this flask to the burette and from here all along the free rubber tube and the puncturing needle. This is the position in which the user must puncture the heart, since the entire system is filled with distilled water and atmospheric air pollution is not possible. It is also necessary in this case to fill the pericardial cavity with distilled water for the same purpose. Moving down the flask creates a negative pressure in the burette and in the free rubber tube, suctioning whatever is found inside the heart. Clamp the free rubber tube in this position. Gas will ascend to the upper part of the burette and then the two physical phases will be separated. To collect the sample, it is only necessary to apply a vacutainer and to open the stopcock.

2.3 GAS SAMPLING FROM BUBBLES

Disposable insulin syringes (BD Plastipak U-100 insulin) are used and their contents are promptly injected into a vacutainer. One new syringe and one new vacutainer are used for each bubble.

2.4 TRANSPORT AND STORAGE OF GAS SAMPLES

Vacutainers are kept upside-down at room temperature with one blank per sample, or a total of at least 3 blanks.

2.5 GAS ANALYSIS

Gas analysis is conducted by gas chromatography. Samples are injected manually into the analyzer (Varian 450-GC) with the use of a block-pressure syringe (Supelco A-2 series). The temperature of the injector is set at 230˚C. This analyzer is equipped with a Varian CP7430 column composed of two different sub-columns in tandem: a (Q) PoraBOND Q column, for separation of CO2 and hydrocarbon compounds up to 4 carbons, and a (M) Molsieve 5 A column, for separation of permanent gases (such as oxygen, nitrogen, argon, etc). To detect these compounds, it is necessary to have both a thermal-conductivity detector (TCD) and a flame-ionization detector (FID) disposed one after the other. The TCD is a universal detector for permanent gases. Its temperature is fixed at 80°C, while the temperature filament is 160°C. The FID is a selective hydrocarbon destructive detector. Because of its destructive nature, the FID must always be placed after the TCD. The temperature for the FID is fixed at 230°C. Samples are run for 25 minutes with an isothermal temperature of 45°C and electronically controlled flux with a fixed pressure of 13.1 psi on the head column. Helium is used as the carrier gas.

3 EXPERIMENTAL MODELS

We have studied the gas amount and composition on three experimental (NZWR) models: experimental putrefaction, air embolism model and compression/decompression model. Based on these models a semi-quantitative method has been established to evaluate the presence and abundance of intravascular and tissular gas in carcasses during necropsy with forensic aim.

During necropsy of the rabbits (with decomposition codes 1 and 2) of the putrefaction experimental model, no atmospheric air was found to enter into vessels as a result of dissection (Fig 3.2). Massive presence of gas within the cardiovascular system of rabbits with decomposition codes 1 and 2 in our experimental models should be interpreted as a systemic "in vivo" gas embolism (Fig 3.2). Rabbits dying during or after compression and decompression showed a higher quantity and wider distribution of gas within the vascular system compared to rabbits which died due to experimental air embolism (Fig 3.2).

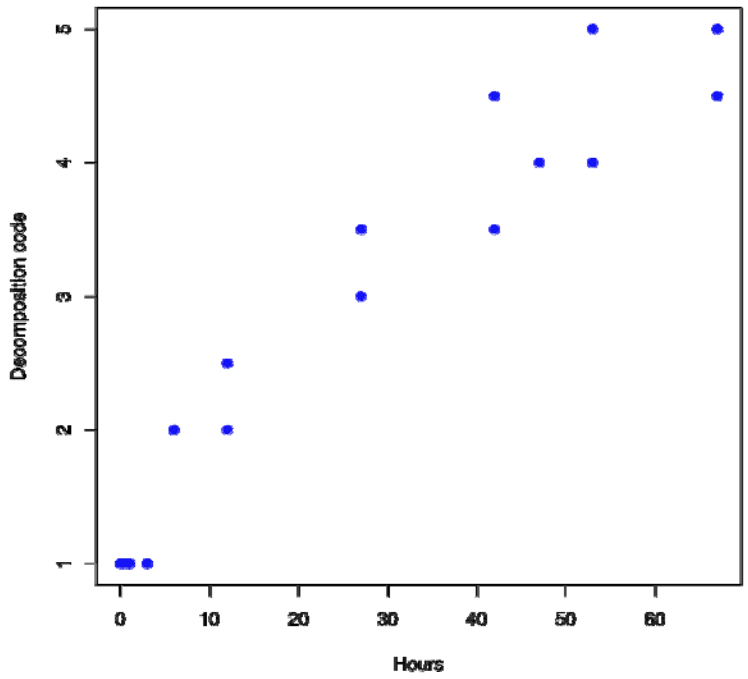

Fig. 3.1: Correlation between PM time (hours) and decomposition codes with a Pearson coefficient correlation value of 0.9591 (P<0.001).

