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PULMONARY FUNCTION EVALUATION DURING NORMAL VENTILATION IN BOTH CAPTIVE AND WILD BOTTLENOSE DOLPHINS (*Tursiops truncatus*)

Tesi di laurea in

Adattamenti degli animali all'ambiente marino

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“We need to respect the oceans and take care of them as if our life depended on it. Because they do.”

Silvia Earle

CONTENTS

1.ABSTRACT 2

2.INTRODUCTION 3
- 2.1 Marine mammal's adaptation 3
  - 2.1.1 Lung functions 4
  - 2.1.2 Oxygen storage 6
  - 2.1.3 Alveoli collapse 6
  - 2.1.4 Decompression sickness (DCS). 9
  - 2.1.5 Rete mirabile 10
  - 2.1.6 Other strategies for deep dives 11
- 2.2 *Tursiops truncatus*, model animal to be studied. 13
  - 2.2.1 Scientific classification 14
  - 2.2.2 Body description 14
  - 2.2.3 Range description 15
  - 2.2.4 Habitat and ecology 15
- 2.3 Reason of this study 16
  - 2.3.1 Wild or captive? 16

3.AIM OF THE STUDY 18

4.MATERIALS AND METHODS 19
- 4.1 Animals 19
  - 4.1.1 Captive dolphins 19
  - 4.1.2. Wild dolphins 21
- 4.2 Equipment 23
  - 4.2.1 Data acquisition of differential pressure 25
- 4.3 Data processing and statistical analysis 26
  - 4.3.1 Statistic design 26
  - 4.3.2 ANOVA assumptions 27
  - 4.3.3 Analysis of variance - ANOVA 27
  - 4.3.4 Post hoc test 29
  - 4.3.5 Pearson correlation test 30

5.RESULTS 31
- 5.1 General results 32
- 5.2 Differences Captive-Wild 36
- 5.3 Pearson correlation test results 37

6.DISCUSSION 39
- 6.1 ANOVA assumptions discussion 39
- 6.2 Analysis of variance - ANOVA discussion 40
- 6.3 Post hoc test discussion 40
- 6.4 Pearson correlation test discussion 41

7.CONCLUSIONS 43

8.ACKNOWLEDGEMENTS 44

9.REFERENCES 45

10.APPENDIX 49

1.ABSTRACT

In this work, pulmonary functions of eight adult bottlenose dolphins (*T. truncatus*) during normal ventilation have been evaluated (maximal chuff events have not been included). Breath duration, respiratory flow rate, tidal volume and frequency have been measured, data collection involved both male and female dolphins and both captive and wild animals. It came to light that these animals are capable of 5.19 L (maximum recorded 13.11 L) of tidal volume gas exchange during normal ventilation, with an average respiratory frequency of 3.39 breaths*min^{-1} (range between 2.12-4.65 breaths*min^{-1}, minimum recorded 0,79 breaths*min^{-1}). They are capable of generating great flow too, especially during expiration (23.73±15.41 L*s^{-1}, max recorded 134.14 L*s^{-1}). With this work I provided new data for respiratory physiology of bottlenose dolphin and I looked for differences between wild and captive animals but, despite the interesting findings obtained, which show a statistical difference between the two groups, I am not going to draw any concrete conclusion from these data, even if some differences in tidal volume and flow rate can be related to size and subspecies.

2. INTRODUCTION

Life appeared on Planet Earth more than 3.8 billions of years ago (S. J. Mojzsis et al. 1996) and since the first biochemical reactions, unicellular organisms had to adapt at the changing conditions of the primordial oceans.

From this very ancient forms of life, step by step life started the great process we call evolution, which permitted organisms to colonize every corner of the world (W. Westheide et al. 2011). From the oceans, life spread over the world, reaching intern fresh water, lands, deserts, mountains and even glaciers and volcanoes, and for each of these steps, bacteria fungi, plants and animals had to face new physical and chemical conditions, and adapt themselves (T.M. Williams 1999).

Sometimes these living beings, even if perfectly adapted to this new environment, decided to move to another one. For example: marine phanerogams, vascular plants adapted to live on land which moved to water (G. Pasqua et al. 2010), flightless birds, which forgot how to fly and unicellular algae, living on land in a symbiotic relation with fungi, called lichens (B. McCune et al. 1992).

And of course marine mammals too.

2.1 Marine mammals' adaptation

Marine mammals probably represent the most fascinating of these re-adaptations. We are talking about animals completely adapted to live on land, which moved back to water less than 60 millions years ago (T. M. Williams 1999).

Moving from a terrestrial to an aquatic environment demands a complete behavioural, morphological and physiological modification. Since water is 800 times denser and 60 times more viscous than air (P. Dejours 1987), this transition undoubtedly challenged the mechanical and physiological systems of ancestral marine mammals, including different locomotor mechanisms, different hunting technique and, above all a new breath-holding lifestyle.

Some of these animals, the cetaceans, nowadays spend the whole life in water, feeding, mating and breeding without moving on ground (S. Hadoram et al. 2006).

It is extremely fascinating to study the physiological adaptations needed by these animals to survive in such a hostile habitat, especially for a not gills equipped animal. In this study I want to investigate into the most important adaptation occurred to mammals to spend their whole life into water: lung function and physiology of respiration.

While diving, marine mammals must balance the metabolic costs of active swimming (while hunting or migrating) with the conservation of limited oxygen reserves. Lungs are the organs involved in the oxygen assumption from the atmosphere and they present some adaptations which cannot be encountered in terrestrial animals.

2.1.1 Lung volumes

Lung capacities of marine mammals seem to be larger than terrestrial mammals especially if compared with the body mass index of these animals (G. L. Kooyman 1973).

In the case of *T. truncatus* for example, the lungs occupy 37% of the total thoracic cavity volume (M. A. Piscitelli 2010) and the importance of this lung size has been also related with an increase in buoyancy, which enables these mammals to rest at sea (G. L. Kooyman 1973).

The relatively large lung size of dolphins permits the lungs to be a site of respiratory gas exchange throughout a rapid breathing and short-duration shallow dives (M. A. Piscitelliet al. 2010), but not for deeper dives (see below).

Terminology of lung's volumes (figure 2.1):

- Inspiratory Reserve Volume (IRV): the maximal volume that can be inhaled from the end-inspiratory level;
- Tidal Volume (TV): that volume of air moved into or out of the lungs during quiet breathing;

- Expiratory reserve volume (ERV): the maximal volume of air that can be exhaled from the end-expiratory position;
- Residual Volume (RV): the volume of air remaining in the lungs after a maximal exhalation;
- Inspiratory capacity (IC): the sum of IRV and TV;
- Functional Residual Capacity (FRC): the volume in the lungs at the end-expiratory position;
- Vital Capacity (VC): the volume of air breathed out after the deepest inhalation;
- Residual Volume (RV): the volume of air remaining in the lungs after a maximal exhalation;
- Total Lung Capacity (TLC): the volume in the lungs at maximal inflation, the sum of VC and RV.

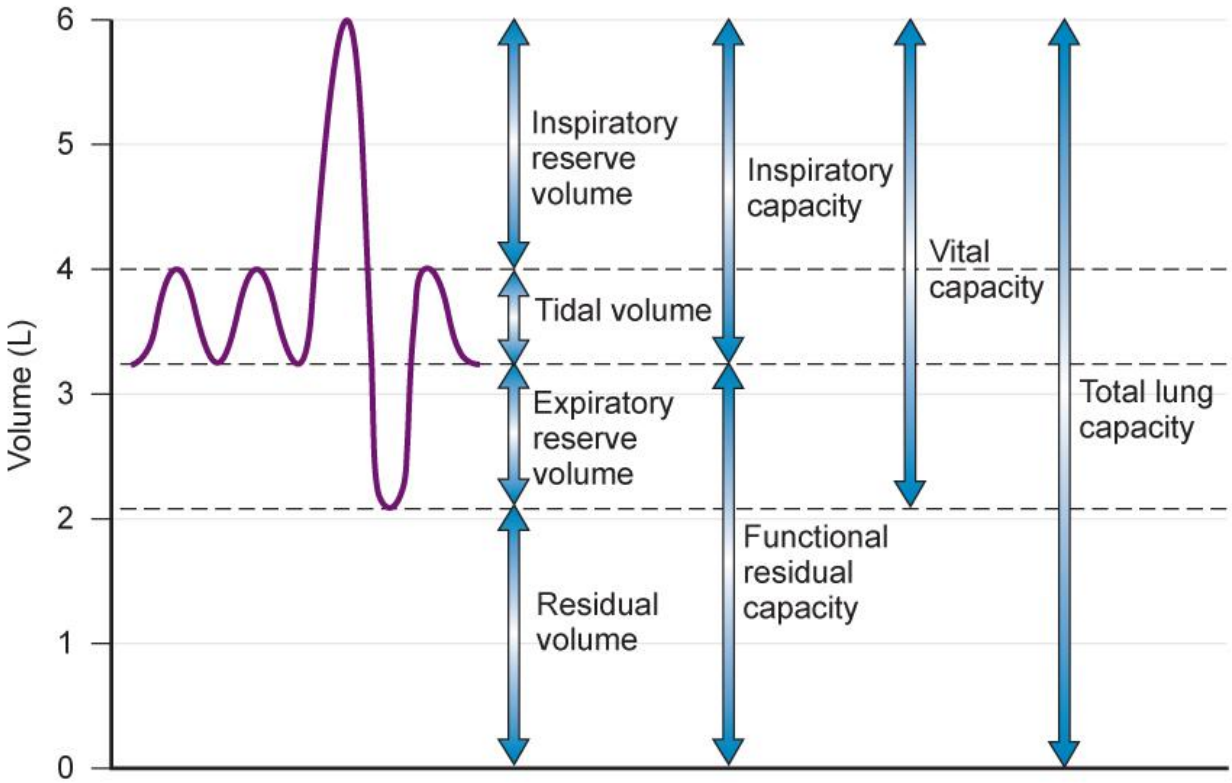


Figure 2.1: Lung volumes terminology (source The Cliveland Clinic foundation (www.clevelandclinicmeded.com)

2.1.2 Oxygen storage

The importance of the lung as an oxygen store seems to be relevant only to those species characterized by a low breath-hold tolerance. In fact, in their case the oxygen concentration available in the fully inflated lungs is from four times to equal than in the blood (G. L. Kooyman 1973). On the other hand, in those species with a large breath-hold tolerance the lung oxygen store is smaller.

2.1.3 Alveoli collapse

A lot of experiments indicate that during deep dives, gas exchange between blood and lungs is low, and one of the most reasonable suggestions is the collapse of the alveoli. All the breath-holding animals capable of deep dives experience alveolar collapse, from marine birds to marine mammals.

According to Pascal's and Boyle's laws, as depth increases, air volume into the lungs decreases (R. Resnick et al. 1966) (figure 2.2).

To study the depth at which alveolar collapse occur on a living marine mammal is not easy. It was first predicted from intramuscular nitrogen tensions measured in two bottlenose dolphins trained to complete a series of repetitive deep dives (up to 100 m depth). The results of this study suggested that alveolar collapse was complete at approximately 70 m (S. H. Ridgway et al. 1979).

After this first approach, more practical and based on field sampling investigation has been performed. A trained and free-swimming bottlenose dolphin appeared to experience alveolar collapse at a depth of approximately 80 m (R. C. T. Skrovan et al. 1999). It has been possible to observe it thanks to a time-depth recorder and video camera arranged on the dolphin's back.

At this depth in fact, the dolphin showed a different behaviour, it began gliding rather than actively swimming, and this change in locomotor behaviour, which has been measured also in other diving cetaceans (T. M. Williams et al. 2000) (D. P. Nowacek et al. 2001) (P. L. Tyack et al. 2006) (P. J. O. Miller et al. 2004), has been linked to reduced buoyancy due to compression of air into the lungs.

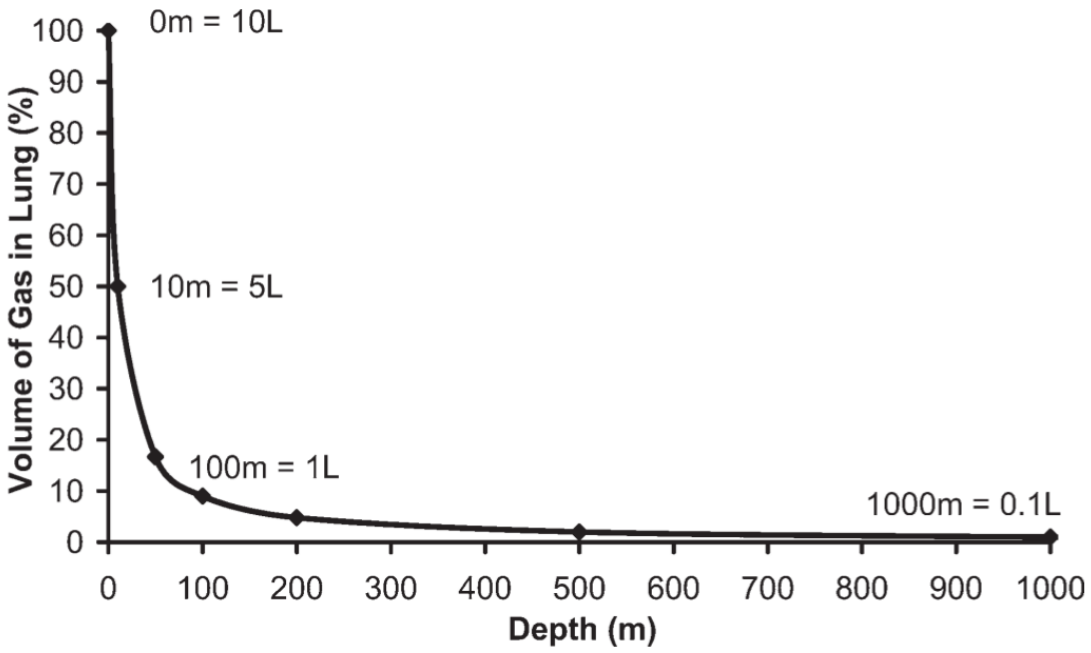


Figure 2.2: Relation between depth and gas volume. Note that air volume decreases by 50% within the first 10 m of descent; below 100 m the rate of change in air volume decreases dramatically (source A.Taylor 1994)

Alveolar collapse starts at depth as shallow as 10 m (P. Bagnoli et al. 2011), and is made possible by the particular adaptation of the upper airways in marine mammals.

Primary bronchus and bronchioles are more strong and stiffened (B. L. Bostroma et al. 2008) and these animals also have a very compliant rib cage (figure 2.3) which permits air from the alveoli to move into the rigid upper airways, once alveoli themselves collapsed because of pressure (P. F. Scholander 1940).

Recovering from lung shunt and alveoli collapse is also possible thanks to the presence of alveolar cells called pneumatocytes. There two different pneumatocytes: Type I and Type II.

While the Type I pneumatocytes are involved in the gas exchange between the alveoli and the blood and covering more than 90% of alveolar surface (K. Bensch et al. 1964), pneumatocytes Type II are involved in the secretion of pulmonary surfactant, important to decrease the alveolar surface tension (B. Rustow et al. 1993). It avoids any electrostatic attraction between the internal surface of the alveoli and, when external pressure allows it, air can easily flow back into the alveolar cavity.

