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REPRODUCTIVE PHYSIOLOGY OF THE RESIDENT POPULATION OF FIN WHALES IN THE GULF OF CALIFORNIA

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"REPRODUCTIVE PHYSIOLOGY OF THE RESIDENT POPULATION OF FIN WHALES IN THE GULF OF CALIFORNIA"

Presentada por el alumno:

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Apellido paterno	materno	nombre(s)

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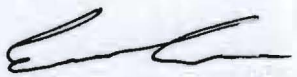

M en C. ERICA CARONE

Nombre y firma del alumno

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DEDICATION

Al mio piccolo Enea Tui,
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ακούω τον ήχο των σεισμών

κι από την γη που άνοιξε

περνάω στον κάτω κόσμο

να βρω όλα τα χαμένα

ίσως να βρω κι εσένα

Αχ, να σε δω, Ορφέας Περίδης

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GLOSSARY

Anestrus: Reproductive phase of not exhibiting estrus. Can occur both post calving and post breeding.

Antibody: Proteins of high molecular weight that are produced normally by specialized B cells after stimulation by an antigen and act specifically against the antigen in an immune response.

Capital breeders: Species that fuel reproduction from energy gained earlier and stored prior to use.

Blubber: Layer located between the epidermis and the fascia of the underlying muscle.

Corpus luteum: An endocrine structure that forms from the remnants of the ovarian follicle after the egg is released. The corpus luteum secretes progesterone and other progestins, which support the uterine lining in support for blastocyst implantation.

Cross reactivity: Antibody ability to have immunoreactivity with more than one antigen.

Enzyme-linked immunosorbent assay (ELISA): Assay where antigens immobilized on a microplate well are incubated with the sample and the concentration of the biomarker of interest quantified using an enzyme-linked anti-immunoglobulin antibody.

Estrus: the period during which female mammals will permit copulation

Extraction: Method by which components of interest in a sample are isolated, removed or concentrated, making it free of interference from other particulates found within the matrix (fecal, urine, blood or saliva).

Fatty acids (FA): Main component of lipids, composed by long-chain carboxylic acids subdivided into two categories based on whether they contain C=C double bonds: saturated fatty acids (without double bonds) and unsaturated fatty acids (with double bonds).

Field metabolic rate: Measurement of the metabolic rate of a free- living animal in the wild.

Glucocorticoids (GC): A class of steroid hormones produced in the adrenal cortex and involved in carbohydrate metabolism. The two primary glucocorticoids are cortisol and corticosterone, which are often released in response to stressful stimuli.

Income breeders: Species that fuel reproductive expenditure by simultaneous feeding.

Interbirth intervals: The time between the birth of two successive offspring or calves.

Lipids: Any of a class of organic compounds that are fatty acids or their derivatives and are insoluble in water but soluble in organic solvents. They include many natural oils, waxes, and steroids.

Metabolism: Biochemical processes that occur within a living organism. Most specifically to the breakdown of food and its transformation into energy.

Monounsaturated fatty acids (MUFA): Fatty acids that have one bond in the fatty acid chain with all of the remainder carbon atoms being single-bonded.

Monsoon: seasonal shift in the prevailing wind direction, that usually brings with it a different kind of weather.

Mysticetes: One of the two recent (non-fossil) cetacean suborders. They lack functional teeth compared with the other suborder Odontoceti, which are toothed whales. They feed on small marine organisms, by means of a highly specialized filter-feeding apparatus made up of baleen plates (“whalebone”) attached to the gum of the upper jaw. Furthermore, they have paired blowhole, symmetrical skull, and absence of ribs articulating with the sternum.

Neutral lipids: Molecules lacking charged groups. Triacylglycerols and steryl esters comprise the major part of neutral lipids. These storage lipids accumulate when cells are with an excess of nutrients.

Nutritional condition: State of body components (like fat and proteins) that influences an animal's future fitness.

Perfusion: The passage of blood, a blood substitute, or other fluid through the blood vessels or other natural channels in an organ or tissue.

Polar lipids: Molecules with polar or charged groups. They form the main component of cell membranes.

Polyunsaturated fatty acids (PUFA): Polyunsaturated fatty acids have two or more double bonds in its carbon chain, necessary to build cell membranes, the covering of nerves and proper blood clotting, muscle movement and inflammation. There are two main types of polyunsaturated fats: omega-3 fatty acids and omega-6 fatty acids. The numbers denote the distance between the beginning of the carbon chain and the first double bond.

Population: Geographical entity within a species, distinguished either ecologically or genetically.

Prostaglandins: Constitute a group of 20-carbon derived from fatty acids

Pseudopregnancy: A condition resembling pregnancy that occurs in some mammals, marked by persistence of the corpus luteum and usually following infertile copulation

Reproductive cycle: Period for all states of reproduction of an individual that in mammal females is the period from ovulation through conception and pregnancy to birth and lactation to the next ovulation.

Rorqual: Baleen whales distinguished by the presence of ventral grooves extending from the chin and lower jaw margins down the throat to the belly. These allow the mouth cavity to expand into the throat required to do gulp-feeding.

Specificity: In assay terminology, the ability of the antibody to discriminate between antigens.

Steroid hormones: Group of hormones with three six-carbon rings plus one conjugated five-carbon ring. In vertebrates, the precursor of all steroid hormones is cholesterol.

Stress: Reaction of the body to a factor that causes alteration to homeostasis.

Triacylglycerols (TAG): Molecules with a glycerol (carbohydrate) backbone attached to three acyl groups. They represent a concentrated source of metabolic energy.

Thyroid hormones: hormones that are produced by the thyroid gland and act to increase the basal metabolic rate, affect protein synthesis, and increase sensitivity to catecholamines.

RESUMEN

El rorcual común del Golfo de California constituye una población residente, genéticamente aislada del resto de la población del Pacifico Norte. Su pequeño tamaño poblacional y la escasa información disponible sobre sus dinámicas en un mar semicerrado, recalcan la importancia de realizar estudios sobre la reproducción y sus implicaciones fisiológicas. Debido a las variaciones oceanográficas del Golfo durante el año, se hipotizó una estacionalidad en la actividad reproductiva de esta población. Para lo anterior, se validaron y cuantificaron la progesterona y testosterona en 84 biopsias de rorcual común. Los lípidos neutros fueron separados del resto de los lípidos para investigar si la reproducción estacional tiene un efecto en el almacenamiento energético de esta población. Las hembras lactantes, que exhibieron bajas concentraciones de progesterona, no mostraron el contenido lipídico más bajo comparado con las otras categorías reproductivas. Esto sugiere que las hembras no ayunan durante esta etapa. Las hembras con elevada concentración de progesterona mostraron una marcada separación de las demás hembras y se consideraron potencialmente preñadas-en ovulación. El modelo estacional mostró un elevado pico de la testosterona en tardo verano. Este patrón fue soportado por el primer registro de comportamientos de cortejo y por la detección de una hembra preñada, con elevada progesterona, en verano y recapturada el año siguiente con una cría. Al igual que las poblaciones migratorias de misticetos, es probable que la reproducción sea estacional y que ocurra en verano/otoño, o sea durante el periodo con menor productividad. La presencia de una estacionalidad se vio reflejada en una diferencia significativa de los lípidos neutros en las hembras potencialmente preñadas-en ovulación. Por el contrario, en el análisis de componentes principales se observó un bajo porcentaje de explicación (40.27%), lo cual no permitió la separación de los ácidos grasos neutros por categoría reproductiva y temporadas. Estos resultados confirman el carácter estable de le capa más externa de la grasa subcutánea en termino de contenido lipídico y sugieren el uso de este tejido solo para inferir cambios de los depósitos de energía a largo plazo. Por último, se investigaron por primera vez las hormonas del estrés en la grasa subcutánea de rorcual común. Sin embargo, los resultados obtenidos no cumplieron con los requisitos de las pruebas de validación y la

cuantificación no se llevó a cabo. Este estudio representa un valioso aporte en las futuras políticas de gestión de esta población.

ABSTRACT

Fin whales in the Gulf of California constitute a resident population, genetically isolated from the rest of the North Pacific Ocean. Its small population size and the scarce information available about its dynamics in a semi-enclosed sea, underline the importance of conducting studies about its reproduction and its physiological implications. Given the variations of oceanographic conditions of the gulf through the year, I hypothesized a seasonality in the reproductive activity of this population. To test this, I validated and quantified progesterone and testosterone in 84 fin whale blubber samples. Blubber's neutral lipids (which serve as major energy storage form), were separated from the rest of the lipids to investigate whether seasonality has an effect in energy storage of fin whales. Lactating females, which exhibited low progesterone concentrations, did not have the lowest lipid content compared to other reproductive classes. This suggests that females do not fast during this state. Females with extremely high progesterone concentrations showed strong evidence of separation from the other females and were considered to be likely pregnant-ovulating. A seasonal model of testosterone concentrations showed a high peak during the late summer. This trend was supported by the first documentation of courtship events, and by the confirmation of a pregnant female with high progesterone concentration during summer and re-sighted with a calf the following year. The breeding in this fin whale resident population is likely to be seasonal, as it is in migratory baleen whales, but occurring during the summer/autumn, when the lowest productivity in the Gulf of California occurs. This seasonality was reflected by a significant difference of neutral lipids in likely pregnant-ovulating females. Conversely, the low percentage (40.27%) of explained variance in the principal component analysis did not allow the separation of neutral fatty acids profiles between reproductive categories and seasons. These results confirm the stable character of the outer blubber layer in terms of lipid content and suggest the use of this tissue only to infer long term changes in energy reserves. Finally, stress hormones were studied for the first time in fin whale blubber, but results did not comply with the parameters of the validation tests, and the quantification was not carried out. This study represents a valuable input to assist in future management policies of this protected population.

1. INTRODUCTION

Baleen whales have been historically considered to migrate every year between high-latitudes regions, where they spend feeding during the summer, to tropical or sub-tropical areas, called wintering grounds, which are used for mating and calving (Lockyer & Brown, 1981). Feeding activity takes place opportunistically also during winter (Gendron *et al.*, 2001; Lesage *et al.*, 2017), but biomass intake is lower than during summer (Lockyer, 1981a). Models of basal rate and energy storage indicated that fin whales obtain 60% of their food at high latitudes and 40% during the displacement between feeding and breeding grounds (Brodie, 1975). According to the seasonal migration pattern, a general fattening is observed in winter (detected by an increase of birth and the highest energy content of tissue)(Lockyer and Waters, 1986; Víkingsson, 1990; Víkingsson, 1995). Models suggest a doubling of body weight in humpback whales (*Megaptera novaeangliae*) and fattening of about 50 - 30 % of body weight in blue (*Balaenoptera musculus*) and fin whales (*Balaenoptera physalus*) during the Antarctic feeding season (Lockyer, 1981a). This increase on weight occurs in all reproductive classes and supports the long migratory journey and the entire reproductive process of these populations.

Under seasonal variation in food resources availability, the maximization of the feeding activity is essential for fertility. Studies in different species of mammals demonstrated that reproduction is activated only in better-nourished individuals, whereas, when the energetic costs of foraging exceed the calories gained, non-vital functions, like body growth and reproduction, are delayed or suppressed (Hileman *et al.*, 2000; Bronson, 2009). In females a minimum level of stored fat is necessary to activate ovulation and menstrual cycles, while in males interstitials cells regress and spermatogenesis does not take place (Widdowson, 1981; Frisch & McArthur, 2016). In marine mammals, and especially in cetaceans, it is difficult to investigate the direct effect of food availability on fertility. However, an increase of fecundity was detected in fin whale females by the observance of an active corpus luteum, over a period characterized by an increase in food abundance (Lockyer, 1986).

Proper storage of energy is also essential during the costly energy process of gestation. Pregnant baleen whales are, in fact, the reproductive class that gain the

largest amount of fat in southern winter feeding grounds (Lockyer, 1981; Vikingsson, 1990; Víkingsson, 1995). During this state, studies showed that females have a higher field metabolic rate due to the development of the fetus (Nordøy *et al.*, 1995). In addition, the consumption of large amounts of food in high-productivity areas, enables females to fast throughout the 6-7 months lactation period (Oftedal, 1997) when requires 3-5 times more energy than gestation (Young, 1976).

Capital breeding is the strategy to use energy stores accumulated prior to breeding. (Stephens *et al.*, 2006). Although this strategy is traditionally attributed a response to adverse environmental conditions for ectotherm organisms (Bonnet *et al.*, 1998), studies highlighted that the concept describes wide provisioning strategies both in ectotherm and endotherms (Drent, 1980). On the other hand, income breeders use energy that is acquired on a continual basis, including during the reproductive period (Stephens *et al.*, 2009). The infraorder of Cetacea shows a large range of possible strategies within each taxon. Thus, while most of the odontocetes, such as the sperm whale (*Physeter microcephalus*), are considered “income breeders” owing to a continuous feeding activity through the year (Irvine *et al.*, 2017), migratory species of mysticetes show a shift toward the reliability of energy gathered at some previous time, and stored until later use (Stephens *et al.*, 2006).

Advances in research techniques, such as satellite telemetry and genetic analyses, highlight the complexity of whale migration patterns and how boundaries between feeding and breeding are not always decidedly marked. Studies showed individuals suspending their migration and remaining at either high or low latitudes throughout the year (Payne and Webb, 1971; Aguilar, 2002; Geijer *et al.*, 2016). Fin and blue whales have been observed year round et their probable breeding grounds off the Azores (Silva *et al.*, 2013) Costa Rica Dome (Reilly & Thayer, 1990), as well as at their summer feeding area in the California Current System (Scales *et al.*, 2017; Busquets-Vass *et al.*, 2017). Fin whales were also reported in each hemisphere at higher latitudes (50° - 60° north or south) during colder months, indicating that not all individuals performed seasonal migrations (Edwards, 2015). Finally, different studies described the existence of genetically distinct resident populations of mysticetes (Fujino, 1960; Best, 1977; Bérubé *et al.*, 2002).

All these discoveries reveal a complex general framework of cetacean dynamics and raise questions on the strategies adopted by mysticetes that do not fit in traditional patterns. The permanence of a group of individuals in a given area may be related to the presence of available food throughout the year and this may drive the adoption of distinct reproductive strategies. To better understand those dynamics diverting from traditional movement patterns and reproductive strategies, studies should be focused on ascertained resident populations.

To date, reproductive strategies in populations of resident baleen whales are still unclear. In the Mediterranean Sea, the occurrence of fin whale newborns year-round, with a peak between September and January, suggests a breeding season not well defined for that resident population (Notarbartolo di Sciara *et al.*, 2003). Similarly, year-round calving and ovulation frequency on the inshore Bryde's whales (*Balaenoptera edeni*) off South Africa, proved that this resident population is an aseasonal breeder (Best, 2001). In contrast, the resident humpback whale population in the Arabian Sea showed seasonal reproductive habits lasting from January to May (Mikhalev, 1997).

All these observations underline that more research is needed on these resident populations of mysticetes and how the mechanisms connecting reproduction and fat metabolism remain poorly understood. In the present dissertation, reproductive aspects of the resident fin whales of the Gulf of California (Mexico) were investigated for the first time to determine its reproductive strategy and associated physiological aspects. To do this, sex steroid hormones were quantified in blubber biopsies to assess 1) reproductive categories in females and 2) seasonal changes in males. To examine the effect of such reproductive strategy on the nutritional condition, lipid content in the blubber was quantified for each reproductive class founded in this study. Finally, a section was dedicated to an explorative study on hormones involved in possible nutritional stress responses with a focus on resident habits.

2. BACKGROUND

2.1. Fin whale

The fin whale is the second largest of the rorquals included in the family Balaenopteridae, reaching lengths of 27m (Mackintosh, 1942). Currently, the Society of Marine Mammalogy accepts the existence of three subspecies: *B. p. physalus* in the Northern Hemisphere (Linnaeus, 1758), *B. p. quoyi (*Fischer, 1829) in the Southern Hemisphere, and the pygmy fin whale, *B. p. patachonica (*Burmeister, 1865). Nevertheless, recent genetic studies indicate that the North Pacific and North Atlantic fin whales may not be the same subspecies (Archer *et al.*, 2013) and further genetic analyses are being reviewed to make the case for a fourth new subspecies (NOAA, 2019).

Although fin whales are a cosmopolitan mysticete (Edwards, 2015), this species showed a gap in distribution at the equator (20°N - 20°) during the whaling era as well as during the post-whaling era (Fig. 1). The data show a preference for offshore waters of the temperate and subpolar zones and although they feed mainly on euphausiids and copepods (Nemoto, 1959), it is considered a generalist species that includes schools of herring, capelin and anchovies in its diet (Mitchell, 1975; Kawamura, 1982).

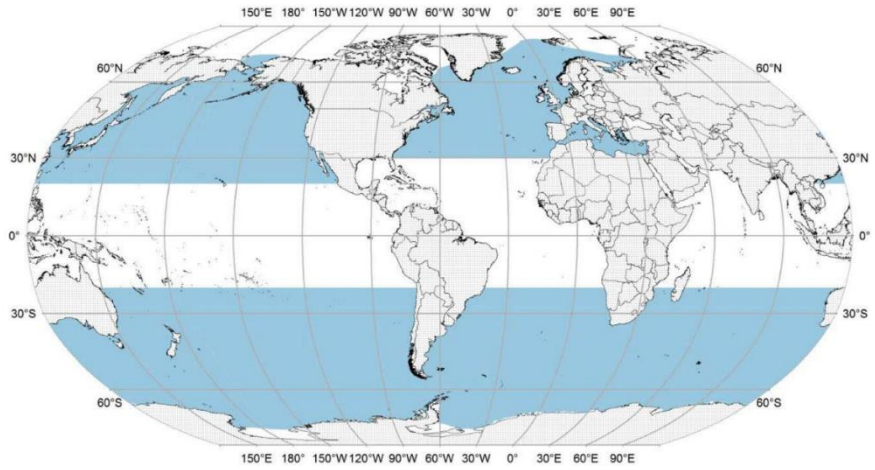

Figure 1. Global distribution of fin whale (source: Edwards et al., 2015)

The reproductive cycle of fin whale is biennial and synchronized with their annual feeding cycle (Lockyer, 1984). Earlier studies of captured migratory populations from the Atlantic and Pacific oceans showed that both sexes reach sexual maturity between 3 - 15 years old (Rice, 1963; Lockyer, 1972; Aguilar *et al.*, 1988). The mating season duration varies among populations, and conception probably occurs during winter (Mizroch *et al.*, 1984). Females usually give birth to a single calf after a gestation period of about 11 months (Lockyer, 1984a) (Lockyer, 1984b), and nurse them for 7-11 months before weaning (Mizroch *et al.*, 1984).

After fin whales were object of intensive harvesting since the 19th century, they were protected from commercial whaling since 1986 by the moratorium of the International whaling Commission (IWC). Currently, this species is commercially hunted in Greenland, Iceland, Norway, and Japan but populations in the North Atlantic and North Pacific are increasing which indicates that births are exceeding mortality (NOAA, 2019). Thus, recently, fin whales have been changed from Endangered to Vulnerable status in the IUCN list (IUCN., 2018). Despite the recovery, the slow rate of reproduction of those animals coupled with natural or manmade impacts are causes of concern. Within these, morbillivirus (Mazzariol *et al.*, 2012; Jo *et al.*, 2017) and high levels of contaminants e.g. persistent organic pollutants (Pinzone *et al.*, 2015), phthalates (Fossi *et al.*, 2012), and heavy metals (Wise *et al.*, 2015) are unpredictable emerging threats that may be affecting cetaceans populations.