$$E\left[Gas\right] = \alpha + \tau_2 T_2 + \tau_3 T_3 + \beta_1 T_1 HOURS + \beta_2 T_2 \log\left(1 + HOURS\right) + \beta_3 T_3 \log\left(1 + HOURS\right)$$

Parameter	*Estimation (SE)*	*P-value*
α	-0.797 (2.090)	0.7061 (NS)
τ_2 (effect of T_2 compared to T_1 at time 0)	11.986 (2.913)	< .001
τ_3 (effect of T_3 compared to T_1 at time 0)	33.112 (2.739)	< .001
β_1 (growing rate T_1 *in log-hours*)	0.409 (0.051)	< .001
β_2 (growing rate T_2 *in log-hours*)	6.228 (0.793)	< .001
β_3 (growing rate T_2 *in log-hours*)	1.051 (0.808)	0.2047 (NS)

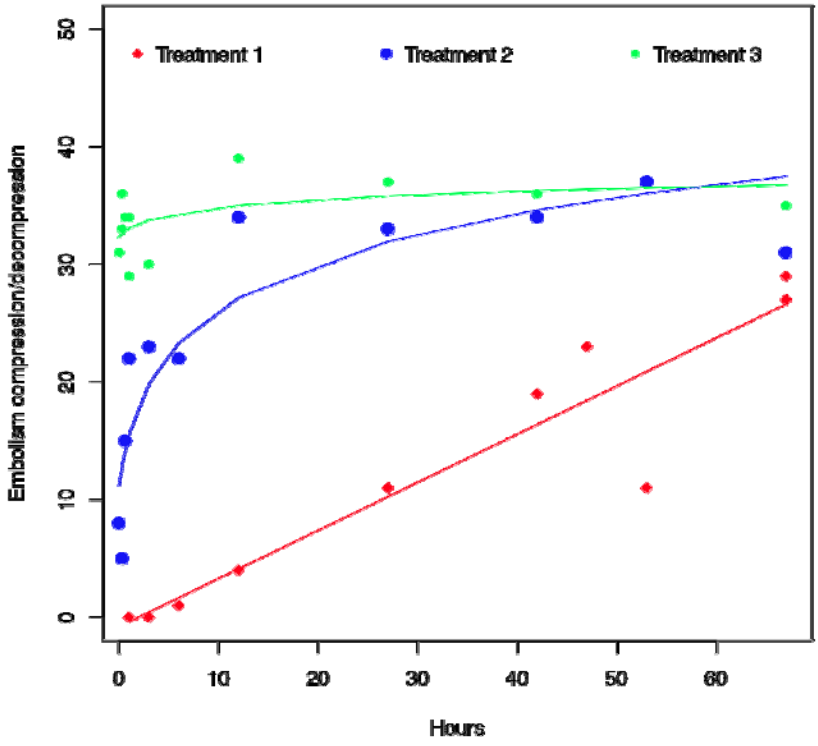

Fig. 3.2: Correlation between gas score and PM time (hours) for the different treatments, where treatment 1 is putrefaction, treatment 2 is air embolism, and treatment 3 is compression/decompression model.

Putrefaction gases in rabbits were composed of presence of hydrogen and / or high levels of CO_2, low concentration of nitrogen (mostly lower than 40%) and very small quantities of oxygen when present. CH_4 and SH_2 were randomly present in trace levels (Fig 3.3). Gas composition from samples obtained in codes 1 and 2 rabbits from air embolism and decompression experiments were very similar being composed of 70%-80% Nitrogen and 20%-30% CO_2, and therefore quite difficult to make a difference (Fig 3.4 and 3.5).