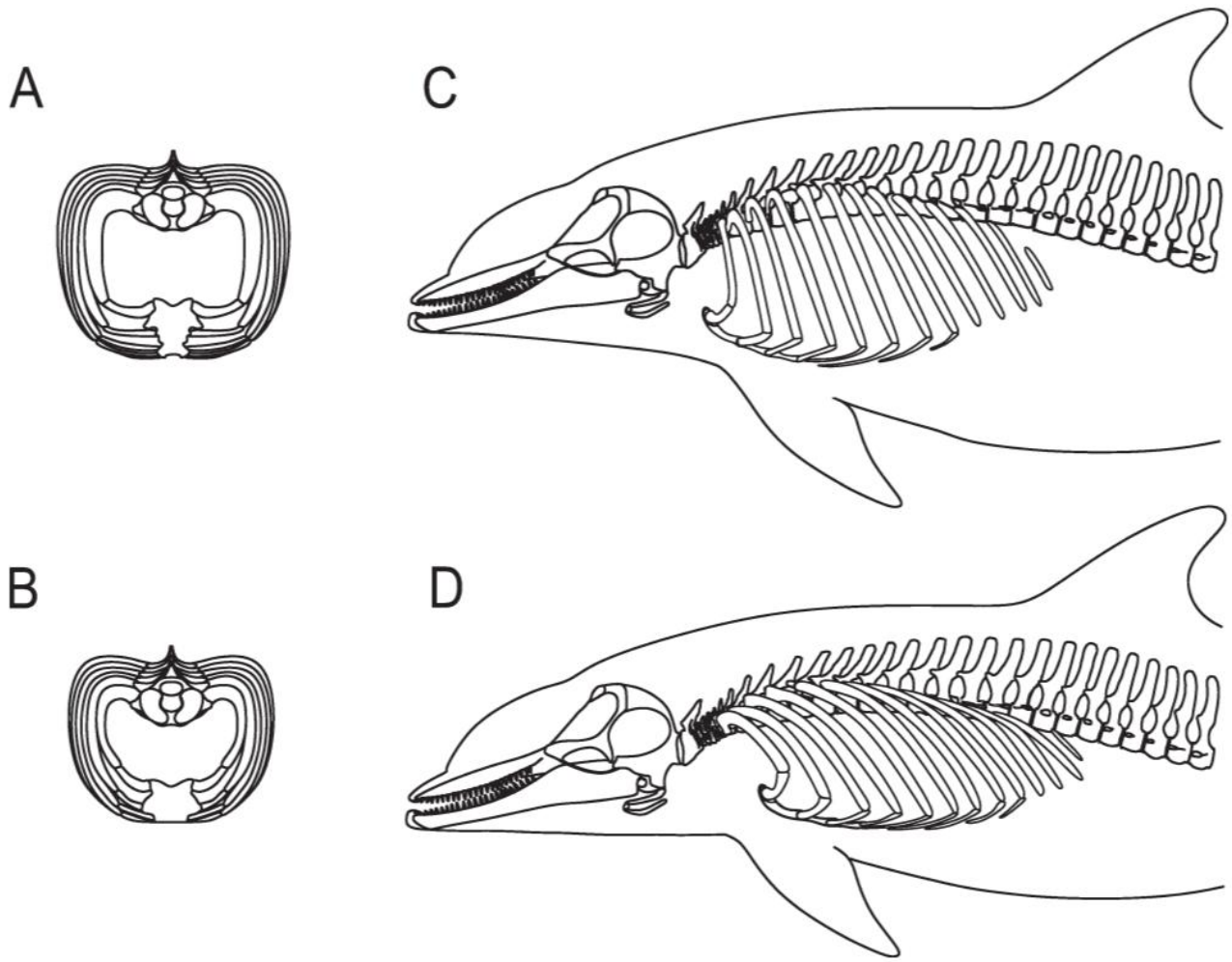


Figure 2.3: The range of thoracic mobility in T. truncatus. (A) and (C) show the most expanded posture, while (B) and (D) show the most collapsed posture (source P. B. Cotten et al. 2008)

Since gas exchange is not allowed on the upper airways, consequence of the alveoli collapse is the decrease of gas exchange rate (B. L. Bostroma et al. 2008).

For this reason, it is correct to assume that the contribution of the lung as an oxygen storage site is inversely related to dive depth and lung collapse not only effect availability of oxygen. The decreasing of gas exchange rate limits absorption of nitrogen too, which permits to blood nitrogen levels to remain below the threshold for decompression sickness.

Since deeper divers do not rely on their lungs as an oxygen store at depth, the lungs of deeper diving cetaceans should be smaller, in relation with the body mass, than those of shallow-diving species (P. F. Scholander, 1940).

2.1.4 Decompression sickness (DCS).

The air is a gas mixture made approximately by 78% nitrogen, 21% oxygen, 0.03% carbon dioxide and other rare gases (A. Poli et al. 2012). Even though most of animal organisms do not need nitrogen, it is absorbed by organs and tissues depending on its partial pressure in the atmosphere.

Breath holding animals like marine mammals or marine birds breathe air at 1 atm, so the gas pressure within the organism is balanced with the gas pressure of the atmosphere.

During a long diving session, nitrogen stored within the lungs will be absorbed ad higher concentrations (since the partial pressure increase because of deep). This event implies the increase of nitrogen concentration into the tissues of the animal. And It will of course decrease with decreasing of the pressure, while the animal returns to the surface (S. Gasparotto 2009).

The total value of absorbed nitrogen is due to depth and duration of dive: the deeper and longer these animals will dive, the more nitrogen will be accumulated in their body.

As already said, animals need oxygen for their metabolic process. Nitrogen is an inert gas which does not affect the metabolism and which can be eliminate through the breath ventilation.

Decompression sickness (also known as DCS) is well known among the divers all around the world. This sickness occurs because of the gas bubbles generated into the tissues (S. Gasparotto 2009).

While going back to the surface, environmental pressure decreases and the nitrogen previously absorbed by the lungs will be released as real gas bubbles. These bubbles can occur in any tissue, into the blood, the bones or the brain, with all the consequences that may follow.

As already discussed in paragraph 2.1.3, alveolar compression and pulmonary shunt help the provenience of these tissues, but it is not the only defence of these animals.

These animals know how dangerous can be to perform a rapid return to surface. Ethology studies' results suggests that some of these animals, like penguins (A. Poli et al. 2012) or marine turtles (M. E. Lutcavage et al. 1987) perform a repetitive dive pattern, characterised by a rapid descend and followed by a slow ascend (figure 2.4).

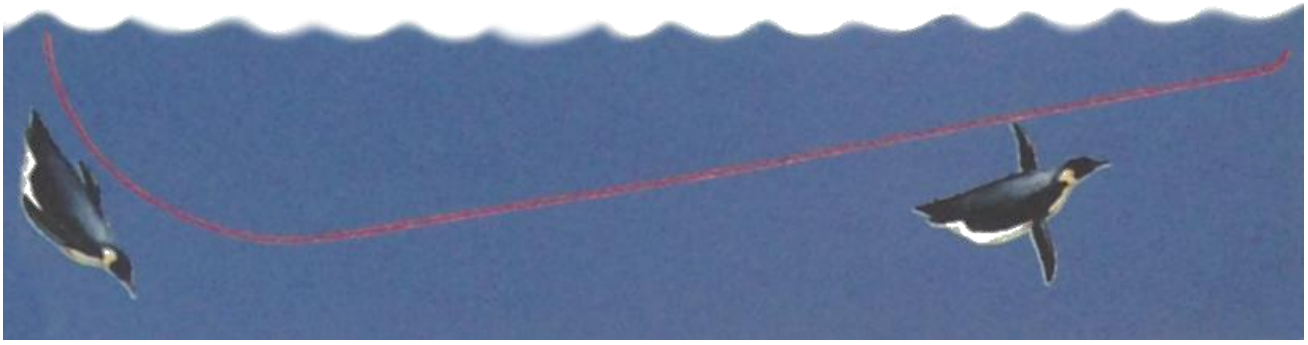


Figure 2.4: schematic dive pattern of the emperor penguin Aptenodytes forsteri (source "Fisiologia degli animali marini", A. Poli, E. Fabbri 2012)

2.1.5 Rete mirabile

Another more sophisticated instrument that breath-holding animals can use to avoid DCS is represented by the so called "Rete Mirabile" (Latin for "wonderful net"; plural retia mirabilia). It is a complex of arteries and veins lying very close one to each other (in a sort of net) which utilizes counter current blood flow and permits to exchange ions, heats, or gasses between vessel walls so that the two bloodstreams within the rete maintain a gradient with respect to temperature, or concentration of gases or solutes (C. J. Pfeiffer et al. 1990).

This thick and dense net of arteries also provides a spread of bigger gas bubbles in smaller and less dangerous ones (A. Poli et al. 2012), in fact one of this retia mirabilia is located near the most threatened organism by gas bubbles, the brain (E. L. Nagel et al. 1968) (figure 2.5).

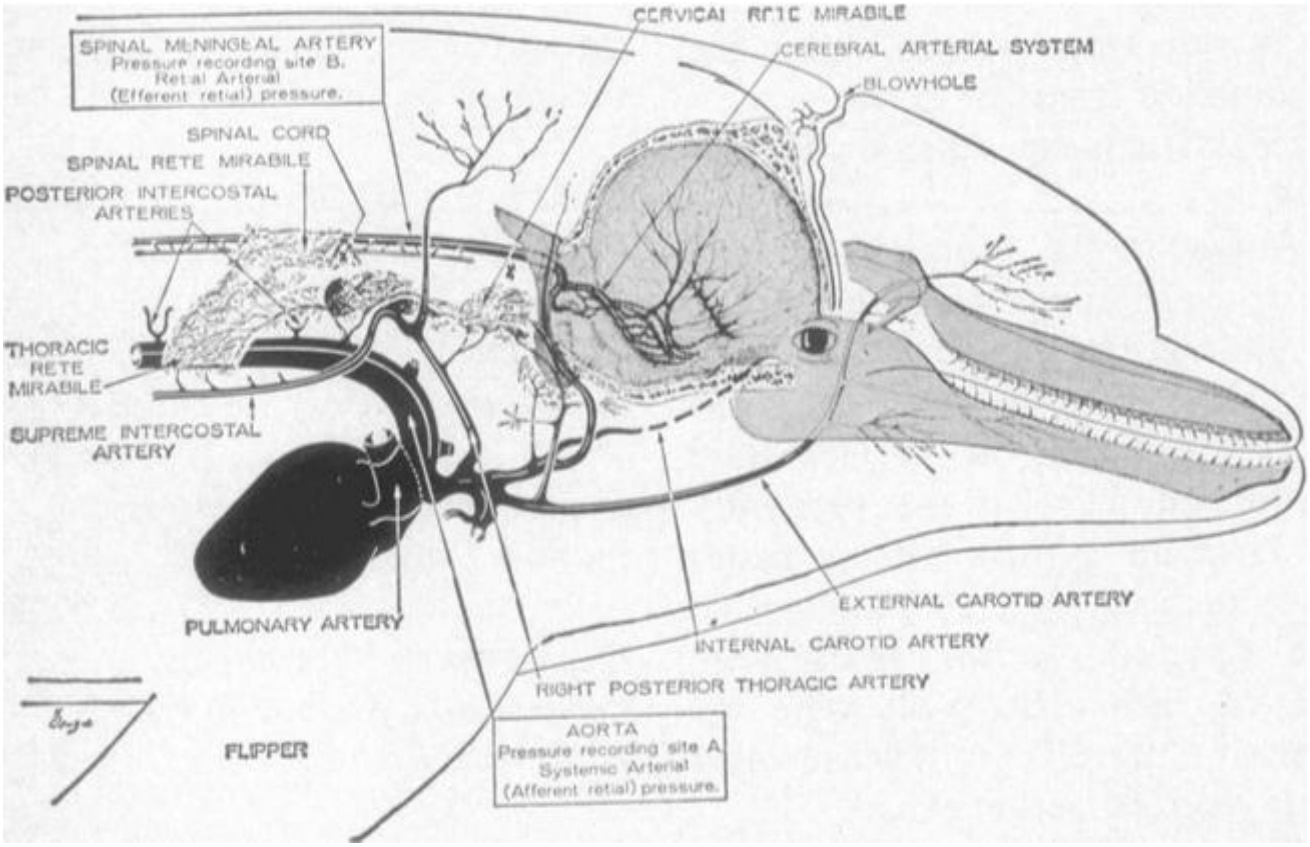


Figure 2.5: Scheme of the thoracic retial complex of bottlenose dolphin (source Nagel et al. 1968)

2.1.6 Other strategies for deep dives

Once realized the lung capacity is not strictly connected with diving possibility, the question is: how can these animals carry out these extreme performances? Which other physiological adaptations permit them to store enough oxygen to dive and hunt efficaciously?

Under these conditions, the animals must rely on other oxygen storage like blood muscles to support aerobic metabolism. It is known in fact that deeper diving species have larger blood volumes which permit to store more oxygen thanks to higher presence of haemoglobin, but these animals can also rely on another Oxygen-binding pigment: the myoglobin (M. L. L. Dolar et al. 1999). While the haemoglobin is related to the blood, myoglobin is found in muscle tissues in almost all mammals, but a higher myoglobin concentration is found in aquatic, air-breathing vertebrates (S. R. Noren et al. 2000). For

example, its concentration is 12 times higher in weddel seal (*Leptonychotes weddellii*) and 20 times higher in sperm whales (*Physeter macrocephalus*) than in humans (A. Poli et al. 2012).

Optimizing the use of both blood and muscle oxygen stores allows aquatic breath-holding and deep diver animals to perform long diving periods while holding their breath (R. W. Davisa 2004). More complex adaptation regards the entire cardio vascular apparatus of breath-holding animals to avoid (or at least postpone) the aerobic dive limit. To avoid waste of oxygen while performing long and deep dives, these animals can associate to reduction in heart rate (bradycardia) (A. S. Blix et al. 1983) an extreme peripheral vasoconstriction (P. J. Butler 1988).

During this phase, peripheral tissues and organs like muscles are cut of the blood stream (oxygen stored by the myoglobin is enough for their metabolism). In this way the blood stream is related only to the most important organs (like brain and heart) permitting to save energy and oxygen. In this way breath these aquatic animal can dive longer periods using oxygen store and aerobic metabolism.

That moment in which oxygen storage is exhausted is called aerobic dive limit (ADL) and from now on anaerobic metabolism starts, with the creation of great amount of lactic acid. Great concentrations of lactic acid causes acidosis, cramps and even lethal spasms. To escape this occurrence marine mammals must spend a long period of normal breathing after a long dive session. With a lengthened ventilation and thanks to Cori cycle, they can convert lactic acid in pyruvic acid.

2.2 *Tursiops truncatus*, model animal to be studied.

I focused my efforts to quantify the lung functions of the most common among all the cetaceans: the bottlenose dolphin *Tursiops truncatus* Montagu 1821 (figure 2.6).

It is presumably the most familiar of the small cetaceans for many reasons: its presence in many delphinariums and aquariums all around the world, its frequent appearance in media and movies and of course its costal occurrence (T. A. Jefferson et al. 2008).