Previous studies pointed out the presence of three genetically isolated and resident populations of fin whales in the world: 1) the Eastern China Sea (Fujino, 1960) , 2) Mediterranean Sea (Bérubé *et al.*, 1998) and 3) Gulf of California (Bérubé *et al.*, 2002). Several studies have been conducted to determine its population size with discordant results in the Gulf of California population (Gerrodette and Palacios, 1996; Díaz-Guzmán, 2006). A new study based on aerial surveys, genetic markers, and photographic capture/recapture estimates a population of around 300 individuals (95%-CI:150 - 420) (Pardo *et al.*, 2016). The resident life-cycle strategy indicate the gulf productivity fulfills the population’s prey requirements year-round despite a seasonal variable regime (Adams & Comrie, 2003) that favors high

biological production during winter/spring, but more oligotrophic conditions during summer/autumn (Álvarez-Borrego & Lara-Lara, 1991).

Fin whales are more frequently sighted close to shore during winter and spring than during summer (Tershy *et al.*, 1993; Urbán-Ramírez *et al.*, 2005). Although the habitat used by fin whales in the Gulf of California is not well defined, it is possible that they aggregate during summer in some areas that remain productive due to upwelling triggered by strong tidal regimes or other specific topographical features, capable of sustaining a rich macrofaunal community throughout the year (Brusca *et al.*, 2005; Urbán-Ramírez *et al.*, 2005) the seasonal movements appear to be related to the patchy distribution of its main prey (Urbán-Ramírez *et al.*, 2005), which is the dominant euphausiid species, *Nyctiphanes simplex* (Brinton and Townsend, 1980; Gendron, 1992; Tershy *et al*., 1993; Del Ángel Rodríguez, 1997;.Gómez-Gutiérrez *et al.*, 2012) Thus, although fin whales occur throughout the entire Gulf of California, there are some important gathering regions that have been identified such as the Ballenas Channel, Kino Bay, Santa Rosalia, Loreto Bay, and La Paz Bay (Pardo *et al.*, 2015).

The reproductive behavior of fin whale resident population in the Gulf remains unknown. During 3 years of monthly surveys (1983-1986) in the Ballenas Channel (Fig. 2), only 1% of sightings included mother/calf pairs (Tershy *et al.*, 1990). Calves can be seen year-round throughout the Gulf, similar to what has been observed for the resident population of the Mediterranean Sea (Notarbartolo di Sciara *et al.*, 2003). To date, however, the lack of information about the body length of calves observed has prevented the verification of seasonality in calving or mating events within the Gulf of California.

2.2. Reproduction in large whales

The reproductive cycle is defined as the normal minimum time period for all states of reproduction in the female from ovulation through conception and pregnancy to birth and lactation followed usually by a short rest period (Boyd *et al.*, 1993). Fin whales generally have a 2-years reproductive cycle (Fig. 2) comprising a gestation

period of about 11 months and a lactation period between 7- 11 months, followed by a period of anestrus (Laws, 1961). The cycle starts in winter at low latitudes with ovulation and conception leading to pregnancy, during which the female migrates to summer feeding grounds in higher latitudes. The female returns to low latitudes to give birth in winter next year (Mizroch *et al.*, 1984). The calf is weaned during the migration to high latitudinal waters for feeding during the summer of the second year (Mackintosh, 1965;.Lockyer, 1984a) The timing of events in the reproductive cycle for all mysticetes is geared to optimize the seasonal changes in environmental conditions to benefit the ecology of the species and favor maximal survival of the young (Stephens *et al.*, 2006). Reproductive interval (meaning the actual time between the end of one cycle and the start of another) may be extended or shortened because of environmental, nutritional and anthropogenic factors (Boyd *et al.*, 1993) varying among species and/or even among populations (Urian *et al.*, 1996).

Unlike odontocetes, in which aspects of behavior and physiology have been also documented from studies in specimens in captivity, reproductive biology of mysticetes have been mainly studied from dissections of carcasses and subsequent morphological and histological samples (Marsh & Kasuya, 1984; Read & Hohn, 1995). From the latter, it has been observed that the ovaries are both functional, and during the ovulation, the ripe egg is released from the ovary with the subsequent development of the corpus luteum, which shrinks if conception does not take place or continues to function if pregnancy occurs. In either event, the corpus luteum eventually changes into a smaller corpus albicans, and unlike other mammals, it remains in the form of a permanent body associated with the ovulatory scar on the ovary (Boyd *et al.*, 1993).Thus, record of corpora lutea have been used to infer the number pregnancies in the life of an individual (Ivashin, 1984; Marsh and Kasuya, 1984; Atkinson and Yoshioka, 2007).

Males of most mysticete species display seasonal reproductive cycle which closely coincides with estrus in females. In this case, seasonal changes in testes and in seminiferous tubules have been used to mark an increase of spermatogenesis activity and therefore of the breeding activity (Lockyer, 1984a). However, knowledge

derived from these approaches are restricted to opportunistic sampling (harvesting activities or standings) that regulate the timing, location, and composition of the sample sets (Read, 1990; Zeh *et al.*, 1995). For this reason, traditional approaches to studying physiology of large whales are gradually replaced by new non-lethal techniques (Hunt *et al.*, 2013).

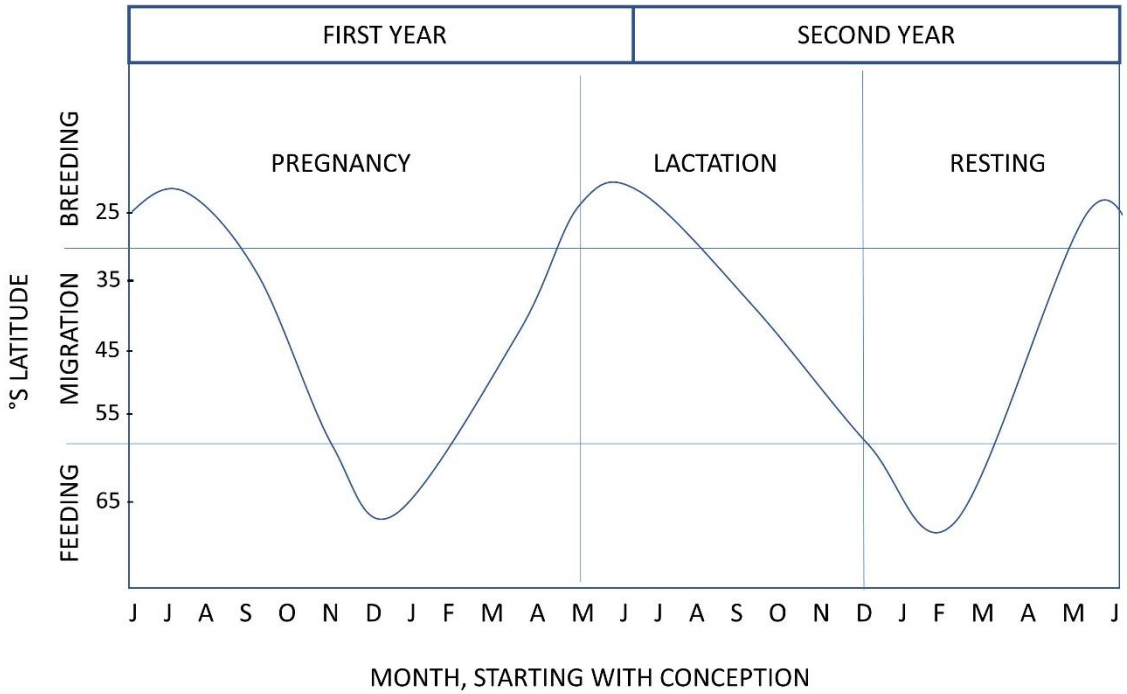

Figure 2. Schematic reproductive cycle of the southern hemisphere female fin whale. The fin whale has a 2-year reproductive cycle comprising a gestation period of about 11 months and a lactation period of about 6 to 7 months, followed by a period of anestrus (image modified from Lockyer *et al*., 1984)

2.3. Biomarkers in blubber

Animals allocate energy resources for storage in different parts of the body. One of the most important tissue in marine mammals is the *blubber*, a dense vascularized layer of fat under the skin (Iverson, 2009). It is composed of adipocytes, but in contrast to other adipose tissues, these cells are surrounded by a rich net of collagen and elastic fibers that gives blubber a firm character. Furthermore, it is characterized

by specialized shunts called arterio-venous anastomoses, which allow larger and swifter blood flow than would be possible through capillaries alone and are important to the thermoregulation process (Iverson, 2009).

Blubber represents a substantial proportion of the total body mass of a marine mammal, exceeding 30% in some cases (McLellan *et al.*, 2002), which is greater than the 4 – 8% adipose found in the average healthy wild terrestrial mammals (Pond, 1993). The importance of this tissue lies in its polyfunctionality since not only is it an important site of storage but also participates in thermoregulation, buoyancy, protection of the internal organs and in hydrodynamic locomotion (Noren and Wells, 2009; Liwanag *et al.*, 2012). The thickness and the biochemical composition of this layer can vary greatly not only among species (Koopman, 2007) but also along the body of the individual (Lockyer & Waters, 1986). In fin whales, for instance, anterior ventral blubber contains less lipid, more protein and more ash than posterior dorsal blubber (Lockyer *et al.*, 1984). Blubber characteristics may differ also according to gender (West *et al.*, 1979), age (Koopman *et al.*, 1996), nutrition, reproductive categories and among species (Aguilar and Borrell, 1990a ; Samuel and Worthy, 2004).

2.3.1. Hormones

Blubber is a tissue containing important biomarkers that can be used to extract useful information on the physiological state of an individual (Iverson, 2009). In this context, some studies, through the quantification of lipophilic steroid hormones, provided a clearer picture of several endocrinological mechanisms in marine mammals (Atkinson and Yoshioka, 2007; Hunt *et al.*, 2013; Atkinson *et al.*, 2015).

Reproduction responds to a complex interaction between environmental factors and the hypothalamic-pituitary-gonad axis that activate the production of hormones that play a reproduction function (Nelson *et al.*, 1990). Within these, progesterone and testosterone have been well studied in terrestrial mammals. Progesterone is mainly produced by the ovaries and on a smaller scale by the adrenal glands. Like all other

steroid hormones, it is synthesized from pregnenolone, which itself is derived from cholesterol (Fig. 3) (Nelson, 2005). Progesterone is an hormone involved in menstrual cycle and pregnancy (Graham & Clarke, 1997). The menstrual cycle occurs in three phases: follicular, ovulatory and luteal. The first half of the cycle is the follicular phase, and the second is the luteal phase. Midway through the cycle, ovulation occurs, which is known as the ovulatory phase (Atkinson & Yoshioka, 2007).

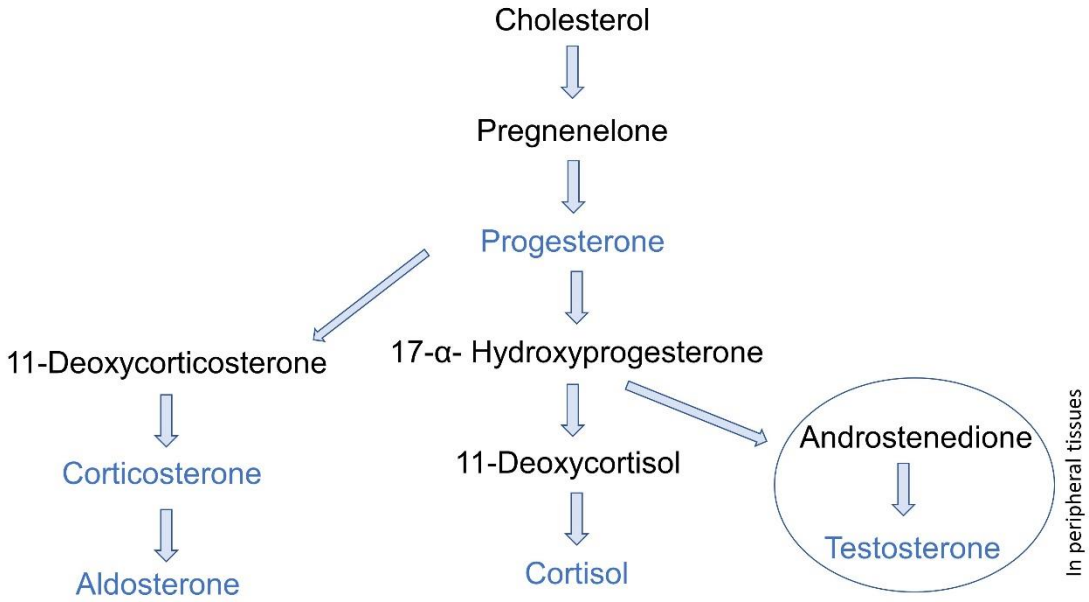

Figure 3. Simplified pathways of steroid biosynthesis in adrenal glands (image modified from Nelson, 2005).

During the follicular phase, progesterone level is low, whereas in the luteal phase, the step when corpora lutea is active, progesterone level starts to raise preparing the uterus for the implantation (Fig. 4)(Nelson, 2005). In the corpus luteum, lutein cells uptake cholesterol and other precursors and return synthesized progesterone into the circulatory system. There, the majority of it is bound to albumin or transcortin to protect it from kidney and liver metabolism whereas the unbound fraction (5-10%) enters in target cells and through cytoplasmic receptors interacts in transcription of

genes (Pineda & Dooley, 2003). During pregnancy, progesterone is produced also by the placenta, and its circulating levels are higher than levels in menstrual cycle in order to maintain quiescence of the uterus until the fetus is mature (Graham & Clarke, 1997). Although progesterone present complex patterns, for both odontocetes and mysticetes, its concentrations in pregnant females are dramatically higher than those of non-pregnant females (Atkinson & Yoshioka, 2007; Pérez *et al.*, 2011).

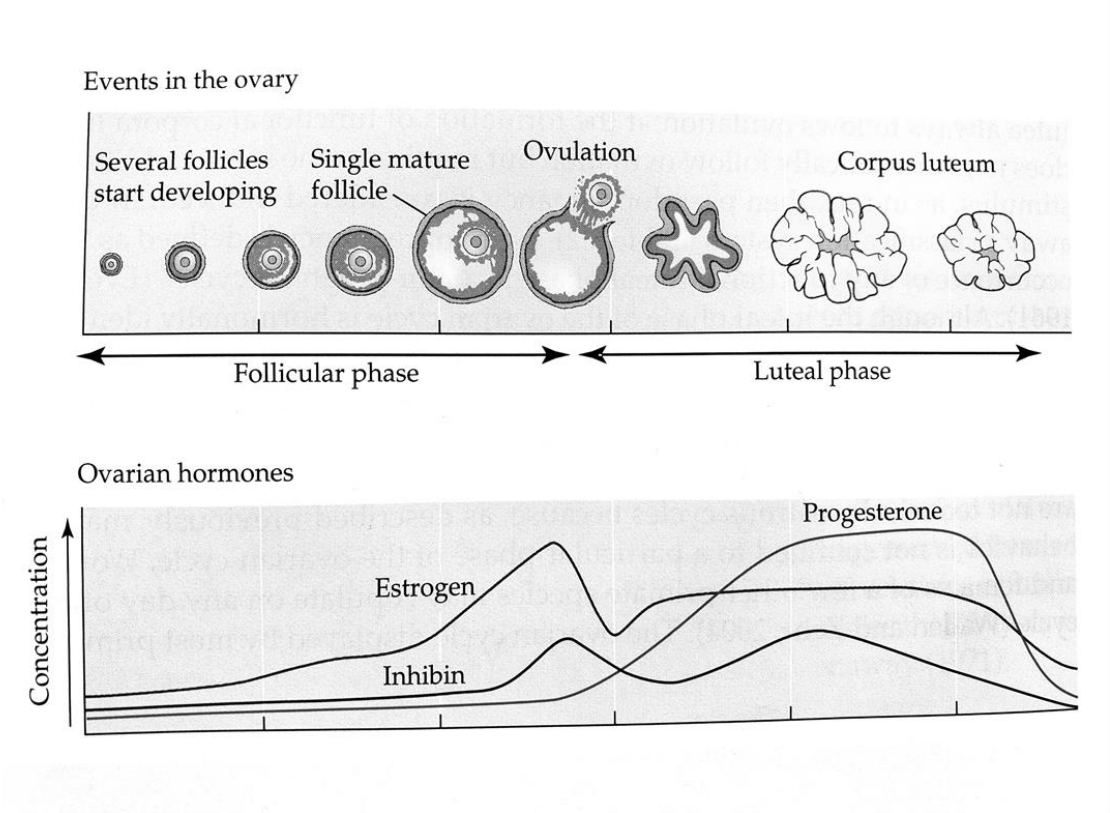

Figure 4. Human menstrual cycle (Source: Nelson, 2005)

Testosterone, is mainly produced by the Leydig cells, is locally released in paracrine fashion into the seminiferous tubules, where it interacts with the germinal epithelium to promote sperm development (Hu *et al.* 1987; Behre and Nieschlag, 2012). Additional testosterone is taken up by the circulatory system and transported to cells throughout the body, where it exhibits many of the anabolic effects associated with general secondary sexual characteristics (Pineda & Dooley, 2003). In terrestrial mammals, changes in its concentrations respond to various factors such as sexual

maturation, competition, female estrus, and stress (Dixson & Anderson, 2004; Hansen, 2009). Although little is known on the functions of testosterone in cetaceans, it has been used to discern between mature and immature males and to detect seasonal variations associated with changes in testis size and sperm production (Daoquan *et al.*, 2006; Kellar *et al.*, 2009). In cetaceans, progesterone and testosterone concentrations have been quantified in serum, urine, saliva, milk, feces, baleen, earplug, muscle, blow and blubber(Yoshioka *et al.*, 1994; Amaral, 2010; Trumble *et al.*, 2013; Hunt *et al.*, 2017; Cates *et al.*, 2019).Despite the great potential of studies through blubber biopsies, these hormones have been mainly quantified in the blubber of odontocetes and few species of mysticetes (Table1).

Besides sexual hormones, steroid hormones related with stress conditions have been quantified in blubber (Amaral, 2010). One of the hallmarks of the stress response is the activation of the hypothalamic–pituitary–adrenal (HPA) axis, and the release of glucocorticoids (GC) which induce a variety of behavioral and physiological effects (Atkinson *et al.*, 2015). The two primary GC are corticosterone and cortisol. Most animals produce mainly either corticosterone or cortisol but, but rarely are both GC produced in large quantities (Nelson, 2005). In cetaceans, presence of cortisol has been detected in bottlenose dolphins (*Tursiops truncatus*) short-beaked common dolphins (*Delphinus delphis*), belugas (*Delphinapterus leuca*) and blue whales (*Balaenoptera musculus*) blubber (Kellar *et al.*, 2015; Trana *et al.*, 2015; Champagne *et al.*, 2016; Champagne *et al.*, 2018; Atkinson *et al.*, 2019). GC secretion is often considered an obligatory response to noxious stimuli, however recent studies indicates that some free living species seasonally modulate GC release but is still unknown if these changes are caused by photoperiod, temperature, food availability or other factors (Romero, 2002; Atkinson *et al.*, 2015). Furthermore, some studies have detected high levels of CG during pregnancy (Valenzuela-Molina *et al.*, 2018b) probably due to a competition by progesterone for cortisol receptors that would lead to competitive inhibition of cortisol and subsequent stimulation of ACTH production (Smith & Thomson, 1991). In cetaceans, this has been observed in feces of blue and right whales (*Eubalaena glacialis*) where females classified as pregnant had higher concentrations of corticosterone than resting and lactating females (Valenzuela Molina *et al.*, 2018; Hunt *et al*., 2006).