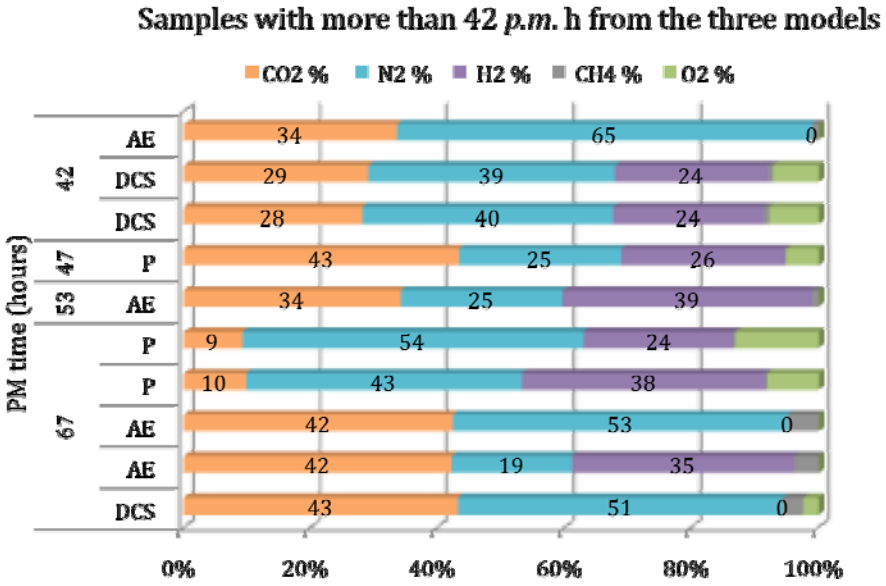

Fig. 3.3: Right Heart gas sample composition taken after more than 42 PM hours in the three models. Right heart gas sample composition of each animal *vs.* PM time illustrating the contribution of each gas to the total amount in percentage.

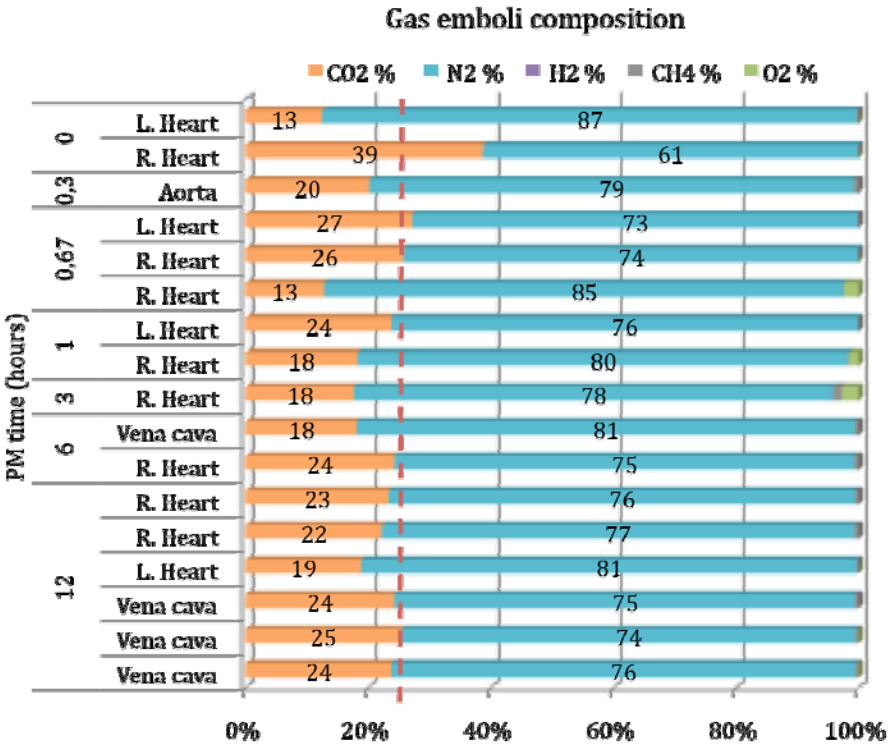

Fig. 3.4: Gas emboli composition from different localizations (heart, Vena cava and Aorta) *vs.* PM time illustrating the contribution of each gas to the total amount in percentage for the first 12 PM hours