There are three recognised subspecies of bottlenose dolphins: Atlantic bottlenose dolphin (*T.t. truncatus*), Pacific bottlenose dolphin (*T.t. gilli*) and Black Sea bottlenose dolphin (*T.t. ponticus*).

Figure 2.6: an example of bottlenose dolphin swimming (© Gaby Barathieu)

2.2.1 Scientific classification

Kingdom:	Animalia
Phylum:	Chordata
Subphylum:	Vertebrata
Class:	Mammalia
Order:	Cetacea
Suborder:	Odontoceti
Family:	Delphinidae
Genus:	*Tursiops*
Species:	*T.* truncatus
Subspecies #1:	*T. truncatus truncatus*
Subspecies #2:	*T. truncatus gilli*
Subspecies #3:	*T. truncatus ponticus*
Common name*:*	bottlenose dolphin

2.2.2 Body description

Bottlenose dolphin is characterized by its medium-sized, compact body, with a sharp short rostrum, the moderately curved dorsal fin and mouth's edge looking like a smile (figure 2.7).

Adult body mass goes from 220-500 kg and length ranges from 2-3.8 m, with geographical variation (D. Bloch et al. 2000). According to recent studies, body size seems to vary inversely with water temperature in many parts of the world (R. S. Wells et al. 2009).

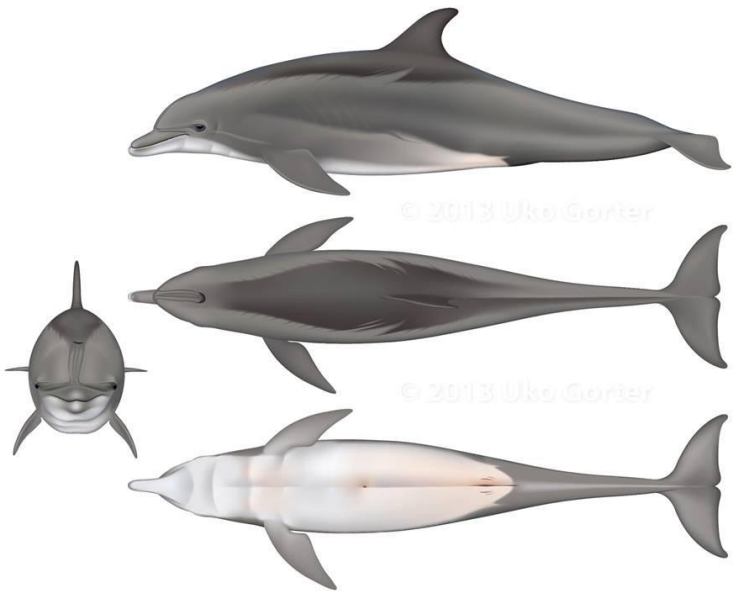


Figure 2.7: body scheme of bottlenose dolphin Tursiops truncatus (© Uko Gorter)

Pigmentation can change among individuals, but it is generally light grey to black shadows dorsally, with a light belly (P. S. Hammond et al. 2009).

2.2.3 Range description

Common bottlenose dolphins have a worldwide distribution through tropical and temperate latitudes (R. S. Wells et al. 2009) (figure 2.8). Even if primarily costal, bottlenose dolphins can also be found in pelagic waters (R. S. Wells et al. 2009). Generally, they do not exceed the 45°except in southern New Zealand and in northern Europe (C. Nichols et al. 2007). Bottlenose can be located close to Faroe Islands (62° N - 7° W) (D. Bloch et al. 2000) and sometimes in the Baltic Sea and in Norway (R. S. Wells et al.1999).

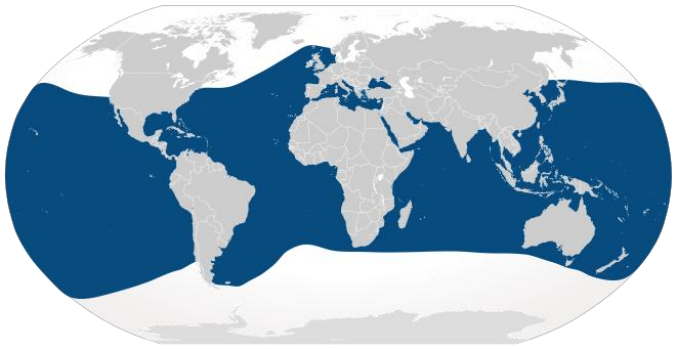


Figure 2.8: Tursiops truncatus range (©Cypron Map Series)

2.2.4 Habitat and ecology

As already said, common bottlenose dolphins can live in inshore and offshore oceanic waters where distinct ecotypes are known. (S. Leatherwood et al. 1990). They are commonly associated with many other cetaceans, including both large whales and other dolphin species (R. S. Wells et al. 1987).

While the offshore form is apparently less restricted in range and movement, in many inshore areas bottlenose dolphins maintain a multi-generational long-term home ranges, even if in some locations near the extremes of the species range they are migratory (M. Panayotova et al. 2015).

The inshore form frequents estuaries, bays, lagoons and other shallow coastal regions, occasionally ranging far up into rivers, on the other hand, most of offshore dolphins are residents around oceanic islands (D. Bloch et al. 2000).

They mostly inhabit waters with surface temperatures ranging from about 10°C to 32°C (R. S. Wells et al.1999) and they consume a wide variety of prey species, mostly fish and

squid (M. K. Stolen et al. 2002). They sometimes eat shrimps and other crustaceans (C. Blanco et al. 2001) (M.B. Santos et al. 2001).

2.3 Reason of this study

Even if arranged in a least concern by the IUCN red list, a deep knowledge of respiratory ability is important to work efficiently on conservational projects regarding “breath holding” animals.

Here an example: The global warming is a problem we cannot keep overlook. Due to increasing of temperature, animals all around the world are moving to seek new and more suitable regions (G. R. Walther et al. 2002). As regards oceans, the increasing of temperature at the surface force some groups of fish to move on deeper and colder water. Due to the fact dolphins feed on this group of fish, the question is: are they able to dive deeper enough to hunt this fish efficaciously? Their lung capacity permits them to keep feeding on these areas or do they have to move somewhere else?

2.3.1 Wild or captive?

From several years, few species of marine mammals have been held in captivity for educational, and business goals. It is our responsibility to conduct valuable research and combine the possibility to study these animals (e.g. veterinarian and physiology investigations) while giving the opportunity to everybody to enjoy them.

I developed this project after a 6 months’ experience at the Oceanogràfic research centre of Valencia. During my internship I had the great opportunity to work with these extraordinary animals. The aim of this study is to describe the lung capacity of these animals, their active volume exchange and frequency of respiration.

Nevertheless, due to small size of these animals (small if compared with other cetaceans like whales), the same data collection protocol can be applied with wild dolphins too, in fact I add to my results data collected by my co-workers who sampled wild animals in Florida. Once described both results (from captive and wild dolphins) my question is: is it worth to hold these animals in captivity for these kind of researches or can we obtain the same results from wild dolphins? This question can also be reconsidered: is it useful to bother

wild dolphins for these studies or is it better to involve only trained animals?

This work does not demand to answer all this question, but to set some step for further and deeper investigation.

3.AIM OF THE STUDY

The main part of this work is a statistical description (average and standard deviation) of lung volume capacity, generated flow, breath duration and breath frequency of each dolphin I tested at the Oceanogràfic of Valencia. I led the same analysis and I sought the same results with data collected from wild dolphins by the Sarasota Dolphin Research Program.

Once described both results (from captive and wild dolphins) my question is: is it worth to hold these animals in captivity for this kind of research or can we obtain the same results from wild dolphins? This question can also be reconsidered: is it useful to bother wild dolphins for these studies or is it better to involve only trained dolphins?

To answer these questions, I compared wild animals and captive animals with a 2-way analysis of variance. I also checked if different sizes of the animals or other characteristics could lead to different results.

4.MATERIALS AND METHODS

For this work I included only tests (or part of them) during which the animals were performing normal lung ventilation, without previous apnoea session, esophageal probe or other invasive devices and elements of disturb.

4.1Animals:

In this work included data collected from wild and captive dolphins, involving 8 different animals (7 *T. truncatus truncatus* and 1 *T. truncatus ponticus)* both male and female, and eventually I obtained 22 good trials. Due to differences in data collection I describe the two (captive dolphins and wild ones) separately.

4.1.1Captive dolphins:

Animals:

I worked along with trainers in order to obtain these data. In a total of 13 dolphins hold at the Oceanogràfic's delfinarium, 3 dolphins of different age and size were involved in this project: 2 male bottlenose dolphins (*Tursiops truncatus*) and 1 female of subspecies *Tursiops truncatus ponticus*.

I analysed a total of 15 trials

- F 1 → 4 tests
- M 2 → 6 tests
- M 3 → 5 tests

Data collection

Thanks to the trainers' efforts, these animals were trained to rest close to the rim of the pool, where trainers could seat and arrange the pneumotachometer above the blowhole. Thanks to this special training, participation by the dolphins was voluntary (the animals were not restrained and could refuse to participate or withdraw at any point during the spirometry test).

The animals I worked with were completely relaxed during tests. This approach allowed data collection on lung function and respiratory mechanics in dolphins that were in a relaxed and a normal physiological state (but still under a variety of circumstances). In fact, they allowed placement of the appropriate equipment without demonstrate annoyance. This is extremely important to have reasonable data because stress can effect lung ventilation.

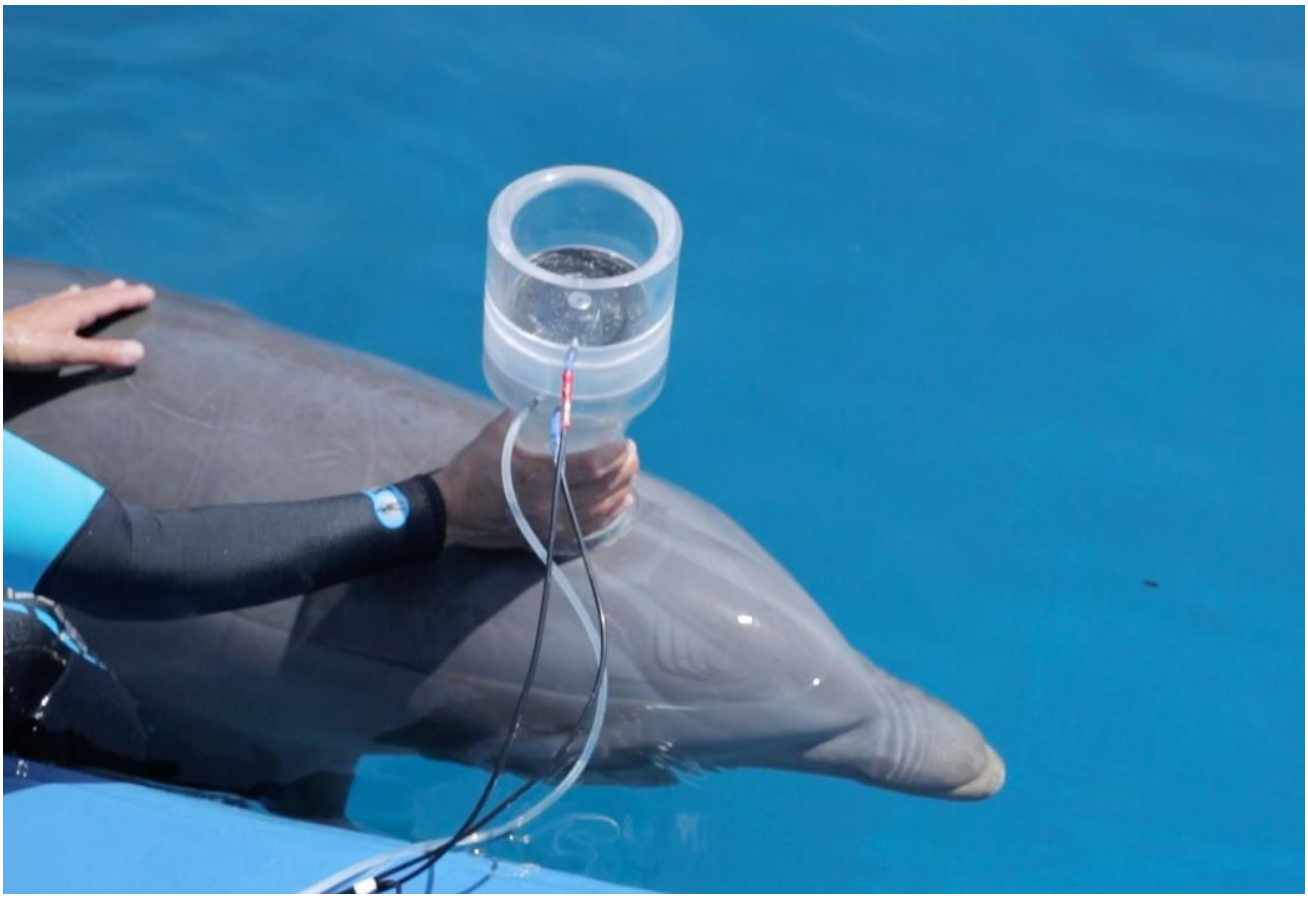


Figure 4.1: data collection at the Oceanogràfic's Delphinarium (© Fundacion Oceanogràfic)

Each experiment (trial) consisted of one animal asked for staying stationary in the water (rest position) close to the edge of the pool and breathing spontaneously into the equipment while continuous measurements were made (figure 4.1). In this way we wanted to collect data from each breath event.

During trial with captive dolphins it can happen that trainers miss some breaths: we called

these events “leak”. Leaks happen basically because of movement of the animals.

For every single breath event, we had to add a note into the software used to display the trial (LabChart, see paragraph 4.2) to indicate the start point of the event and the quality of it (“leak” or “good”).

At the end of every test, the pneumotachometer was cleaned with a 3% solution of and aldehyde based disinfectant (Korsolex® PAA, BODE Chemie GmbH, HARTMANN GROUP, Valencia, España), to avoid potential spread of bacteria or viral infections.

4.1.2 Wild dolphins:

Data from wild dolphins have been collected in Sarasota, Florida during May 2016. by the Sarasota Dolphin Research Program under National Marine Fisheries Service.

Animals

In the expedition research lead by Sarasota Dolphin Research Program, a total of 17 dolphins had been captured, including males, females and calves too. After a screening of all trials I chose trials collected from 2 male and 3 female adult dolphins for a total of 7 tests:

- F 209 → 1 test
- F 33 → 1 test
- M 178 → 2 tests
- M 188 → 2 tests
- F 233 → 1 test

Data collection

The same equipment has been used to test wild dolphins, but these animals were absolutely not trained to be studied. It obligated researchers to catch them with nests, restrain them but still free to breathe voluntarily into the equipment. These animals were captured also for other research projects, and therefore lifted on the deck.

Trials I used for this work had been collected while dolphins were restrained into the water

(figure 4.2), in order not to include differences on results coming from a dolphin breathing in or out the water (to avoid introduction of variance of results because of absence of water pressure on animal body).

Due to great stress these animals are subjected to and, seen how equipment can annoy these not trained animals, experimental design demands the collection of 50% of breath events, positioning the pneumotachometer every two breaths.