Since the understanding behind GC production is still unclear in free living species, the study of other hormones in cetaceans is growing. For example aldosterone, which is produced principally by the cells of the zona glomerulosa of the adrenal cortex (Funder, 2002), has been assayed. During a stress response, an increase of aldosterone is stimulated in order to stabilize the blood pressure and to restore cardiovascular homeostasis in the face of alterations in ionic and osmotic balance that occur due to increased catabolic activity (induced by elevated GC) (Atkinson *et al.*, 2015). In fecal samples of right whales, aldosterone concentration showed similar pattern to those reported for GC providing further evidence of elevated adrenal activation and showing its utility as a complementary biomarker to GC (Burgess *et al.*, 2017). Since an increase of GC (such as cortisol and corticosterone) represent a general response to natural and anthropogenic stressors, some studies have coupled the quantification of steroid hormones with the thyroid hormones to determine the presence of a nutritional stress condition, being strongly related to energy availability in the organism. Thyroid hormones are thyroxine (T_4) and triiodothyronine (T_3), which are produced by the thyroid gland in response to TSH stimulation from the anterior pituitary. Since both T_3 and T_4 are fat soluble, they diffuse rapidly across cell membranes, but they need carrier proteins to travel through the blood. They are involved in different processes such as increased heart rate, improved brain development, enhanced basal metabolic rate and regulation of bones metabolism (Norman & Litwack, 1997). Recent studies in avian and mammalian species showed a negative correlation between the GC and thyroid hormone levels in the feces (Wasser *et al.,* 2010). According to this relationship, in nutritional stress conditions, the GC will increase and conversely the thyroid hormones will drop, thus lowering the metabolic rate and the total energy usage. In marine mammals, thyroid hormones have been so far detected in blood and feces of orcas (*Orcinus orca*) (Ayres *et al.*, 2012), in blood of bottlenose dolphins (Aubin *et al.*, 1996; Fair *et al.*, 2011; West *et al.*, 2014), belugas (Aubin & Geraci, 1989), Amazon river dolphins (Robeck *et al.*, 2019), harbor seals (Oki & Atkinson, 2004), Hawaiian monk seals (Gobush *et al.*) and in Steller sea lions (Mashburn & Atkinson, 2007; Jeanniard du Dot *et al.*, 2009).

In fin whales, sex steroid hormones have been quantified in serum of individuals caught in the North Atlantic during the summers of 1981-1989 (Kjeld *et al.*, 1992). Based on concentrations found, it was possible to differentiate between young sexually immature females, mature non-pregnant females, and pregnant females, as well as the temporal change in testosterone in males. Steroid and thyroid hormones have been detected also in baleen of this species (Hunt *et al.*, 2016) and a novel study showed a relationship between baseline cortisol levels of the ear plug and corresponding to the 20th century whaling data counts from fin whale (Trumble *et al.*, 2018). Nevertheless, no study has been conducted in live organisms so far.

Table 1. Studies on sex hormones in blubber of odontocetes and mysticetes

Species	Hormone	Author
Odontocetes		
Delphinapterus leucas	Progesterone	Biancani *et al.,* 2009
Tursiops truncatus	Progesterone, Testosterone	Pérez *et al.*, 2011 Boggs *et al.*, 2019 Galligan, *et al.*, 2019
Delphinus delphis	Progesterone, Testosterone	Kellar *et al.,* 2009
Delphinus capensis	Progesterone	Trego & Kellar, 2007
Lissodelphis borealis	Progesterone	Kellar *et al.,* 2006
Lissodelphis obliquidensis	Progesterone	Kellar *et al.,* 2006
Stenella longirostris	Progesterone	Kellar & Trego, 2007
Stenella guianensis	Progesterone, Testosterone	Rocha, 2001
Phocenoides dalli	Progesterone	Kellar & Trego, 2007
Mysticetes		
Balaenoptera acutorostrata	Progesterone	Mansour *et al.,* 2002
Balaenoptera bonaerensis	Progesterone	Inoue *et al.*, 2019
Balaenoptera musculus	Progesterone,	Atkinson *et al*., 2019
Balaena mysticetus	Progesterone	Kellar *et al.*, 2014

Megaptera novaeangliae	Progesterone	Vu *et al.*, 2015 Pallin *et al.*, 2018 Cates *et al.*, 2019

2.3.2. Lipids

Blubber has also been used to assess the nutritional condition as well as seasonal changes in prey quality and environmental temperature changes on the organisms. In particular, studies have mainly focused on thickness, total lipids and fatty acids (FA)(Ackman *et al.*, 1965; Budge *et al.*, 2006; Koopman, 2007; Miller *et al.*, 2011). Lipid deposition in adipose tissue occurred predominantly in form of neutral lipids, in particular, as triacylglycerols (TAGs), which consist of three fatty acids esterified with a glycerol backbone (Fig. 5). Hence, during digestion, triacylglycerols are hydrolyzed in the gut by lipases to fatty acids and monoglycerides and released in the bloodstream. These fatty acids can be used either for energy or storage as TAGs in adipose tissue (Pond, 1993). When needed, FA may be mobilized from adipose tissue by the action of hormone-sensitive lipase (HSL), which is activated by glucagon and adrenaline (epinephrine) and inhibited by insulin (Kalo & Kemppinen, 2003). In this way, most FA travel up the food chain intact. In particular, blubber is characterized by high levels of long- chain polyunsaturated fatty acids (PUFAs) which are the most abundant in marine environment in the forms of ω-3 and ω-6 (Fraser *et al.*, 1989). Nevertheless, some FA, in addition to being acquired through the diet, may also be produced de novo by the organism. Between them, FA with 16 or 18 carbon atoms and usually one double bond are the most common (Iverson, 2009).

Lipid metabolism also play an important role in many reproductive physiological processes. Hence, beside cholesterol being the precursor of steroid hormones (Strauss & Barbieri, 2013), studies have demonstrated that n-6 and n-3 PUFAs can influenced the reproductive processes through a variety of mechanisms. For instance, PUFAs provide the precursors for prostaglandin synthesis and can modulate the expression patterns of many enzymes involved in both prostaglandin

and steroid metabolisms (Wathes *et al.*, 2007). Nevertheless, these types of studies have been mostly conducted in humans or terrestrial mammals. Thus, the link between lipids and reproductive regulators must be further explore in marine mammals.

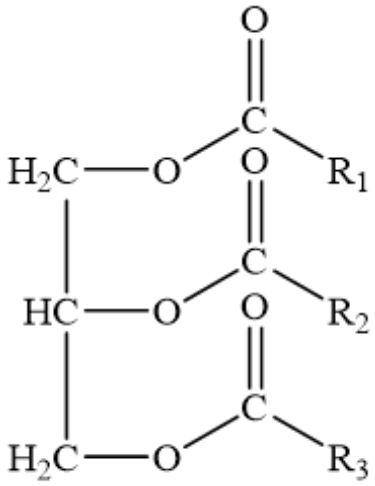

Figure 5. General structure of a triacylglycerol

In marine mammals, studies based on harvested, stranded, or by-catch animals (Lockyer, 1984), have enabled researchers to analyze the entire thickness of the blubber layer. Currently, research on free ranging cetaceans aims to get samples through a variety of non-lethal, minimally invasive approaches. Between these, biopsy sampling is becoming a preferred protocol for sampling skin and blubber, thus allowing the study not only of stranded animals and in some cases in decomposition conditions but also of healthy individuals. Despite these advantages, studies of lipidic components in blubber biopsies resulted in controversy due to the heterogenous structure of the blubber. Hence, some studies showed that the lipid content of the external blubber is stable and showed no variation with age, reproductive state or sex, whereas the internal layer is highly variable (Lockyer *et al.*, 1984; Aguilar and Borrell, 1990; Olsen and Grahl-Nielsen, 2003) In terms of FA the outer layer showed higher monounsaturated fatty acids (MUFAs) concentration, while the inner layer showed significantly larger amounts of saturated fatty acids

(SFAs) and PUFAs (Ackman *et al.*, 1965; Samuel and Worthy, 2004). The presence of PUFAs in the inner layer is important energetically, as it would not be efficient to deposit or request these FA in a less active outer layer (Koopman *et al.*, 1996).

The unique layer that may reflect the nutritive reserves of the individual is the deepest layer, whereas the external layer plays a primary role in buoyancy and thermoregulation. In addition, previous results from biopsies of wild cetaceans showed lower percentage of lipid from blubber biopsies than would be expected from blubber sampled via necropsy. For example, Krahn *et al.*, (2001), reported that the mean lipid levels in gray whale (*Eschrichtius robustus)* blubber sampled by biopsy (10 ± 1.0%) were substantially lower than those found for subsistence animals sampled by necropsy (43 ± 2.7%). Due to these results, different explanations were proposed: 1) lipid loss occurs as adipocytes are burst due to the force of the dart, 2) lipids are washed away when the dart falls into the water and 3) if the dart hits the body with an oblique angle it collects more epidermis and connective tissue than blubber (Krahn *et al.*, 2001; Krahn *et al.*, 2004; Ryan *et al.*, 2013).

These studies suggest that the blubber from a biopsy is an inappropriate tissue to assess the nutritional condition. However Waugh *et al.* (2013) proposed that the intensity of the gradient of % lipids throughout the blubber layer may be dependent on the nutritional demands on the individual whale. This hypothesis is consistent with the results presented by Aguilar and Borrell (1990) in which of a total of 82 fin whale males and non-lactating females, there was no evidence of stratification between layers whereas lactating females showed a gradient of lower lipid in the inner layers compared to the outer layer reflecting the intensification of lipid metabolism during lactation (Iverson *et al.*, 1995; Grahl-Nielsen *et al.*, 2000). In addition, in blue whale males, changes in total lipids as well as in FA composition were detected in blubber biopsies during the winter season in the Gulf of California (Espino Perez, 2009). This result demonstrates that the biopsy samples may are useful to detect long term variations in feeding activity. Besides the use of FA as dietary tracers, the outer layer shows to be useful to investigate physiological aspects of the individuals. Birkeland *et al.*, (2005) for instance, found that FA composition of newborn calves of beluga whale differed significantly from the

blubber of their respective mothers due to differences in metabolic requirements**.** Furthermore, Herman *et al.*, (2008, 2009) used the combination of specific FA founded in biopsy samples to age live free-ranging humpback and killer whales.

These evidences highlight that the physiological mechanisms behind lipid metabolism in blubber are still unclear in cetaceans and that the usefulness of certain tissues to study marine mammals depend on the scope of the study.

3. JUSTIFICATION

Understanding the reproductive physiology of protected species is important in the development of more effective conservation policies and management tools. It is especially crucial for small populations occupying restricted areas such as islands, mountain lakes or semi-enclosed seas, which make them more vulnerable to environmental changes and anthropogenic disturbances (Willi *et al.*, 2006). Fin whales inhabiting the Gulf of California are geographically and genetically isolated (Bérubé *et al.*, 2002). Despite the efforts to study this population, basic aspects about its reproduction and physiology remain unknown. This depends not only on the lack of knowledge about breeding-site fidelity but also on the difficulty to cover most inaccessible areas of the Gulf throughout the year for sampling. For this reason, the implementation of new techniques to study this population are crucial to complement our understanding of its physiology and anthropogenic and ecological effects on its viability. Finally, due to its residency throughout the year, this population represents an important bioindicator of changes that may occur in the Gulf of California.

4. HYPOTHESIS

Given the seasonal wind changes on regime that dominates the oceanographic habitat of the Gulf of California, I hypothesized that the resident fin whale population has a seasonal reproductive cycle, that reflects in their hormone and lipid concentrations in their blubber biopsies.

5. OBJECTIVE

To assess the seasonal reproductive strategy of the resident fin whale population of the Gulf of California using blubber biomarkers from biopsies.

5.1. Specific objectives

- Validate the presence of steroid (progesterone, testosterone, cortisol, corticosterone and aldosterone) and thyroid (triiodothyronine and thyroxine) hormones in blubber of fin whales.

- Determine the female reproductive categories using progesterone concentrations and records.

- Assess the seasonal variations in hormones and lipid compositions.

- Estimate lipid content, and percentages of neutral and polar fractions in blubber of fin whales by sex, females' reproductive categories and seasons.

- Quantify and qualify neutral fatty acids in blubber by sex, reproductive categories and seasons.

6. MATERIALS AND METHODS

6.1. Samples collection

Samples and data were collected under permits issued by *Secretaría de Medio Ambiente y Recursos Naturales* (SGPA/DGVS 08021/06, 00506/08, 09760/08, 01110/15, 00255/16 and 00987/17, issued to D. Gendron., and SGPA/DGVS//036624/17 and 002162/18, issued to M.A. Pardo.; CITES export permits: MX 88860). We collected 84 skin/blubber biopsies of fin whales (Fig. 6) spanning the period of 2007-2009 (n = 33) and 2015-2017 (n = 51).

Samples were collected in collaboration with CICESE-ULP and Prescott College Kino Bay Station, in in five main aggregation regions in the Gulf of California: La Paz Bay, Loreto Bay, Santa Rosalía, Kino Bay, and Ballenas Channel (Fig. 7). All biopsies were collected using a dart of 40 mm long assembled on an arrow with a stopper that limits the depth of penetration and fired from a crossbow at a distance of about 10 m from the whale, into the animal's mid-lateral region. In order to prevent contamination between samples and infection to the animals, each dart was washed with 50% chlorine solution, then with 70% ethanol solution, and at the end exposed to a blowtorch flame (Costa-Urrutia *et al.*, 2013). Samples were wrapped in aluminum foil and stored in liquid nitrogen (210 - 196°C) before being processed.

Figure 6. Example of a biopsy-dart sample obtained via crossbow showing epidermis (black tissue) and blubber (white tissue) obtained from a fin whale (Source: Laboratorio de Ecología de Cetáceos)

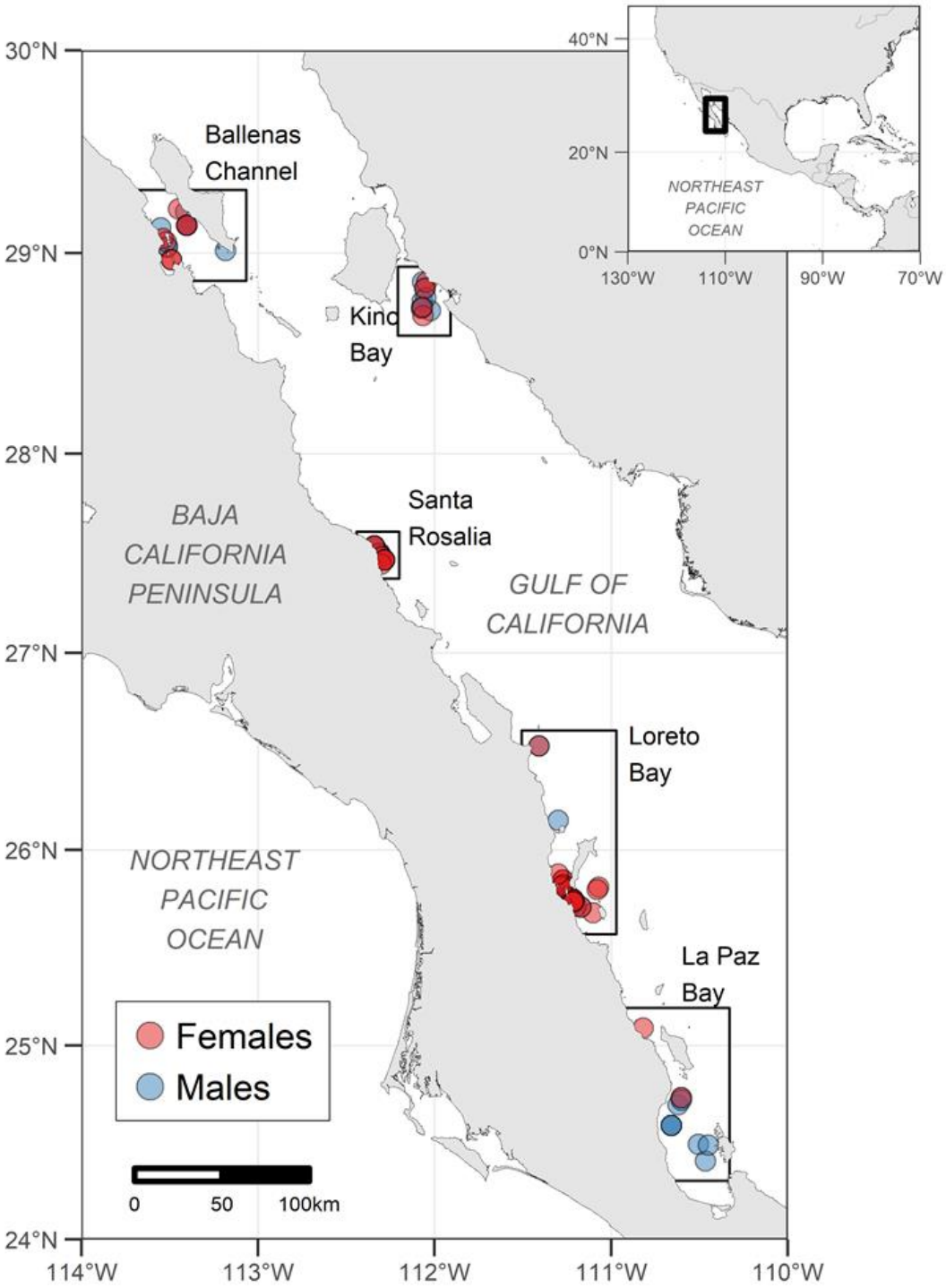

Figure 7. The Gulf of California in the Northeast Pacific Ocean. Colored dots show the geographic distribution of blubber biopsy samples from female (red) and male (blue) fin whales, used in this study.

6.2. Whale identification

Each biopsied individual was photographed and identified by the combination of dorsal fin profile, pigmentation patterns and the presence of permanent scars (Fig. 8) (Agler *et al.*, 1990). We obtained a series of photographs of both sides whenever was possible. To facilitate the photo-identification, individuals were separated into 6 categories based on the profile of the dorsal fin (See Appendix I) using the methodology proposed by (Agler *et al.*, 1990), with modifications. When conditions were not suitable to recognize the dorsal fin shape (e.g. picture angle, lack of proper lighting), the individual was classified as “undetermined” (Type F; see Appendix I).

Figure 8. Fin whale photo-identified using the dorsal fin profile and the lighter skin mark (Photo on left side: Prescott College. Photo on right side: CICESE-ULP)

6.3. Samples preparation

Biopsies collected in the mid lateral dorsal region were 10 - 35mm long. For each biopsy, skin samples were stored in 96% alcohol and sent to the Molecular Ecology Laboratory of the Universidad Autónoma de Baja California-UABC (Ensenada, Mexico) for the sex determination using the method described in Bérubé and Palsbøll (1996). Blubber was used for hormones and lipid analyses. Since we assumed that the physiological daily variation in hormones and lipids is not detectable in the outer blubber layer, when more than one sample from the same individual was collected on the same day, we processed the duplicate samples and values were averaged. Hormone analyses were carried out in the Endocrinology

Laboratory of the University of Alaska Fairbanks (Juneau, Alaska) whereas lipidic analyses were performed at the Microalgae Laboratory of the Centro de Investigaciones Biológicas del Noroeste, CIBNOR S.C. (La Paz, Mexico).

6.4. Hormone analysis

6.4.1. Extraction

Hormone extractions from blubber were carried out using the methods described by Mansour *et al.* (2002) with modifications (Atkinson *et al.*, 2019). Approximately 0.060 - 0.15 g of blubber sample was grinded manually with 500 µL of ethanol. After centrifugation (2500 rpm for 15 min; Beckman Coulter GS-6R Centrifuge), the supernatant was collected, and the pellet was resuspended in 500 µl of ethanol. Once dried, 2 ml of a 4:1 mixture of acetone and ethanol were added to the samples. Then, samples were mixed with a vortex (1 min; VWR Vortex-Genie 2), centrifuged (2500rpm for 15 min), and evaporated. The same procedure was done first with 1 mL of ether and then with 2 mL of a 1:1 mixture of hexane and acetonitrile, twice. The final extracts were then frozen at -20°C until analyzed.

6.4.2. Quantification

Prior to quantifying concentrations, samples were re-suspended in methanol, dilution depended on the hormone analyzed. Progesterone for females, testosterone for males and cortisol, corticosterone, aldosterone, total thyroxine (T4) and triiodothyronine (T3) were quantified for both sexes. For all hormones considered we used commercially available enzyme immunoassay kits from Arbor Assays. Total concentration was calculated as the fraction of the relative concentration related to the total weight of the sample and reported in ng g^{-1}. All samples were processed and quantified in duplicates and were rerun if the coefficient of variation was higher than 10%.