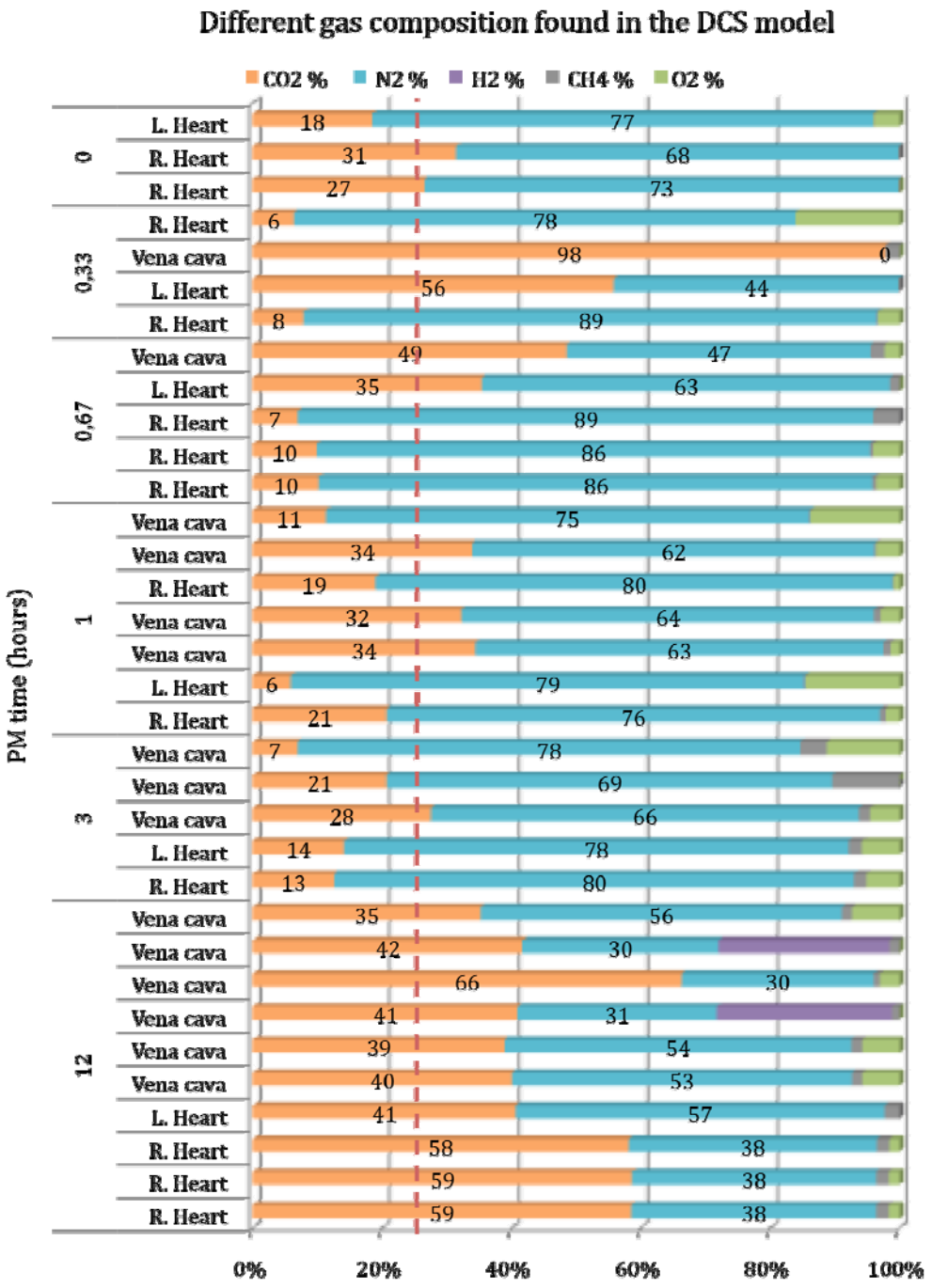

Fig. 3.5: Gas sample composition of samples from the DCS model that did not match with the observed tendencies. Gas composition is illustrated with the contribution of each gas to the total amount in percentage.

4 STRANDED CETACEANS

We studied gas abundance and composition in 93 marine mammals belonging to 18 different species. We found that the presence of bubbles detected within the cardiovascular system and tissues during the necropsy of stranded cetaceans is a common finding related to "*in vivo* and / or *postmortem*" process (Fig 4.2 and 4.3).