Nevertheless, researchers still noted every breath on the computer, in order to keep collecting data regarding breath frequency.

Figure 4.2: data collection with wild dolphins (©the Sarasota Dolphin Research Program under National Marine Fisheries Service, Scientific Research Permit No. 15543)

4.2 Equipment:

A custom-made Fleisch type pneumotachometer (Mellow Design, Miami, FL, USA, figure 4.3) utilizing a low-resistance laminar flow matrix (Z9A887-2, Meriam Process Technologies, Cleveland, OH, USA, figure 4.4) was placed over the blowhole of the dolphins. Differential pressure across the flow matrix was measured using a differential pressure transducer (MPX-2.5 mbar type 339/2, Harvard Apparatus, Holliston, MA, USA), connected to the pneumotachometer with two, 310 cm lengths of 2 mm i.d. firm-walled flexible tubing.

Figure 4.3: custom-made Fleisch type pneumotachometer (Mellow Design, Miami, FL, USA)

Figure 4.4: low-resistance laminar flow matrix (Z9A887-2, Meriam Process Technologies, Cleveland, OH, USA)

Breath events generate two different pressure patterns on the two sections of the pneumotachometer determinate by the flow matrix. During expiration there is a positive pressure between the flow matrix and the blow-hole (figure 4.5), while a negative pressure is recorded while inspiration expiration (figure 4.6). The pneumotachometer was calibrated using a 7.0 L calibration syringe (Series 4900, Hans-Rudolph Inc, Shawnee, KS, USA). The signal was integrated and the flow determined assuming a linear response between differential pressure and flow. The linear response of the pneumotachometer was confirmed by calibrating with the 7.0 L syringe immediately before and after each trial, through a series of pump cycles at various flows. The pump cycles allowed us to determinate the relationship between differential pressure and flows for the expiratory and inspiratory phases.

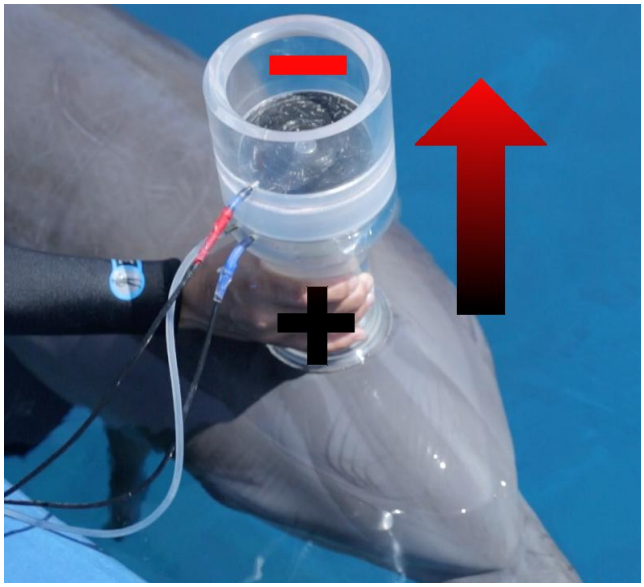


Figure 4.5: expiration scheme and flow direction (© Fundacion Oceanogràfic)

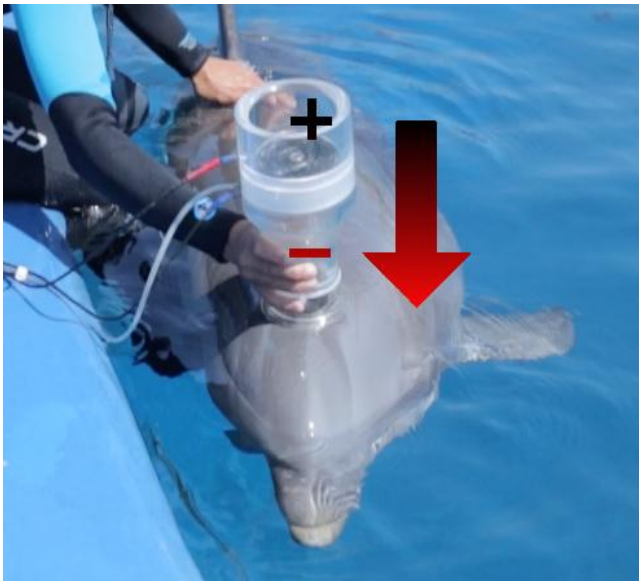


Figure 4.6: inspiration scheme and flow direction (© Fundacion Oceanogràfic)

4.2.1 Data acquisition of differential pressures

Differential pressure transducers were connected to an amplifier (Tam-A, Harvard Apparatus). The data from the transducers were captured at 400 Hz using a data acquisition system (Powerlab 8/35, ADInstruments, Colorado Springs, CO, USA), and displayed on a laptop computer running LabChart (v8.1.3, ADInstruments, figure 4.7). All differential pressure transducers were zeroed immediately before and after each trial.

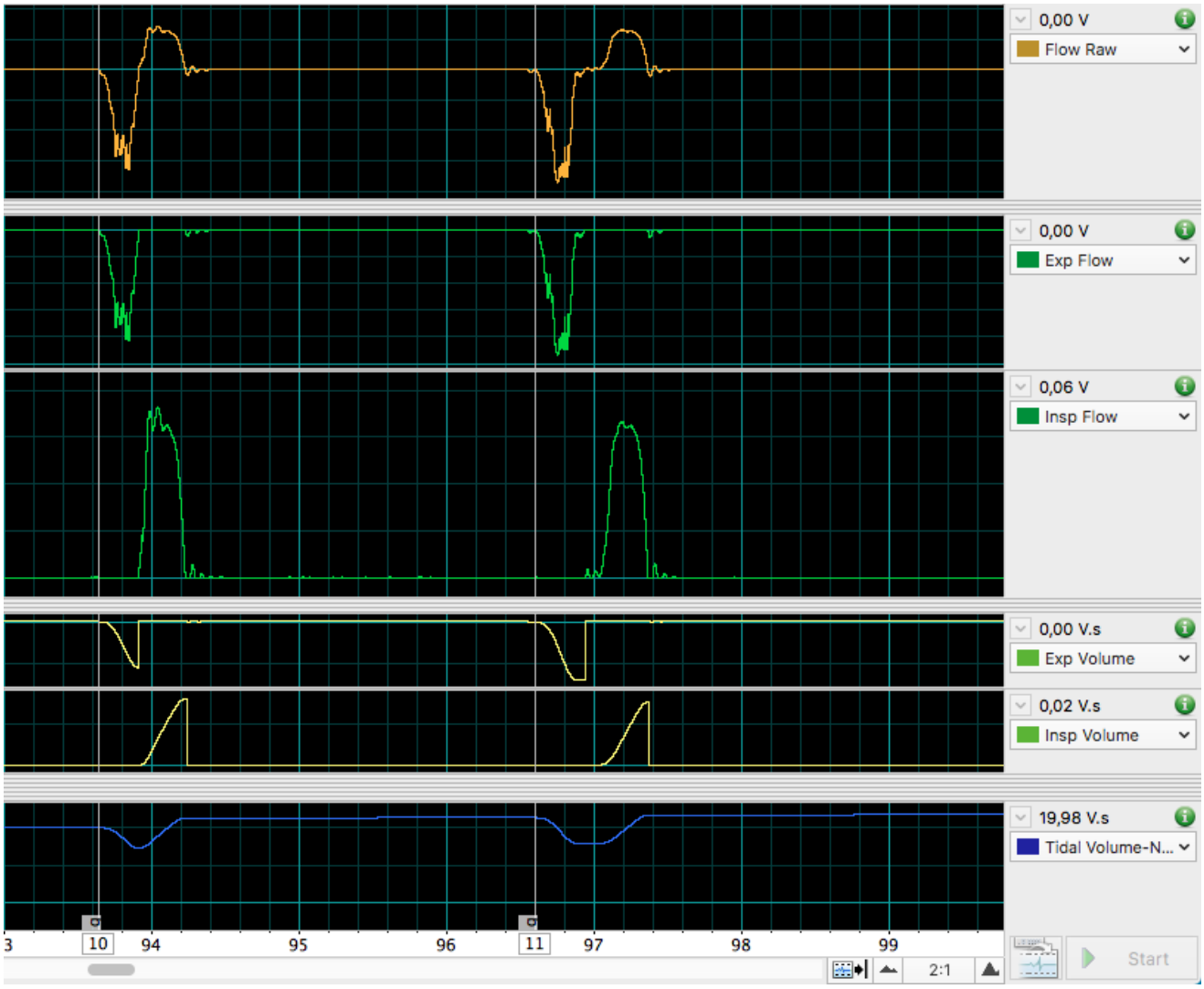


Figure 4.7: "Test number35-subject: F1 –Valencia Oceanogràfic" displayed on LabChart

4.3 Data processing and statistical analysis

All gas volumes were converted to standard temperature pressure dry STPD (P. H. Quanjer et al. 1993). During the trial analysis, led with LabChart, inhaled air volume was corrected for ambient temperature and relative humidity, while exhaled air was assumed saturated at 37°C. Once alienated all the channel and the note, it has been possible to obtain the data-pad and start working with Excel (graphs) and R software (statistical analysis).

4.3.1 Statistic design:

FACTORS:

- Factor A → 2 fixed level (captive and wild dolphins),
- Factor B(A) → random, individuals involved

REPLICATIONS:

- Each breath event collected.

VARIABLES:

- Even if software gives more, for this is study I took in consideration only 6 variables: Frequency (as breath-holding time expressed in seconds), Expiration and Inspiration Duration (s), Max Expiration and Max Inspiration Flow ($L*s^{-1}$), and Tidal Volume (L).

The presence of gaps (due to leaks event for captive dolphins or due to experimental design for wild dolphins) does not influence the breath frequency results, but does not allow to study frequency along with all the other variables (Duration, Flow, Volume). I lead two different analyses, reorganizing my DataPad in function of variables of interest.

I analysed my data using R software, and I began with a descriptive analysis, to obtain an average value for each one of variables for all the animals.

On the second part I sought differences in lung functions between animals. Potentials differences between wild and captive groups should be associated at different stress of the animals. I led a two-way ANOVA for each of my variables. In the following paragraphs I

reported the “Frequency” analysis as an example. All analysis led, R scripts and results in attachment (10. APPENDIX).

4.3.2 ANOVA assumptions

Before leading the analysis of variance, the ANOVA assumptions must be checked:
→ normal distribution: I verified this assumption with the Shapiro-Wilks test. When P value is >0,05 null hypothesise (H_0) can be accepted, which means that distribution of my data is normal.

```
> shapiro.test(br_holding_pause)

        Shapiro-Wilk normality test

data:  br_holding_pause
W = 0.83496, p-value < 2.2e-16
```

→ homogeneity of variance: I verified this assumption with the Bartlett test. When P value is >0,05 null hypothesis (H_0) can be accepted, which means that variances are homogeneous.

```
> bartlett.test(br_holding_pause~CW, data=Dolphin.frequency)

        Bartlett test of homogeneity of variances


data:  br_holding_pause by CW
Bartlett's K-squared = 18.642, df = 1, p-value = 1.577e-05
```

4.3.3 Analysis of variance - ANOVA

Analysis of variance (ANOVA) is a statistic technique which permits to compare two or more groups of data studying variability within groups and between groups. Thanks to this analysis the total variability of a dataset can be split into the different component of the dataset, to seek different sources of variances.

In other words, the objective of the ANOVA test is to analyse if there is a (statistically) significant difference in variables, between different animals or groups of animals in my dataset.

As already mentioned above, I am going to examine the relationship between:

X → "CW" (Captive/Wild) and "animals", which are my explanatory variables

Y → frequency, duration, flow, tidal volume, which are my response variables

ANOVA is going to compare means of variables among the animals, and check if differences are statistically significant. Here are my null and alternative hypothesis:

- Null Hypothesis: variables means for all animals are equal —> there is no relationship between animal's different means values, which we can write as follows:

H_0: $U_1 = U_2 = U_3 = U.. = U_n$

- Alternative Hypothesis: not all variables means for animals are equal —> there is a relationship animals different means values:

H1: not all U are equal

Hence, the question is: are the variations between the groups of animals means due to true differences about the variables means or just due to sampling variability?

To answer this question, ANOVA calculates a parameter called F statistic, which compares the variation among animals means to the variation within animals.

F statistics = Variation among sample means / Variation within groups.

Thanks to the F statistics we can see if the variation among sample means dominates over the variation within groups, or not.

For this work I used the R code "aov" to lead the ANOVA.

```
>anova.frequency<aov(br_holding_pause~CW*animal,data=
dolphinfrequency)
> summary(anova.frequency)
             Df Sum Sq Mean Sq F value   Pr(>F)
CW            1    636   635.5   5.308   0.0218 *
animal        6   8489  1414.8  11.816 3.54e-12 ***
Residuals   386  46218   119.7
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
```

4.3.4 Post hoc test

ANOVA does not tell me which groups (animal) are different from the others, so after the analysis of variance, I had to check which groups show a significant difference.

To determine which groups are different from the others I need to conduct a POST HOC TEST. These tests compare the results and seek differences which determinates differences shown by the ANOVA.

There are many post hoc tests available for analysis of variance, and for this work I choose the Tukey-Kramer test, also known as HSD test (Honestly Significant Difference) using the R code “HSD.test”:

```
> HSD.frequency<-HSD.test(br_holding_pause, CW:animal, 386,
119.7, group=TRUE)
> HSD.frequency
$statistics
      Mean       CV MSerror      HSD r.harmonic
  17.71625 61.72965   119.6 9.715195   23.53951

$parameters
   Df ntr StudentizedRange alpha  test    name.t
  385   8         4.310073  0.05 Tukey CW:animal

$means
             br_holding_pause       std   r     Min     Max
Captive:F 1          18.48407 12.701650  81  1.7750 71.4400
Captive:M 2          12.91204  6.509433 119  1.5900 36.0975
Captive:M 3          21.48939 17.933659  74  2.4625 78.8750
Wild:F 209           23.85568  9.481221  11 10.4475 38.0100
Wild:F 223           13.24364  6.079031  35  3.3650 26.0350
Wild:F 33            26.74312  3.733109   8 22.0300 33.8725
Wild:M 178           15.74708  5.061729  36  7.2375 27.1925
Wild:M 188           28.31550  7.620214  30 14.9725 43.5100

$comparison
NULL

$groups
           trt    means   M
1 Wild:M 188   28.31550   a
2 Wild:F 33    26.74312  ab
3 Wild:F 209   23.85568 abc
4 Captive:M 3  21.48939 abc
5 Captive:F 1  18.48407  bc
6 Wild:M 178   15.74708 bcd
7 Wild:F 223   13.24364  cd
8 Captive:M 2  12.91204   d
```

At a later time, I wanted to check if there are statistical differences between the two groups of animal: Captive and Wild:

```
> HSD.frequency<-HSD.test(br_holding_pause, CW, 386, 119.7,
group=TRUE)
> HSD.frequency
$statistics
      Mean       CV MSerror      HSD r.harmonic
  17.71625 61.72965   119.6 2.353766   166.9036

$parameters
   Df ntr StudentizedRange alpha  test name.t
  385   2         2.780549  0.05 Tukey     CW

$means
         br_holding_pause       std   r   Min    Max
Captive          16.87576 12.844346 274 1.590 78.875
Wild             19.63537  9.013641 120 3.365 43.510

$comparison
NULL

$groups
       trt    means M
1 Wild     19.63537 a
2 Captive  16.87576 b
```

4.3.5 Pearson correlation test

I eventually led a Pearson correlation coefficient test, which is a test giving a value between -1 and +1 included, and its aim is to describe a plausible linear dependence between two variables X and Y (where 1 is total positive linear correlation, 0 is no linear correlation, and −1 is total negative linear correlation).