6.4.3. Validation

Validation tests ensure that the assay works properly and that there are no substances present in the extracts disturbing the binding properties of the antibodies used. In this study, to validate the assays, we employed parallelism and accuracy tests as described in Atkinson *et al.*, 2019. In a pooled sample (derived by combining equal volumes of a subset of samples), done by randomly combining 20 samples for males and 18 for females. Parallelism is a way of determining if the assay is measuring what it should be measuring and informs on the dilution of sample to use for the assay. This test is based on the simultaneous run of pooled samples with the standard curve and the determination of parallelism between these two curves. When sample dilutions assumed a non-parallel displacement, samples cannot be run in the assay system because it does not demonstrate immunoactivity of endogenous antigen similar to the assay standards. In contrast, samples with a low concentration of immunoactive antigen demonstrated limited parallelism and in this case the sample could only be run 'neat' (without dilution). The accuracy test represents the level at which the measured hormone concentration matches the concentration of standard hormone added to the sample pool. This test allows the detection of the potential interference from other metabolites in the samples and is expressed as a linear regression formula (I):

$$y=mx + b,$$

where y=amount of hormone observed, x=amount of hormone expected, m=slope of the line and the multiple correlation coefficient was squared to produce the coefficient of determination (R^2). A slope much higher or much lower than 1 represents an over- or under-estimation of the hormone, respectively. Finally, the recovery was calculated by the following formula (II):

$$\% \text{ Recovery} = (\text{Amount Observed}/\text{Amount Expected}) * 100$$

6.5. Lipid analysis

Since previous studies showed stratification in terms of lipids between outer, intermedium and the inner layer of blubber (Ackman *et al.*, 1975), an exploratory study was performed to investigate whether there was a difference through the biopsy layers (see Appendix II).

6.5.1. Extraction

Blubber subsamples were weighted and cut into smaller pieces in order to mix with the solvents and were transfer in glass tubes. Total lipids were extracted by mixing 2 mL of chloroform, 1 mL of methanol, and 0.15 mL of water to achieve a proportion of 8:4:0.6 according to (Folch *et al.*, 1957) method. Lipid oxidation was prevented by adding 0.10% of antioxidant (BHT) per weight of lipids, and mixing with a vortex for 30 seconds. Vials were further transferred to a tube rack inside an ultrasonic bath with ice cubes and sonicated for 40 minutes. After 0.6 mL of water was added and mixed with vortex, a two-phase solution was formed with the final proportions of 8:4:3. The lower phase that contained the lipid fraction was removed by double pipetting with Pasteur pipettes and transferred into a clean tube. The solvent was evaporated under a flow of nitrogen. Finally, lipids were resuspended in 500µL of chloroform and stored in a freezer (-20°C).

6.5.2. Separation

The separation of neutral and polar lipids was achieved using a column of silica gel coupled to a burette. The column was prepared with a plug of fiberglass at the bottom of the Pasteur pipet and filled with silica gel, reaching a height of 3.5 cm. Silica gel was previously activated using a muffle furnace (at 450 °C) for at least 8 hours and then hydrated with 6% distilled water per weight of silica. The extract with chloroform was slowly added into the pipet, and 10 mL of chloroform flowed through

the burette towards the column at a constant dripping flow and collected into a new tube. For the polar fraction, the same process was repeated by adding 15 mL of methanol in the burette towards the column and collecting it into a new tube. Lipid fractions were evaporated under a nitrogen flow and resuspended in 2mL of chloroform. the lipid content of each fraction was determined gravimetrically with 1 mL of the recovered extract transferred into a previously weighed vial. Each half extract (1mL) was weighted three times and averaged to improve accuracy before being multiplied by two (2mL) to obtain the final weight of each fraction. Lipid content for each sample was obtained by the following formula:

$$\text{Neutral or polar percentage (\%)} = \frac{\text{Lipid fraction weight (mg)} \times 100}{\text{Sample weight (mg)}}$$

6.5.3. Fatty acids

Fatty acids (FA) were hydrolyzed from their original, more complex molecules and transformed into more-volatile methyl esters (FAME) derivates. An aliquot of the total lipid extract was transmethylated by adding 1.5mL of a mixture of 95% methanol and 5% of hydrochloric acid in each tube and placed in a water bath for 140 minutes (85°C) to obtain fatty acid methyl esters (FAME)(Sato & Murata, 1988). After this step, 1 mL of heptane was added into the solution, mixed with a vortex, and the upper organic phase was transferred into a new glass tube. This step was done twice for each sample. The heptane phase was evaporated under a nitrogen flux and FAMEs were re-suspended in 50 µL of heptane. Then FAME samples were transferred into 0.2-mL crimp vials prior to injection in the gas chromatograph. To analyze FAME samples, a gas chromatograph coupled to a flame ionization detector (Agilent 7820) was used. FAME determination was based on the retention time of each component and compared with a Supelco 37 FAME mix (Sigma). We quantified FAMEs comparing areas with those obtained with a calibration curve obtained at different concentrations of the Supelco 37 component FAME mix (Sigma).

The area of each peak was integrated and interpolated with a calibrated curve that related 5 known concentrations (5, 10, 20, 40, and 80 µg mL-1) of each standard of the Supelco 37 component FAME mix (Sigma). The relative contribution of individual fatty acids (FA) was expressed as percentage of the total FA concentration. Only fatty acids that were found in all samples and amounts greater than or equal to 0.1% were used in the analysis. Samples that had not produced a clear, accurate chromatogram were excluded from the analysis.

6.6. Statistical analysis

The first step was to estimate the probability distributions of progesterone and testosterone to obtain their ranges and central tendencies. A preliminary diagnosis of the progesterone frequency distribution (see Appendix III) showed a possible bimodal pattern in the logarithmic scale, with frequent extreme high values. Therefore, we performed a Bayesian mixture model of normal distributions on the progesterone observations (Marin *et al.*, 2005; Dahl, 2006), to evaluate if there was evidence for such bimodal structure, and to estimate the probability that the progesterone values come from each of the clusters detected by the model. For the testosterone, whose frequency distribution did not show apparent bimodal structure (see Appendix III), we made a simple Bayesian estimation of the mean (Gelman *et al.*, 2013). Both models were based on logarithmic likelihoods of the hormone concentrations.

Determination of reproductive category for females was based on the progesterone model and sightings for which was possible to classify as follows: 1) “lactating” observed with a calf, 2) “likely ovulating/pregnant” with high progesterone concentration, 3) “pregnant” with high progesterone concentration and re-sighted with a calf and 3) “unknown” with low progesterone concentration and without sighting history.

In order to address the potential seasonality of fin whale reproductive cycle in the Gulf of California, we stated the testosterone concentrations as a sinusoidal function of the day of the year, whose parameter probabilities were estimated through

Bayesian regression analysis (Gelman *et al.*, 2013). A log-normal likelihood was stated for the testosterone observations, with uninformative priors. Given the limited amount of observations throughout the year cycle, we decided to complete the yearly series by replicating the observations made during winter as the first part of a hypothetical second year. This was based on the idea that it is not possible that very high values of testosterone maintain indefinitely, and because the predominantly low concentrations observed during the first part of the year suggest it was a transition period. Conversely, due to the role of progesterone in the different female reproductive state, seasonality in progesterone concentrations was not investigated.

The lipid data (total, neutral, and polar lipids) were analyzed to determine if significant differences were found between sex and females' reproductive categories. Furthermore, data were grouped in two main seasons: warm (July - August) and cold (January - May) season. When residuals were deemed inaccurate by a normality test, nonparametric Kruskal- Wallis H test was used. To test a relationship between neutral lipids percentage and progesterone concentration, we used linear regression. Since the residuals did not meet the required assumptions, we transformed them calculating the log(x+1).and the log(y+1).

To explore whether differences among seasons or reproductive categories in fatty acids, a multivariate ordination using a principal component analysis (PCA) was performed. In this analysis, only fatty acids with concentrations higher than 0.1% were used as explanatory variables for the variation of whale samples. Samples were grouped according to season (cold or warm) and to reproductive categories (pregnant, likely pregnant/ovulating, lactating, unknown, and males). Analyses were performed using R programming in the R-studio GUI environment.

7. RESULTS

7.1. Whale identification

Of the photo-identified individuals sampled, 34 were females, and 44 were males. Of the females, 3 were lactating, 1 confirmed pregnant, 12 likely ovulating/pregnant, and 18 resting/immature.

7.2. Blubber variability

Four individuals (Table 2) had duplicated samples taken on the same day or week, and thus values for hormones and lipids were averaged before statistical analysis. Individual B (bold) had duplicated samples but one-year apart. These were considered as two distinct values (Table 2).

Table 2. Concentrations for progesterone, testosterone (as ng g^{-1} of blubber extracted) and total lipids (%) in duplicated samples

Date	Individual	Progesterone	Testosterone	Total Lipids
7/08/2015	A	2.0	-	56.8
7/08/2015	A	3.4	-	54.9
6/08/2015	**B**	**173.3**	-	89.6
24/07/2016	**B**	**3.5**	-	41.8
23/07/2016	C	1.1	-	38.9
23/07/2016	C	0.7	-	35.4
26/07/2016	C	0.4	-	33.0
24/07/2016	D	0.8	-	65.9

25/07/2016	D	0.5	-	68.1
23/07/2016	E	-	0.94	53.4
23/07/2016	E	-	1.23	49.7

7.3. Hormones

7.3.1. Validation of hormones

Progesterone and testosterone assays were successfully validated. Serial dilutions of pooled extracts showed parallelism with the standard curves of progesterone and testosterone (Fig. 9). The slopes of the regressions between added and measured masses were close to a hypothetical 1:1 ratio, which suggests that the method was highly accurate in measuring the hormone concentrations from the samples. The slope was 0.83 for progesterone (95%-CI: 0.78 – 0.87) and 0.83 for testosterone (95%-CI: 0.76 – 0.9). Additionally, the high Bayesian R-squared (BR^2) observed support strong linearity between the standard and blubber extracts, which indicates that the assays were measuring primarily the same antigen in both groups. The BR^2 reached 0.998 in progesterone (95%-CI: 0.994 – 0.999) and 0.997 in testosterone (95%-CI: 0.985 – 0.997) (Fig. 10). The recovery efficiency was 111% (CV=21.7%) for progesterone and 91.7% (CV=23.9%) for testosterone. For cortisol, corticosterone, aldosterone and thyroid hormones results obtained did not complied with the parameters of the validation tests (see Appendix IV) and the quantification was not carried out.

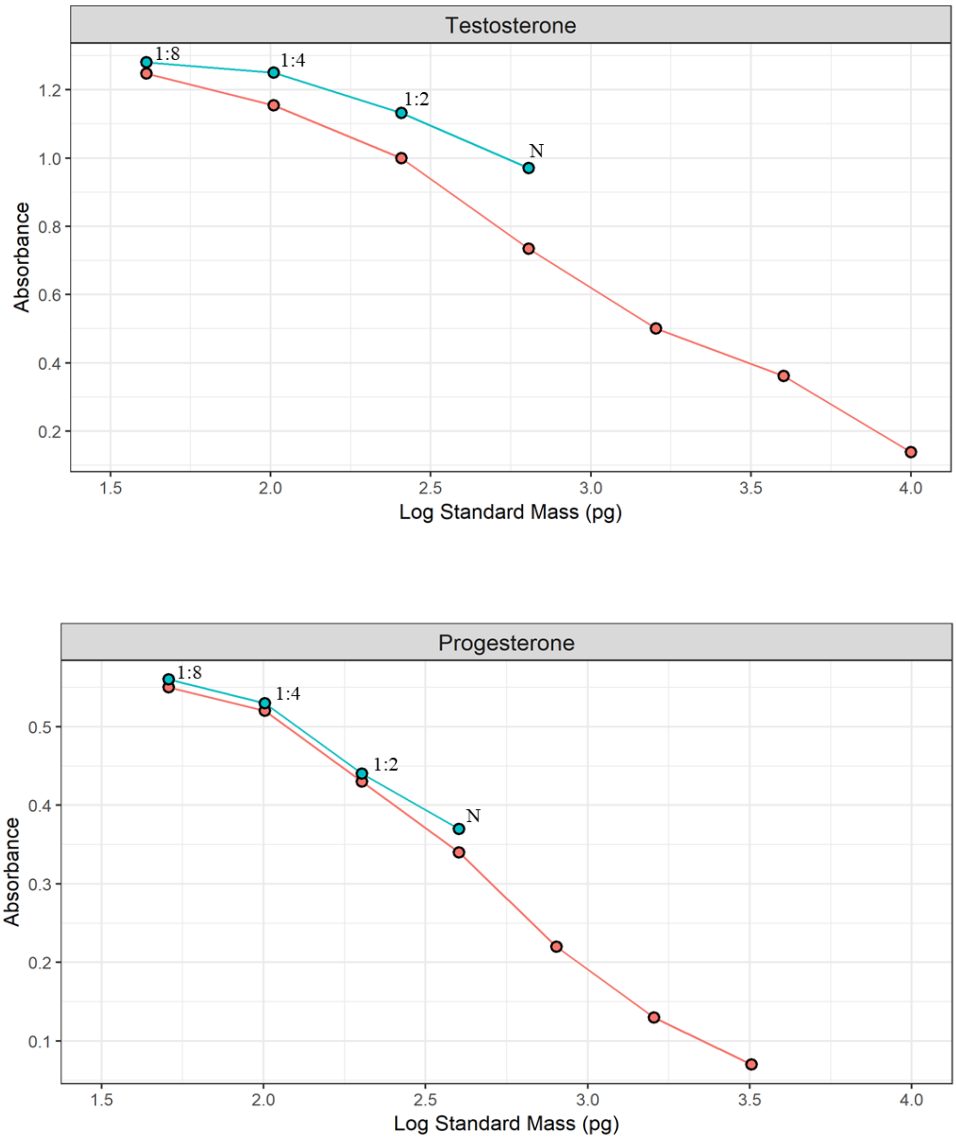

Figure 9. Serial dilutions of samples showing parallelism with the standards of testosterone (upper panel) and progesterone (lower panel). The standard curves are indicated by the orange circles, while the blubber dilutions are indicated by blue circles.

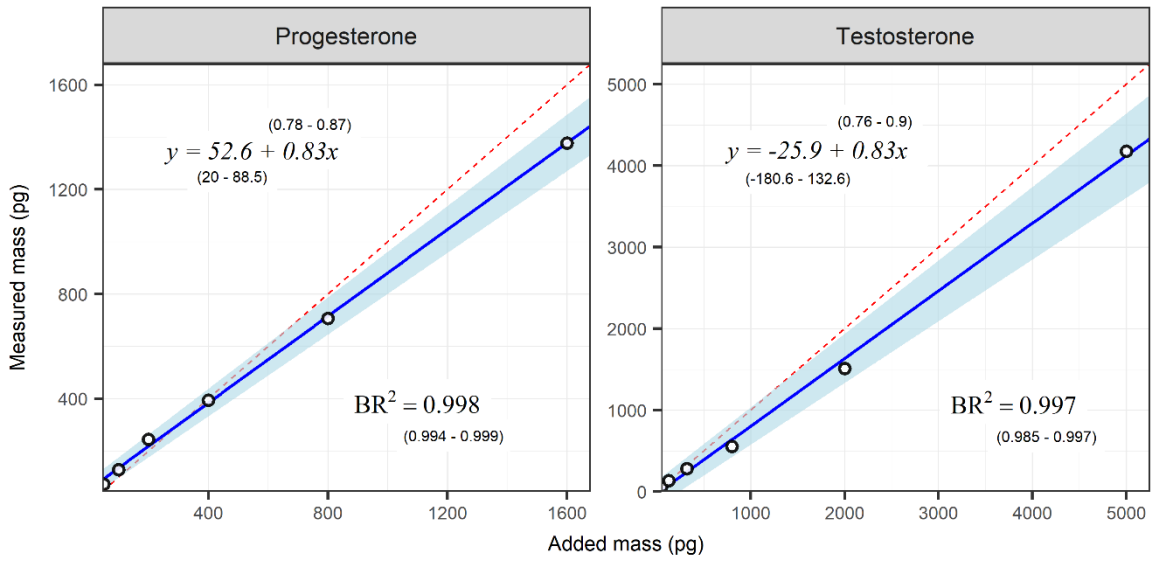

Figure 10. Accuracy tests to compare the slope of measured vs. added (i.e. theoretical) masses of progesterone (left) and testosterone (right) in fin whale's blubber.

7.3.2. Concentrations of sex hormones in the blubber

The results of the mixture model of normal distributions strongly supported a bimodal structure in progesterone concentrations. The first normal distribution of the mixture corresponded to the lowest values, with a median of 1.56 ng g^{-1} (95%-CI: 0.97 - 2.76), whereas the distribution of the higher values had a median of 27.12 ng g^{-1} (95%-CI: 8.55 – 64.78) (Table 3), with a slightly broader posterior probability (Fig. 11).

The overlap between the curves was barely noticeable, limited to the tails of the distributions, and beyond the extreme limits of the 95%-credible intervals of both clusters (Fig. 11). This separation was confirmed by the probabilities of cluster source for the range of the data (Fig. 11), which showed that the overlap only occurs at intermediate values, with probabilities less than 0.1. At that point, the probability that lower values come from cluster B is virtually 0, and vice versa. The results show that 61% (95%-CI: 34 - 82) of the females were likely to come from cluster A (lower

values) and 39% (95%-CI: 18 - 66) from cluster B (higher values) (Fig.11). The testosterone exhibited a median of 0.88 ng g^{-1} (95%-CI: 0.59 – 1.32) (Table 3).

Table 3. Posterior distribution summary of progesterone and testosterone means in blubber of fin whales from the Gulf of California. A Gelman-Rubin convergence statistic close to one indicates good convergence of chains. The N_{eff} is the effective sample size as the percentage of the total iterations retained from all chains. **P4** represent Progesterone and **T** the testosterone.