There is a direct relationship between decomposition codes and increasing amount of bubbles (Fig 4.1-3). Postmortem processes were analytically related to the presence of hydrogen and/or high levels of CO_2 as demonstrated in the rabbit experimental models (Fig 4.4). To try to avoid these putrefactive masking gases, necropsy and gas sampling must be performed as soon as possible, before 24 hours PM as recommendation but preferably within 12 hours PM.

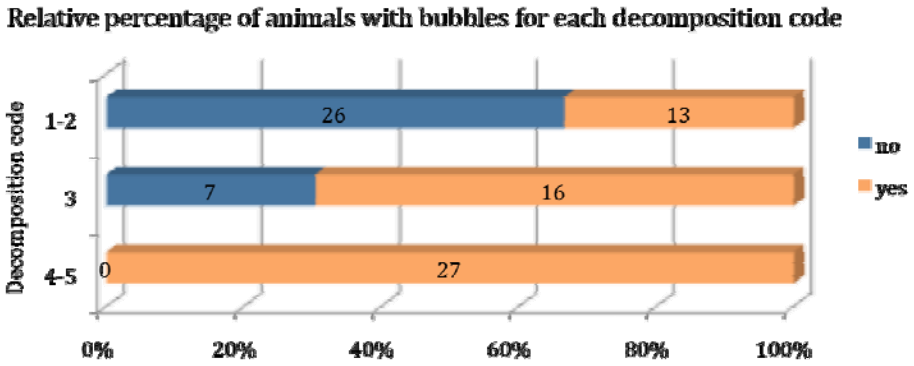

Fig. 4.1 Number and relative percentage of animals with bubbles (in orange) compared to those without bubbles (in blue) regarding to decomposition code.

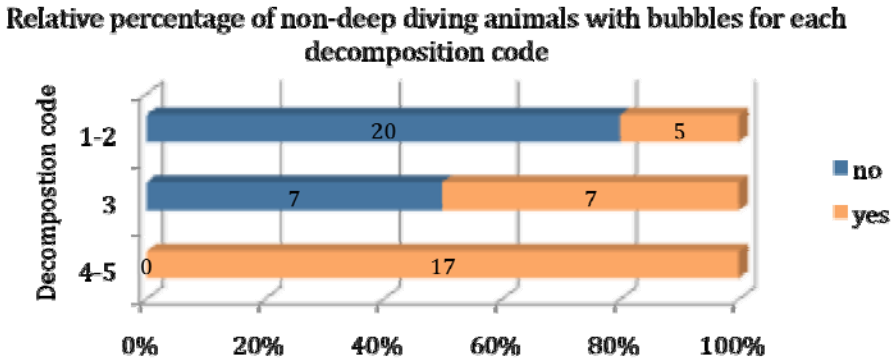

Fig. 4.2: Number and relative percentage of non-deep diving animals in which bubbles were observed (in orange) compare to those in which bubbles were absent (in blue) attending to decomposition code.

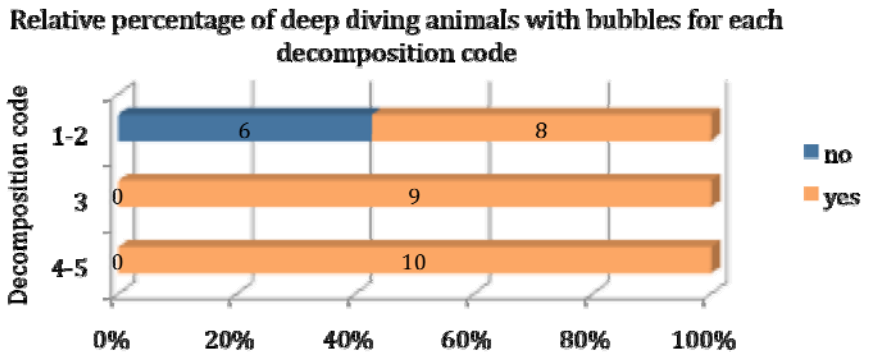

Fig. 4.3: Number and relative percentage of deep diving animals in which bubbles were observed (in orange) compare to those in which bubbles were absent (in blue) attending to decomposition code.