For this test I included all the variables and the characteristics of dolphins and, once more, I led a different test for the frequency variable because of different datasets.

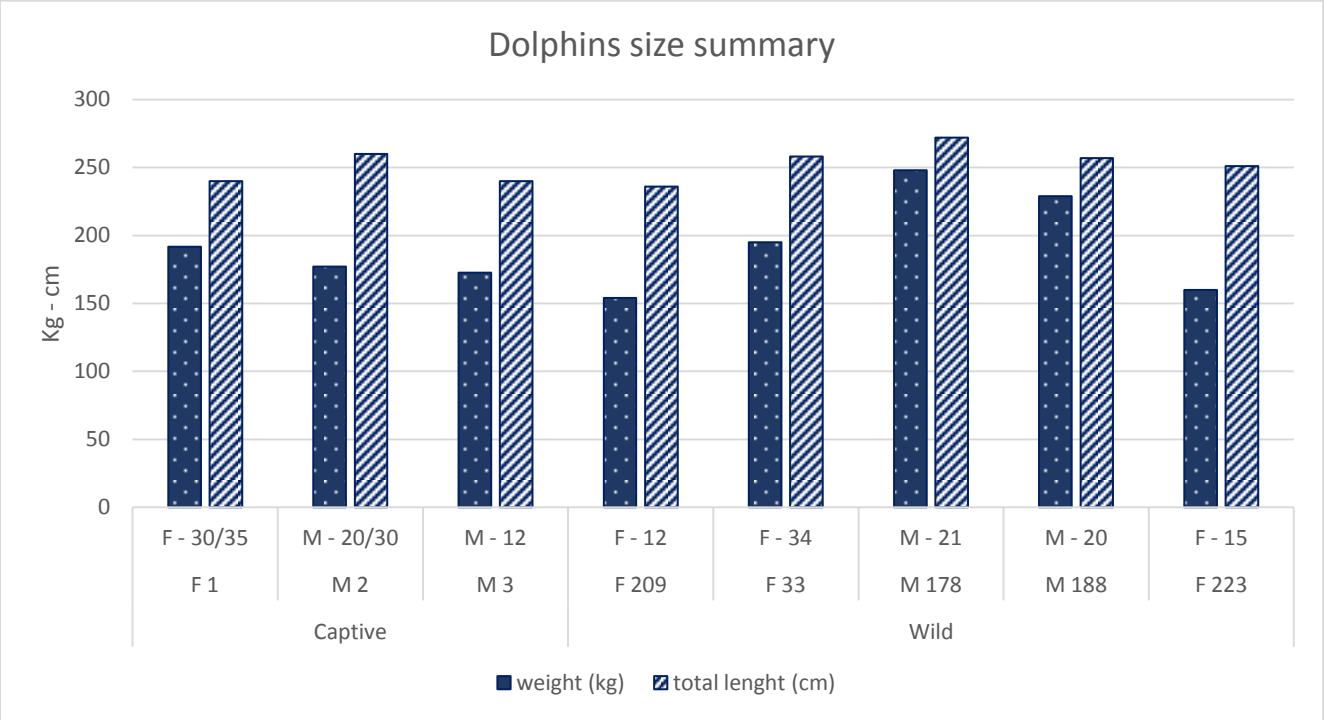
```
> corr_Pearson<-rcorr(cbind(CW, animal, sex, age.year,
weight.kg, total.lenght.cm, BMI.class, exp_dur,insp_dur,
exp_flow, insp_flow,tidal_vol),type="pearson")
> corr_Pearson
```

5.RESULTS

To comprehend how animals breathe, it is useful to know the characteristics concerning age, size and sex of the animals (table 5.1, graph 5.1, note[1]).

CW	Animal	Date of measurement	Sex	Age (year)	Weight (kg)	Total length (cm)	Body mass index	BMI class
Captive	F 1	March 2015	F	30/35	191,6	240	0,798	C
Captive	M 2	May 2016	M	25/30	177,2	260	0,682	B
Captive	M 3	May 2016	M	12	172,6	240	0,719	B
Wild	F 209	May 2016	F	12	154	236	0,653	B
Wild	F 33	May 2016	F	34	195	258	0,756	C
Wild	M 178	May 2016	M	21	248	272	0,912	D
Wild	M 188	May 2016	M	20	229	257	0,891	D
Wild	F 223	May 2016	F	15	160	251	0,637	A

Table 5.1: all dolphins ID



Graph 5.1: age, sex, weight and total length of animals tested

[1] Coefficient "BMI class" conversion:
0,6<x<0,7 → A
0,7<x<0,8 → B
0,8<x<0,8 → C
0,9<x<1 → D

5.1 General results

All graphs here reported are made on the averages of data recorded during the sampling phase. During this phase, in a total of 22 tests, 330 breath events have been collected through the pneumotachometer (table 5.2). Differences in breath collection efficiency (89.9% of captive dolphins and 51.64% of wild animals) are due to experimental design discussed in paragraph 4.

	Captive			Wild				
Animal	F1	M2	M3	F 209	F 33	M 178	M 188	F 223
Number of tests	4	6	5	1	1	2	2	1
Total breaths	92	126	79	13	9	39	32	35
Collected breaths	80	109	76	7	5	22	14	17
% of breath collection	87,0%	86,5 %	96,2 %	53,8 %	55,6%	56,4 %	43,8 %	48,6 %

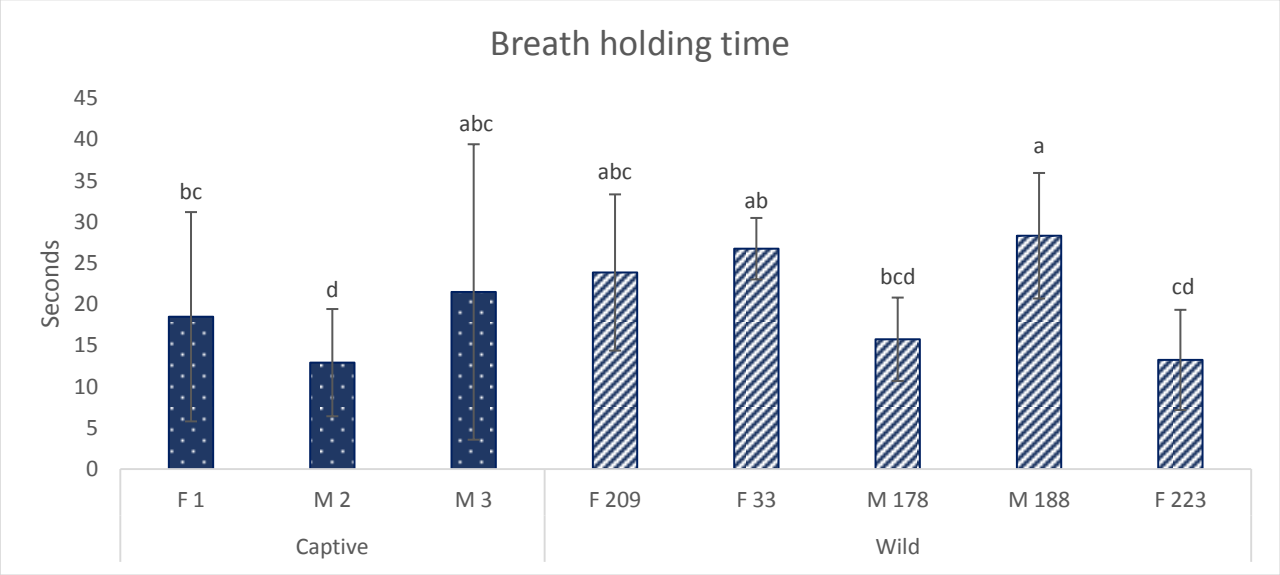
Table 5.2: distribution of trials and of breath events collected among the animals involved in this study

Averages of all the variables considered in this study are reported in table 5.3 with the respective standard deviation.

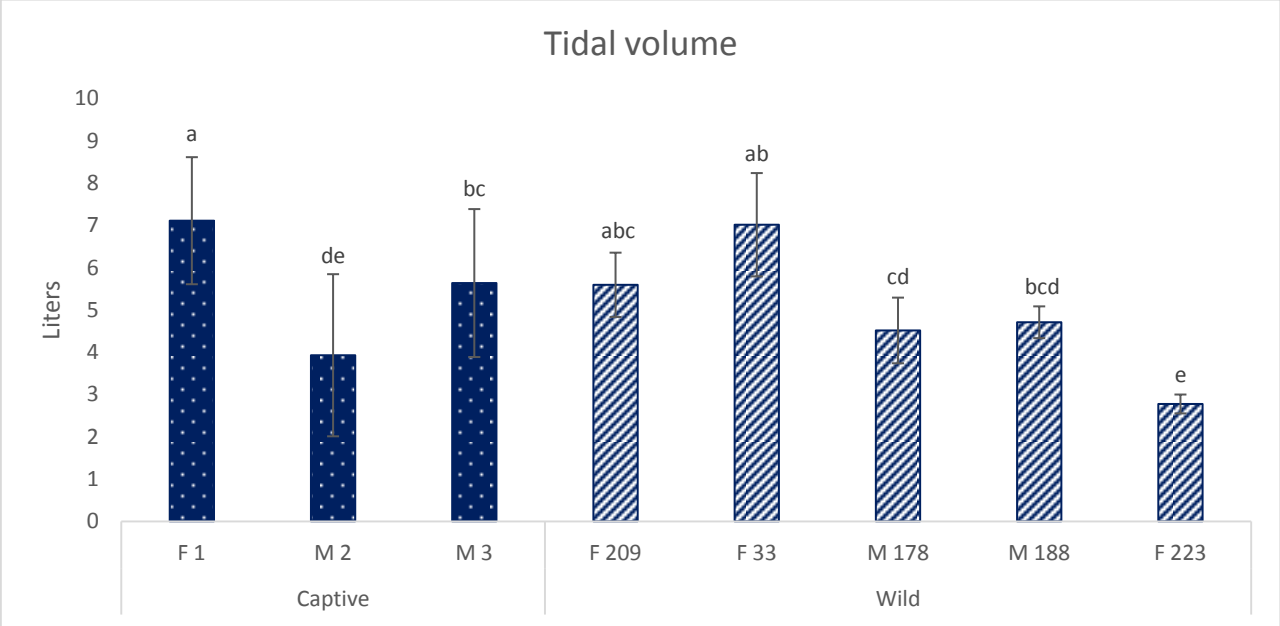
Animal	Br_holding_pause (s)	Exp_dur (s)	Insp_dur (s)	Exp_flow (L*s−1)	Insp_flow (L*s−1)	Tidal_vol (L)
F 1	18,48±12,70	0,34±0,06	0,36±0,07	-42,20±19,50	27,57±4,82	7,11±1,50
M 2	12,91±6,51	0,41±0,14	0,34±0,08	-17,73±8,21	15,25±5,24	3,93±1,92
M 3	21,49±17,93	0,50±0,16	0,47±0,12	-18,78± 5,64	17,27±5,27	5,64±1,75
F 209	23,86±9,48	0,44±0,13	0,61±0,23	-21,62± 2,67	15,29±1,22	5,59±0.76
F 33	26,74±3,73	0,44±0,05	0,57±0,08	-21,45± 3,14	17,31±2,02	7,01±1,22
M 178	15,75±5,06	0,40±0,04	0,52±0,22	-19,42± 3,49	13,55±2,62	4,51±0,78
M 188	28,32±7,62	0,42±0,07	0,44±0,04	-17,07± 2,09	15,21±1,53	4,71±0,38
F 223	13,24±6,08	0,47±0,14	0,47±0,22	-9,48± 0,84	12,05±2,58	2,78±0,22

Table 5.3: average and standard deviation of all variables taken in consideration for this study

Graph 5.2 shows that most of animals' frequencies are characterised by a considerable standard deviation. It confirms that frequency is an easily influenced variable, and that can explain why there are no relations between frequency and animals' characteristics.



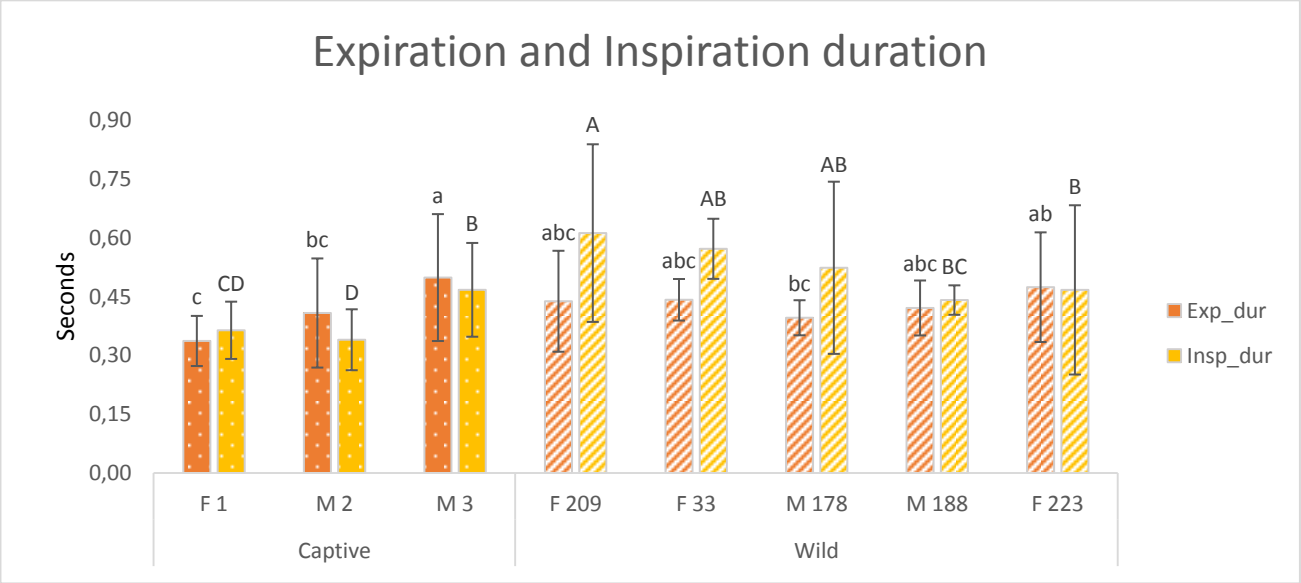
Graph 5.2: breath frequency of all animals tested