	Mean	SD	2.5%	25.0%	50.0%	75.0%	97.5%	$\hat{R}$	N_{eff} (%)
P4									
Cluster A	1.58	1.30	0.97	1.33	1.56	1.84	2.76	1.00	92.86
Cluster B	26.05	1.66	8.55	19.43	27.12	36.24	64.78	1.00	100
T									
	0.88	1.23	0.59	0.77	0.88	1.01	1.32	1.00	65

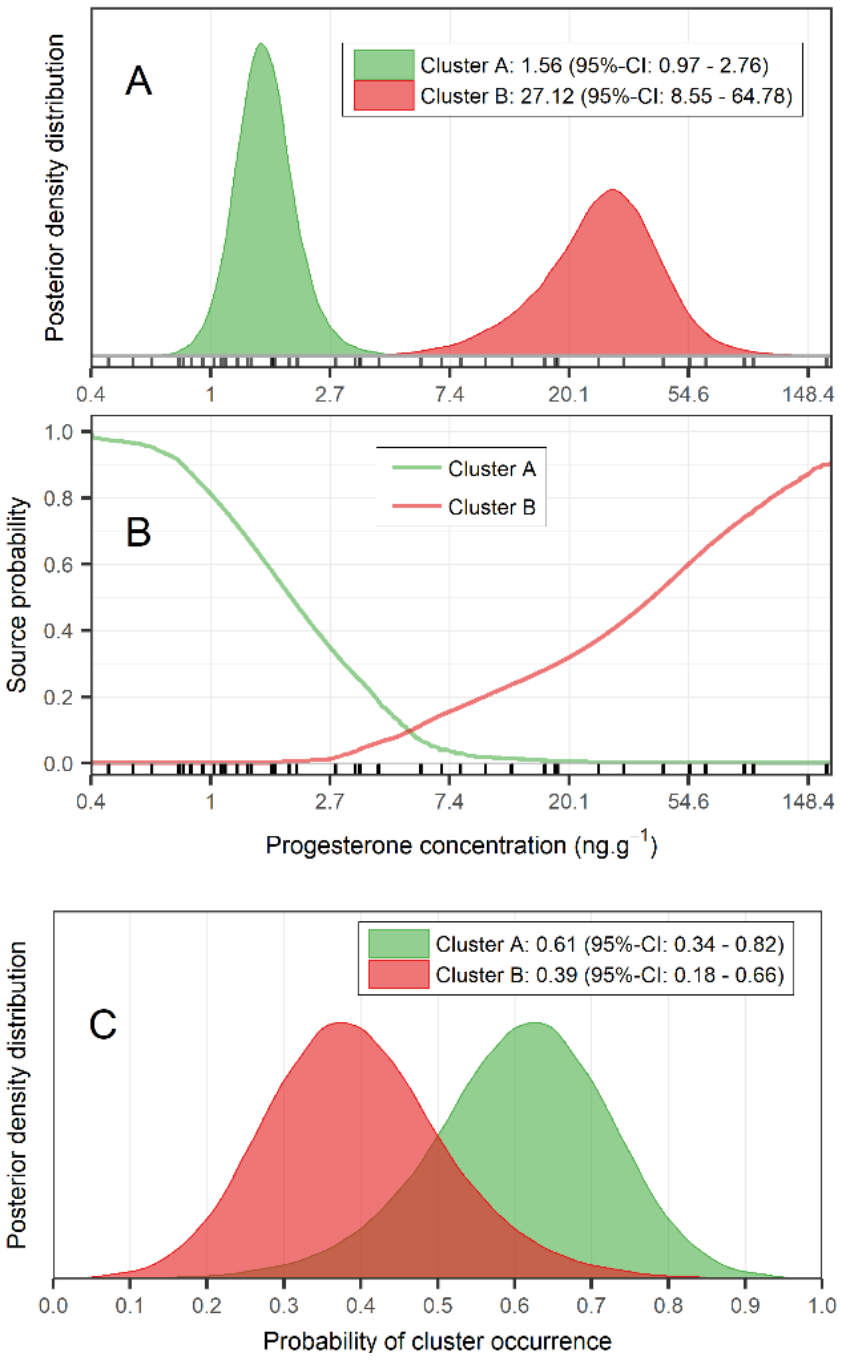

Figure 11. Panel A. Posterior probability distributions of progesterone concentrations (logarithmic scale) for the two clusters estimated by the Bayesian mixture model. **Panel B.** Posterior probability that any value within the observed range of concentrations comes from either Cluster A or Cluster B. **Panel C.** Posterior probability of cluster occurrence for each cluster.

7.3.3. Seasonality

The seasonal model of testosterone concentrations showed a well-defined predicted high peak during late summer (August), and lower concentrations during late winter (February/March) (Fig. 12). The probability of positive amplitude in the sinusoidal curve was 100%, indicating high certainty on the curve trend. The variability explained by the model was relatively low (Bayesian R-squared: 0.29; 95%-CI: 0.13 – 0.42), but expected, since our model could not take into account inter-individual variability, and there was a lack of observations during parts of the cycle.

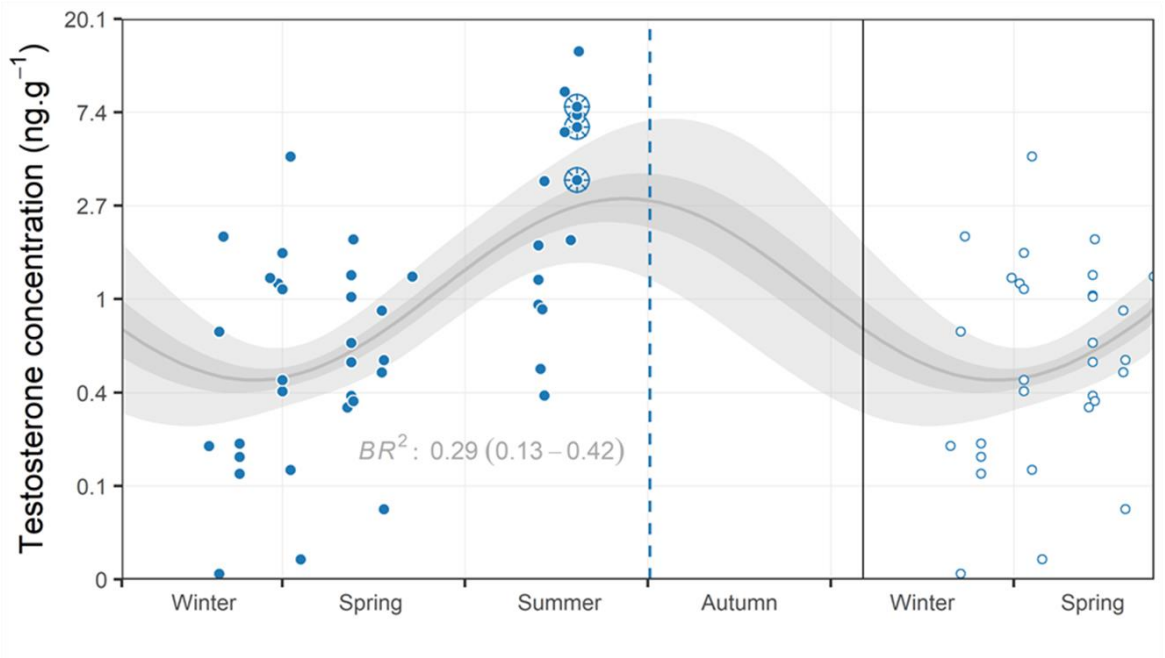

Figure 12. Seasonal trend (gray sinusoidal curve) of testosterone concentrations in fin whale blubber biopsies in the Gulf of California, Spanning 2007-2009 and 2015-2017. The Y-axis is in logarithmic scale. Color-filled dots represent the original observations, and the empty dots represent the repetitions to complete the yearly series (see Methods). Observations surrounded by sliced circles represent animals involved in the first courtship event (2015). The vertical blue dashed line shows the day of the second courtship (2018; one female followed by two competing males, of unknown hormone concentrations).

Sightings of biopsied fin whales allowed the detection of some cases that suggested a seasonal pattern in the reproductive cycle of the population and confirmed the results of the seasonal model of testosterone concentrations. The female C003 was observed during summer (August 2015) in the Ballenas Channel (Fig. 11) with the highest progesterone concentration of all females biopsied (173.4 ng g^{-1}). The same female was observed twice the next summer (July 2016) in Loreto Bay accompanied by a calf, presumably lactating, with a low progesterone concentration in blubber (3.5 ng g^{-1}). Another female (C021) was observed during summer (August 2015) in the Ballenas Channel with a calf (a biopsy could not be collected). Almost six months later, during winter (February 2016), she was re-sighted and biopsied in Kino Bay without the calf, exhibiting a low progesterone concentration (0.76 ng g^{-1}), which suggests that weaning probably occurred before winter. Finally, in the Ballenas Channel, our team witnessed two courtship events during summer, never described before for this species. On August 12, 2015, (Fig. 13) an individual was followed by three other fin whales at an anomalous high-sustained speed. The individuals of the group were seen at least twice side-lunging and obstructing each other's path. Fortunately, we were able to collect biopsies from the four animals, and genetic analysis confirmed that the leading animal was a female exhibiting a medium progesterone concentration of 18.26 ng g^{-1} (Cluster B; Fig. 11), whereas the other three animals were males with high testosterone concentrations (7.86, 6.32, and 3.58 ng g^{-1}), above the highest limit of the 95%-CI of the testosterone posterior probability. In addition, on September 17, 2018, we observed, another courtship event between one female and two males (sex genetically confirmed) in the same area. As with the previous event, males were chasing the female, breaching alternately, and obstructing each other's path (Fig 13). On numerous occasions, both males turned on their longitudinal axes while surfacing, showing one of their flippers and half of the fluke (Fig. 13).

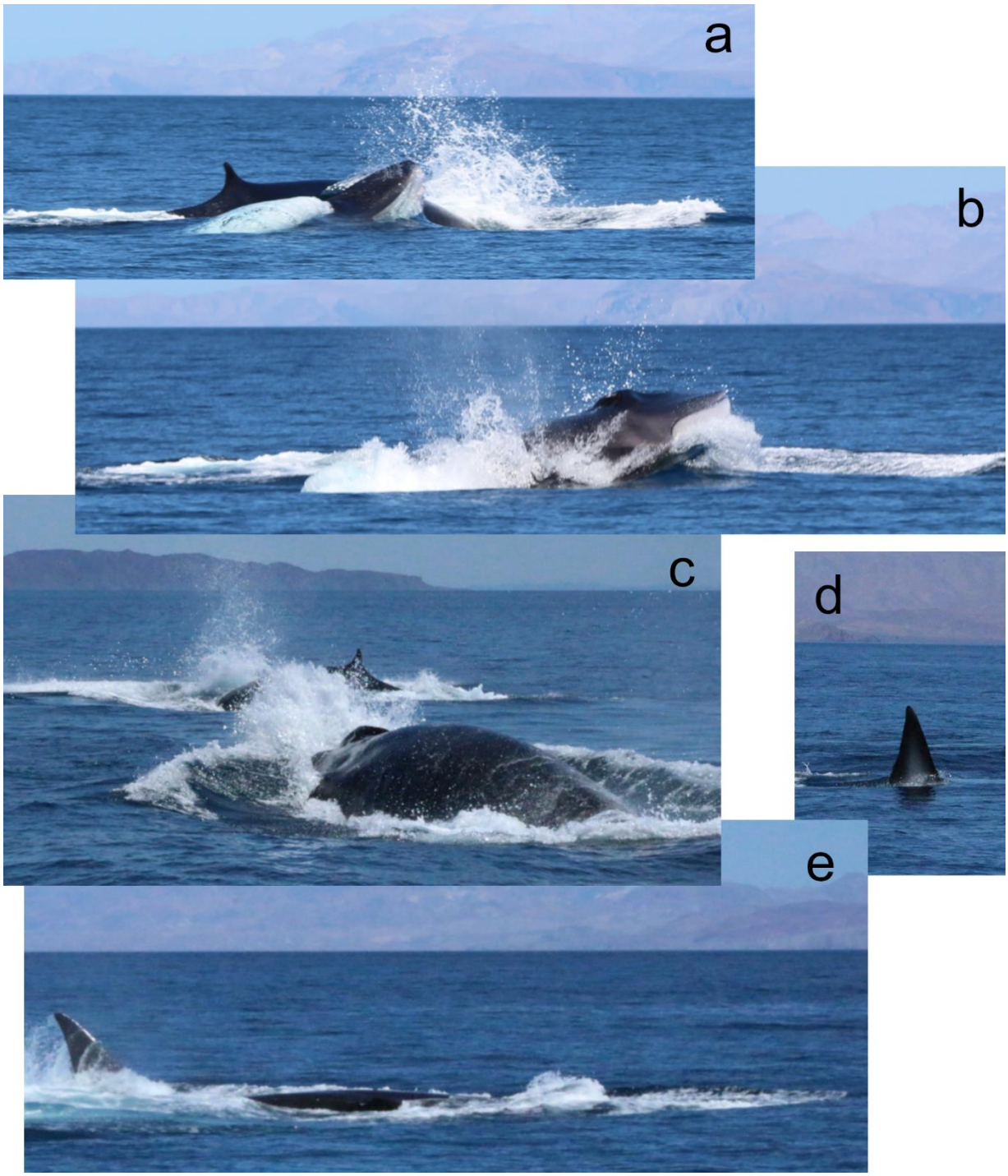

Figure 13. Two fin whale males during a presumed courtship display for a female (not shown) that always leaded their main path. They made fast and strong breaching displays very close to each other (a). Many times, the entire head was visible above the surface (b). They often irrupted each other's path (c), and rolled

on their longitudinal axes, showing half of the fluke (d and e) and a flipper out of the water. (Photographs by G. Busquets-Vass)

7.4. Lipids

Biopsies exhibited high variability was in lipid content without an apparent gradient from the innermost to the outermost layer (see Appendix II). This result allowed to increase the number of biopsies to compare by using only the first cm of tissue under the skin. Samples showed a loss of moisture content of around 50-60% after lyophilization.

7.4.1. Lipid variation related to sex and reproductive categories

Females' total lipid content (58.9 ± 35.9, n=39) showed no significant difference than males' (53.2 ± 31.5, n=45) ($p>0.05$). Moreover, when females were ranked according to their reproductive status (Fig. 14), confirmed pregnant females and likely pregnant-ovulating females showed a slightly higher percentage of neutral lipids than unknown and lactating, similarly to what observed in other studies, but no significant differences were found among groups ($p>0.05$).

The relationship between reproductive status and neutral lipid fraction in females was explored by plotting progesterone values against neutral lipids (Fig. 15). The regression model showed a slope significantly different from zero ($p=0.009$), but both variables had a low correlation coefficient ($R^2 = 0.16$).

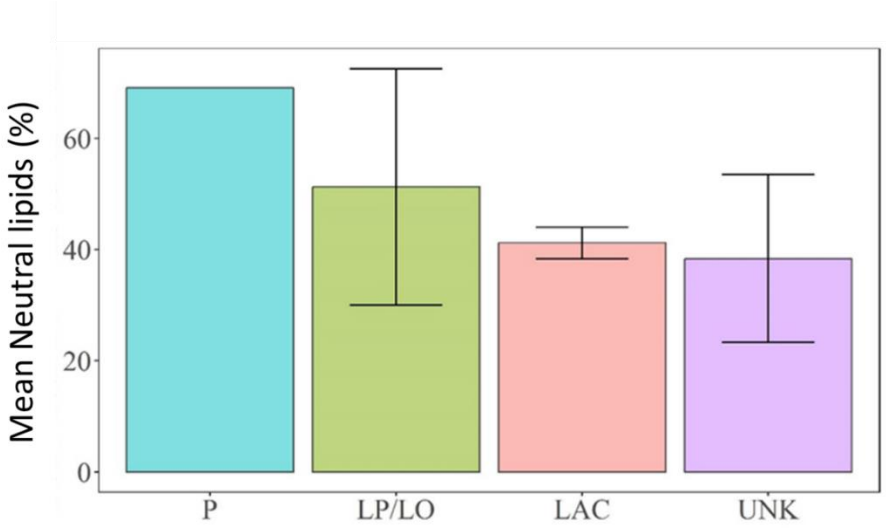

Figure 14 Average neutral lipid percentage in blubber by female category: P=pregnant (n=1), LP/LO Likely Pregnant/Ovulating (n=12), Lac= lactating (n=3), UnK= unknown (n=23)

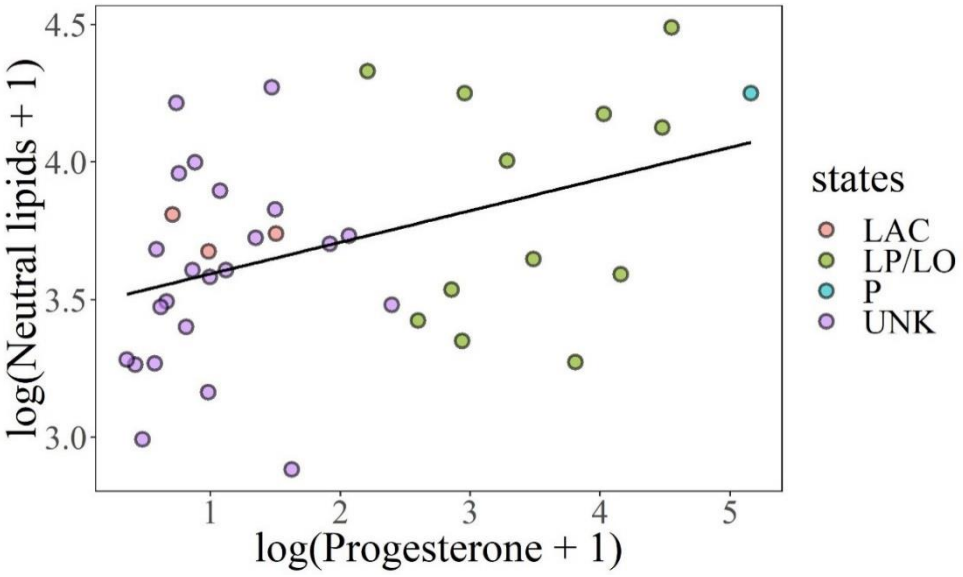

Figure 15 scatterplot representing the relationship between neutral lipids percentages and progesterone concentrations. Variables were Log(x+1) transformed. Points are colored according to the reproductive category: P=Pregnant (n=1), LP/LO=Likely Pregnant-Ovulating (n=12), Lac=Lactating (n=3), Unk=Unknown (n=23)

7.4.2. Seasonality

Neutral and polar fractions showed no significant differences between warm and cold seasons (p>0.05) neither in females nor in males. Nevertheless, once data were plotted, neutral lipids of females seemed slightly higher during the warm season (Fig. 16). Therefore, female's data were separated into the two main groups obtained by hormones analyses: Likely Pregnant-Ovulating and unknown. Likely Pregnant-Ovulating females showed significantly higher neutral lipid content during the warm season compared with the colder season (p = 0.014). Conversely, no significant seasonal difference was detected in the Unknown group (p > 0.05) (Figure 17).