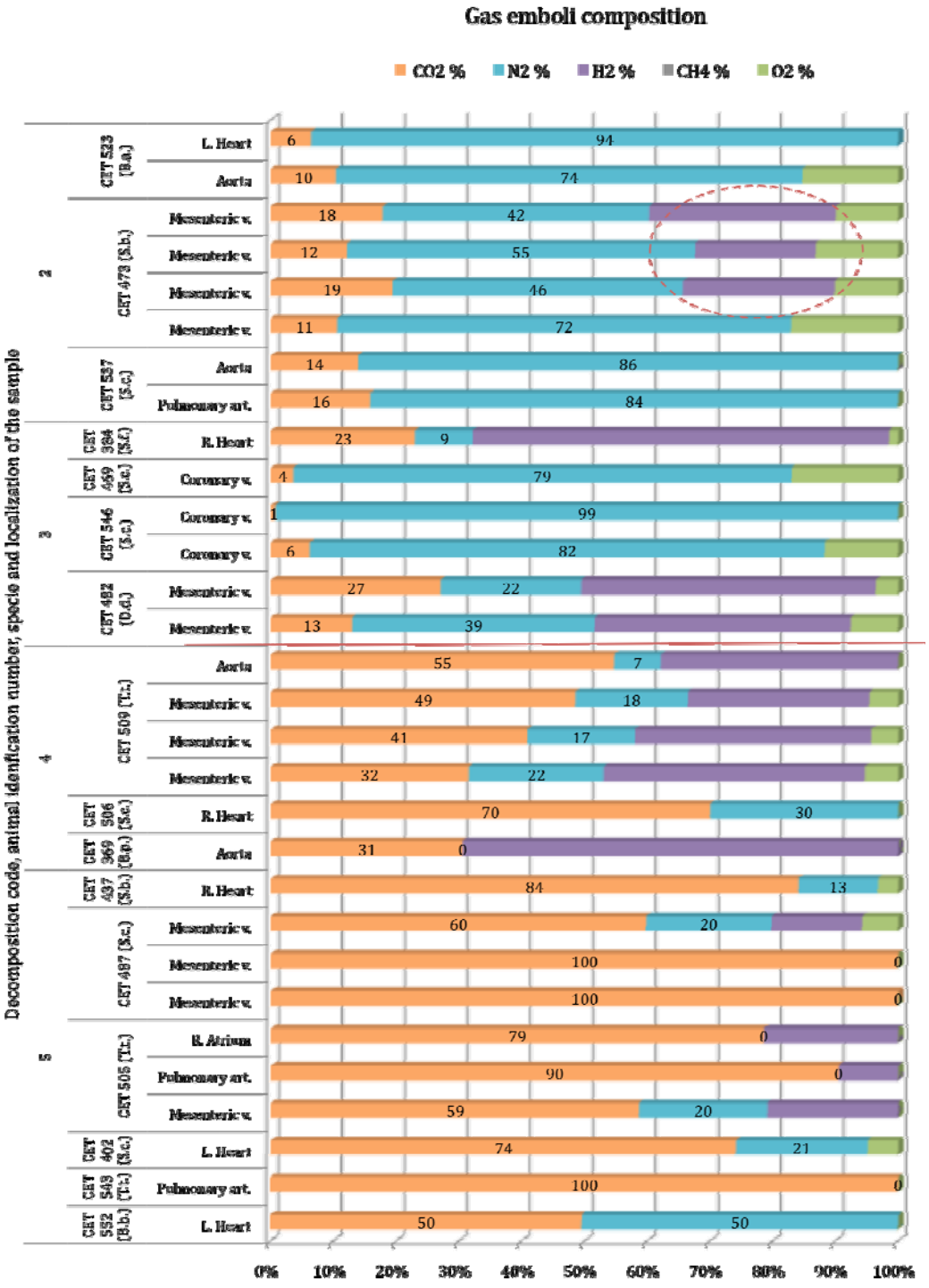

Fig. 4.4: Gas embolism composition sampled form different localizations and with different decomposition codes, illustrating the contribution of each gas to the total amount in percentage µmol. The solid red line is separating decomposition codes 4 and 5 from the rest since they have high amounts of CO_2 compare to samples from fresher decomposition codes where the main compound in nitrogen. The circle in enclosing samples with gas composition different from their group.