Graph 5.3: Tidal volume gas exchange of all animals tested

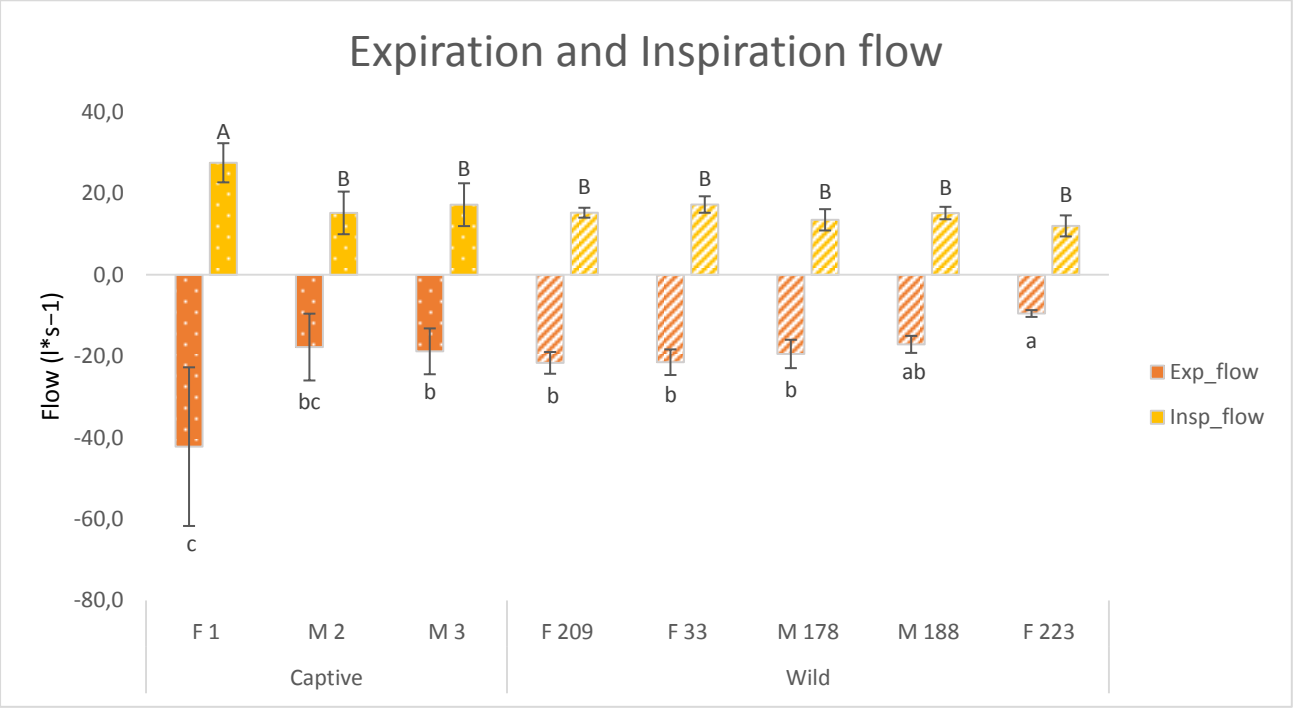
Tidal volume averages (graph 5.3) show that “F 223” which is the animal with the smaller Body Mass Index (BMI) is characterized by the smaller tidal volume capacity. Nevertheless, the other animals do not follow the same pattern.

Concerning inspiration duration (graph 5.4), the smaller values have been recorded for the captive dolphins, while no trend can be found regarding expiration duration. Age, size or sex do not influence the breath duration.



Graph 5.4: expiration and inspiration duration of all animals tested

Regarding flow generated during expiration and inspiration (graph 5.5), significant difference has been recorded between F1 animal and the other dolphins tested. Despite F1 is the only *Tursiops truncatus ponticus* tested, this fact can be of interest.



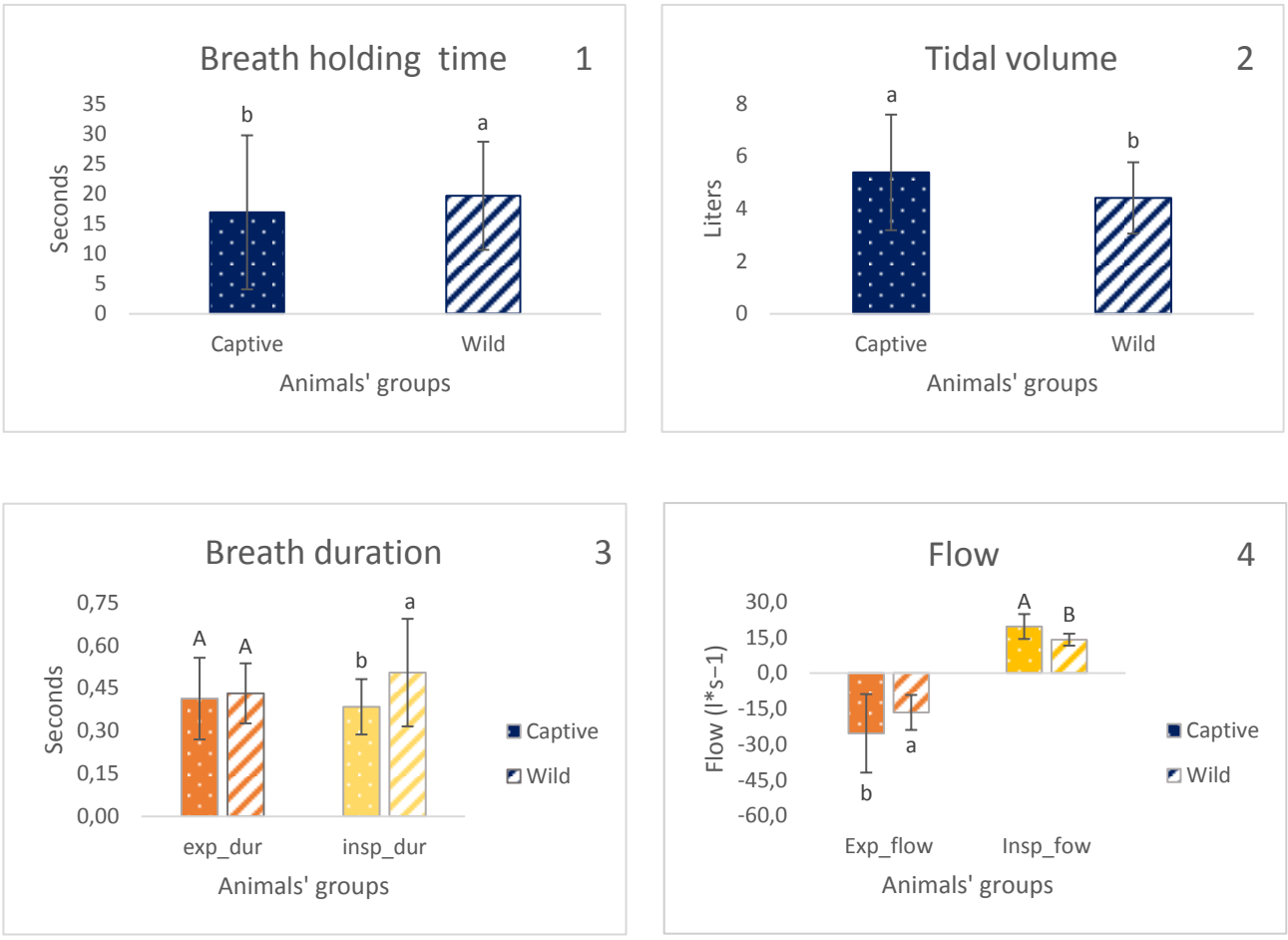
Graph 5.5: Expiration and inspiration flow of all animals tested

5.2 Differences Captive-Wild

All graphs here reported are made on the averages of data divided between Captive and Wild dolphins. Averages of all the variables considered in this study are reported in the table 5.4 with the respective standard deviation.

Groups	Br_holding_pause (s)	Exp_dur (s)	Insp_dur (s)	Exp_flow (L*s−1)	Insp_flow (L*s−1)	Tidal_vol (L)
Captive	16.88±12.84	0.41±0.14	0.38±0.11	-25.42±16.52	19.54±7.36	5.38±2.20
Wild	19.64±9.01	0.43±0.10	0.50±0.19	-16.62±5.24	14.01±2.50	4.41±1.36

Table 5.4: average and standard deviation of all variables taken in consideration divided between Captive and Wild dolphins



Graph 5.6: significant differences between captive and wild dolphins have been observed in breath frequency (1) tidal volume (2) and in both expiratory and inspiratory flow (4). The same has been observed with inspiratory duration but not for expiratory duration (3)

As regard the comparison between the two groups Captive and Wild (graph 5.6), the results show a significant difference among all the variables exception made for the expiration duration.

5.3 Pearson correlation test results

Frequency (table 5.5):

```
>  corr_Pearson<-rcorr(cbind(CW,  animal,  sex,  age.year,  weight.kg,
total.lenght.cm, BMI.class,br_holding_pause),type="pearson")
> corr_Pearson
```

All variables taken in consideration except for breath frequency (table5.6):

```
> corr_Pearson<-rcorr(cbind(CW, animal, sex, age.year, weight.kg,
total.lenght.cm, BMI.class, exp_dur,insp_dur,exp_flow, insp_flow,
tidal_vol),type="pearson")
> corr_Pearson
```

Table 5.5: Pearson correlation test results (frequency dataset)

	CW	animal	sex	age.year	weight.kg	total.lenght.cm	BMI.class	br_holding_pause
CW		0.0000	0.0029	0.0000	0.0000	0.0000	0.0000	0.0335
animal	-0.21		0.0000	0.0000	0.0282	0.0000	0.0000	0.7485
sex	-0.15	0.93		0.0000	0.0000	0.0000	0.2377	0.6852
age.year.	-0.27	-0.45	-0.28		0.0000	0.0000	0.0000	0.0045
weight.kg	0.46	-0.11	0.21	0.25		0.0000	0.0000	0.0238
total.lenght.cm	0.38	0.34	0.52	0.27	0.51		0.0000	0.0005
BMI.class	0.31	-0.25	0.06	0.33	0.93	0.25		0.0001
br_holding_pause	0.11	-0.02	-0.02	-0.14	0.11	-0.17	0.19	

Table 5.6: Pearson correlation test results (all variables except for frequency)

	CW	animal	sex	age.year	weight.kg	total.lenght.cm	BMI.class	Exp_dur	Insp_dur	Exp_flow	Insp_flow	Tidal_vol
CW		0.0019	0.0164	0.0003	0.0000	0.0000	0.0000	0.3335	0.0000	0.0000	0.0000	0.0009
animal	-0.17		0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.3595	0.0000	0.0000	0.0000
sex	-0.13	0.95		0.0000	0.6967	0.0000	0.0003	0.0000	0.9111	0.0000	0.0000	0.0000
age.year	-0.20	-0.55	-0.40		0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0201
weight.kg	0.47	-0.24	0.02	0.29		0.0000	0.0000	0.0015	0.1020	0.0052	0.1559	0.0534
total.lenght.cm	0.35	0.35	0.51	0.29	0.41		0.0491	0.5385	0.1067	0.0000	0.0000	0.0000
BMI.class	0.31	-0.44	-0.20	0.38	0.92	0.11		0.0000	0.2638	0.0000	0.0000	0.0000
Exp_dur	0.05	0.32	0.24	-0.39	-0.17	-0.03	-0.24		0.0000	0.0000	0.0000	0.0902
Insp_dur	0.35	0.05	0.01	-0.37	0.09	-0.09	0.06	0.36		0.9048	0.0540	0.0000
Exp_flow	0.23	0.56	0.51	-0.40	-0.15	0.37	-0.36	0.34	0.01		0.0000	0.0000
Insp_flow	-0.31	-0.57	-0.54	0.37	0.08	-0.49	0.32	-0.22	-0.11	-0.66		0.0000
Tidal_vol	-0.18	-0.38	-0.38	0.13	0.11	-0.48	0.32	0.09	0.28	-0.73	0.74	

6.DISCUSSION

During trials, all breath events have been noted on LabChart, and a total of 394 breath events have been used to study breath frequency. The average respiratory frequency during trials show a breath every 17.72±11.87 s (3.39 breaths*min^{-1}, range between 2.12-4.65 breaths*min^{-1}). Breath events are extremely fast. A total duration average of 0.82±0.22 s has been recorded. Inspiration events ranged from 0.34 to 0.61 s, while expiration events ranged from 0.34 to 0.50 s.

A total of 328 breath events have been collected through the pneumotachometer. Inspiratory flow ranged from 2.64 to 37.95 L*s^{-1} while expiratory flow ranged from 4.10 L*s^{-1} to 134.14 L*s^{-1}. The average maximum inspiratory flow of spontaneous breaths was 18.48±7.05 L*s^{-1}, which was a little bit lower than the maximum expiratory flow of 23.73±15.41 L*s^{-1} in absolute value. The animal with the higher expiration flow level was F1 animal (*T. truncatus ponticus*).

As regards the tidal volume I measured an average of 5.19±2.10 L of gas exchange during normal breath. Again, the animal with the largest tidal volume recorded during this study was F1 animal: 13.11 L).

Results concerning respiratory frequency, flow rate and tidal volumes are in accordance with a previous published study (see references: A. Fahlman et al. 2015).

6.1 ANOVA assumptions discussion

According to results of Shapiro-Wilks test and Bartlett test led for my variables, P value is never >0.05, so null hypothesis (H_0) cannot be accepted in both cases.

It means that distribution of my data is not normal and variances are not homogeneous, and this is probably due to the small size of samples.

If a dataset violates one or more ANOVA test assumptions, the results of the analysis may be misleading or incorrect. For example, if the assumption of normality is violated (or if outliers are present) the ANOVA may not be the most powerful test among all, and this

could influence the test while detecting a true difference among the population means (or not).

Employing a transformation of my tests did not change the results of these preliminary tests, and even if a nonparametric test may result more powerful, I decided to keep carry on with the ANOVA test. I will take in consideration these presuppositions while discussing the ANOVA results.

6.2 Analysis of variance - ANOVA discussion

Looking at the ANOVA results, I can see that p-value is <0.05 for almost all of my variables. Differences among all dolphins are statistically significant for all variables, while differences among Captive/Wild dolphins are statistically significant for all variables but “frequency” and “expiration duration”.

Looking at the F statistic results, I can deduce that the numerator (the variation of variables means among different animals and group of animals) is larger than the variation of variables within each test, hence I can conclude that for our confidence interval I accept the alternative hypothesis H_1 that there is a significant relationship between variables and animals tested and between variables and these two groups of animals (C/W).

What came to light from this test is that there are a lot of differences among animals, and it could influence results among groups of animals too.

Nevertheless, the small size of the data can also influence the ANOVA results, not only the preliminary tests. In other words, these results do not allow me to establish that testing trained animals can produce different results than data collected with wild dolphins.

6.3 Post hoc test discussion

As already said before, there are a lot of differences among animals. Concerning duration of breaths, expirations seems be faster as younger as the animals is.

Expiration and inspiration flows suggest that differences are due to subspecies. But since F1 animal is the only *T. truncatus ponticus* tested in this work, it is hard to assume it for certain, and more and deeper investigations are needed.

Regarding tidal volume, even if the smaller dolphin is the one characterized by the smaller volume gas exchange, there are no strong evidences that body max index influences this variable.

With this test I also checked differences between wild and captive animals.