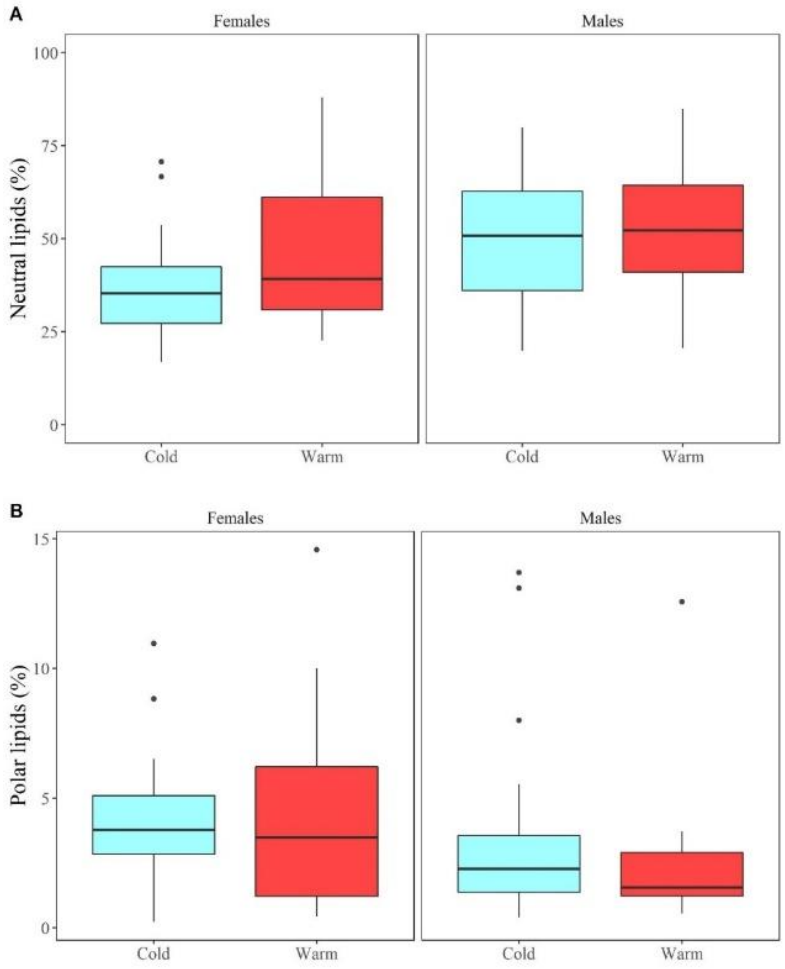

Figure 16. Percentages of neutral (**panel A**) and polar lipids (**panel B**) by females (n= 39) and males (n= 45)

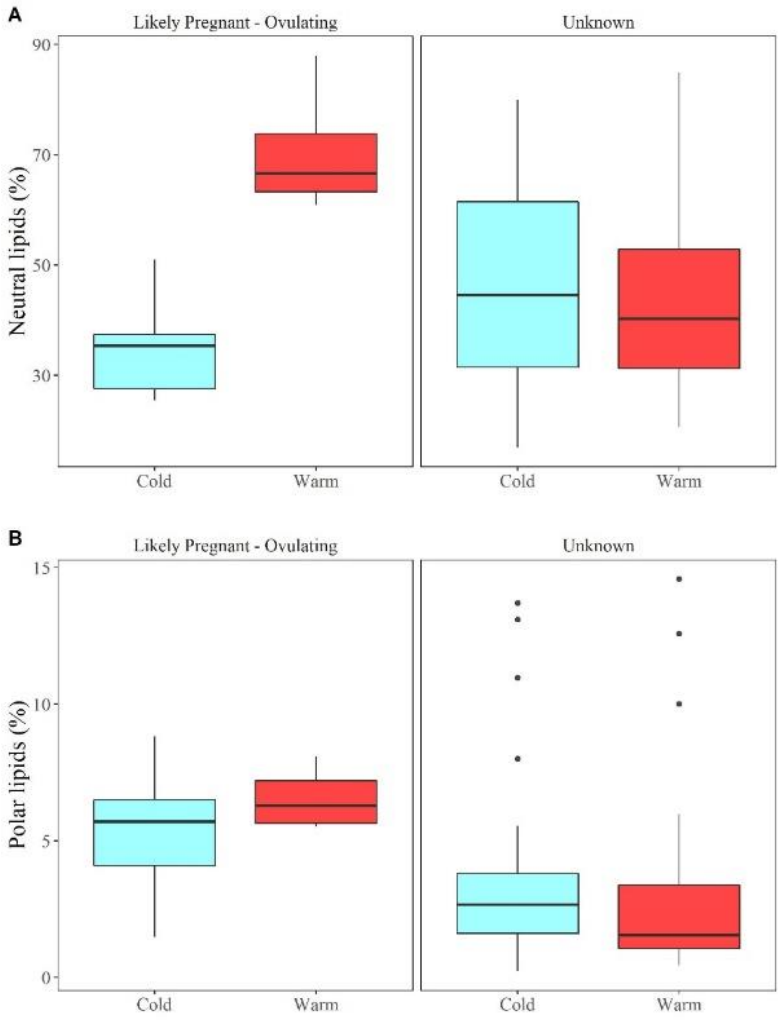

Figure 17. Percentages of neutral (**panel A**) and polar lipids (**panel B**) by cold and warm season in Likely Pregnant-Ovulating (cold season n=5, warm season n=7) and Unknown state (cold season n=15, warm season= 8)

7.4.3. Fatty acids

Neutral lipids detected in blubber biopsies of fin whales from the Gulf of California comprised 32 fatty acids. The FAs of neutral fraction were dominated by MUFAs, followed by PUFAs and SFAs in both sexes (Table 4). In both males and females, the most abundant fatty acid was 18:1n9, followed by 16:1n7 and 16:0.

Table 4. Neutral fatty acids detected in blubber biopsies of five groups of fin whales: Males (G1), Pregnant Female (G2), Likely Pregnant-Ovulating Females (G3), Unknown females (G4) and Lactating Females (G5).

Saturated	G1	G2	G3	G4	G5
12:0	trace	trace	trace	trace	trace
14:0	3.6± 0.6	2.7	3.2 ± 0.5	3.3± 0.7	1.8 ± 0.9
15:0	0.6 ± 0.4	0.4	0.6 ± 0.3	0.8 ± 0.5	0.7 ± 0.2
16:0	14.9 ± 3.7	12.2	10.4 ± 1.2	13.5 ± 2.8	13.2 ± 1.7
17:0	0.9 ± 0.3	1.3	0.9 ± 0.7	0.8 ± 0.4	1.0 ± 0.5
18:0	2.2 ± 1.0	2.7	2.3 ± 0.9	1.3 ± 0.9	1.5 ± 1.2
19:0	trace	trace	trace	trace	trace
20:0	0.2 ± 0.2	0.2	0.3 ± 0.2	0.3 ± 0.2	0.4 ± 0.3
22:0	trace	trace	trace	trace	trace
Monounsaturated					
14:1	0.6 ± 0.3	0.6	0.5 ± 0.1	0.5 ± 0.2	0.6 ± 0.2
16:1n9	0.4 ± 0.2	0.2	0.4 ± 0.2	0.5 ± 0.3	1 ± 0.3
16:1n-7	16 ± 2.5	13	12.1 ± 6.4	13.2 ± 3.6	16.6 ± 1.2
16:1n-5	0.3 ± 0.2	0.5	0.3 ± 0.3	0.4 ± 0.4	0.2 ± 0.05
17:1	2 ± 0.3	0.9	1.9 ± 0.2	0.8 ± 0.4	0.9 ± 0.1
18:1n-9	21.8 ± 4.9	25.1	22.7 ± 5.5	25.7 ± 4.4	33.7 ± 2.9
18:1n-7	6.5 ± 2.2	5.6	6.3 ± 1.5	6.1 ± 2.5	4.6 ± 1.4
18:1n-5	0.1 ± 0.1	0.2	0.2 ± 0.1	0.2 ± 0.1	0.1 ± 0.05

20:1n-9	3.2 ± 0.6	2.9	2.8 ± 0.6	3.0 ± 0.8	1.5 ± 0.5
20:1n-7	0.1 ± .0.08	0.1	0.1 ± 0.06	0.1 ± 0.07	0.1 ± 0.05
22:1n-11	0.1 ± 0.03	0.2	0.2 ± 0.02	0.2 ± 0.04	0.01 ± 0.0
22:1n-9	trace	trace	trace	trace	trace
22:1n-7	trace	trace	trace	trace	trace
Polyunsaturated					
16:2n-4	0.3 ± 0.2	0.2	0.2 ± 0.2	0.2 ± 0.1	0.3 ± 0.1
18:2n-6	3.4 ± 0.4	3.7	3.4 ± 0.2	2.3 ± 0.4	2.9 ± 0.2
20:2(n-6)	0.2 ± 0.02	0.3	0.1 ± 0.01	0.3 ± 0.01	0.3 ± 0.01
18:3(n-6)	0.1 ± 0.1	0.2	0.2 ± 0.1	0.1 ± 0.2	0.1 ± 0.2
18:3(n-3)	0.7 ± 0.5	0.1	0.9 ±0.3	0.9 ±0.9	1.3 ± 0.2
20:4(n-6)	3 ± 0.1	2.3	2.1 ± 0.2	2 ± 0.2	1 ± 0.2
20:3(n-3)	0.8 ± 0.02	0.2	0.3 ± 0.01	0.2 ± 0.02	0.2 ± 0.02
20:5(n-3)	6.8 ± 2.1	8	6.7 ± 1.9	7.2 ± 1.7	6.8 ± 2.1
22:5(n-6)	0.1± 0.1	0.1	0.1± 0.1	0.1± 0.1	0.1 ± 0.2
22:6(n-3)	8 ± 1.5	9.8	6.7 ± 1	10.7± 2.2	13.4. ± 1.4

The PCA analysis resulted in two main axes that explain 40.27% and 28.24% of the sample variance respectively (Figure 18). The 18:1n-9 fatty acid had the most substantial contribution to overall variance, which does not seem to be season dependent. Indeed, when the reproductive state is depicted in the PCA (Figure 18-A) then, the 18:1n-9 fatty acid characterizes the lactating category. Furthermore, other reproductive status does not seem to have substantial differences in the fatty acid composition of their neutral lipids, as shown by the overlap in Figure 18-B.

Moreover, PCA suggests a trend in feeding patterns throughout the seasons, where different fatty acids are assimilated (Figure 18-B). In particular, during the cold season, two fatty acids (16:1n7, 16:0) characterize the samples, whereas during the warm season, the 18:1n7 seems predominant.

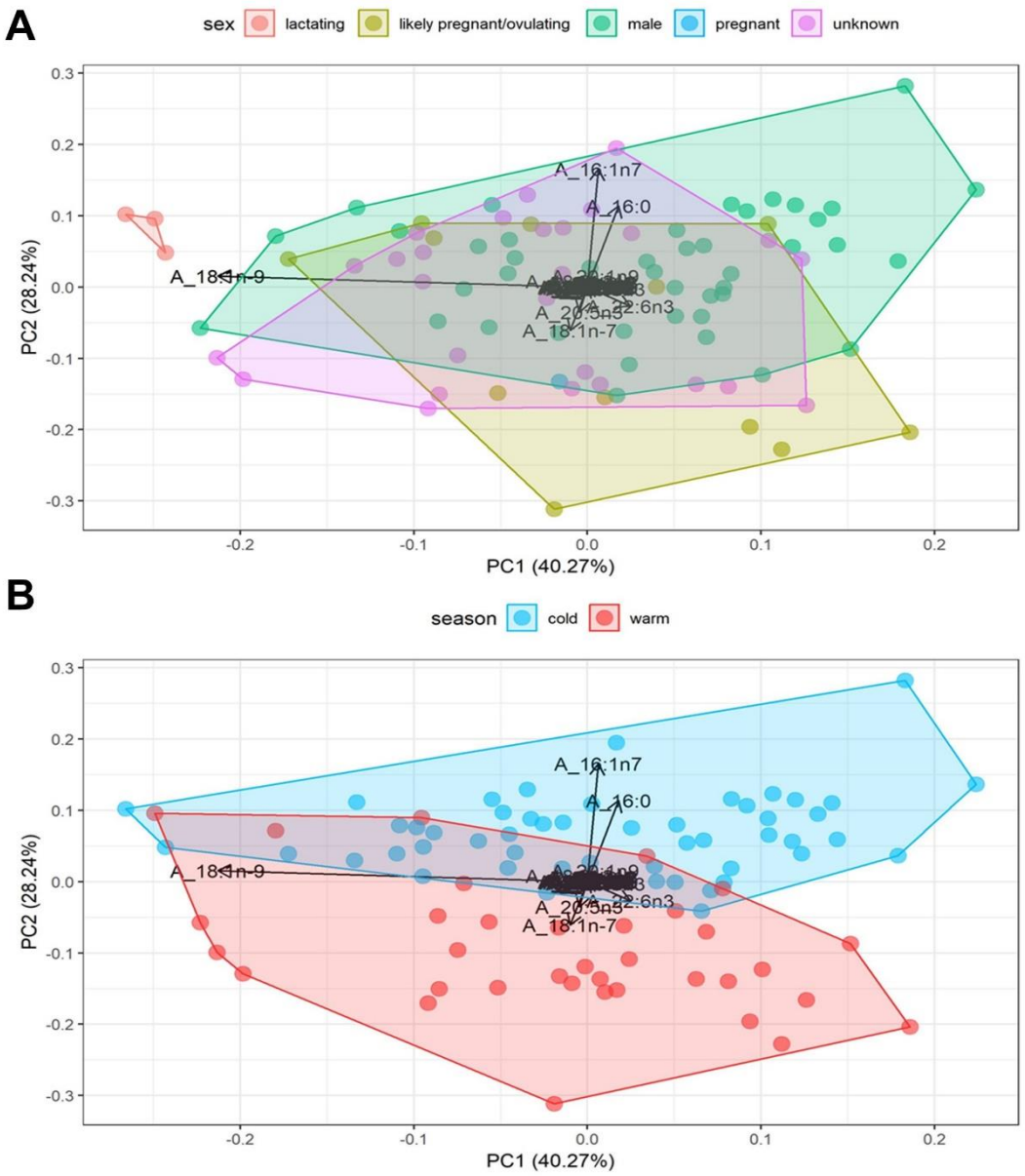

Figure 18 PCA of fin whales' fatty acids on the basis of A) season and B) reproductive state

8. DISCUSSION

This study represents the first analysis of reproductive hormones in free-ranging fin whales. Furthermore, contrary to what has been observed in other resident mysticete populations, here we report a seasonal reproductive cycle for the population of the Gulf of California. The detected trend of reproductive hormones was supported by the first documentation of courtship events and the recapture of females at different reproductive state. The seasonal reproductive cycle combined with its small population size and residency habits, underlines its vulnerability to environmental changes.

Nevertheless, the lack of long sighting histories for most of the individuals sampled prevented us from matching age classes with hormone concentrations and lipidic composition. Despite the residency of this population, many factors are responsible for the poor knowledge of its basic physiological mechanisms and the complexity to get recapture sighting data. First, its small population size distributed in many remote areas makes it difficult and very expensive to cover the entire area with sampling surveys throughout the year. In addition, in winter, strong northerly winds, also associated with high marine productivity, are frequent and last for several days or weeks. In summer, coastal fin whale sightings decrease (Tershy et al 1993), probably due to changes in the patchy distribution of its main preys. Finally, the possibility of obtaining samples from dead animals is also low. For instance, the stranding network that operates in this area has only reported 7 confirmed stranded fin whales from 2012 to 2019, often along the less accessible shore of the northern gulf.

8.1. Variability in blubber

The use of blubber, through non-lethal sampling techniques allows the assessment of the reproductive status not only of hunted or sick individuals, but at the population level. Blood was a matrix widely used to assess endocrine status in animals but the collection of blood from mysticetes is limited to freshly dead individuals which makes

it difficult to measure baseline values of hormones. Since blubber is a complex matrix characterized by different metabolic activities along its thickness, studies in marine mammals have revealed differences in fatty acids, contaminants concentrations, and adipocyte size (Ackman *et al.*, 1975; Iverson, 2009; Waugh *et al.*, 2013). However, even if the lipidic composition of blubber and its distribution has stimulated interest and has been studied in different species, the inability to investigate the entire layer of blubber obtained by non-invasive techniques leave unsolved questions about the real pathway of macromolecules through this tissue. At the same time steroid hormones, due their lipophilic properties, enter from capillaries into the fat cells via molecular diffusion (Deslypere *et al.*, 1985), but there is no information about their accumulation and the enzymatic activity once they reach the blubber. Moreover, the time lag between the production of sex steroids and their deposition in blubber, and whether this tissue is representative of the current status of individuals is still uncertain and under debate.

Another important aspect that should be considered is the vertical variability in the blubber biopsy. Results of our pilot study on lipidic distribution through three biopsies confirm the macroscopic heterogeneity of blubber reported in previous studies (Ackman *et al.*, 1975; Lockyer *et al.*, 1984). As for steroid hormones, neither testosterone (Kellar *et al.*, 2009) nor progesterone (Kellar *et al.*, 2006) was found to be significantly different through the blubber in different species of delphinids. In contrast, in belugas whale (*Delphinapterus leucas*), cortisol concentration increased with blubber depth, with highest concentrations in the inner layer (Trana *et al.*, 2015). These studies were done on odontocetes, but there is still no information about steroid deposition in mysticete blubber. Thus, due to the greater blubber thickness of large whales, a different concentration of steroid hormones through the tissue can be expected with highest concentration in the inner layer due to a higher bloodstream in this area. We only will be able to corroborate if stratification in steroid hormones occurs throughout the tissue of fin whales until the entire thickness of blubber samples from fresh carcasses is analyzed. Meanwhile, to obtain comparable results in steroid analyses, it may be preferable to use the same biopsy subsample layer.

The presence of a complex metabolic activity through the blubber was reflected also by the weak correlation between progesterone and neutral lipids for females' reproductive categories reported in the present work. This result may be explained by a higher and growing energetic demand of gestating females than no pregnant females. Even if circulating lipids and hormones follow different intracellular pathways, it is likely that lipids in the outer layer reflect the incorporation of a major accumulation of energetic reserves in parallel with a constant diffusion of progesterone from blood into the blubber during the pregnancy. However, the small sample size for some categories, in particular Pregnant, Likely Pregnant-Ovulating and Lactating females prevent us from detecting a stronger significant correlation and higher sampling size is needed to confirm this result. Moreover, the incorporation of the Unknown category to the analysis add a high degree of variability since in this group are enclosed individuals (resting and immature females) with different energetic demands. Aguilar and Borrell, (1990a) showed that immature female fin whales from North Atlantic had higher lipid content than resting females.

8.2. Sex hormones

This is the first study quantifying progesterone and testosterone in free-ranging fin whales. Because hormone metabolism and blubber structure vary among species, it is crucial to validate the hormone measuring technique for each new species considered. Although knowledge of fin whale physiology is still limited, our validation for progesterone and testosterone was successfully carried out, and the results strongly support the use of blubber biopsies of fin whales to investigate reproductive aspects of the population and to detect long term changes.

In this study, the differences observed between replicates of the same individuals may be linked to the topographical heterogeneity of the blubber over the body (Niæss *et al.*, 1998; Strandberg *et al.*, 2008; Gómez-Campos *et al.*, 2015). Anatomical measurements and biochemical analyses in fin and sei whales, for example, indicated that the posterior dorsal blubber probably represents an

important fat energy storage depot, having the highest lipid contents and the thickest blubber. In contrast, the anterior ventral blubber may not be so important in this role and contains higher concentration of proteins (Lockyer *et al.*, 1985). Because steroid hormones exhibit affinity to lipids, it is likely that differences in lipidic content along the body affects the hormone concentrations. Nevertheless, differences between replicate samples of the same individual were minimal, which is coherent with the progesterone concentrations observed in dolphins (Kellar *et al.*, 2006). Therefore, a bias in the body site where the biopsies were taken along the dorsal edge of the whale, regarding sex hormones, should not compromise the accuracy of the designation of reproductive status.

8.2.1. Females

The highest progesterone concentration observed in the present study was observed in summer by a female for which pregnancy was confirmed by its re-sighting a year later accompanied with a calf. This is the first quantification of blubber progesterone in a pregnant fin whale. The wide range of progesterone values observed in the present study (Fig. 11) is in agreement with the high variability observed in North Atlantic fin whale serum (Kjeld *et al.*, 1992), and in blubber of other cetacean species (Mansour *et al.*, 2002; Atkinson & Yoshioka, 2007; Pérez *et al.*, 2011; Atkinson *et al.*, 2019). Even if the comparison of absolute progesterone values between studies are not comparable, due to differences in extraction and quantification processes as well as species variations, a similar trend between pregnant and non-pregnant was observed in all species of cetaceans studied so far (Table 6).

The presence of a group of females with high progesterone concentrations (Fig.11) may be explained by the luteal phase of the ovarian cycle, pseudopregnancy or by the gestation. In this sense, the progesterone concentration of the female involved in the first courtship (2015) would reinforce the existence of this sexually active group of females (cluster B) in our mixture model. The intermedium progesterone concentration, close to the lower limit of the cluster B, could indicate that this female

was in estrous (ovulating period) or recently conceived from a previous mating event during the same season. Nevertheless, is still unknown which phase of the ovarian cycle may be detected through progesterone in large whales. Kjeld *et al.* (1992), identified three groups of females in the North Atlantic fin whale, based on progesterone values in serum: 1) with low progesterone values (young sexually immature females); 2) with intermediate values and 3) with high values (pregnant females). Because the first female group has similar length and age the author suggests the second group are mostly females recently maturated (although an ovulation or a recent conception cannot be either discarded. In contrast to serum, blubber is considered a matrix that represents only long-term changes (i.e. on the order of weeks to months, time frame of reproductive changes) (Kellar *et al.*, 2014). So, considering that progesterone levels decrease after ovulation if conception does not occur, it is likely that high progesterone concentrations in blubber reflect only pregnancy, which would explain the clearly distinction of two groups in our study, in accordance with previous studies on the same tissue (Mansour *et al.*, 2002; Pallin *et al.*, 2018) and in discordance with the serum results of Kjeld (1992).