A small amount of intravascular bubbles is not an uncommon gross finding in fresh or very fresh stranded cetaceans showing a gas composition of 70% Nitrogen and 30% CO_2 (Fig 4.4). This observation is highly consistent with physiological *"in vivo* silent bubbles", more frequently observed in stranded deep divers (Fig 4.3, 4.6). This may also be an evidence of predisposition of these species to suffer from decompression, attending to our results (Fig 4.6). At necropsy, quantity of bubbles in decomposition codes 1 and 2 stranded cetaceans is more important than the merely presence versus absence of bubbles (Fig 4.5 and 4.6). High amount and widely distributed intravascular gas bubbles in these animals with gas composition of around 70% Nitrogen and 30% CO_2 would be related to "*antemortem"* bubble formation / growth involved in physiopathological processes (Fig 4.4).

Gas analysis is not a conclusive diagnosis for DC like Sickness in stranded cetaceans, but may contribute complementarily with other data obtained by imaging analytical techniques, a systematic necropsy, histopathology, microbiology, toxicology and other possible future analyses to reach a definitive diagnosis of decompression disease.

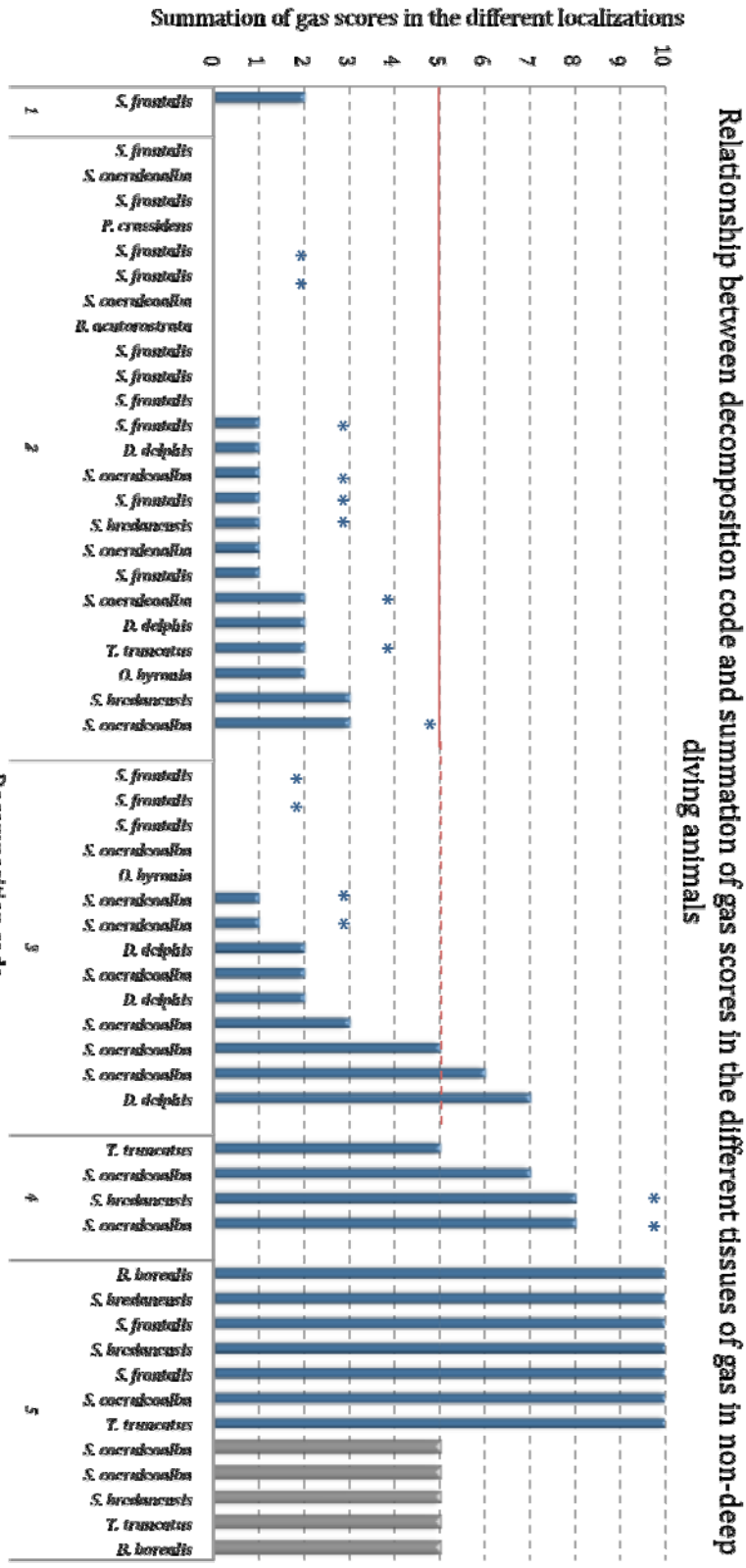

Fig. 4.5: Cumulative gas score in the different tissues and veins. Blue bars represent the maximum potential summation of gas presence and grey bars represent symbolically those animals so decomposed that veins could no be distinguished from the rest of the tissues.

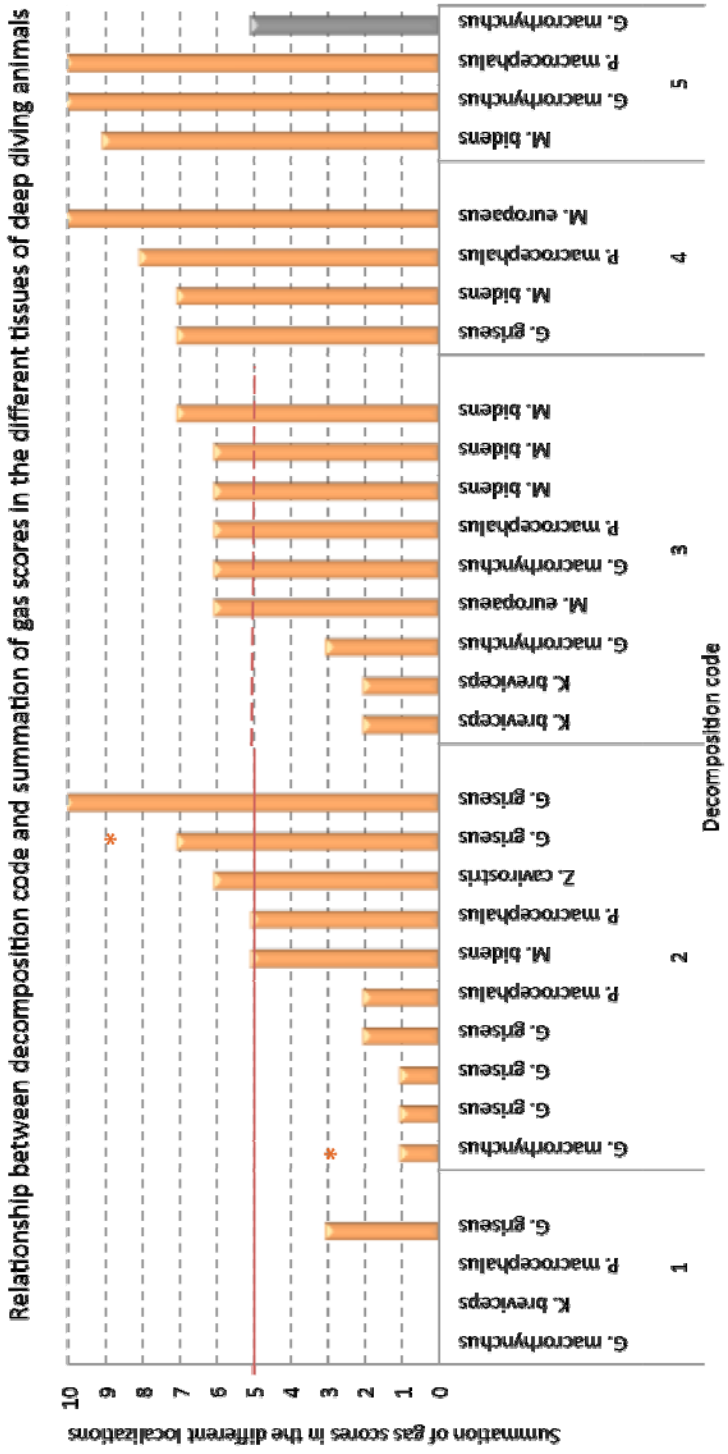

Fig. 4.6: Cumulative gas score in the different tissues and veins. Orange bars represent the maximum potential summation of gas presence and grey bars represent symbolically those animals so decomposed that veins could no be distinguished from the rest of the tissues.

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