Captive animals have a breath frequency faster than wild animals. Due to stress wild animals are forced during the experiment, I supposed to found a different result, but the slower breath frequency can be linked with the different lifestyle lead by wild animals. They are used to swim every day much more than captive animals, hunting and migrating, and that can influence their resistance and their performances (the same difference occurs between a well-trained athlete and a normal person).

Wilde and captive animals have the same breath expiration duration, but captive animals have a faster inspiration. Since inspiration is active and expiration is passive in these animals (Fahlman et al. 2015) this result could be linked with different breath between captive and wild animals, but this dataset cannot uncover the reason of this difference.

Also the expiration and inspiration flow show differences between captive and wild, but this is reasonably linked with the presence of a *T. truncatus ponticus* (F1) among captive animals, as already discussed before.

It came to light that, apart from expiration duration, which seems to be the same for both groups, all the other variables show statistical differences between these two groups. Once more time, the great variability among all dolphins, the unbalanced dataset and its small size can influence these results. We should also take in consideration the presence of one *T. truncatus ponticus* among the Captive dolphins, which can influence this result.

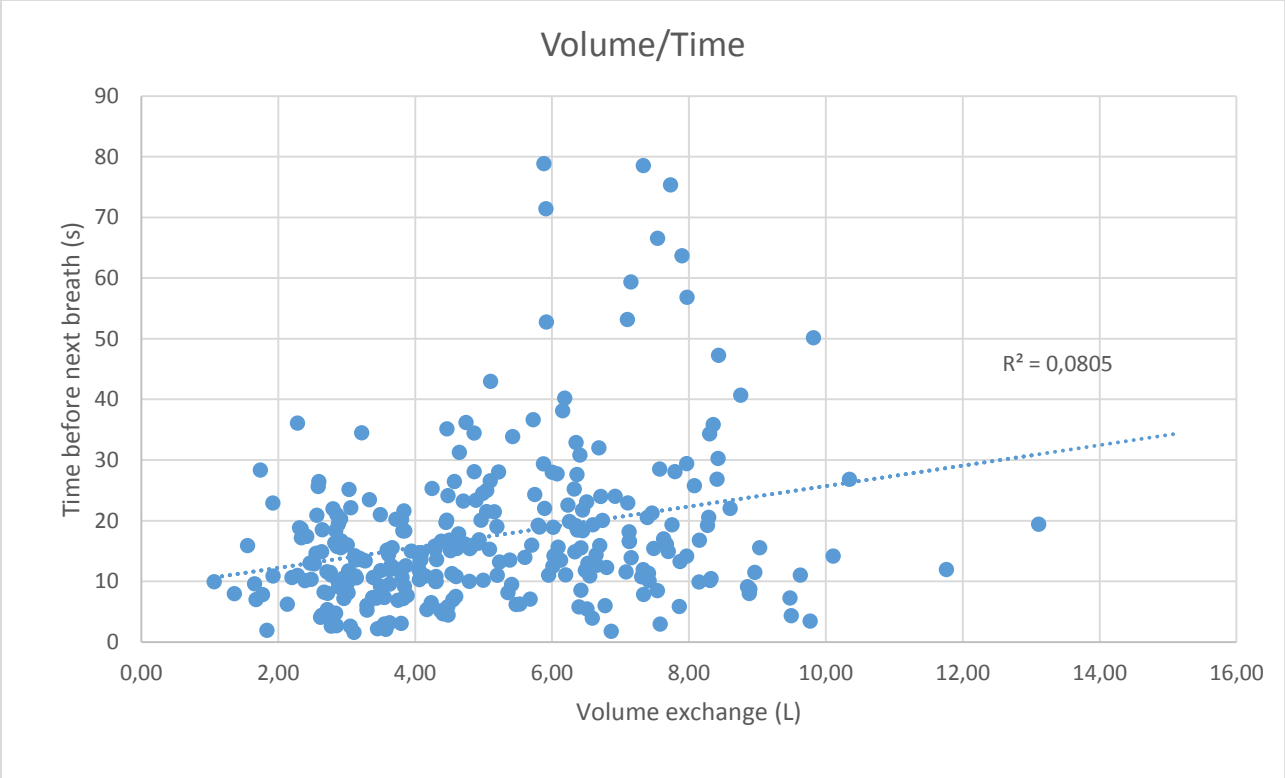
6.4 Pearson correlation test discussion

This last test has been led to seek correlations between all variables. Apart from variables which are obviously correlated, like expiration volume and tidal volume (-0.94) or inspiration volume and tidal volume (+0.92), this test does not show significant correlation between the other variables tested.

Breath frequency probably is the most easily influenced variable among variables taken in consideration. In fact, this test does not show correlation between frequency and any of the characteristics of animals (age, weight, length).

There is a small correlation between age and expiration duration (-0.39) and inspiration duration (-0,37). Total length and Tidal volume show a small relation (-0.48) too, as already shown before.

As a later consideration, it should be plausible to think that animals hold breath in function of the volume of air exchanged: the more air they exchange, the more they will wait before the next breath event. Despite there is a positive correlation between these two variables, which fits my hypothesis, this correlation is not significant (R^2 0,08) (graph 6.1).



Graph 6.1: X → volume of gas exchanged at (n) event, Y → time between (n) and (n+1) event. No significant relationship matched

7.CONCLUSIONS

Despite the interesting findings obtained with the ANOVA test, which show a statistical difference between captive and wild dolphins trials, and a potential relationship between some animals' characteristics (size and subspecies) and variables of interest, I am not going to draw any concrete conclusion from these data, and the question if it is worth to capture wild dolphins for these specific investigations still remains without an answer.

While it is impossible with such a poor model and dataset to obtain tangible conclusions to the main question of this work, I provide new data for respiratory physiology of bottlenose dolphin, and I guess we should take the descriptive results as a confirm and an implementation of previous studies led on this field of research, which could be useful for veterinarian efforts and conservation strategies.

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10 APPENDIX

5.1 ANOVA assumptions

Frequency:

```
> shapiro.test(br_holding_pause)

        Shapiro-Wilk normality test

data:  br_holding_pause
W = 0.83496, p-value < 2.2e-16

> bartlett.test(br_holding_pause~CW, data=Dolphin.frequency)

        Bartlett test of homogeneity of variances


data:  br_holding_pause by CW
Bartlett's K-squared = 18.642, df = 1, p-value = 1.577e-05
```

Expiration duration:

```
> shapiro.test(exp_dur)

        Shapiro-Wilk normality test

data:  exp_dur
W = 0.88735, p-value = 8.035e-15

> bartlett.test(exp_dur~CW,data=Dolphin.main)

        Bartlett test of homogeneity of variances

data:  exp_dur by CW
Bartlett's K-squared = 12.747, df = 1, p-value = 0.0003567
```

Inspiration duration:

```
> shapiro.test(insp_dur)

        Shapiro-Wilk normality test

data:  insp_dur
W = 0.81361, p-value < 2.2e-16

> bartlett.test(insp_dur~CW,data=Dolphin.main)

        Bartlett test of homogeneity of variances

data:  insp_dur by CW
Bartlett's K-squared = 41.957, df = 1, p-value = 9.331e-11
```

Max expiration flow:

```
> shapiro.test(exp_flow)

        Shapiro-Wilk normality test

data:  exp_flow
W = 0.77001, p-value < 2.2e-16

> bartlett.test(exp_flow~CW,data=Dolphin.main)

        Bartlett test of homogeneity of variances

data:  exp_flow by CW
Bartlett's K-squared = 80.841, df = 1, p-value < 2.2e-16
```

Max inspiration flow:

```
> shapiro.test(insp_flow)

        Shapiro-Wilk normality test

data:  insp_flow
W = 0.93502, p-value = 8.759e-11

> bartlett.test(insp_flow~CW,data=Dolphin.main)

        Bartlett test of homogeneity of variances

data:  insp_flow by CW
Bartlett's K-squared = 73.309, df = 1, p-value < 2.2e-16
```

Tidal volume gas exchange

```
> shapiro.test(tidal_vol)

        Shapiro-Wilk normality test

data:  tidal_vol
W = 0.96247, p-value = 1.811e-07

> bartlett.test(tidal_vol~CW,data=Dolphin.main)

        Bartlett test of homogeneity of variances

data:  tidal_vol by CW
Bartlett's K-squared = 18.992, df = 1, p-value = 1.313e-05
```

5.2Analysis of variance- ANOVA

Frequency:

```
> anova.frequency<-aov(br_holding_pause~CW*animal,data=Dolphin.frequency)
> summary(anova.frequency)
             Df Sum Sq Mean Sq F value   Pr(>F)
CW            1    636   635.5   5.308   0.0218 *
animal        6   8489  1414.8  11.816 3.54e-12 ***
Residuals   386  46218   119.7
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
```

Expiration duration:

```
> anova.main<-aov(exp_dur~CW*animal, data=Dolphin.main)
> summary(anova.main)
             Df Sum Sq Mean Sq F value   Pr(>F)
CW            1  0.017 0.01723   1.123     0.29
animal        6  1.081 0.18011  11.743 6.68e-12 ***
Residuals   320  4.908 0.01534
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
```

Inspiration duration:

```
>anova.main<-aov(insp_dur~CW*animal, data=Dolphin.main)
> summary(anova.main)
             Df Sum Sq Mean Sq F value   Pr(>F)
CW            1  0.733  0.7327   56.34 6.09e-13 ***
animal        6  0.961  0.1602   12.32 1.74e-12 ***
Residuals   320  4.161  0.0130
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
```

Max expiration flow:

```
> anova.main<-aov(exp_flow~CW*animal, data=Dolphin.main)
> summary(anova.main)
             Df Sum Sq Mean Sq F value   Pr(>F)
CW            1   3940    3940   31.46 4.41e-08 ***
animal        6  33648    5608   44.78  < 2e-16 ***
Residuals   320  40074     125
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
```

Max inspiration flow:

```
> anova.main<-aov(insp_flow~CW*animal, data=Dolphin.main)
> summary(anova.main)
             Df Sum Sq Mean Sq F value Pr(>F)
CW            1   1561  1561.2   71.49  1e-15 ***
animal        6   7710  1285.0   58.84 <2e-16 ***
Residuals   320   6988    21.8
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
```

Tidal volume gas exchange

```
> anova.main<-aov(tidal_vol~CW*animal, data=Dolphin.main)
> summary(anova.main)
             Df Sum Sq Mean Sq F value  Pr(>F)
CW            1   48.0   48.04   18.55 2.2e-05 ***
animal        6  564.8   94.14   36.36 < 2e-16 ***
Residuals   320  828.6    2.59
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
```

5.3 Post hoc test, all animals

Frequency:

```
> HSD.frequency<-HSD.test(br_holding_pause, CW:animal, 386, 119.7, group=TRUE)
> HSD.frequency
$statistics
      Mean       CV MSerror      HSD r.harmonic
  17.71625 61.72965   119.6 9.715195   23.53951

$parameters
   Df ntr StudentizedRange alpha  test    name.t
  385   8         4.310073  0.05 Tukey CW:animal

$means
             br_holding_pause       std   r     Min     Max
Captive:F 1          18.48407 12.701650  81  1.7750 71.4400
Captive:M 2          12.91204  6.509433 119  1.5900 36.0975
Captive:M 3          21.48939 17.933659  74  2.4625 78.8750
Wild:F 209           23.85568  9.481221  11 10.4475 38.0100
Wild:F 223           13.24364  6.079031  35  3.3650 26.0350
Wild:F 33            26.74312  3.733109   8 22.0300 33.8725
Wild:M 178           15.74708  5.061729  36  7.2375 27.1925
Wild:M 188           28.31550  7.620214  30 14.9725 43.5100

$comparison
NULL

$groups
           trt    means   M
1 Wild:M 188   28.31550   a
2 Wild:F 33    26.74312  ab
3 Wild:F 209   23.85568 abc
4 Captive:M 3  21.48939 abc
5 Captive:F 1  18.48407  bc
6 Wild:M 178   15.74708 bcd
7 Wild:F 223   13.24364  cd
8 Captive:M 2  12.91204   d
```

Expiration duration:

```
> HSD.exp_dur<-HSD.test(exp_dur, CW:animal, 320, 0.01534, group=TRUE)
> HSD.exp_dur
$statistics
       Mean       CV MSerror       HSD r.harmonic
  0.4161662 29.76089 0.01534 0.1411349   14.33842

$parameters
   Df ntr StudentizedRange alpha  test    name.t
  320   8         4.314916  0.05 Tukey CW:animal

$means
               exp_dur        std   r    Min    Max
Captive:F 1  0.3369375 0.06392982  80 0.1900 0.6000
Captive:M 2  0.4082339 0.13923705 109 0.2075 1.3025
Captive:M 3  0.4986184 0.16190038  76 0.1725 0.9725
Wild:F 209   0.4382143 0.12879016   7 0.3050 0.6925
Wild:F 223   0.4741176 0.13986026  17 0.2550 0.8525
Wild:F 33    0.4420000 0.05298585   5 0.3525 0.4775
Wild:M 178   0.3961250 0.04473221  20 0.3050 0.4900
Wild:M 188   0.4210714 0.07009413  14 0.3525 0.6250

$comparison
NULL
```

```
$groups
           trt     means   M
1 Captive:M 3  0.4986184   a
2 Wild:F 223   0.4741176  ab
3 Wild:F 33    0.4420000 abc
4 Wild:F 209   0.4382143 abc
5 Wild:M 188   0.4210714 abc
6 Captive:M 2  0.4082339  bc
7 Wild:M 178   0.3961250  bc
8 Captive:F 1  0.3369375   c
```

Inspiration duration:

```
> HSD.insp_dur<-HSD.test(insp_dur, CW:animal, 320, 0.0130, group=TRUE)
> HSD.insp_dur
$statistics
       Mean       CV MSerror       HSD r.harmonic
  0.4068369 28.02537   0.013 0.1299252   14.33842

$parameters
   Df ntr StudentizedRange alpha  test    name.t
  320   8         4.314916  0.05 Tukey CW:animal

$means
              insp_dur        std   r    Min    Max
Captive:F 1  0.3641250 0.07303448  80 0.1900 0.6850
Captive:M 2  0.3399771 0.07775458 109 0.1950 0.5950
Captive:M 3  0.4673355 0.11966086  76 0.2850 0.9850
Wild:F 209   0.6121429 0.22659356   7 0.3925 0.9650
Wild:F 223   0.4672059 0.21594430  17 0.2650 1.1200
Wild:F 33    0.5720000 0.07667708   5 0.4575 0.6475
Wild:M 178   0.5236250 0.21967002  20 0.3125 1.3575
Wild:M 188   0.4412500 0.03758004  14 0.3875 0.5125

$comparison
NULL

$groups
           trt     means  M
1 Wild:F 209   0.6121429  a
2 Wild:F 33    0.5720000 ab
3 Wild:M 178   0.5236250 ab
4 Captive:M 3  0.4673355  b
5 Wild:F 223   0.4672059  b
6 Wild:M 188   0.4412500 bc
7 Captive:F 1  0.3641250 cd
8 Captive:M 2  0.3399771  d
```