The wide range of progesterone values in cluster B also may be linked to a high variability in the timing of conception during the breeding season, or simply to its lower sample size. In various mammals, progesterone serum levels gradually rise through the gestation (Bedford *et al.*, 1972; Boyd *et al.*, 1993). Nevertheless, the progesterone trend and whether its concentration in blubber reflects different gestation states are still unclear in cetaceans. Bottlenose dolphin and killer whale showed higher progesterone concentration in serum during early and mid-pregnancy compared to late pregnancy (Katsumata *et al.*, 2006; Bergfelt *et al.*, 2011; Robeck *et al.*, 2018). In blubber of three dolphin species, no difference was found in progesterone concentrations with respect to fetal length (Kellar *et al.*, 2006). In mysticetes however, as mentioned above, blubber is highly variable in thickness and composition, and no studies have investigated progesterone throughout the entire gestational period.

Even if the physiological processes that drive progesterone production in cetaceans are not clear, we hypothesized, based on studies in captive odontocetes (Bergfelt *et al.*, 2011), a rapid decrease of the hormone during parturition. This was confirmed

by the low progesterone concentrations found in lactating females in this study. Furthermore, since fin whales show long calving intervals of at least two years (Mizroch *et al.*, 1984), we assumed that progesterone production, in resting females, was similar to that of lactating females, according to the pattern reported previously in blue whale feces and blubber (Valenzuela *et al.*, 2018; Atkinson *et al.*, 2019). These animals would be represented by the cluster A (Fig.11) identified in this study.

Table 5. Progesterone concentration in the blubber of cetaceans. Concentrations for progesterone are reported as ng g^{-1} of blubber extracted.

Species	Pregnant	Non-pregnant	Author
Tursiops truncatus	54.82 ± 22.86	6.16 ± 3.62	Pérez *et al.,* 2011
Globicephala melas	45.28 ± 28.40	-	Pérez *et al.,* 2011
Delphinus delphis	261 ± 29	13.7 ± 1.8	Kellar *et al.,* 2006
Lissodelphis borealis	312 ± 44	15.0 ± 7.5	Kellar *et al.,* 2006
Lissodelphis obliquidens	161	12.1 ± 8.4	Kellar *et al.,* 2006
Balaenoptera acutorostrata	132 ± 22	-	Mansour *et al.* 2002
Megaptera novaengliae	254.65 ± 293.94	2.06 ±1.12	Pallin *et al.*, 2018
Balaenoptera musculus	25.5 ± 3.1	< 5.8	Atkinson *et al.*, 2019
Balaenoptera physalus	173.36	1.58 ± 1.30	Present study

8.2.2. Males

In mysticete males, the reproductive behavior in the breeding grounds is diversified and could be characterized in some cases by the exhibition of a certain aggressiveness toward conspecifics, courtship (exhibited with vertical head up postures, spy-hopping and tail bobbing) and for some species by a complex

vocalization (Donnelly, 1967; Payne and McVay, 1971; Clapham, 1996). In terrestrial mammals the increase of aggressive behavior (Bouissou, 1983) or in the song in birds (Nottebohm *et al.*, 1987) is often associated with an increase in testosterone. Little is known about the physiological change in cetaceans, and in particular male hormonal fluctuations associated to the reproductive behavior.

The high testosterone concentrations observed in the present study during late summer coincide with the pattern found in the serum of North Atlantic fin whales, when testosterone was more than fourfold during late summer (August-September) than during early summer (June) (Kjeld *et al.*, 1992). Nevertheless, that migratory population showed this rise in the summer feeding grounds, several months before the mating that takes place in winter (December-January). This also has seen in other cetacean species. In North Atlantic minke whale and in in North Pacific humpback whale, increasing testosterone concentrations were observed prior to the mating season (Kjeld *et al.*, 2004; Cates *et al.*, 2019). According to several studies in migratory species, this increment in testosterone observed while they are still in their feeding areas at high latitudes, could suggest a physiological preparation before reaching the reproductive areas (Kjeld *et al.*, 2004; Vu *et al.*, 2015). Furthermore, in humpback whales an increase in testosterone towards the end of the feeding season would trigger the start of males singing (Herman, 2017; Cates *et al.*, 2019).

Little data prior to summer (May-June) prevented from the detection of this male preparation status associated to the highest testosterone concentrations. Thus, it is possible that testosterone concentration during late spring or early summer could be higher than the ones observed in summer in the present study. However, the observation of two courtship displays in August and September may discard the scenario of a long preparatory phase in the males of the Gulf of California population, and rather suggests the start of the mating season during the late summer. Because mating season generally occurs during several months (Evans, 1987), it is possible that testosterone concentrations keep increasing during autumn. Due to the lack of autumn samples in the present study, and the unknown time lag in detection of these hormones in the blubber, I cannot infer the precise range of the mating season. Nevertheless, low values of testosterone during winter (Fig. 12,)

suggest mating occurs before winter. Our results highlight the lack of complete understanding on how reproductive hormone levels vary in breeding grounds. Each species is a unique case possibly caused by a mixture of abiotic and biotic factors that influence the physiology and even populations of the same species. The present study is the first report a fin whale courtship behavior associated with high testosterone concentration in males involved in mating behavior. This is similar to testosterone levels observed in terrestrial mammals and contribute the understanding of reproductive physiology of cetaceans' males (Atkinson & Yoshioka, 2007).

8.3. Stress hormones

Exposure to short-term stressors (acute stress) stimulates the release of some hormones and may be beneficial for organisms (Möstl & Palme, 2002; Atkinson *et al.*, 2015), functioning as a physiological mechanism for mobilizing energy stores and triggering appropriate behaviors (Boonstra, 2004). If the stimulus persists and related hormones continue to release (chronic stress), the result may be a weakening of vital functions causing illness, decreased reproduction and/or death (Boonstra, 2005). In some phases of the reproductive process, the production of stress hormones is required since GC play an important role in energy mobilization (Romero, 2002). Male mammals show a peak in baseline GC secretion during the mating season since this behavior often entails increased energy costs associated with territorial defense and competition (Romero, 2002). Similarly, in females, from an energetic perspective, gestation and lactation are the most metabolically expensive periods of a female's life history with changes in GC regulation (Reeder & Kramer, 2005). In some mammals (i.e. rats), GC levels increase during the middle and late gestation period and decline at parturition to levels that are still somewhat elevated relative to males and resting or non-reproductive females (Atkinson, 2004). As for cetaceans, GC metabolite concentrations were found to vary significantly in feces of North Atlantic right whale (*Eubalaena glacialis*) with sex and reproductive category, being highest in pregnant females and mature males, intermediate in lactating females, and lower in immature individuals (Hunt *et al.*, 2006). Blue whale

feces also have the highest concentrations of corticosterone in pregnant females (Valenzuela-Molina *et al.*, 2018a) highlighting the complexity of a status where the female not only provides the metabolic demands of the fetus but also prepares the organism for lactation (Nelson, 2005). In contrast, Kellar *et al.*, (2015) found no evidence that blubber cortisol concentrations varied as a function of sexual maturity in short-beaked common dolphins.

Blubber may reflect stress over a longer period and provides a mean for measuring hormone concentrations that is not associated with stress due to sampling collection (Trana *et al.*, 2015). However, despite reported measurement of GC from blubber of several marine mammals (Atkinson *et al.*, 2015), there are currently no published studies describing extraction and measurement of aldosterone and thyroid hormones in blubber. The unsuccessful validation of stress hormones, including GC, of fin whale blubber analyzed in the present study may be due to:

Technical procedures. The extraction method used is generally dependent on the properties of the component of interest. Our analysis method for fin whale blubber was based on the method described by Mansour *et al.* (2002), which was previously tested in other marine mammal species (Atkinson *et al.*, 2019; Cates *et al.*, 2019). This technique involves the use of ethanol, which is a polar solvent and turned out to be efficient for the extraction of steroid hormones (Daughaday, 1959). Similarly, although thyroid hormones, have not been detected yet in adipose tissue, they have been successfully extracted through polar solvents in other tissues and excretions (Wasser *et al.*, 2010). The unsuccessful validation could be also linked to the cross reactivity in the antigen assay used. However, the validation carried out by Hunt *et al.*, (2017) in fin whale baleen, used the same manufacturer (Arbor assay), so in this case a problem in the specificity of the assay can be ruled out for this species. Eventually, the effects of degradation of GC and thyroid hormones may suggest false result trends and should be investigated in future. Many metabolites, in fact, degrade over time due to various processes (Elliott & Peakman, 2008). In beluga whale blubber, for instance, cortisol concentration was observed to be lower in degraded samples. Although in our study biopsies were immediately stored in liquid nitrogen, most samples were stored for more than 10 years and a control sample

quality has not been standardized for fin whale. Understanding how hormone concentrations vary over time will certainly help to interpret the results and optimize future sampling protocols.

Physiological aspects. Concentrations can be different between sexes and reproductive status. In the present study, validation tests were applied separately to males and females. However, it is possible that our sample set contained few females or males of a certain reproductive state, in which stress hormones levels are more elevated, so the population was not well represented, making it difficult to detect these stress hormones.

Tissue. As mentioned above, blubber does not represent a direct target tissue for steroid and thyroid hormones. To further complicate this issue, the recent in vitro study of Galligan *et al.* (2018), proposed that, similarly to what happens in adipose tissue in terrestrial mammals (Deslypere *et al.*, 1985; Prins *et al.*, 1996; Li *et al.*, 2015), blubber could serve as an endocrine organ that actively metabolizes or synthesizes cortisol, which would make this tissue an inappropriate tissue for monitor physiological status under stress conditions. However, the Galligan *et al.* (2018) study include only three bottlenose dolphin samples, all of which came from stranded (i.e., stressed) animals, so the generalization of these results to other physiological status or species requires more investigation. Besides these aspects, it is important to consider that, in different species of cetaceans, blubber reflects only *qualitative* changes in circulating steroid hormone profiles (Kellar *et al.*, 2006; Pérez *et al.*, 2011; Trana *et al.*, 2015; Cates *et al.*, 2019) and is not considered as a good *quantitative* predictor of circulating levels of stress hormones. Champagne *et al.* (2016) reported a significant relationship between cortisol levels in serum and in blubber in bottlenose dolphins, but serum cortisol concentration explained only 57% of the variation in blubber cortisol. To explain these differences, they proposed different rates of cortisol concentration change in serum and blubber. In addition, each tissue has different rates and degrees of incorporation of hormones which in turn may be influenced by a variety of factors, including perfusion, growth rate and metabolic activity, hormone solubility and environmental conditions (Bortolotti *et al.*, 2008). In pigs (*Sus scrofa domesticus*), plasma progesterone enters in the adipose tissue within 1-2 days (Hillbrand & Elsaesser, 1983). In cetaceans, the only study

that investigated progesterone entry in the adipose tissue was done under controlled environmental conditions through the application of a stress protocol to bottlenose dolphins (Champagne *et al.*, 2018). These authors observed that cortisol can be detected within 2 h in blubber and 3.5 - 5 h in fecal samples. Failure in the detection of stress hormones in the present study may be also linked to a concentration below threshold method detection. A study on baleen of fin whale showed that reproductive hormones concentrations are three times higher than stress hormones with cortisol barely detectable (Hunt *et al.*, 2017). This pattern is consistent with relative concentrations typically seen in mammalian plasma (Hunt *et al.*, 2017). Furthermore, the low cortisol concentration found in baleen is consistent with the serum concentration observed in 21 fin whales caught off the west and southwest coast of Iceland during summer, which was found to be about 17 times lower than the mean morning level in humans and 3 times lower than in odontocetes (Kjeld, 2001). These observations lead to consideration that not only blubber may reflect a long-term production (e.g. months for sexual hormones), but also that baseline levels of stress hormones are lower in fin whale than in other species.

Ecological aspects. The absence of an annual long-distance migration in a resident population of mysticetes could have endocrinological effects. In general, during the annual migration of baleen whales, the adults are not feeding and therefore rely on energy stored to fuel the 8–9 month journey exposing the animals to a certain level of stress due to starvation (Corkeron and Connor, 1999; Braithwaite *et al.*, 2015). So, a relatively constant foraging activity through the year associated with a low consumption of energy stored (see Results) in the Gulf of California fin whale population could be associated with a lower synthesis in stress hormones levels. Future studies should incorporate blubber samples of a migratory population of fin whales to test this hypothesis.

8.4. Seasonality

Evidence obtained in the present study and the estimated duration of gestation and lactation in other fin whale populations (Lockyer, 1984a; Mizroch *et al.*, 1984), allow

us to propose an hypothetical reproductive cycle for the resident fin whale population in the Gulf of California (Fig. 19). Mating activity would occur during the late summer/autumn, part of gestation and lactation would take place around winter/spring, and weaning would follow during summer/autumn, depending on when conception occurs.

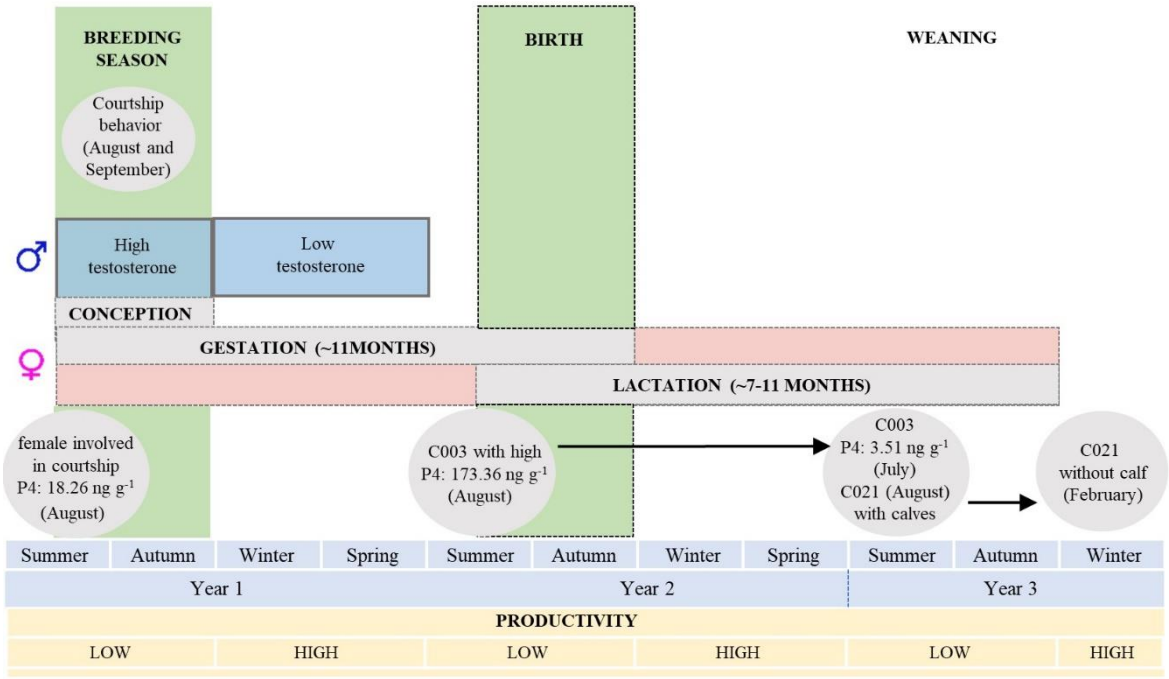

Figure 19. Schematic reproductive cycle of the resident fin whale population of the Gulf of California, based on the seasonal hormone analysis of this study. The sighting histories of pregnant and lactating females, weaning, and courtship events observed. Black dashed lines represent a period of uncertainty during which each event is proposed. P4 is progesterone concentration observed in females C003 and C021.

In marine and terrestrial mammals, a seasonal reproductive cycle implies a limited birth period, usually as an adaptation response to seasonal environment variability (McGuire *et al.*, 2010). In migratory whale species, the calving season seems to be driven partially by the necessity to avoid thermal stress for the newborns, giving birth in warm, low-latitude waters (Gaskin, 1982). Comparing to high latitudes, the sea surface temperature in the Gulf of California in winter is relatively warm (20.37 ±

1.92) (Escalante *et al.*, 2013). In fact, during this season, the Gulf of California and overall surroundings waters around the Peninsula of Baja California are used as an important calving areas by several migratory whale species, such as the gray whale (Rice *et al.*, 1982), the humpback whale (Urbán & Aguayo, 1987), and the blue whale (Gendron & Ugalde De La Cruz, 2012). Therefore, I discard temperature stress as a driving factor influencing the seasonal reproductive cycle of the Gulf of California fin whales.

Seasonal reproductive strategies have also been observed among animals that live in more stable temperature conditions (Clutton-Brock & Harvey, 1978). In these cases, food availability may be an important limiting factor for any biological activity related to reproduction. As reported for the resident fin whales of the Mediterranean Sea (Relini *et al.*, 1992; Canese *et al.*, 2006), those in the Gulf of California are observed feeding during all year (Ladrón de Guevara *et al.*, 2008). The absence of a long fasting period seems to be confirmed by the observation of no significant differences of polar and neutral fraction between seasons (except for likely pregnant-ovulating females). However, its main prey in the gulf is the euphausiid *Nyctiphanes simplex* (Tershy, 1992; Gendron, 1992; Del Ángel Rodríguez, 1997), which is most abundant in winter/spring and decreases in summer/autumn (Brinton & Townsend, 1980; Gómez-Gutiérrez *et al.*, 2012), when fin whales shift their diet to small pelagic fish (Tershy, 1992; Gendron *et al.*, 2001). This change in the gulf conditions may be reflected partially by the seasonal differences of the most abundant fatty acids detected in the present study. High amounts of the fatty acid 16:1(n-7) and 16:0, typical of diatoms, in fact, were observed in cold season whereas the 18:1 (n-7) and 20:5 (n-3) seems to be higher in the warm season. Thus, although this population does not strictly fast as other migratory populations do, resources may not be enough year-round (in terms of nutritional quality). This is in particular important for the strong energetic demands of lactating females, for which the energetic cost can be double that of the gestation and fetal development period (Lockyer, 1981a). In most mysticete migratory populations, females fast or feed infrequently during lactation, often showing poor body condition (Aguilar & Borrell, 1990), and relying on the energy reserves stored during the feeding season. However, two studies conducted in the Gulf of California revealed that whereas most

lactating females of blue whale that spend winter in this area show poor body conditions (Gendron, 2002; Casillas-Lopez, 2016) none of the lactating fin whale females showed this condition (Arcos Diaz, 2018). This good body condition observed in females coupled with a constant lipidic composition through the year observed in the present study in all sampled population (see section 8.5), suggest that females do not fast during lactation in the Gulf of California. Based on the present results, it is likely that early- or mid-lactation, when calves are completely dependent on the mother's milk (Oftedal, 1997), would start slightly before the most productive period of the year, that is the winter/spring (Álvarez-Borrego & Lara-Lara, 1991). Thus, the lactation period for the female C003 probably began in autumn, and therefore the weaning would probably have taken place during the following summer/autumn after we observed her with her calf. This is supported by the sighting of the female C021 with her calf in late summer (August), and its posterior re-sighting six months later during winter (February) without the calf (Fig.17). This is analogous to the pattern of migratory rorquals, whose calves are weaned just before or immediately after the female's arrival to the feeding grounds (Oftedal, 1997). All these observations support the hypothesis that prey availability or quality could be crucial in the regulation of the reproductive cycle of the resident fin whale population of the Gulf of California.

8.5. Metabolic aspects of reproduction

Total lipid values for each reproductive category revealed a lower composition of lipidic component in the blubber of the fin whale of the Gulf of California than the North Atlantic population reported by Aguilar and Borrell (1990). This could be linked with the temperature of the area in which the species lives. Some authors suggest that thermoregulation, in fact, does not depend only on the blubber thickness but also on the lipid content and its patterns in the blubber (Strandberg *et al.*, 2008; Bagge *et al.*, 2012). In this sense, for example, the bowhead whales (*Balaena mysticetus*), which spends its entire life in the Arctic and sub-Arctic waters, showed one of the most abundant percentage of lipids in their blubber (75.8 ± 1.6; Hoekstra *et al.*, 2002). In contrast low percentage of lipids found in blubber of fin whales of

the Gulf of California (see Results), may be influenced by a relatively constant warm waters they inhabit. Another aspect that could play an important role in the blubber composition may be related to the nutritional quality of their prey. In this regard, future comparative studies of the nutritional quality of the main prey between resident and migratory population is needed. Moreover, in a resident mysticete population, the blubber layer may not serve the same role of long-term energy storage found in capital breeders such as most baleen whales, therefore occupying less space in body and containing a lower percentage of lipids. Finally, is important to interpret with caution these comparative results since they come from different blubber sampling methods, layers, without any specification of the thickness considered, and from different species.

Energy use during reproduction has received considerable attention due to its implications in the reproductive strategy. In this sense, biological and environmental factors, such as changes in the oceanographic conditions or the overfishing, might modulate or alter the preferred prey distribution or abundance, leading to suboptimal nutritional conditions for reproduction, with long-term effects in breeding season and in rate at which pregnancies were conceived and sustained (Croxall, 1992; Trites & Donnelly, 2003; Ayres *et al.*, 2012)

The energy expended during reproduction is very high for baleen whale females (Lockyer and Brown, 1981). In particular, gestation and lactation are the periods that require the greatest consumption of the energy stored (Gittleman & Thompson, 1988). In general, pregnant females show large increases in energy requirements (Young, 1976; Gittleman and Thompson, 1988; Lydersen, 1995b) during which not only does the lipid content of blubber reach a maximum during pregnancy, but its thickness and the mass of several tissues that also serve as fat stores tend to be greater than in other females (Lockyer, 1981; Mackintosh and Wheeler, 1929). A pregnant blue whale, for example, is thought to store 45,000kg of blubber during the feeding season, most of which is converted into milk for her calf during calving season (Lockyer, 1981b). Here, the single confirmed pregnant female found in this study showed one of the highest values in lipid content and the high lipid composition of the unconfirmed pregnant female group, support this significant energy stored period in females. Furthermore, particular attention deserves the

marked difference of neutral lipid fraction between seasons with a significant increase in the warm season. This, according to the reproductive cycle proposed in this study, would coincide with the late gestation period and the start of the lactation. Nevertheless, is important to take into account that since the most productive season in the Gulf is the cold season and a delay in lipid deposition in the outer layer of the blubber must be considered, it is highly probable that these values need to be considered as a result of the energy stored from previous season. This interesting result differs from the North Atlantic fin whale population, in which the mean lipid content of the blubber did not correlate with the fetus length from pregnant females (n=8) (Aguilar & Borrell, 1990). Nevertheless, the authors reported only one season (June-August) so it is likely that a change in lipid content was not detected for this reason.

The average nutritional requirements are even higher for lactating females (Hadjipieris and Holmes, 1966; Millar, 1975, 1977; Bowen *et al.*, 2001). Lactating females have been shown to have the lowest body fat condition and blubber lipid content (Lockyer 1986; Aguilar and Borrell 1990). During mid to late lactation, in fact, baleen whales produce milk containing 300–400 (g/kg) fat, 110–150 protein, and probably 10–20 sugar (Oftedal, 1997). Most of the energy transferred to calves is via lipids, restricting the need for maternal gluconeogenesis. The amounts of milk transferred have not been measured but can be predicted based on mammary gland mass and calf growth rates, based on the assumptions that cetacean mammary glands produce 0·5–1·3 kg milk kg^{-1} of mammary mass, and that cetacean calves require 2–4 kg milk kg^{-1}gain, blue whales are predicted to produce about 220 kg milk d^{-1} (range of estimates 110–320 kg d^{-1}; Oftedal, 1997).

The energetic cost of the lactation in marine mammals may be supported by a constant food intake or, in some species by the catabolism of maternal tissues (Oftedal, 1993; Oftedal, 1997). In this study, lactating females did not show lower values of lipids as observed in the North Atlantic population in which the average total lipid percentage (of the blubber layers) of lactating females were lower (57.5% cv=15.7%) than the other reproductive categories (pregnant= 81.4%, cv= 6.8%; immature= 77.5%, cv= 7.6%; resting= 60.8%, cv= 27.9%) (Aguilar & Borrell, 1990).

In the Gulf of California the prevalence of lactating females with a regular body condition (Arcos Diaz, 2018) combined with an intermedium content in total and neutral lipids in the blubber reinforce the hypothesis that these female fin whales do not fast completely during lactation. Nevertheless, the low number (n=3) of lactating females sampled do not permitted us to further investigate on the energy expenditure of lactation and variation in lipid content in this resident population.

Energy reserves may affect the duration of lactation. In southern elephant seals, for example, the duration of lactation was positively correlated with the postpartum mass of mothers (Arnbom *et al.*, 1997). As for cetaceans, in general, the difference between non migratory odontocetes and migratory mysticetes is reflected in the lactation strategies with less lipid milk content and longer lactation period in the former compared to higher lipid content and shorter lactation period in the latter (Berta *et al.*, 2015). In the case of this resident fin whales, we do not have sightings for the same females during the entire lactation and the observation of the lactating female C021, re-sighted six month later without a calf, does not confirm the total duration of the lactation since the calf could have been weaned anytime between the two sightings. However, we cannot exclude that, due to the absence of migration, lactation is extended one or more months than the migratory populations.

While female reproductive success is mainly limited by gestation and lactation costs, male reproductive success, is limited by access to receptive females (Lockyer, 1984a). In several terrestrial and marine mammals, hypophagia occurs during the mating season, namely the time of year when energy expenditure by males is greatest. This, lead to an energy depletion and in some case to a loss of body mass (Miquelle, 1990; Deutsch *et al.*, 1990; Mysterud *et al.*, 2008). Similarly, migratory populations of mysticetes, as typical capital breeders, rely on energy previously stored before a reproductive period involving fasting. However, there is no specific information on the energy expenditure of fin whale males during this process. In the present study, we found no differences in males between seasons. This result could be linked in part by a constant feeding activity through the year and in part by with the stable character of the outer blubber layer which does not reflect short term variation in nutritional condition. Indeed, although reproductive cost in male remain

unknown, reproductive effort occurs in a shorter period (4-5 months) than lactation and gestation (respectively 7-11 months and 11 months).

Finally, the low percentage (40.55%) of explained variance in principal component analysis did not allow the separation of fatty acids profiles between reproductive categories and season. This could be related with the small sample size for the categories considered that conceals the differences caused by physiological state or a change in diet. However, it is important to underline the predominance of several fatty acids in the warm (18:1 (n-7); 20:5 (n-3)) and in cold (16:1(n-7); 16:0) season which may be indicators of a seasonal variation in diet.

9. SUMMARY

Contrary to what has been observed in the resident fin whale population of the Mediterranean Sea, here we report a seasonal reproductive cycle for the fin whale population of the Gulf of California. This seasonality is influenced by regional prey abundance variability, that even if is sufficient to ensure year-round feeding activity for most of the population (males, resting and immature females), it likely is not enough (in terms of quantity or quality) during warm season to sustain a long reproductive period for categories with high energy demands (lactating and pregnant females). A short reproductive period (3-4 months) in a restricted area and a small population size make this population more vulnerable to environmental changes and anthropogenic disturbances. In fact, changes in survival rate of the calves (Lockyer, 1984a), prey availability (Lockyer, 1986), or fertility (Hohn *et al.*, 2007) (e.g. for exposure to contaminants) may negatively affect the reproductive success of a population, and more so if it does not breed continually during the year. This also highlights the importance of specific measures that should be considered in the conservation plan of this population.

10. CONCLUSIONS

- The resident fin whale population in the Gulf of California has a seasonal reproductive cycle.

- Blubber represents a useful tissue to quantify sex steroid hormones in fin whale and more research is needed on the stress hormones in this species.

- The outer layer of blubber represents a useful tissue to detect long term changes (months) in nutritional condition, such as the gestation.

11. RECOMMENDATIONS

I recommend that future studies continue to use blubber for sex steroid hormone analysis of free ranging fin whales, whereas more study is needed about stress hormones in this species. However, in order to get a more precise insight into its reproductive dynamics, future studies must incorporate photogrammetry analyses to account for individual size at varying hormone concentrations, as well as to increase the time series monitoring to detect more details of the seasonal cycle, and detect possible inter-annual anomalies from it. Finally, results for stress hormones and lipids, highlight the importance to increase the investigation about the physiological mechanism of this species and to compare living strategies between resident and migratory populations.

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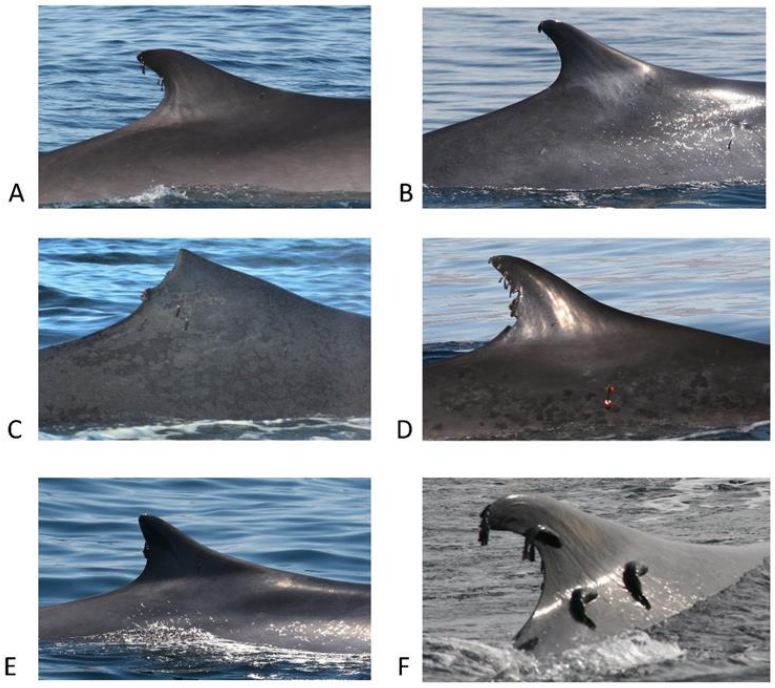
13. APPENDICES

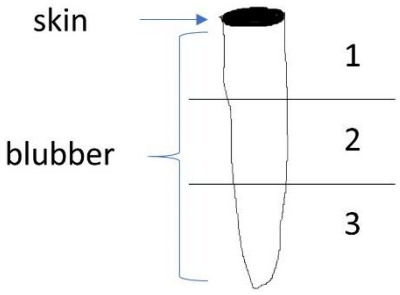
Appendix I

In the present were recognized 6 categories of dorsal fin in the fin whale of the Gulf of California: a) hooked dorsal fin with wide base (Type A), b) hooked dorsal fin with tight base (Type B), c) dorsal fin without tip (Type C), d) marked dorsal fin (Type D), e) dorsal fin with triangular shape (Type E), f) undetermined (Type F).



Appendix II

Three biopsies (A, B, C) of around 2.5 cm of the outermost layer of the blubber were cut into three equal parts (1,2,3) to verify the homogeneity of this tissue along the



sample. For each part was analyze neutral (NL) and polar (PL). Results showed a high heterogeneity in terms of lipid content through the biopsies:

A1: 83.24% NL, 3.55%PL; B1: 60.02 % NL 1.12%PL; C1: 45.03% NL 1.5% PL

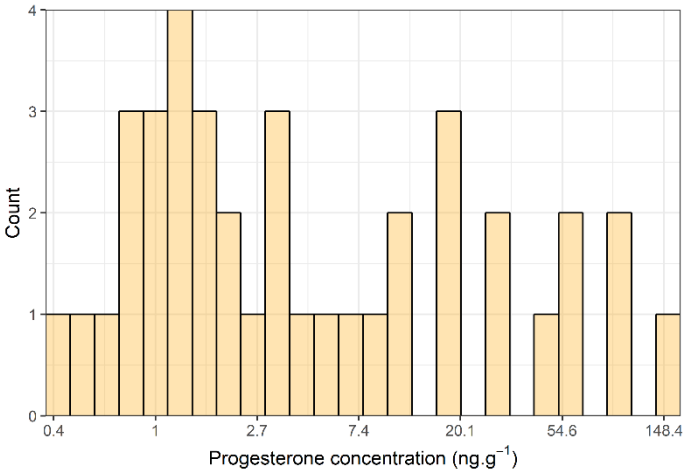
A2: 19.86% NL, 2.13%PL; B2: 50.43 % NL 2.3 %PL; C2: 49.67% NL 1.2% PL

A3: 6.7% NL, 0.9%PL; B3: 56.78% NL 2.0 %PL; C3: 56.83 % NL 1.1% PL

Appendix III

Mixture model of progesterone concentrations.

Apparent bimodal pattern of progesterone:



Algebraic representation:

The logarithmic scale of progesterone concentrations (*H*) at each biopsy (*i*) are stated as coming from a normal distribution:

$$\log(H_i) \sim N(\mu_{H_i}, \tau_{H_i})$$

Its stochastic means (μ) and precisions (τ) are grouped in a known number of clusters (*C*), with fixed effects on them. A half-normal likelihood was stated for the means, with a broad standard deviation, and an uninformative distribution for the precision:

$$\mu_{P_i} = \tilde{\mu}_{C_i} \; ; \quad \tau_{P_i} = \tilde{\tau}_{C_i}$$

The clusters have a categorical distribution, whose stochastic parameter represents the probability of the source for each observation (i) of each cluster (C):

$$C_i \sim Cat(p_C)$$

The prior for such probability of cluster source (*p*) was a Dirichlet for both clusters:

$$p_C \sim Dir(C)$$

JAGS code:

"model {

We have hormone log-progesterone values

that we suppose are generated by a mixture

of two different normal distributions (i.e. clusters).

We don't know which datum came from each cluster.

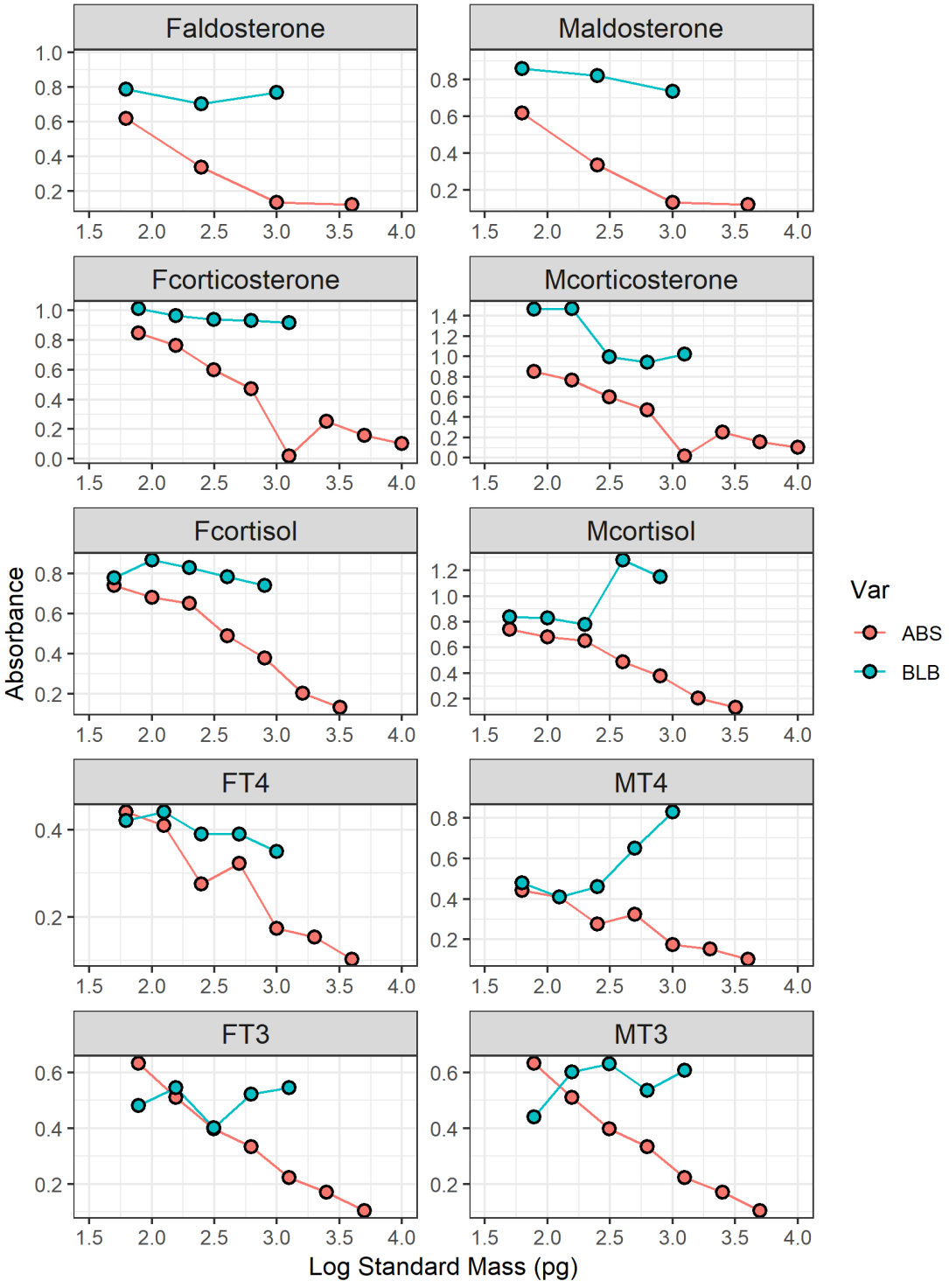
Our goal is to estimate the probability that each

score came from each of the two clusters,

```
# That is, an ordered vector of the posterior values of

# p_cluster[1] and p_cluster[2]. We are also interested

# in the means and SDs of the normal distributions

# that describe those clusters, and the probability of

# occurrence of each cluster, which are simply the

# posteriors of the same parameter (p_cluster).

 for (i in 1:n_prog) {
   log_prog[i] ~ dnorm(mu_log_prog[i], tau_log_prog[i])   # Mixture of normals
   mu_log_prog[i] <- mu_cluster[cluster[i]]               # Fixed means per cluster
   tau_log_prog[i] <- tau_cluster[cluster[i]]            # Fixed precision per cluster
   cluster[i] ~ dcat(p_cluster[1:n_cluster])             # Categorical likelihood
 }
 # Fixed effects on clusters:
 for (j in 1:n_cluster) {
   mu_cluster[j] ~ dnorm(0, 1.0E-10)                     # Half-normal
   tau_cluster[j] ~ dunif(0.2, 2.1)                    # Uniform
   sd_cluster[j] <- sqrt(1/tau_cluster[j])               # Define precision
 }
 # Priors:
 p_cluster[1:n_cluster] ~ ddirch(ones_rep_n_cluster)    # The Dirichlet prior

}"
```

Appendix IV

Serial dilutions of samples showing no parallelism with the standards of aldosterone, corticosterone, cortisol, T4 and T3. The standard curves are indicated by the orange circles, while the blubber dilutions are indicated by blue circles.



Accuracy. In females:

a) The slope was 0.55 and the BR^2 0.98 in aldosterone

b) The slope was 0.86 and the BR^2 0.97 in corticosterone

c) The slope was 0.97 and the BR^2 0.99 in cortisol

d) The slope was 1.98 and the BR^2 0.97 in T4

e) The slope was 1.66 and the BR^2 0.95 in T3

Whereas in males:

a) The slope was 0.69 and the BR^2 0.99 in aldosterone

b) The slope was 0.81 and the BR^2 0.94 in corticosterone

c) The slope was 0.89 and the BR^2 0.97 in cortisol

d) The slope was 1.59 and the BR^2 0.99 in T4

e) The slope was 1.13 and the BR^2 0.98 in T3