Max expiration flow:

```
> HSD.exp_flow<-HSD.test(exp_flow, CW:animal, 320, 125, group=TRUE)
> HSD.exp_flow
$statistics
       Mean        CV MSerror      HSD r.harmonic
  -23.72831 -47.11815     125 12.74021   14.33842

$parameters
   Df ntr StudentizedRange alpha  test    name.t
  320   8         4.314916  0.05 Tukey CW:animal

$means
               exp_flow        std   r       Min      Max
Captive:F 1  -42.202027 19.4965602  80 -134.1430 -15.5226
Captive:M 2  -17.730322  8.2103211 109  -52.2127  -4.1009
Captive:M 3  -18.776763  5.6368885  76  -38.1857  -6.4375
Wild:F 209   -21.616286  2.6714761   7  -24.3453 -16.9384
Wild:F 223    -9.478041  0.8372365  17  -10.8686  -8.0308
Wild:F 33    -21.450060  3.1432425   5  -26.6052 -18.2560
Wild:M 178   -19.420195  3.4861111  20  -26.1131 -13.8454
Wild:M 188   -17.070579  2.0939333  14  -19.9624 -13.4201
```

```
$comparison
NULL

$groups
           trt      means  M
1 Wild:F 223    -9.478041  a
2 Wild:M 188   -17.070579 ab
3 Captive:M 2  -17.730322 ab
4 Captive:M 3  -18.776763  b
5 Wild:M 178   -19.420195  b
6 Wild:F 33    -21.450060  b
7 Wild:F 209   -21.616286  b
8 Captive:F 1  -42.202027  c
```

Max inspiration flow:

```
> HSD.insp_flow<-HSD.test(insp_flow, CW:animal, 320, 21.8, group=TRUE)
> HSD.insp_flow
$statistics
      Mean      CV MSerror      HSD r.harmonic
  18.48066 25.2645    21.8 5.320468   14.33842

$parameters
   Df ntr StudentizedRange alpha  test    name.t
  320   8         4.314916  0.05 Tukey CW:animal

$means
            insp_flow      std   r     Min     Max
Captive:F 1   27.56518 5.265773  80 15.7922 37.9482
Captive:M 2   15.24656 4.816418 109  5.9098 28.9005
Captive:M 3   17.26553 5.239989  76  2.6447 37.9498
Wild:F 209    15.29310 1.216802   7 14.1466 17.5146
Wild:F 223    12.05164 2.020913  17  8.2048 16.6263
Wild:F 33     17.30656 2.617193   5 14.3210 20.7547
Wild:M 178    13.54809 1.531599  20 10.6493 16.2328
Wild:M 188    15.21160 2.584979  14 11.9162 21.7357

$comparison
NULL

$groups
           trt    means M
1 Captive:F 1  27.56518 a
2 Wild:F 33    17.30656 b
3 Captive:M 3  17.26553 b
4 Wild:F 209   15.29310 b
5 Captive:M 2  15.24656 b
6 Wild:M 188   15.21160 b
7 Wild:M 178   13.54809 b
8 Wild:F 223   12.05164 b
```

Tidal volume gas exchange

```
> HSD.tidal_vol<-HSD.test(tidal_vol, CW:animal, 320, 2.59, group=TRUE)
> HSD.tidal_vol
$statistics
     Mean      CV MSerror      HSD r.harmonic
  .191565 30.99928    2.59 1.833882   14.33842

$parameters
   Df ntr StudentizedRange alpha  test    name.t
  320   8         4.314916  0.05 Tukey CW:animal
```

```
$means
            tidal_vol       std   r     Min      Max
Captive:F 1   7.110006 1.5027878  80 4.29920 12.75865
Captive:M 2   3.927850 1.9172760 109 1.06310 13.10885
Captive:M 3   5.635255 1.7497061  76 2.35805  9.81870
Wild:F 209    5.593321 0.7610616   7 4.13780  6.34785
Wild:F 223    2.775518 0.2237973  17 2.29455  3.08965
Wild:F 33     7.014670 1.2219571   5 5.42370  8.60125
Wild:M 178    4.514482 0.7761526  20 3.43400  6.23145
Wild:M 188    4.708404 0.3753459  14 3.83545  5.21840

$comparison
NULL

$groups
           trt    means   M
1 Captive:F 1  7.110006   a
2 Wild:F 33    7.014670  ab
3 Captive:M 3  5.635255  bc
4 Wild:F 209   5.593321 bcd
5 Wild:M 188   4.708404 bcd
6 Wild:M 178   4.514482  cd
7 Captive:M 2  3.927850  de
8 Wild:F 223   2.775518   e
```

5.4 Post hoc test, Captive- Wild.

Frequency:

```
> HSD.frequency<-HSD.test(br_holding_pause, CW, 386, 119.7, group=TRUE)
> HSD.frequency
$statistics
      Mean       CV MSerror      HSD r.harmonic
  17.71625 61.72965   119.6 2.353766   166.9036

$parameters
   Df ntr StudentizedRange alpha  test name.t
  385   2         2.780549  0.05 Tukey     CW

$means
         br_holding_pause       std   r   Min    Max
Captive          16.87576 12.844346 274 1.590 78.875
Wild             19.63537  9.013641 120 3.365 43.510

$comparison
NULL

$groups
       trt    means M
1 Wild     19.63537 a
2 Captive  16.87576 b
```

Expiration duration:

```
> HSD.exp_dur<-HSD.test(exp_dur, CW, 320, 0.01534, group=TRUE)
> HSD.exp_dur

$statistics
       Mean       CV MSerror        HSD r.harmonic
  0.4161662 29.76089 0.01534 0.03415467   101.7988

$parameters
   Df ntr StudentizedRange alpha  test name.t
  320   2         2.782331  0.05 Tukey     CW

$means
           exp_dur        std   r    Min    Max
Captive  0.4126321 0.14308486 265 0.1725 1.3025
Wild     0.4310317 0.09704467  63 0.2550 0.8525

$comparison
NULL

$groups
       trt     means M
1 Wild     0.4310317 a
2 Captive  0.4126321 a
```

Inspiration duration:

```
> HSD.insp_dur<-HSD.test(insp_dur, CW, 320, 0.0130, group=TRUE)
> HSD.insp_dur
$statistics
       Mean       CV MSerror        HSD r.harmonic
  0.4068369 28.02537   0.013 0.03144193   101.7988

$parameters
   Df ntr StudentizedRange alpha  test name.t
  320   2         2.782331  0.05 Tukey     CW

$means
          insp_dur       std   r   Min    Max
Captive  0.3837925 0.1051492 265 0.190 0.9850
Wild     0.5037698 0.1885310  63 0.265 1.3575
```

```
$comparison
NULL

$groups
       trt     means M
1 Wild     0.5037698 a
2 Captive  0.3837925 b
```

Max expiration flow:

```
> HSD.exp_flow<-HSD.test(exp_flow, CW, 320, 125, group=TRUE)
> HSD.exp_flow
$statistics
       Mean        CV MSerror      HSD r.harmonic
  -23.72831 -47.11815     125 3.083135   101.7988

$parameters
   Df ntr StudentizedRange alpha  test name.t
  320   2         2.782331  0.05 Tukey     CW

$means
          exp_flow       std   r       Min     Max
Captive  -25.41812 16.517064 265 -134.1430 -4.1009
Wild     -16.62037  5.235798  63  -26.6052 -8.0308

$comparison
NULL

$groups
       trt     means M
1 Wild     -16.62037 a
2 Captive  -25.41812 b
```

Max inspiration flow:

```
> HSD.insp_flow<-HSD.test(insp_flow, CW, 320, 21.8, group=TRUE)
> HSD.insp_flow
$statistics
      Mean      CV MSerror      HSD r.harmonic
  18.48066 25.2645    21.8 1.287555   101.7988

$parameters
   Df ntr StudentizedRange alpha  test name.t
  320   2         2.782331  0.05 Tukey     CW

$means
         insp_flow      std   r    Min     Max
Captive   19.54442 7.362355 265 2.6447 37.9498
Wild      14.00614 2.503560  63 8.2048 21.7357

$comparison
NULL

$groups
       trt    means M
1 Captive  19.54442 a
2 Wild     14.00614 b
```

Tidal volume gas exchange

```
> HSD.tidal_vol<-HSD.test(tidal_vol, CW, 320, 2.59, group=TRUE)
> HSD.tidal_vol
$statistics
      Mean       CV MSerror       HSD r.harmonic
  5.191565 30.99928    2.59 0.4438001   101.7988

$parameters
   Df ntr StudentizedRange alpha  test name.t
  320   2         2.782331  0.05 Tukey     CW
```

```
$means
         tidal_vol      std   r     Min      Max
Captive   5.378172 2.201292 265 1.06310 13.10885
Wild      4.406630 1.356765  63 2.29455  8.60125

$comparison
NULL

$groups
       trt    means M
1 Captive  5.378172 a
2 Wild     4.406630 b
```

5.5 Pearson correlation test

```
> corr_Pearson<-rcorr(cbind(CW, animal, sex, age..year., weight..kg., total.lenght..cm., BMI.class,
exp_dur,insp_dur,exp_flow,insp_flow,tidal_vol),type="pearson")
> corr_Pearson
```

	CW	animal	sex	age.year.	weight.kg.	total.lenght.cm.	BMI.class	exp_dur	insp_dur
CW	1.00	-0.17	-0.13	-0.20	0.47	0.35	0.31	0.05	0.35
animal	-0.17	1.00	0.95	-0.55	-0.24	0.35	-0.44	0.32	0.05
sex	-0.13	0.95	1.00	-0.40	0.02	0.51	-0.20	0.24	0.01
age..year.	-0.20	-0.55	-0.40	1.00	0.29	0.29	0.38	-0.39	-0.37
weight.kg.	0.47	-0.24	0.02	0.29	1.00	0.41	0.92	-0.17	0.09
total.lenght.cm.	0.35	0.35	0.51	0.29	0.41	1.00	0.11	-0.03	-0.09
BMI.class	0.31	-0.44	-0.20	0.38	0.92	0.11	1.00	-0.24	0.06
exp_dur	0.05	0.32	0.24	-0.39	-0.17	-0.03	-0.24	1.00	0.36
insp_dur	0.35	0.05	0.01	-0.37	0.09	-0.09	0.06	0.36	1.00
exp_flow	0.23	0.56	0.51	-0.40	-0.15	0.37	-0.36	0.34	0.01
insp_flow	-0.31	-0.57	-0.54	0.37	0.08	-0.49	0.32	-0.22	-0.11
tidal_vol	-0.18	-0.38	-0.38	0.13	0.11	-0.48	0.32	0.09	0.28

	exp_flow	insp_flow	tidal_vol
CW	0.23	-0.31	-0.18
animal	0.56	-0.57	-0.38
sex	0.51	-0.54	-0.38
age.year.	-0.40	0.37	0.13
weight.kg.	-0.15	0.08	0.11
total.lenght.cm.	0.37	-0.49	-0.48
BMI.class	-0.36	0.32	0.32
exp_dur	0.34	-0.22	0.09
insp_dur	0.01	-0.11	0.28
exp_flow	1.00	-0.66	-0.73
insp_flow	-0.66	1.00	0.74
tidal_vol	-0.73	0.74	1.00

n= 328

P

	CW	animal	sex	age.year.	weight.kg.	total.lenght.cm.	BMI.class	exp_dur
CW		0.0019	0.0164	0.0003	0.0000	0.0000	0.0000	0.3335
animal	0.0019		0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
sex	0.0164	0.0000		0.0000	0.6967	0.0000	0.0003	0.0000
age.year.	0.0003	0.0000	0.0000		0.0000	0.0000	0.0000	0.0000
weight.kg.	0.0000	0.0000	0.6967	0.0000		0.0000	0.0000	0.0015
total.lenght.cm.	0.0000	0.0000	0.0000	0.0000	0.0000		0.0491	0.5385
BMI.class	0.0000	0.0000	0.0003	0.0000	0.0000	0.0491		0.0000
exp_dur	0.3335	0.0000	0.0000	0.0000	0.0015	0.5385	0.0000	
insp_dur	0.0000	0.3595	0.9111	0.0000	0.1020	0.1067	0.2638	0.0000
exp_flow	0.0000	0.0000	0.0000	0.0000	0.0052	0.0000	0.0000	0.0000
insp_flow	0.0000	0.0000	0.0000	0.0000	0.1559	0.0000	0.0000	0.0000
tidal_vol	0.0009	0.0000	0.0000	0.0201	0.0534	0.0000	0.0000	0.0902

	insp_dur	exp_flow	insp_flow	tidal_vol
CW	0.0000	0.0000	0.0000	0.0009
animal	0.3595	0.0000	0.0000	0.0000
sex	0.9111	0.0000	0.0000	0.0000
age.year.	0.0000	0.0000	0.0000	0.0201
weight.kg.	0.1020	0.0052	0.1559	0.0534
total.lenght.cm.	0.1067	0.0000	0.0000	0.0000
BMI.class	0.2638	0.0000	0.0000	0.0000
exp_dur	0.0000	0.0000	0.0000	0.0902
insp_dur		0.9048	0.0540	0.0000
exp_flow	0.9048		0.0000	0.0000
insp_flow	0.0540	0.0000		0.0000
tidal_vol	0.0000	0.0000	0.0000	

```
>   corr_Pearson<-rcorr(cbind(CW,   animal,   sex,   age..year.,   weight..kg.,   total.lenght..cm.,
BMI.class,br_holding_pause),type="pearson")
> corr_Pearson
```

	CW	animal	sex	age.year.	weight.kg.	total.lenght.cm.	BMI.class	br_holding_pause
CW	1.00	-0.21	-0.15	-0.27	0.46	0.38	0.31	0.11
animal	-0.21	1.00	0.93	-0.45	-0.11	0.34	-0.25	-0.02
sex	-0.15	0.93	1.00	-0.28	0.21	0.52	0.06	-0.02
age.year.	-0.27	-0.45	-0.28	1.00	0.25	0.27	0.33	-0.14
weight.kg.	0.46	-0.11	0.21	0.25	1.00	0.51	0.93	0.11
total.lenght.cm.	0.38	0.34	0.52	0.27	0.51	1.00	0.25	-0.17
BMI.class	0.31	-0.25	0.06	0.33	0.93	0.25	1.00	0.19
br_holding_pause	0.11	-0.02	-0.02	-0.14	0.11	-0.17	0.19	1.00

n= 394

P

	CW	animal	sex	age.year.	weight.kg.	total.lenght.cm.	BMI.class
CW		0.0000	0.0029	0.0000	0.0000	0.0000	0.0000
animal	0.0000		0.0000	0.0000	0.0282	0.0000	0.0000
sex	0.0029	0.0000		0.0000	0.0000	0.0000	0.2377
age.year.	0.0000	0.0000	0.0000		0.0000	0.0000	0.0000
weight.kg.	0.0000	0.0282	0.0000	0.0000		0.0000	0.0000
total.lenght.cm.	0.0000	0.0000	0.0000	0.0000	0.0000		0.0000
BMI.class	0.0000	0.0000	0.2377	0.0000	0.0000	0.0000	
br_holding_pause	0.0335	0.7485	0.6852	0.0045	0.0238	0.0005	0.0001

	br_holding_pause
CW	0.0335
animal	0.7485
sex	0.6852
age.year.	0.0045
weight.kg.	0.0238
total.lenght.cm.	0.0005
BMI.class	0.0001
br_holding_pause	